SYNTHESIS AND BIOLOGICAL EVALUATION OF NOVEL DRUG CANDIDATES TO ADDRESS DRUG RESISTANCE IN TUBERCULOSIS AND FUNGAL DISEASES

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SYNTHESIS AND BIOLOGICAL EVALUATION OF NOVEL DRUG CANDIDATES TO ADDRESS DRUG RESISTANCE IN TUBERCULOSIS AND FUNGAL DISEASES

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Pharmacy at the University of Kentucky

By
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Lexington, Kentucky

2018

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ABSTRACT OF DISSERTATION

SYNTHESIS AND BIOLOGICAL EVALUATION OF NOVEL DRUG CANDIDATES TO ADDRESS DRUG RESISTANCE IN TUBERCULOSIS AND FUNGAL DISEASES

Tuberculosis (TB) and fungal infections are two of the most lethal infectious diseases worldwide due to the emergence of drug-resistant *Mycobacterium tuberculosis* (*Mtb*) and fungal strains that can resist the most potent antimicrobial drugs currently employed. Due to the rise of these drug resistant strains, effective treatment options for these two infections are limited. This dissertation aims at exploring novel drug scaffolds to help combat drug resistance in TB and fungal infections.

TB caused by the pathogenic *Mtb* is, alongside with human immunodeficiency virus acquired immunodeficiency virus (HIV), the deadliest infectious disease worldwide with approximately 2-3 billion people infected yearly. The situation has become increasingly intensified due to the emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) *Mtb* strains. Aminoglycoside (AG) antibiotics such as amikacin and kanamycin A (KAN) are heavily relied upon for the treatment of MDR- and XDR-*Mtb* strains. However, the success rate for the treatment of these MDR- and XDR-TB cases is decreasing as a result of increased KAN resistance. It was reported by the Centers for Disease Control and Prevention (CDC) that upregulation of the enhanced intracellular survival (*eis*) gene was the cause of resistance to KAN in a large portion of *Mtb* clinical isolates. Our lab previously demonstrated that Eis is an AG acetyltransferase that can inactivate AGs via chemoenzymatic modification of the AG scaffolds. As Eis has been shown to acetylate a wide variety of AG scaffolds, the development of novel AGs that can completely escape the action of Eis remains highly challenging. Therefore, we suggested an alternative therapeutic approach involving inhibiting Eis enzyme and still employing the current FDA-approved KAN. As exemplified by the clinically successful combination of penicillin and β-lactamase inhibitors, we hypothesized that an Eis inhibitor may be used as adjuvant therapy in combination with KAN to treat MDR- and XDR-tuberculosis. Using high-throughput screening, we were able to identify several small-molecule scaffolds capable of inhibiting Eis. We performed structure activity relationship (SAR) studies using purified Eis enzyme and optimized lead compounds.
Additionally, we also showed that co-administration of Eis lead inhibitors with KAN led to recovery of KAN activity against a KAN-resistant *Mtb* cell line that overexpressed Eis.

Invasive fungal infections are on the rise due to an increased population of critically ill patients as a result of HIV infections, chemotherapies, and organ transplantations. Unlike antibiotics that are greatly diverse in categories and mechanisms of action, our current antifungal drug repertoire is greatly limited and insufficient in addressing the problem of drug-resistant fungal infections. Thus, there is a growing need for novel antimycotics that are safe and effective. We report a number of lead compounds with potent antifungal activity. The MIC values of these compounds were as low as 0.02 µg/mL against the fungal strains tested. Our compounds are derived from the ebselen core structure, which has been shown to be safe in multicenter clinical trials. Notably, fungal cells treated with our compounds showed the accumulation of ROS, which may further contribute to the growth inhibitory effect against fungi. This study provides new lead compounds for the development of antimycotic agents.

KEYWORDS: Antitubercular Agents, SAR, Eis inhibitors, Aminoglycosides, Kanamycin
SYNTHESIS AND BIOLOGICAL EVALUATION OF NOVEL DRUG CANDIDATES
TO ADDRESS DRUG RESISTANCE IN TUBERCULOSIS AND FUNGAL
DISEASES

By

Huy X Ngo

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Director of Graduate Studies

October 17, 2018
To my parents:

Công cha như núi Thái Sơn
Người mẹ như nước trong nguồn chảy ra
Một lòng thợ me kính cha
Cho tròn chữ hiếu mới là đạo con
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TABLE OF CONTENTS

ACKNOWLEDGEMENTS ........................................................................................................................................ iii

LIST OF TABLES ............................................................................................................................................... xvi

LIST OF FIGURES ........................................................................................................................................ xviii

LIST OF ABBREVIATIONS .......................................................................................................................... xxiii

PREFACE ..................................................................................................................................................... xxviii

Chapter 1 ..................................................................................................................................................... 1

Introduction: tuberculosis (TB), current TB drug targets, and resistance associated with TB .............................................. 1

1.1. INTRODUCTION TO TB ...................................................................................................................... 1

1.1.1. History of TB .................................................................................................................................. 1

1.1.2. Biology and pathology of TB ...................................................................................................... 7

1.2. CURRENT TB DRUG TARGETS ......................................................................................................... 12

1.2.1. Cell Envelope ............................................................................................................................... 13

1.2.2. Translation ................................................................................................................................... 14

1.2.3. Transcription and DNA Replication .......................................................................................... 15

1.3. RESISTANCE RELATED TO TB ...................................................................................................... 16

1.3.1. Efflux pumps ............................................................................................................................... 16

1.3.2. Gene mutations ........................................................................................................................... 17

1.3.2a. Mutations or modifications of drug targets ............................................................................. 17

1.3.2b. Inability to activate prodrugs ................................................................................................... 18
1.3.3. Enzymatic modifications and inactivation of drugs ......................................... 19

1.4. ACKNOWLEDGEMENTS ................................................................................... 20

1.5. AUTHORS’ CONTRIBUTIONS .......................................................................... 21

Chapter 2 ............................................................................................................................ 22

Combating Eis-mediated kanamycin resistance of *M. tuberculosis* by novel pyrrolo[1,5-\(a\)]pyrazine-based Eis inhibitors ......................................................................................... 22

2.1. ABSTRACT ........................................................................................................... 22

2.2. INTRODUCTION ................................................................................................. 22

2.3. RESULTS AND DISCUSSION ............................................................................ 24

2.3.1. Synthesis .......................................................................................................... 24

2.3.2. Biochemical and biological evaluation ............................................................ 26

2.3.2.1. Eis inhibition ................................................................................................. 26

2.3.2.2. Activity of Eis inhibitors in *Mtb* cell culture ............................................. 33

2.3.2.3. Mammalian cytotoxicity ............................................................................... 35

2.3.3. Structural biology studies ................................................................................ 37

2.4. CONCLUSION ...................................................................................................... 41

2.5. MATERIALS AND INSTRUMENTATION ........................................................ 42

2.6. METHODS ............................................................................................................ 43

2.6.1. Chemical methods ......................................................................................... 43

2.6.1.1. Synthesis of compound 4 .............................................................................. 43

2.6.1.2. General procedure for benzylation (e.g., Preparation of compound 5) ...... 43

2.6.1.3. General procedure for cyclization (e.g., Preparation of compound 8) ......... 45

2.6.1.4. General procedure for *N*-alkylation (e.g., Preparation of compound 1a) ..... 46
3.3.4. Selectivity of inhibitors towards Eis over other AACs...............................112
3.3.5. Effect of inhibitors on KAN MIC for M. tuberculosis ..............................113
3.3.6. A crystal structure of Eis-CoA-inhibitor 39b complex confirms mechanism of inhibition..................................................................................................................115
3.3.7. Mammalian cytotoxicity of Eis inhibitors ..............................................120
3.4. CONCLUSION ..............................................................................................122
3.5. MATERIALS AND INSTRUMENTATION ..................................................122
3.5.1. Materials and instrumentation for chemistry experiments ......................122
3.5.2. Materials and instrumentation for biochemistry and biology experiments ...124
3.6. METHODS ..................................................................................................125
3.6.1. Chemical methods ..................................................................................125
3.6.1.1. General procedure A for the preparation of the N-alkylisatin compounds 56b, 56d, 56h, 57b, 57c, 57d, 57f, and 57h.................................................................125
3.6.1.2. General procedure B for the preparation of compounds 58b, 58d, 58h, 59b, 59c, 59d, 59f, and 59h.................................................................129
3.6.1.3 General procedure C ..........................................................................133
3.6.2. Biochemical, biological, and biophysical methods ..................................149
3.6.2.1. Eis chemical library screening .........................................................149
3.6.2.2. Hit validation ......................................................................................150
3.6.2.3. Inhibition kinetics ..............................................................................151
3.6.2.4. Mode of inhibition ............................................................................151
3.6.2.5. Selectivity of inhibitors towards Eis over other AACs .......................152
3.6.2.6. Mycobacterium tuberculosis MIC values determination ..................152
3.6.2.7. Crystallization, diffraction data collection, and structure determination and refinement of EisC204A-CoA-inhibitor 39b complex ............................................. 153
3.6.2.8. Mammalian cytotoxicity assays .......................................................... 155
3.7. ACKNOWLEDGEMENTS ............................................................................ 156
3.8. AUTHORS’ CONTRIBUTIONS ..................................................................... 157

Chapter 4 ............................................................................................................. 158

Eis inhibitors with potential synergistic bactericidal activity with Kanamycin against Mycobacterium tuberculosis ................................................................. 158

4.1. INTRODUCTION .......................................................................................... 158
4.2. RESULTS AND DISCUSSION ..................................................................... 160
  4.2.1. High-throughput screen ......................................................................... 160
  4.2.2. Chemistry ............................................................................................... 160
  4.2.3. Eis inhibitory activity ........................................................................... 163
  4.2.4. Activity of thieno[2,3-d]pyrimidine Eis inhibitors against Mtb K204 cells .. 168
  4.2.5. Antitubercular activity against Mtb MC26020 ....................................... 168
4.3. CONCLUSION ............................................................................................... 169
4.4. MATERIALS AND INSTRUMENTATION ..................................................... 169
4.5. METHODS ................................................................................................... 171
  4.5.1. Chemical methods ............................................................................... 171
    4.5.1.1. General procedure A for the preparation of compounds 17-24 ......... 171
    4.5.1.2. General procedure B for the preparation of compounds 25-32 ........ 175
    4.5.1.3. General procedure C for the preparation of compounds 33-40 ........ 179
    4.5.1.4. General procedure for S-alkylation of compounds 33-40 ............. 182
Chapter 5 ..........................................................................................................................232

Development of ebsulfur analogues as potent antibacterials against methicillin-resistant
Staphylococcus aureus .....................................................................................................232

5.1. ABSTRACT ......................................................................................................... 232

5.2. INTRODUCTION ............................................................................................... 233

5.3. RESULTS AND DISCUSSION .......................................................................... 236

5.3.1. Chemistry .......................................................................................................236

5.3.2. Biochemistry and Biology .............................................................................239

5.3.2.1. Evaluation of compounds 2a-4n as antibacterial agents .........................239

5.3.2.1a. Evaluation of compounds 2a-4n against various S. aureus strains .........244

5.3.2.1b. Evaluation of compounds 2a-4n against various non-S. aureus strains...247

5.3.2.2. Evaluation of time-kill curve of compounds 2a, 3b, and 3c .....................249

5.3.2.3. Activity against biofilms of S. aureus and S. epidermidis .........................252

5.3.2.4. Evaluation of the hemolytic potential of compounds 2a, 2h, and 3c ......255

5.3.2.5. Evaluation of mammalian toxicity potential of ebselen and compounds 2e,
2h, 2k, 2l, 3c, 3i, and 3k .........................................................................................256
5.3.2.6. Evaluation of ebselen and compounds 2a and 3b,c as bacterial membrane disruptors..................................................................................................................257

5.3.2.7. Detection of reactive oxygen species (ROS) production............................260

5.4. CONCLUSION.................................................................................................... 262

5.5. MATERIALS AND INSTRUMENTATION...........................................................263

5.6. METHODS ......................................................................................................264

5.6.1. Chemical methods.......................................................................................264

5.6.1.1. Preparation of compound 1........................................................................264

5.6.1.2. Preparation of compounds 2a-o following procedure A.............................264

5.6.1.3. Preparation of compounds 3a-4n following procedure B...........................274

5.6.2. Biochemical and biological methods.........................................................287

5.6.2.1. Bacterial strains.........................................................................................287

5.6.2.2. Determination of MIC values against bacterial strains..............................288

5.6.2.3. Time-kill curves.........................................................................................288

5.6.2.4. Biofilm disruption......................................................................................289

5.6.2.5. Determination of hemolytic activity .........................................................290

5.6.2.6. Determination of mammalian cytotoxicity ..............................................290

5.6.2.7. Cell membrane permeabilization assay....................................................291

5.6.2.8. Assay for ROS production..........................................................................292

5.7. ACKNOWLEDGMENTS ...............................................................................293

5.8. AUTHORS’ CONTRIBUTIONS ......................................................................293

Chapter 6..................................................................................................................294
Introduction: fungal diseases, current antifungal agents, and resistance associated with fungal diseases ..............................................................................................................................294

6.1. INTRODUCTION TO FUNGAL DISEASES .......................................................... 294

6.2. CURRENT ANTIFUNGAL AGENTS ..................................................................... 297

6.3. RESISTANCE ASSOCIATED WITH FUNGAL DISEASES ................................. 304

6.4. ACKNOWLEDGMENTS .................................................................................... 305

6.5. AUTHORS’ CONTRIBUTIONS .......................................................................... 305

Chapter 7 ..................................................................................................................306

Identification of ebsulfur analogues with broad-spectrum antifungal activity ..............306

7.1. ABSTRACT ........................................................................................................ 306

7.2. INTRODUCTION ................................................................................................ 307

7.3. RESULTS AND DISCUSSION .......................................................................... 312

7.3.1. Antifungal activity ........................................................................................ 312

7.3.2. Time-kill curves ............................................................................................ 324

7.3.3. Hemolytic assay ............................................................................................ 327

7.3.4 Mammalian cytotoxicity assay ....................................................................... 329

7.3.5. ROS production ............................................................................................ 332

7.4. CONCLUSION .................................................................................................... 335

7.5. MATERIALS AND INSTRUMENTATION ......................................................... 335

7.5.1. Antifungals and equipment .......................................................................... 335

7.5.2. Fungal strains ............................................................................................... 336

7.5.3. Mammalian cells .......................................................................................... 337

7.6. METHODS ......................................................................................................... 337
7.6.1. Determination of MIC values against fungal strains .........................................337
7.6.2. Mammalian cytotoxicity assays ........................................................................339
7.6.3. Assay for reactive oxygen species (ROS) production .....................................340
7.7. ACKNOWLEDGMENTS ......................................................................................340
7.8. AUTHORS’ CONTRIBUTIONS ...........................................................................341

Chapter 8 .....................................................................................................................342
Ongoing research ......................................................................................................342

8.1. EXPANSION OF THE THIENO[2,3-D]PYRIMIDINE LIBRARY ..................342
8.1.1. Preparation of compounds 3-4 ....................................................................343
8.1.2. Preparation of compounds 7-8 ....................................................................345
8.1.3. Preparation of compounds 9-13 .................................................................346

8.2. EBSELEN ANALOGUES ..................................................................................349
8.2.1. Preparation of 2-Iodobenzamides (compounds 15-19) .................................350
8.2.2. Preparation of isoselenazolone compounds (compounds 20-23) ...............352

Chapter 9 .....................................................................................................................355
Conclusion and future directions ..............................................................................355

9.1. EIS INHIBITORS ...............................................................................................355
9.2. EBSULFUR/EBSELEN ANALOGUES .................................................................357

APPENDIX A ............................................................................................................358

APPENDIX B .............................................................................................................449

APPENDIX C .............................................................................................................505

APPENDIX D .............................................................................................................632
LIST OF TABLES

Table 2.1, Inhibition of Eis-catalyzed KAN acetylation (IC$_{50}$ values) by the pyrrolo[1,5-$a$]pyrazine derivatives as well as effect of these molecules on KAN MIC values for Mtb H37Rv and KAN-resistant Mtb K204. ............................................. 29

Table 2.2, X-ray diffraction data collection and refinement statistics for the EisC204A-CoA-inhibitor 2k* ternary complex structure. ................................................................. 85

Table 3.1, IC$_{50}$ values of Eis inhibitors and MIC$_{KAN}$ values for $Mtb$ H37Rv and $Mtb$ K204 when treated in combination with Eis inhibitors......................................................... 97

Table 3.2, IC$_{50}$ values and MIC values of tested compounds. .................................................... 99

Table 3.3, $K_i$ values determined using regression analysis for Eis inhibitors displaying an IC$_{50}$ value <1 µM using NEO as the substrate. ................................................................. 112

Table 3.4, MIC$_{KAN}$ values for Mtb H37Rv and K204 with varying concentrations of cpds 36b and 39b. ............................................................................................................... 114

Table 3.5, X-ray diffraction data collection and refinement statistics for the EisC204A-CoA-inhibitor 39b ternary complex structure. ......................................................... 154

Table 4.1, IC$_{50}$ and MIC values of compounds. ................................................................. 165

Table 4.2, Activity of compounds on $Mtb$ K204 cells............................................................... 168

Table 5.1, MIC values determined for all compounds and for the control antibacterial agent (AMK) against various S. aureus strains............................................................... 240

Table 5.2, MIC values determined for all compounds and for the control antibacterial agent (AMK) against various non-S. aureus bacterial strains........................................... 242
Table 5.3, MIC values determined for all compounds 2a, 2h, and for the control antibacterial agent (AMK) against additional non-S. aureus bacterial strains.

.............................................................................................................................................................................. 243

Table 7.1, MIC values determined for compounds 1-4n and for five control antifungal agents (AmB, FLC, ITC, POS, and VOR) against various yeast strains and filamentous fungi........................................................................................................................................314

Table 7.2, log P values for all compounds studied. ...................................................................................................................... 323
LIST OF FIGURES

Figure 1.1, Pie chart representing the current global distribution of TB prevalence........ 2
Figure 1.2, Structures of the current anti-tubercular drugs based on their therapeutic classification (groups 1-5)................................................................. 4
Figure 1.3, Bar graph indicating the incidence of global TB...................................... 5
Figure 1.4, PubMed publication record over the years for Mtb and ESKAPE pathogens.. 6
Figure 1.5, Schematic representation of the Mtb cell envelope showing the major components discussed herein................................................................. 8
Figure 1.6, A schematic of a Mtb cell showing where the current drug targets affect the cell, including the cell envelope, protein/RNA/DNA synthesis, and other areas. ................................................................................................. 13
Figure 1.7, Crystal structure of Eis (turquoise) bound to tobramycin......................... 20
Figure 2.1, A. Structures of all compounds generated in this study. B. Synthetic scheme used to prepare the compounds in panel A. ........................................... 26
Figure 2.2, Mammalian cytotoxicity of selected compounds (1e*, 1i*, 2c*, 2h*, and 2i*). ........................................................................................................... 36
Figure 2.3, Cytotoxicity of KAN against A549, HEK-293, and J774A. mammalian cell lines.......................................................... ........................................... 37
Figure 2.4, A. Crystal structure of EisC204A-CoA-inhibitor 2k* complex (PDB ID 5TVJ).  ........................................................................................................... 39
Figure 2.5, A zoom-in view of the inhibitor/aminoglycoside binding site showing the superimposition of Eis inhibitors and TOB from several crystal structures..

Figure 2.6, Representative IC50 curves.

Figure 3.1, Stepwise cone diagram showing the winnowing of 23,000 compounds to the four Eis inhibitors that restored KAN activity in \textit{Mtb} K204.

Figure 3.2, Structures of all molecules used in this study along with their origin.

Figure 3.3, Synthetic schemes used for the synthesis of 24 compounds generated in this study.

Figure 3.4, IC\textsubscript{50} curves for purchased compounds A. 9a, B. 10k, C. 11b, D. 11c, E. 11d, F. 11e, G. 14a, H. 16a, and I. 25a against Eis by using NEO as the substrate.

Figure 3.5, IC\textsubscript{50} curves for purchased compounds A. 37b, B. 37d, C. 37g, D. 37i, E. 39b, and F. 39c against Eis by using NEO as the substrate.

Figure 3.6, IC\textsubscript{50} curves for purchased compounds A. 39i and B. 50d, against Eis by using NEO as the substrate. Insets show the double reciprocal plots for the fitted \textit{Ki} values.

Figure 3.7, IC\textsubscript{50} curves for purchased compounds that displayed an IC\textsubscript{50} value $\geq$1 $\mu$M with NEO. A. 11b, B. 11c, C. 14a, D. 37b, E. 37d, F. 37g, G. 37i, H. 39b, I. 39c, J. 39i, and K. 50d against Eis by using KAN as the substrate.

Figure 3.8, IC\textsubscript{50} curves for synthesized compounds A. 36b, B. 37b, C. 38b, D. 39b, E. 43b, F. 48b, G. 52, and H. 53 against Eis by using NEO as the substrate.
Figure 3.9, IC₅₀ curves for synthesized compounds A. 34b, B. 36b, C. 37b, D. 37h, E. 38b, F. 39b, G. 43b, H. 43d, and I. 45b against Eis by using KAN as the substrate.

Figure 3.10, IC₅₀ curves for synthesized compounds J. 46b, K. 48b, L. 48c, M. 48d, N. 48f, O. 48h, P. 49b, Q. 52, and R. 53 against Eis by using KAN as the substrate.

Figure 3.11, A. Crystal structure of one of the six monomers of the EisC204A-CoA-inhibitor 39b complex.

Figure 3.12, Mammalian cytotoxicity of selected compounds (34b, 37b, 39b, and 43d) alone or in the presence of KAN against A. A549, B. HEK-293, and C. J774A.1 cells.

Figure 3.13, Mammalian cell cytotoxicity of KAN in a concentration range of 2.0-500 µM against A. A549, B. HEK-293, and C. J774A.1 cell lines.

Figure 4.1, Structures of the synthesized thieno[2,3-d]pyrimidine compounds.

Figure 4.2, Synthetic scheme to generate the thieno[2,3-d]pyrimidine library. A. Reactions to prepare the thieno[2,3-d]pyrimidine. B. Reactions to prepare the amido side chains.

Figure 5.1, Synthetic scheme for the preparation of compounds 2a-o, 3a-o, and 4e, 4f, and 4n following two different experimental procedures (A and B).

Figure 5.2, Representative time-kill studies of ebselen, AMK, 2a, 3c, and 3b against MRSA S22 (strain W).
Figure 5.3, Bar graphs showing the ability of selected compounds to reduce the amount of biofilm observed for A. S. aureus ATCC 6538 (strain A) and B. S. epidermidis ATCC 35984 (strain AC). ................................................................. 254

Figure 5.4, Hemolytic activity of ebselen, 2a, AMK, 3c, and 2h on mouse red blood cells. ................................................................................................................... 256

Figure 5.5, Mammalian cytotoxicity of ebselen, 2e, 2h, 2k, 2l, 3c, 3i, and 3k. .............. 257

Figure 5.6, Effect of ebsulfur (2a) and its analogues 3b and 3c on cell membrane integrity of S. epidermidis ATCC 35984 (strain AC). .................................................. 259

Figure 5.7, Effect of ebsulfur (2a) and its analogues 3b and 3c on intracellular ROS production by S. epidermidis ATCC 35984 (strain AC). ................................. 261

Figure 6.1, A. Schematic showing that fungi can infect not only humans, but affect humans at multiple points of the food web. ...................................................... 297

Figure 6.2, Timeline showing the introduction to market (year of introduction into parentheses) of the antifungals for human use (top) and for use on crops (bottom) discussed in this chapter. ................................................................. 299

Figure 6.3, Bar graphical representation of the number of individuals living with and deaths caused by fungal infections. ......................................................... 303

Figure 7.1, Chemical structures of our library featuring ebselen (1), ebsulfur (2a), and 32 ebsulfur analogues. .............................................................. 311

Figure 7.2, Time-kill analysis of ebselen (1) (black inverted triangles), ebsulfur (2a) (white triangle), compound 3a (black squares) at 0, 3, 6, 9, 12, and 24 h. 325

Figure 7.3, Hemolytic assays of ebselen (1), ebsulfur (2a), compound 3a, and compound 3c against murine red blood cells (mRBCs). ............................... 329
Figure 7.4, Mammalian cell cytotoxicity of ebselen (1), and compounds 3a, 3b (, and 3g against A. HEK 293 cell line and B. J774 cell line................................. 332

Figure 7.5, ROS induction assay of ebselen (1), ebsulfur (2a), and compound 3a against C. albicans ATCC 10231 (strain A)................................................................. 334

Figure 8.1, Synthetic scheme to expand the thieno[2,3-d]pyrimidine library. ............... 343

Figure 8.2, Synthetic scheme to generate ebselen analogues. ........................................ 349
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAC</td>
<td>Aminoglycoside N-acetyltransferase</td>
</tr>
<tr>
<td>ABPA</td>
<td>Allergic bronchopulmonary aspergillosis</td>
</tr>
<tr>
<td>AcCoA</td>
<td>Acetyl coenzyme A</td>
</tr>
<tr>
<td>AG</td>
<td>Aminoglycoside</td>
</tr>
<tr>
<td>ACP</td>
<td>Enoyl-acyl carrier protein</td>
</tr>
<tr>
<td>AFG</td>
<td>Anidulafungin</td>
</tr>
<tr>
<td>AMB</td>
<td>Amphotericin B</td>
</tr>
<tr>
<td>AMK</td>
<td>Amikacin</td>
</tr>
<tr>
<td>AMX</td>
<td>Amoxicillin</td>
</tr>
<tr>
<td>AXN</td>
<td>Azoxystrobin</td>
</tr>
<tr>
<td>CAN</td>
<td>Candididin</td>
</tr>
<tr>
<td>CAP</td>
<td>Capreomycin</td>
</tr>
<tr>
<td>CBM</td>
<td>Carbendzim</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control</td>
</tr>
<tr>
<td>CFG</td>
<td>Caspofungin</td>
</tr>
<tr>
<td>CFX</td>
<td>Ciprofloxacin</td>
</tr>
<tr>
<td>CFZ</td>
<td>Clofazimine</td>
</tr>
<tr>
<td>CH₂Cl₂</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>CLA</td>
<td>Clarithromycin</td>
</tr>
<tr>
<td>CLN</td>
<td>Cilastatin</td>
</tr>
<tr>
<td>CLV</td>
<td>Clavulanic acid</td>
</tr>
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</table>
CPA  Chronic pulmonary aspergillosis
Cs$_2$CO$_3$  Cesium Carbonate
CYP  Cytochrome P450
CYS  Cycloserine
DCFH-DA  Dichlorodihydrofluorescein diacetate
DMEM  Dulbecco’s Modified Eagle’s Medium
DMF  Dimethylformamide
DMSO  Dimethyl sulfoxide
DTM  Dimethomorph
ECZ  Epoxiconazole
EIS  Enhanced intracellular survival enzyme
EMB  Ethambutol
ETH  Ethionamide
EtOAc  Ethyl acetate
EtOH  Ethanol
FBS  Fetal bovine serum
FDA  US food and drug administration
FLC  Fluconazole
FLU  Flumorph
FPN  Fenpropidin
FQ  Fluoroquinolone
GPI  Glycosylphosphatidylinositol
GTX  Gatifloxacin
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>H₂O</td>
<td>Water</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>HRMS</td>
<td>High-resolution mass spectrometry</td>
</tr>
<tr>
<td>HTS</td>
<td>High-throughput screening</td>
</tr>
<tr>
<td>IDSA</td>
<td>Infectious Diseases Society of America</td>
</tr>
<tr>
<td>INH</td>
<td>Isoniazid</td>
</tr>
<tr>
<td>IPM</td>
<td>Imipenem</td>
</tr>
<tr>
<td>ITC</td>
<td>Itraconazole</td>
</tr>
<tr>
<td>KAN</td>
<td>Kanamycin</td>
</tr>
<tr>
<td>K₂CO₃</td>
<td>Potassium carbonate</td>
</tr>
<tr>
<td>KS-M</td>
<td>Kresoxim-methyl</td>
</tr>
<tr>
<td>KTC</td>
<td>Ketoconazole</td>
</tr>
<tr>
<td>LEV</td>
<td>Levofloxacin</td>
</tr>
<tr>
<td>LRMS</td>
<td>Low-resolution mass spectrometry</td>
</tr>
<tr>
<td>LZD</td>
<td>Linezolid</td>
</tr>
<tr>
<td>m-CBPA</td>
<td><em>meta</em>-Chloroperoxybenzoic acid</td>
</tr>
<tr>
<td>MCZ</td>
<td>Miconazole</td>
</tr>
<tr>
<td>MDR</td>
<td>Multidrug-resistant</td>
</tr>
<tr>
<td>MDR-TB</td>
<td>Multidrug-resistant tuberculosis</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>MFG</td>
<td>Micafungin</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum inhibitory concentration</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>MOX</td>
<td>Moxifloxacin</td>
</tr>
<tr>
<td>MRSA</td>
<td>Methicillin-resistant <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>MSSA</td>
<td>Methicillin-sensitive <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>Mtb</td>
<td><em>Mycobacterium tuberculosis</em></td>
</tr>
<tr>
<td>NAF</td>
<td>Naftifine</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>NEO</td>
<td>Neomycin</td>
</tr>
<tr>
<td>NH₄OH</td>
<td>Ammonium hydroxide</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NYS</td>
<td>Nystatin</td>
</tr>
<tr>
<td>OFX</td>
<td>Ofloxacin</td>
</tr>
<tr>
<td>PAS</td>
<td><em>para</em>-amino salicylic acid</td>
</tr>
<tr>
<td>PCZ</td>
<td>Propiconazole</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PK</td>
<td>Polyketide</td>
</tr>
<tr>
<td>POS</td>
<td>Posaconazole</td>
</tr>
<tr>
<td>PZA</td>
<td>Pyrazinamide</td>
</tr>
<tr>
<td>PRO</td>
<td>Prothionamide</td>
</tr>
<tr>
<td>RIF</td>
<td>Rifampicin</td>
</tr>
<tr>
<td>RND</td>
<td>Resistance-nodulation-cell division</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>Reverse-phase high performance liquid chromatography</td>
</tr>
<tr>
<td>RR</td>
<td>Rifampicin-resistant</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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</tr>
<tr>
<td>SAR</td>
<td>Structure activity relationship</td>
</tr>
<tr>
<td>STR</td>
<td>Streptomycin</td>
</tr>
<tr>
<td>TB</td>
<td>Tuberculosis</td>
</tr>
<tr>
<td>TBAI</td>
<td>Tetrabutylammonium iodide</td>
</tr>
<tr>
<td>TBC</td>
<td>Tebuconazole</td>
</tr>
<tr>
<td>TER</td>
<td>Terbinafine</td>
</tr>
<tr>
<td>TOB</td>
<td>Tobramycin</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>THZ</td>
<td>Thioacetazone</td>
</tr>
<tr>
<td>TRD</td>
<td>Terizidone</td>
</tr>
<tr>
<td>TDM</td>
<td>Trehalose 6,6’-dimycolate</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
</tr>
<tr>
<td>VIO</td>
<td>Viomycin</td>
</tr>
<tr>
<td>VOR</td>
<td>Voriconazole</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>XDR</td>
<td>Extensively drug-resistant</td>
</tr>
<tr>
<td>XDR-TB</td>
<td>Extensively-resistant tuberculosis</td>
</tr>
<tr>
<td>5-FC</td>
<td>5-Fluorouracil</td>
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</table>
The following dissertation consists of 9 chapters highlighting the most important projects of my Ph.D. studies aimed at exploring novel drug scaffolds to address drug resistance in tuberculosis and fungal diseases. The first 7 chapters are adapted or partially adapted from my previous publications. Chapter 1 focuses on the pathology of tuberculosis (TB), the current anti-TB drug options, and the challenges associated with antitubercular drug resistance; this chapter was partially adapted from a book chapter. (Green K.D., Holbrook S.Y.L., Ngo H.X., and Garneau-Tsodikova S. (2017). Chapter 7: Emerging targets in anti-tubercular drug design. Antibiotic drug discovery: new targets and molecular entities. 141-203. DOI:10.1039/9781782629870-0041.) Chapter 2 describes an SAR study of a novel class of pyrrolo[1,5-a]pyrazine-based Eis inhibitors to overcome Eis-mediated aminoglycosides (AGs) resistance (Garzan A., Willby M.J, Ngo H.X., Gajadeera C.S., Green K.D., Holbrook S.Y.L., Hou C., Posey J.E., Tsodikov O.V., and Garneau-Tsodikova S. (2017). Combating enhanced intracellular survival (Eis)-mediated kanamycin resistance of Mycobacterium tuberculosis by novel pyrrolo[1,5-a]pyrazine-based Eis inhibitors. ACS Infect. Dis. 3(4): 302-309.). Chapter 3 reports the investigation of 1,2,4-triazino[5,6b]indole-3-thioether-based Eis inhibitors, which displays unique interactions with Eis AG binding site. (Ngo H.X., Green K.D., Gajadeera C.S., Willby M.J., Holbrook S.Y.L., Hou C., Garzan A., Mayhoub A.S., Posey J.E., Tsodikov O.V., and Garneau-Tsodikova S. (2017). Potent 1,2,4-triazino[5,6b]indole-3-thioether inhibitors of the kanamycin resistance enzyme Eis from Mycobacterium tuberculosis. ACS Infect. Dis. 4(6): 1030-1040.). Chapter 4 focuses on synthesis and biological
Chapter 1

Introduction: tuberculosis (TB), current TB drug targets, and resistance associated with TB

1.1. INTRODUCTION TO TB

1.1.1. History of TB

"Dr. Waksman! You have discovered a new and powerful weapon in the deadly battle against one of the oldest foes of mankind, tuberculosis. This battle is as old as medical science and we now have a definite impression that at last the enemy is beginning to yield…"

-Harald Cramér, member of the Royal Swedish Academy of Sciences

Professor Cramér congratulated the Ukrainian-American scientist Selman A. Waksman regarding his discovery of streptomycin (STR) at Waksman’s Nobel banquet in 1952. Professor Cramér’s optimism resonated with the rest of the scientific world. STR could penetrate the highly lipophilic cell envelope of *Mycobacterium tuberculosis* (*Mtb*), which was previously considered to be an improbable feat. Tuberculosis (TB), also known as phthisis, consumption, or the white plague, was untreatable for centuries and has devastated humanity since the dawn of time. To cope with the TB epidemic, during the 19th century, many writers, such as John Keats and George Sand, romanticized this disease. In fact, TB was considered
to be artistic, poetic, and fashionable, which influenced many upper-class young ladies to pale their skin to mimic the appearance of the diseased. With the discovery of STR, the world finally possessed an agent that could treat TB. Like polio and leprosy, it was thought that TB would eventually be a banal affliction. Unfortunately, the euphoric feeling about STR gradually evaporated, as various clinical studies reported the rapid development of STR resistance in *Mtb* clinical isolates (typically after two months of treatment) as well as the lack of clinical efficacy in the landmark trial by the British Medical Research Council.7-8 Today, TB still remains a public health threat and is increasingly becoming a global healthcare emergency. According to the latest report from the World Health Organization (WHO), in 2014, about 9.6 million new cases of active TB per year are estimated to occur, leading to 1.5 million casualties.9 Globally, the prevalence of TB is highest in Southeast Asia with 5.4 million people currently living with TB. Along with Southeast Asia, Africa and the Western Pacific make up more than 85% of the global TB prevalence, whereas the Eastern Mediterranean, Europe, and the Americas account for the remaining ~15% (Figure 1.1).9

![Figure 1.1. Pie chart representing the current global distribution of TB prevalence.](image-url)
Furthermore, *Mtb* strains continue to evolve to resist the most clinically useful first-line anti-
tubercular agents (isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA), and ethambutol (EMB)) (Figure 1.2). Multidrug-resistant TB (MDR-TB) is caused by isolates that are resistant to at least INH and RIF. The current rate of MDR-TB is estimated to appear in 3.3% of all new TB cases and 20% of previously treated TB cases. Extensively drug-resistant TB (XDR-TB) is a type of MDR-TB, with additional resistance second-line drugs such as any fluoroquinolone (FQ) and at least one of three injectable drugs (*i.e.*, the aminoglycosides (AGs) kanamycin (KAN) and amikacin (AMK), or the cyclic polypeptide capreomycin (CAP)). From the latest estimation, 9.7% of MDR-TB cases eventually become XDR-TB. Moreover, the number of TB cases that are unresponsive to the most potent drugs continues to rise year after year. As reported by the WHO, the number of RIF-resistant (RR) and MDR-TB cases increased by approximately 10-fold from 2005 to 2014 (Figure 1.3). Hence, there is an immediate clinical necessity for the discovery and development of anti-tubercular compounds with novel drug targets and mechanisms of action.
Figure 1.2. Structures of the current anti-tubercular drugs based on their therapeutic classification (groups 1-5).
Figure 1.3. Bar graph indicating the incidence of global TB of any variety (orange bars), RR/MDR-TB (red bars), and XDR-TB (green bars). The data points for incidence of global total TB and RR/MDR-TB were extracted from the Global Health Observatory data repository. The data points for XDR-TB were calculated from the percentage of XDR-TB related to the MDR-TB data reported from 2011 to 2014 in the annual WHO global TB reports. Before 2010, the percentage of XDR-TB was minimal and not reported.

This story of anti-tubercular drug discovery is mirrored by the PubMed publication log (Figure 1.4). For almost a century after the first published report on TB, very few publications on this respiratory disease (<100 per year from 1853 to 1943) appeared in the literature. Starting from the monumental discovery of STR, there was an explosion of TB-related publications ignited by several new and effective anti-tubercular drugs, such as para-aminosalicylic acid (PAS), INH, PZA, cycloserine (CYS), and KAN, from 1944 to 1952 (Figure 1.2). The introduction of RIF in the 1960s established the efficacy of the combination therapy regimen, including INH, RIF, PZA, and EMB, and eventually shortened the duration
of therapy to as low as 6 months. Due to the initial effectiveness of the first-line agents, there was a gradual decline in interest in TB research up until 1980 when MDR-\textit{Mtb} strains began to emerge. This observation deeply troubled the public and propelled an immense effort in TB research. In fact, the number of publications on TB has exceeded those related to the ESKAPE pathogens (\textit{Enterococcus faecium}, \textit{Staphylococcus aureus} (Sau), \textit{Klebsiella pneumoniae}, \textit{Acinetobacter} spp., \textit{Pseudomonas aeruginosa}, and \textit{Enterobacter} spp.) in recent years.

![PubMed publication record over the years for \textit{Mtb} (red circles), \textit{E. faecium} (orange circles), \textit{S. aureus} (light orange inverted triangles), \textit{K. pneumoniae} (yellow triangles), \textit{Acinetobacter} spp. (green squares), \textit{P. aeruginosa} (brown squares), \textit{Enterobacter} spp. (purple diamonds).](figure.png)

\textbf{Figure 1.4.} PubMed publication record over the years for \textit{Mtb} (red circles), \textit{E. faecium} (orange circles), \textit{S. aureus} (light orange inverted triangles), \textit{K. pneumoniae} (yellow triangles), \textit{Acinetobacter} spp. (green squares), \textit{P. aeruginosa} (brown squares), \textit{Enterobacter} spp. (purple diamonds).

Inspired by this renaissance in TB drug discovery, herein, we will first briefly survey the pathophysiology of TB, the current targets of clinically approved medications, and the resistance mechanisms associated with these drugs. These fundamental topics lay the
groundwork and context for our eventual important discussions of novel anti-tubercular agents to combat drug-resistant *Mtb* strains.

### 1.1.2. Biology and pathology of TB

The genus *Mycobacterium* is so named because of the apparent mold-like growth of the bacteria on the surface of liquid media. Other characteristics of this genus include slow growth, the ability to enter a latent state, a complex cell envelope, and a fairly homogeneous genetic content across isolates. These bacteria are resistant to most staining techniques and are classified as acid-fast bacilli. This resistance to staining is due to the complex and lipocentric composition of the mycobacterial cell envelope, which is also responsible for their high resistance to environmental stresses, including drying and antiseptics. This unique cell envelope also precludes mycobacteria from being concretely classified as either Gram-negative or Gram-positive, since it does not adhere to the strict definition of either class (Figure 1.5). While the bacteria have a peptidoglycan layer, they only become faintly colored by Gram staining.
In *Mtb*, the inner most layer of the *Mtb* cell envelope consists of diacylphosphatidylinositol dimannoside, a fairly unusual lipid. This particular lipid is thought to endow the bacteria with poor membrane fluidity and permeability. The peptidoglycan layer is next and linked to a layer of arabinogalactan, which is in turn attached to a layer of mycolic acids, and finally coated with an outermost layer of phospholipids resulting in a fairly thick overall cell envelope. Amid the cell envelope are found porin proteins that form channels filled with water allowing the passage of hydrophilic molecules. Only one porin has been reported for *Mtb*, an OmpA-like porin, which plays a role in pH adaptation and does not appear to function as a transport channel. Interestingly, when *Mtb* expressed the MspA porin from *Mycobacterium smegmatis* (*Msm*), a non-infectious and fast-growing mycobacterium
commonly used as a model for \textit{Mtb}, it becomes sensitized to $\beta$-lactams, INH, EMB, and STR.\textsuperscript{18} The \textit{Mtb} peptidoglycan layer differs slightly from those of typical Gram-positive and Gram-negative bacteria. In \textit{Mtb}, just like in other bacteria, the peptidoglycan layer consists of alternating units of \textit{N}-acetylglucosamine and \textit{N}-acetylmuramic acid with peptidic side chains of L-alanyl-D-isoglutamyl-\textit{meso}-diaminopimelyl-D-alanine with further amidation of the glutamyl side chain. However, in \textit{Mtb}, the \textit{N}-acetylmuramic acid is further acylated with glycolic acid and cross-linking occurs not only between \textit{meso}-diaminopimelyl groups, but also between \textit{meso}-diaminopimelyl and D-alanyl groups (a detailed structure of the peptidoglycan layer cross-linkages is depicted in Figure 2.11 where a new target is discussed).\textsuperscript{19} The arabinoglycan layer is composed of the furanose forms of arabinose and galactose,\textsuperscript{19,20} typically arranged in a linear galactan chain bearing several branched arabinose chains, each ending in four arabinose dimers, which hold two points of attachment for the mycolic acids.\textsuperscript{21-22} Over 60\% of the mycobacterial cell envelope dry-weight consists of lipids.\textsuperscript{12} The three major components of the lipid content are mycolic acids, cord factor (better known as trehalose 6,6$'$-dimycolate, TDM\textsuperscript{19, 23-24}), and wax-D. Mycolic acids are high molecular weight branched \textit{a}-alkyl-\textit{b}-hydroxy fatty acids that form lipophilic tails of glycolipids or are esterified at the end of the arabinogalactans.\textsuperscript{25} TDM is responsible for the serpentine cord into which \textit{Mtb} cells develop. This lipid is also toxic to mammals and abundantly produced in virulent strains of \textit{Mtb}. Wax-D is the antigen found in Freund’s complete adjuvant, which is used in immunopotentiation.\textsuperscript{12} This unique construction of the cell envelope is associated with the bacterium’s characteristic impermeability and resistance to antibiotics, acidic and basic compounds, osmotic lysis, and lethal oxidations, and it allows
for survival inside the macrophage. Of the ~4,000 encoding genes of *Mtb*, 525 are involved in the synthesis of the cell envelope and 200 are related to fatty acid metabolism.

In addition to the highly specialized cell envelope, *Mtb* also utilizes some metabolic pathways to protect itself from its environment. The fact that *Mtb* can enter a non-replicating state indicates a period of metabolic shutdown. During this phase, treatment of TB becomes difficult since the etiologic agent is not replicating and, therefore, not utilizing the enzymes and pathways targeted by the current anti-tubercular agents, discussed in Section 1.2. While the molecular basis for the metabolic shutdown of *Mtb* is still not completely understood, several recent studies have shed some light on the mechanisms and pathways involved in the ability of *Mtb* to enter this non-replicating and slowed metabolic state. One particular gene found to be involved in this process is *dosR* (*rv3133c*) that is responsible for mediating the hypoxic response and aids the rebound of the bacteria back to a normal metabolic state. Changes in glucose phosphorylation have also been documented to be required in the non-replicating state. The genome of *Mtb* appears to have an abundance of extra metabolic and biosynthetic proteins, which is suggestive of its ability to adapt to its environment. *Mtb* has the potential to synthesize all the essential amino acids, vitamins, and cofactors needed for its survival. It also has the ability to catabolize a wide variety of carbon sources, including, but not limited to, carbohydrates, hydrocarbons, and alcohols.

TB is most commonly acquired by the inhalation of dried particles containing one to three *Mtb* bacilli. When these particles reach the lung, they are phagocytized by macrophages in the
alveoli whereupon most bacteria are usually destroyed. In certain instances, whether caused by a compromised immune system or another underlying condition, the body’s defenses can fail to terminate the inhaled bacilli. In this case, the infection progresses in five distinct steps: (i) The tubercle bacilli reach the alveoli of the lung and are ingested by macrophages, however, some bacilli survive. At this point, the infection is asymptomatic, indicative of a latent infection. (ii) The bacilli replicate in the macrophage, recruiting more macrophages to the area, which then become hosts for the mycobacteria. The macrophages form an early tubercle and excrete cytokines and other enzymes that cause lung-damaging inflammation. (iii) After a few weeks of inflammation, symptoms begin to appear as a result of many macrophages dying and releasing their load of bacilli forming a caseous center in the tubercle. The anaerobic environment is not ideal for the propagation of bacteria and growth is halted. However, many cells survive in dormant or latent states to become activated later. At this point, the lesions can become calcified and the disease progression is stalled. (iv) If the growth of the caseous center continues, the center can become liquefied and bacilli are able to multiply outside of macrophages. (v) The tubercle eventually ruptures from the growth of the bacilli and they are released into the bronchiole. From this location, the bacteria can be disseminated to the rest of the lungs, the blood stream, and the lymphatic system.

Transcriptional analysis of Mtb cultivated in macrophages reveals that several genes related to fatty acid degradation and lipid synthesis are attenuated along with transcriptional regulators, proteins involved in the glyoxylate cycle, citrate synthesis, mycobactin synthesis, and α-crystallin, along with many other genes. Additionally, several genes with unidentified products are also noted. One of the cell envelope components, lipoarabinomannan is essential
for the virulence of *Mtb*. This particular lipoglycan binds the macrophage mannose receptor facilitating entry into the macrophage. It is also responsible for modulating the host immune response and preventing the phagosome-lysosome fusion. All these factors combined lead to intracellular survival and persistence.\textsuperscript{32} The virulence of *Mtb* was also found to be reliant on lipoamide dehydrogenase, which is a core protein in three separate multi-enzyme complexes affecting the biosynthesis of amino acids, acetyl coenzyme A (AcCoA), and reduction of reactive nitrogen intermediates.\textsuperscript{33}

1.2. CURRENT TB DRUG TARGETS

The current drugs targets span three main areas: (i) cell envelope synthesis, (ii) translation, and (iii) transcription and DNA replication (Figure 1.6). The structures of these molecules, which are classified as first-, second-, and third-line agents are drawn in Figure 1.2.
**Figure 1.6.** A schematic of a *Mtb* cell showing where the current drug targets affect the cell, including the cell envelope, protein/RNA/DNA synthesis, and other areas.

### 1.2.1. Cell Envelope

While not among the core treatment regimen of TB, the amino acid-derived CYS and terizidone (TRD) display broad-spectrum activity and inhibit the synthesis of the peptidoglycan layer.\(^{34}\) These amino acid derivatives target alanine racemase and D-alanine:D-alanine ligase, both of which are involved in the synthesis of the peptidoglycan layer.\(^{35}\) β-Lactams (e.g., amoxicillin (AMX)) work in a similar fashion inhibiting transpeptidase and preventing the cross-linking of the peptidoglycan layer.\(^{36}\) The penicillin AMX and the carbapenem imipenem (IPM) are used as third-line TB treatments in combination with the β-lactamase inhibitor clavulanate (CLV) and the carbapenemase inhibitor cilastatin (CLN), respectively, to prevent the degradation of the anti-tubercular compounds. EMB targets an arabinosyltransferase responsible for building the arabinogalactans found in the cell
Ethionamide (ETH), prothionamide (PRO), and INH all inhibit the enoyl-acyl carrier protein (ACP) reductase InhA. All of these nicotinamide derivatives are prodrugs that need to be activated. INH is activated by the catalase-peroxidase hemoprotein KatG. On the other hand, ETH is activated by the monooxygenase EthA to an S-oxide metabolite. Thioacetazone (THZ) is also a prodrug that gets transformed by EthA into its active form, 2-ethyl-4-amidopyridine. While THZ is not a nicotinamide analogue, it targets the same pathway as PRO, ETH, and INH. These drugs specifically target the cyclopropane synthase enzymes, disrupting fatty acid synthesis by forming adducts with NADH and preventing InhA from performing its catalytic activity.

1.2.2. Translation

The AGs AMK, KAN, and STR function by binding tightly to the 16S rRNA in the 30S ribosomal subunit. When bound to the ribosome, they prevent normal protein translation, which inevitably leads to cell death. Clarithromycin (CLA), a macrolide antibiotic, also inhibits the ribosome. However, while AGs bind the 30S ribosomal subunit, CLA binds the 50S ribosomal subunit, which also results in a cessation of protein synthesis. CAP and viomycin (VIO) are cyclic peptides from the tuberactinomycin family, which are known to inhibit protein synthesis by binding the ribosome, hence their usual coupling with AGs. CAP also has significant activity against the persistent form of TB and is thought to have a second target outside of the ribosome. Linezolid (LZD), an oxazolidinone, is responsible for inhibiting protein translation in a completely different way than other drugs. While most compounds bind one of the two large halves (30S and 50S) of the ribosome, preventing
translation from proceeding, LZD binds the 23S rRNA, thereby inhibiting translation in the early stages by preventing formyl-methionine tRNA from binding the complex.49

1.2.3. Transcription and DNA Replication

The FQs used to treat TB, including gatifloxacin (GTX), levofloxacin (LEV), moxifloxacin (MOX), ciprofloxacin (CFX), and ofloxacin (OFX), all trap the ATP-dependent type II topoisomerases (DNA gyrase and topoisomerase IV) in an enzyme-DNA-inhibitor complex. When trapped, the enzymes no longer perform DNA uncoiling, freezing that portion of DNA and shutting down DNA replication, translation of genes in the region, and DNA repair.50 Clofazimine (CFZ) binds the guanine bases of bacterial DNA, preventing DNA from being used as a template and inhibiting bacterial proliferation.51-52 It also increases activity of bacterial phospholipase A2, which causes the release and accumulation of toxic lysophospholipids.53-54 PAS was originally proposed to target dihydropteroate synthase, the usual target of sulfonamide drugs.55 Several years later, it was discovered that in fact PAS inhibits the thymidylate synthase, preventing thymidine from being synthesized and, therefore, disrupting DNA replication.56 RIF and its analogues rifabutin, rifalazil, and rifapentine all inhibit the b-subunit of DNA-dependent RNA polymerase, halting transcription.57-58 It is thought that RIF blocks the transit of the growing RNA chain after the addition of two or three nucleotides. In Escherichia coli (Eco), the suicide gene mazEF is triggered, and the same system is found in Mtb.59
PZA has a poorly understood mechanism of action. As a prodrug, it is activated by the pyrazinamidase/nicotinamidase PncA to pyrazinoic acid (POA), which has been shown to inhibit many functions in low-pH environments that are often found when Mtb is internalized to the macrophage.\textsuperscript{60-61} POA is pumped out of the cell, and if the extracellular environment is acidic enough, POA becomes protonated and reenters the cell at a faster rate than it can be egested. This leads to an increase in intracellular acidity, and this change in pH eventually affects numerous cellular cycles.

1.3. RESISTANCE RELATED TO TB

Mtb resists the action of many drugs. The mechanisms of resistance in Mtb comprise (i) intrinsic cellular properties and (ii) the presence of efflux pumps and several chromosomally encoded resistance genes, as well as mutation of several genes resulting in reduced efficacy of the antibiotics.\textsuperscript{62} It is important to note that Mtb has no reported instances of acquiring resistance mechanisms from mobile genetic elements. In addition to the modes of resistance employed by Mtb, the mycolic acid-rich cell envelope decreases the permeability of many antibiotics and antimicrobial compounds.

1.3.1. Efflux pumps

One mechanism of resistance shared by many bacteria is the presence of efflux pumps. At their basal levels, efflux pumps contribute little to resistance, however, when overexpressed or mutated to have higher affinity for a particular substrate, they can greatly affect resistance, particularly in mycobacteria considering their low rate of influx. Molecularly, efflux pumps
are active transporters and use either an energy source (e.g., ATP) or an ionic gradient to shuttle xenobiotics and other toxic compounds out of the cell. An example of a \textit{Mtb} efflux pump includes P55 (Rv1410c). P55, a member of the major facilitator superfamily of efflux pumps, acts on tetracycline, STR, gentamicin, and netilmicin. Interestingly, other efflux pumps associated with AG resistance are from the resistance-nodulation-cell division (RND) superfamily of proteins.\textsuperscript{63} Additionally, the \textit{p55} gene forms an operon with \textit{p27}, a gene encoding an antigenic lipoprotein. The P55 efflux pump is sensitive to inhibition by verapamil and reserpine, two known efflux pump inhibitors, and is dependent on the cellular proton gradient of the bacteria. In mycobacteria, efflux pumps are also responsible for resistance to FQs (in addition to other methods of resistance (See section 1.3.2a)).\textsuperscript{64-65}

\textbf{1.3.2. Gene mutations}

Genetic mutations occur in two major areas: (i) mutations or modifications of drug targets, including methylation of RNA, and (ii) failure to convert prodrugs into active compounds.

\textbf{1.3.2a. Mutations or modifications of drug targets}

\textit{Mutations}: Anti-tubercular agents intercept the cellular metabolism preventing requisite biomolecules from being made. Most often the interactions of these agents with their protein targets is dependent on a few key interactions. A simple and common mechanism for bacteria to prevent this from happening is the mutation of the target’s binding site. This can happen either naturally during replication error, or through selective pressure. The resulting mutations decrease the ability of anti-tubercular drugs from binding to their respective targets allowing
the bacteria to survive the therapy. This mechanism is commonly found in *Mtb* strains resistant to RIF, EMB, and FQs by single residue mutations in the b-subunit of RNA polymerase, a glycosyltransferase, and/or type II topoisomerases. Slight mutations in the 16S rRNA (e.g., A1401G) often confer resistance to the injectable drugs AMK, KAN, and CAP. Two mutations in the *rrs* gene from *Mtb* encoding three rRNA residue alterations (A1400G, C1401A, and G1483T) were found to cause resistance to KAN.

*Modifications*: An alternative mode of resistance in *Mtb* is the inactivation of rRNA methyltransferase enzymes (e.g., TlyA and GibB). TlyA mutations were documented to hinder the methylation activity of rRNA 2'-O-methyltransferase at nucleotides C1409 of the 16S rRNA and C1920 of the 23S rRNA, thereby causing resistance to CAP and VIO. Mutations in GidB were found to confer minor resistance to STR.

### 1.3.2b. Inability to activate prodrugs

Among the anti-tubercular agents, some prodrugs are found (e.g., INH, PZA, and ETH). These prodrugs are activated by KatG (INH), EthA (ETH), and PncA (PZA). Mutations in the *katG*, *ethA*, and *pncA* genes were shown to reduce the ability of the corresponding enzymes to activate INH, ETH, and PZA, respectively. Interestingly, mutations in promoter regions were additionally found to result in overexpression of drug targets and to prevent binding of the active form of INH and ETH to their targets.
1.3.3. Enzymatic modifications and inactivation of drugs

Perhaps the most prevalent and well-studied mechanism of resistance in non-mycobacteria is drug modification. Modifications can include degradation of the core structures or alteration of the chemical appearance of the antibiotic, to the point where the compound no longer binds or inactivates its target. *Mtb* naturally harbors a chromosomally encoded class A β-lactamase, BlaC, which is constitutively expressed to provide intrinsic resistance to penicillins by hydrolysis of their β-lactam ring.\textsuperscript{81} *Mtb* also contains two genes encoding for AG-modifying enzymes in its genome: *aac(2')-Ic* and *eis* encoding an AG 2'-N-acetyltransferase\textsuperscript{45, 82-83} and the enhanced intracellular survival (Eis) protein, respectively.\textsuperscript{84} Both enzymes acetylate AGs, reducing the ability of these drugs to bind the ribosome. In the case of Eis, increased expression due to mutations in the *eis* promoter or the 5'-untranslated region of the transcriptional activator WhiB7 leads to clinically relevant, low-level resistance to KAN.\textsuperscript{84} Eis has a unique ability to modify AGs at multiple sites and multiple times \textit{in vitro}, completely inactivating these compounds.\textsuperscript{85} Crystallographic studies recently showed that the AG tobramycin (TOB) is able to bind in two distinct modes, allowing for a minimum of two acetylations (Figure 1.7).\textsuperscript{86} Eis was also reported to acetylate CAP at its β-lysine side chain \textit{in vitro}, but a clinical link to CAP resistance still needs to be elucidated.\textsuperscript{87} This particular resistance enzyme has been extensively studied, both for its AG acetylating properties\textsuperscript{88-90} and its other \textit{in vivo} functions.\textsuperscript{91-93} Interestingly, Eis enzyme homologues are found in a slew of bacteria, both from the genus \textit{Mycobacterium} as well as other genera.\textsuperscript{94-97}
Figure 1.7. Crystal structure of Eis (turquoise) bound to tobramycin (red sticks) (PDB ID: 4JD6, at 3.5 Å) showing a density with two possible orientations of the AG.

1.4. ACKNOWLEDGEMENTS

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1.5. AUTHORS’ CONTRIBUTIONS

KDG, SYLH, HXN, SGT performed literature search and wrote the manuscript.

NOTE:

This dissertation summarizes the drug discovery projects that I performed under the supervision of Professor Sylvie Garneau-Tsodikova. It consists of nine chapters. Chapter one and six are the introductions providing relevant background information on the disease states (TB and fungal diseases), current treatment options, and resistance mechanisms of current FDA approved drugs. Chapter eight describes ongoing work that I have contributed. Chapter nine concludes the the findings in these projects and suggests future directions for these projects. Chapter two describes an SAR study and lead optimization of a novel class of pyrrolo[1,5-a]pyrazine-based Eis inhibitors to overcome Eis-mediated AG resistance. Chapter three reports the investigation of 1,2,4-triazino[5,6b]indole-3-thioether-based Eis inhibitors, that display unique interactions with Eis AG binding site. Chapter four focuses on synthesis and evaluation of thieno[2,3-d]pyrimidine Eis inhibitors, which uniquely contain their own antitubercular activity and are potentially synergistic with KAN. Chapter five reports the synthesis ebsulfur-based analogues and the repurpose of these compounds as potent antibacterials to treat infections associated with methicillin-resistant Staphylococcus aureus (MRSA). Chapter seven explores the application of ebsulfur analogues as antifungal agents.
Chapter 2

Combating Eis-mediated kanamycin resistance of *M. tuberculosis* by novel pyrrolo[1,5-\(a\)]pyrazine-based Eis inhibitors

2.1. ABSTRACT

Tuberculosis (TB) remains one of the leading causes of mortality worldwide. Hence, the identification of highly effective antitubercular drugs with novel modes of action is crucial. In this manuscript, we report the discovery and development of pyrrolo[1,5-\(a\)]pyrazine-based analogues as highly potent inhibitors of the *Mycobacterium tuberculosis* (*Mtb*) acetyltransferase Eis, whose upregulation causes clinically observed resistance to the aminoglycoside (AG) antibiotic kanamycin A (KAN). We performed a structure-activity-relationship (SAR) study to optimize these compounds as potent Eis inhibitors both in solution and in mycobacterial cells. A crystal structure of Eis in complex with one of the most potent inhibitors reveals that the compound is bound to Eis in the AG-binding pocket, serving as the structural basis for the SAR. These Eis inhibitors have no observed cytotoxicity to mammalian cells and are promising leads for development of innovative AG adjuvant therapies against resistant *Mtb* strains.

2.2. INTRODUCTION

Tuberculosis (TB), caused by the pathogenic *Mycobacterium tuberculosis* (*Mtb*), is the deadliest global bacterial infection. The number of people infected by *Mtb* is currently
estimated to be 2-3 billion worldwide. The situation is greatly aggravated by the emergence and spread of difficult or impossible to treat multidrug-resistant (MDR) and extensively drug-resistant (XDR) TB. Aminoglycoside (AG) antibiotics such as kanamycin A (KAN) and amikacin (AMK) are used for the treatment of MDR and XDR-TB. However, the successful outcomes for the treatment of these MDR and XDR-\textit{Mtb} strains can be impeded as a result of KAN resistance. It was previously discovered that upregulation of the enhanced intracellular survival (\textit{eis}) gene due to point mutations in its promoter is a cause of resistance to KAN in one-third of KAN-resistant \textit{Mtb} infections in clinic. The development of new AGs or use of \textit{Eis} inhibitors are two potential solutions for overcoming the effect of \textit{Eis} in \textit{Mtb}. We recently demonstrated that \textit{Eis} is an AG acetyltransferase (AAC) found in a variety of bacterial strains that can inactivate AGs via a multiacetylation mechanism. As \textit{Eis} has been shown to multiacetylate a wide variety of molecules, including many AG scaffolds, the development of new AGs to combat its action is not likely to be successful. Some metal salts are inhibitory to \textit{Eis}, but this strategy alone is not pharmacologically relevant. A better approach would be to use inhibitors of \textit{Eis} as adjuvant therapies in combination with KAN to combat or forestall the emergence of KAN resistance through \textit{Eis} upregulation. We recently reported discovery and development of \textit{Eis} inhibitors with isothiazole \textit{S,S}-dioxide heterocyclic core, sulfonamide-based, methyl 4H-furo[3,2-\textit{b}]pyrrole-5-carboxylate, and 3-(1,3-dioxolano)-2-indolinone scaffolds, which yielded compounds that potently inhibited \textit{Eis} \textit{in vitro} and abolished KAN-resistance of the \textit{Mtb} mutant strain K204 that was KAN-resistant due to \textit{Eis} upregulation. We previously reported 25 hit compounds identified by high-throughput screening (HTS) of a library comprised of \~23,000 small molecules that displayed \textit{Eis} inhibitory activities. Here, we pursue one of
these preliminary hits (compound 1a*, Figure 2.1A) and report the chemical synthesis of this compound and that of 47 analogues (Figure 2.1B), along with their biochemical and biological studies. Among compounds in this series, we have generated novel and promising Eis inhibitors that not only efficiently inhibit the purified enzyme, but also restore KAN sensitivity of KAN-resistant Mtb bacteria. We also present a crystal structure of Eis in complex with CoA and one potent inhibitor (compound 2k*), which explains the structure-activity relationship (SAR).

2.3. RESULTS AND DISCUSSION

2.3.1. Synthesis

Compound 1a* and 47 additional analogues 1a-3k with different R₁ and R₂ substituents on the two phenyl rings and either a fully aromatized (indicated by an asterisk after the compound number) or a non-aromatized pyrrolo[1,5-a]pyrazine core were generated for a thorough SAR analysis of Eis inhibition (Figure 2.1B). The synthesis of all compounds started with a reaction between the commercially available pyrrole and 2-chloroethylamine, which afforded compound 4 in quantitative yield. Compound 4 was reacted with different substituted benzoyl chlorides to obtain amides 5-7 in 66-71% yields. The resulting amides were mixed with phosphorus(V) oxychloride to generate cyclized products 8-10. Then, compounds 8-10 were reacted with various substituted 2-bromoacetophenones to obtain the desired non-aromatized products 1a-k, 2a-k, and 3a,d,h,k. In order to generate the aromatized counterparts of these products, compounds 8 and 9 were first aromatized in the presence of Pd/C to generate molecules 11 and 12. Conventionally, Pd/C is a hydrogenation catalyst. In
the absence of hydrogen gas, Pd/C is known to catalyze an oxidative aromatization instead of hydrogenation. More details about this heteroaromatic aromatization were summarized in an excellent review. Compounds 11 and 12 were further reacted with the different substituted 2-bromoacetophenones to furnish the desired fully aromatized analogues 1a*-k* and 2a*-k*. These compounds were evaluated for Eis inhibition using the clinically relevant KAN as the AG substrate (IC_{50} values in Table 2.1).
2.3.2. Biochemical and biological evaluation

2.3.2.1. Eis inhibition

We first tested whether the freshly synthesized parent compound 1a* was indeed a potent Eis inhibitor. Expectedly, the freshly synthesized compound 1a* was found to display potent
inhibition of Eis (IC$_{50}$ = 0.064 ± 0.008 µM) (Table 2.1), which was ~6-fold better than the IC$_{50}$ value of the commercially available compound 1a* (IC$_{50}$ = 0.36 ± 0.03 µM) from our previous HTS (Note: freshly synthesized powder are often more active than HTS library compounds, which may degrade upon storage). The hit scaffold 1a* contains a pyrrolo[1,5-a]pyrazine core, a phenyl ring adjacent to the pyrrolo[1,5-a]pyrazine core (containing R$_1$), and an acetophenone moiety (containing R$_2$). A comparison of the chemical structure of compound 1a* with those of the previously published isothiazole S,S-dioxide-based Eis inhibitors co-crystallized with Eis$^{105}$ suggested that 1a* binds to Eis at the AG binding pocket$^{86}$ similarly to the isothiazole S,S-dioxides. Examination of the Eis crystal structure bound to the AG tobramycin (TOB) (PDB: 4JD6$^{86}$) indicated that the positively-charged pyrrolo[1,5-a]pyrazine core is presumably essential for binding to the negatively-charged AG binding pocket and thus, should not be modified. Based on our previous survey of the hits of this HTS,$^{108}$ we also determined that the phenyl ring adjacent to the pyrrolo[1,5-a]pyrazine core (containing R$_1$) is likely important for Eis inhibition. In fact, replacing the phenyl ring of 1a* with an ethyl group resulted in a 25-fold reduction in the inhibitory activity (IC$_{50}$ = 9.25 ± 1.50 µM)$^{108}$ Also from the crystal structure of Eis in complex with the isothiazole S,S-dioxide-based Eis inhibitors, we have rationalized that this phenyl ring is important due to its snug fit in a hydrophobic binding pocket in the AG binding cavity. On the other hand, the π-electron rich acetophenone moiety (containing R$_2$) and the fully aromatic pyrrolo[1,5-a]pyrazine core were predicted to be crucial for binding due to potential π-π interactions with aromatic residues within the Eis binding pocket. However, it remains unexplored whether and which substitutions at R$_1$ and R$_2$ positions would be beneficial. We hypothesized that: (i) tailor fitting the Eis binding pocket by introducing subtle modifications at R$_1$ and R$_2$ would
lead to the discovery of novel optimized inhibitors from our hit scaffold 1a*, and (ii) disruption of the aromaticity of the pyrrolo[1,5-a]pyrazine core would be detrimental to the binding affinity of the molecule to the Eis binding pocket. In our biochemical analysis, we will first examine the aromatic compounds and then explore their non-aromatic counterparts. Both the aromatic and non-aromatic molecules are divided into two series. In series 1, R₁ was kept constant (R₁ = H), and various substituted acetophenones were installed onto the pyrrolo[1,5-a]pyrazine core (changing R₂). Similarly, in series 2, R₁ was kept constant (R₁ = p-F), and the same various substituted acetophenones were installed onto the pyrrolo[1,5-a]pyrazine core (changing R₂). For the non-aromatic compounds, four additional members were added to a third series (series 3) where R₁ was m,p-di-F.
Table 2.1. Inhibition of Eis-catalyzed KAN acetylation (IC$_{50}$ values) by the pyrrolo[1,5-a]pyrazine derivatives as well as effect of these molecules on KAN MIC values for *Mtb* H37Rv and KAN-resistant *Mtb* K204.

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<th>Cpd #</th>
<th>R$_1$</th>
<th>R$_2$</th>
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<th>IC$_{50}$ (µM)$^a$</th>
<th>H37Rv K204 MIC$<em>{KA}$ KAN MIC$</em>{KAN}$ (µg/mL)</th>
<th>Cpd #</th>
<th>R$_1$</th>
<th>R$_2$</th>
<th>Aromatic</th>
<th>IC$_{50}$ (µM)$^a$</th>
<th>H37Rv K204 MIC$<em>{KA}$ KAN MIC$</em>{KAN}$ (µg/mL)</th>
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<td>yes</td>
<td>0.06 ± 0.02 ≤ 1.25</td>
<td>1.5 ± 0.4 ≤ 10, 10</td>
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<tr>
<td>1f*</td>
<td>H</td>
<td>m-OH</td>
<td>yes</td>
<td>1.8 ± 0.5</td>
<td>0.10 ± 0.04 ≤ 1.25</td>
<td>2f*</td>
<td>p-F</td>
<td>m-OH</td>
<td>yes</td>
<td>8.4 ± 2.8 ≤ 1.25</td>
<td>1.5 ± 0.4 ≤ 10, 10</td>
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<tr>
<td>1g*</td>
<td>H</td>
<td>m-</td>
<td>yes</td>
<td>0.21 ± 0.07</td>
<td>0.10 ± 0.04 ≤ 1.25</td>
<td>2g*</td>
<td>p-F</td>
<td>m-</td>
<td>yes</td>
<td>0.53 ± 0.11 ≤ 1.25</td>
<td>1.5 ± 0.4 ≤ 10, 10</td>
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<td>1h*</td>
<td>H</td>
<td>p-F</td>
<td>yes</td>
<td>0.029 ± 0.007</td>
<td>0.10 ± 0.04 ≤ 1.25</td>
<td>2h*</td>
<td>p-F</td>
<td>p-F</td>
<td>yes</td>
<td>0.13 ± 0.04 ≤ 1.25</td>
<td>1.5 ± 0.4 ≤ 10, 10</td>
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<td>1i*</td>
<td>H</td>
<td>p-Cl</td>
<td>yes</td>
<td>0.56 ± 0.20</td>
<td>0.10 ± 0.04 ≤ 1.25</td>
<td>2i*</td>
<td>p-F</td>
<td>p-Cl</td>
<td>yes</td>
<td>0.18 ± 0.06 ≤ 1.25</td>
<td>1.5 ± 0.4 ≤ 10, 10</td>
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<tr>
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<td>H</td>
<td>p-Br</td>
<td>yes</td>
<td>0.27 ± 0.01</td>
<td>0.10 ± 0.04 ≤ 1.25</td>
<td>2j*</td>
<td>p-F</td>
<td>p-Br</td>
<td>yes</td>
<td>0.50 ± 0.13 ≤ 1.25</td>
<td>1.5 ± 0.4 ≤ 10, 10</td>
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<tr>
<td>1k*</td>
<td>H</td>
<td>p-Me</td>
<td>yes</td>
<td>0.19 ± 0.02</td>
<td>0.10 ± 0.04 ≤ 1.25</td>
<td>2k*</td>
<td>p-F</td>
<td>p-Me</td>
<td>yes</td>
<td>0.08 ± 0.03 ≤ 1.25</td>
<td>1.5 ± 0.4 ≤ 10, 10</td>
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<tr>
<td>3a</td>
<td>m,p-di-</td>
<td>H</td>
<td>no</td>
<td>0.15 ± 0.05</td>
<td>0.10 ± 0.04 ≤ 1.25</td>
<td>3d</td>
<td>m,p-di-</td>
<td>m-Cl</td>
<td>no</td>
<td>0.043 ± 0.06 ≤ 1.25</td>
<td>1.5 ± 0.4 ≤ 10, 10</td>
</tr>
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</table>

Footnotes:

- $^a$ Concentration of each compound in µM.
- $^b$ MIC values in µg/mL.
- $^c$ MIC values in µg/mL.
- * indicates compounds with significant inhibition of Eis-catalyzed KAN acetylation.
**Control without an Eis inhibitor:** 1.25 10
<p>| | | | | | |</p>
<table>
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<tr>
<td>3h</td>
<td>m,p-di-</td>
<td>p-F</td>
<td>no</td>
<td>0.11 ± 0.03</td>
<td>≤ 1.25 10, 10</td>
</tr>
<tr>
<td>3k</td>
<td>m,p-di-</td>
<td>p-Me</td>
<td>no</td>
<td>0.11 ± 0.03</td>
<td>≤ 1.25 10, 10</td>
</tr>
</tbody>
</table>

* IC₅₀ values against purified Eis Mtb enzyme.  
  
* Anti-tubercular activity of KAN against Mtb H37Rv.  
  
* Anti-tubercular activity of KAN against Mtb K204.  
  
* The inhibitor interacted with alamarBlue® resulting in a color change, therefore, it was impossible to determine the MIC using this method.  
  
* In MIC assays, the compounds were tested at concentrations that were 100-fold higher than IC₅₀. When the IC₅₀ value was >1 mM the compounds were tested at 100 µM. The compounds were not toxic to Mtb in the absence of KAN at these concentrations.

We began our analysis of the aromatic compounds by investigating series 1. To probe the *ortho* position of the acetophenone moiety, compound 1b* (R₁ = H, R₂ = o-F) was generated and found to display a ~3-fold reduction in Eis inhibitory activity (IC₅₀ = 0.21 ± 0.05 µM) when compared to the freshly synthesized parent 1a* (*Note: from here on, when comparing to 1a*, we refer to the freshly synthesized 1a*), indicating that ortho substitution was not beneficial. We then explored substitutions at the *meta* position with compounds 1c*-1g*. The *meta*-substituted compound 1c* (R₁ = H, R₂ = m-F) was found to have comparable Eis inhibitory activity (IC₅₀ = 0.06 ± 0.01 µM) to the parent compound 1a*. We systematically increased the size of the halogen substituents in compounds 1d* (R₁ = H, R₂ = m-Cl) and 1e* (R₁ = H, R₂ = m-Br). Interestingly, compound 1d* displayed an IC₅₀ value of 0.025 ± 0.006 µM, which was ~3-fold smaller than that of the parent compound 1a*. On the other hand, compound 1e* (IC₅₀ = 0.34 ± 0.10 µM) was not as potent as compound 1d*, suggesting that the Br substituent was possibly too sterically hindered and thus, not well tolerated in the Eis binding pocket. Compound 1f* (R₁ = H, R₂ = m-OH) (IC₅₀ = 1.8 ± 0.5 µM) was less potent than the parent compound, which suggested that having a highly polar substituent could be disfavored. Alternatively, replacing the hydroxyl group by a methoxy (compound 1g* (R₁ = H, R₂ = m-OMe)) yielded a molecule with improved Eis inhibition (IC₅₀ = 0.21 ± 0.07 µM) when compared to 1f*. Overall, we found that the Cl was the best substituent at the *meta* position.
We pondered whether this trend would translate to the para position, which prompted us to evaluate compounds 1h* (R_1 = H, R_2 = p-F), 1i* (R_1 = H, R_2 = p-Cl), and 1j* (R_1 = H, R_2 = p-Br). Intriguingly, the smaller F substituent was optimal at the para position with an IC\(_{50}\) value of 0.029 ± 0.007 µM contrary to what we observed at the meta position. Intriguingly, inhibitor 1h* displayed an IC\(_{50}\) of 0.029 ± 0.007 µM, which, similarly to the IC\(_{50}\) values for several other inhibitors was smaller than the half of the enzyme concentration used in our assay (0.25/2 = 0.125 µM). This effect has at least three potential explanations: (1) If inhibition of one monomer per Eis hexamer by one inhibitor molecule leads to the loss of activity of the entire hexamer, then the IC\(_{50}\) can, in principle, be as low as 0.125/6 = 0.02 µM; (2) If only a fraction of Eis protein is active in acetylating KAN and binding inhibitors (e.g., due to protein aggregation), then the concentration of active Eis in the assay is an overestimate of the concentration of active enzyme; and (3) If inhibitor binding to Eis causes Eis aggregation and inactivates multiple hexamers, then IC\(_{50}\) is also a fraction of the half of the enzyme concentration in the assay. Mechanism #3 is unlikely, because we do not observe global conformational changes in Eis upon inhibitor binding in crystal structures. Distinguishing among these mechanisms of these highly potent analogues is a goal of ongoing work in the group.

Subsequently, we investigated the effect of R_1 substitutions on the phenyl ring adjacent to the pyrrolo[1,5-a]pyrazine core. As the p-F substitution was one of the best in terms of activity when we varied R_2 in series 1, we first decided to install the p-F substituent at the R_1 position and generated series 2 analogues. Analogue 2a* (R_1 = p-F, R_2 = H) displayed weaker Eis inhibition (IC\(_{50}\) = 0.35 ± 0.07 µM) than did 1a*. Similarly to 1b* (R_1 = H, R_2 = o-F), the
ortho-substituted analogue 2b* (R₁ = p-F, R₂ = o-F) was also not optimal. When we increased the size of the R₂ substituents at the meta position, we observed that the larger halogens led to more potent Eis inhibition (Br = Cl > F) and yielded compounds with IC₅₀ values varying from 0.06 to 0.22 µM. Unlike compound 1e* (R₁ = H, R₂ = m-Br; IC₅₀ = 0.34 ± 0.10 µM), the m-Br substituted analogue 2e* (R₁ = p-F, R₂ = m-Br; IC₅₀ = 0.06 ± 0.02 µM) was much more potent, which pointed to the possibility that changing R₁ from H to p-F could lead to a slight variation in the binding orientation of the molecule, especially near the meta position of the acetophenone ring. Additionally, the m-hydroxy and m-methoxy substitutions, as in the cases of 2f* (R₁ = p-F, R₂ = m-OH) and 2g* (R₁ = p-F, R₂ = m-OMe) either completely abolished Eis inhibitory activity or resulted in moderate inhibition of the enzyme (IC₅₀ = 8.4 ± 2.8 and 0.53 ± 0.11 µM, respectively). This was consistent with the observations made with compounds 1f* (R₁ = H, R₂ = m-OH) and 1g* (R₁ = H, R₂ = m-OMe). For the para-substituted analogues (2h* (R₁ = p-F, R₂ = p-F), 2i* (R₁ = p-F, R₂ = p-Cl) and 2j* (R₁ = p-F, R₂ = p-Br)), similarly to what was observed with series 1, the larger halogen substituents were generally less favorable with activities varying in the range of 0.13–0.50 µM. We also evaluated the para-methylated analogue 2k* (R₁ = p-F, R₂ = Me) and found that 2k* displayed excellent activity with an IC₅₀ value of 0.08 ± 0.03 µM, which was 2-fold better than that of 1k* (IC₅₀ = 0.19 ± 0.02 µM).

Having established the general SAR trends for the aromatic analogues, we next aimed to determine whether their non-aromatic counterparts (1a-k and 2a-k) would exhibit decreased activity due to potential disruption of the π-π interactions with Eis aromatic amino acid...
residues. Indeed, we found that most of the non-aromatic analogues generally displayed less potent Eis inhibition than their aromatic counterparts did. In four out of 22 cases, the aromatic and non-aromatic compounds display nearly equipotent inhibition of Eis. In the case of compounds 1c and 1c* (R_1 = H, R_2 = m-F), the IC_{50} values were virtually the same (IC_{50} = 0.05 ± 0.01 and 0.06 ± 0.01 µM, respectively). Additionally, compounds 2e and 2e* (R_1 = p-F, R_2 = m-Br) were also practically equipotent in terms of Eis inhibitory activities (IC_{50} = 0.07 ± 0.02 and 0.06 ± 0.02 µM, respectively). Compounds 1g and 1g* (R_1 = H, R_2 = m-OMe) (IC_{50} = 0.15 ± 0.04 and 0.21 ± 0.07 µM, respectively) were also similar. For the pair 1i and 1i* (R_1 = H, R_2 = p-Cl) (IC_{50} = 0.31 ± 0.09 and 0.56 ± 0.20 µM, respectively), the non-aromatic counterpart 1i was marginally better. Regardless of whether the analogue in series 2 was aromatic or non-aromatic, it was conclusive that at the meta position of the acetophenone moiety, bigger halogen substituents such as Cl and Br were generally better suited, and at the para position of the acetophenone, the smaller F substituent was the best.

2.3.2.2. Activity of Eis inhibitors in Mtb cell culture

Once our pyrrolo[1,5-a]pyrazine derivatives were optimized for inhibition of the purified Eis enzyme in vitro, we set to confirm whether these compounds could display Eis inhibitory activity in the Mtb culture, by measuring the effect of the compounds on KAN MIC (MIC_{KAN}). Compounds were tested in combination with KAN against the KAN-sensitive H37Rv Mtb strain as a control and against the KAN-resistant Mtb K204, which is H37Rv Mtb bearing a
clinically occurring point mutation in the *eis* promoter leading to overexpression of Eis. Mtb H37Rv has an MIC\textsubscript{KAN} of 1.25 mg/mL, whereas KAN-resistant *Mtb* K204 has an MIC\textsubscript{KAN} of $\geq$ 10 mg/mL. Active compounds were expected to resensitize *Mtb* K204 to KAN. The compounds were generally tested at concentrations that were 100-fold higher than their respective IC\textsubscript{50} values in the enzymatic assays, to correct for the variation in the potency of Eis inhibition. Weakly potent compounds (IC\textsubscript{50} $>$ 1 mM) were tested at 100 mM in the MIC assays. *Mtb* is notorious for its highly lipophilic and complex cell wall, which provides intrinsic resistance to many antibacterial compounds and presents an immense challenge for anti-tubercular drug discovery. Indeed, as shown in our previous Eis inhibitors studies, some of the most potent *in vitro* compounds were not active in *Mtb* cultures. We also cannot exclude low solubility or aggregation of the compounds in the culture media as a reason for poor activity. Herein, we determined the MIC values for KAN (MIC\textsubscript{KAN}) against *Mtb* K204 in the absence or presence of our Eis inhibitors and compared them to the MIC\textsubscript{KAN} of the drug-sensitive *Mtb* H37Rv strain. As anticipated, most compounds caused a reduction in the MIC\textsubscript{KAN} for *Mtb* K204, overcoming KAN resistance. Poor Eis inhibitors such as compounds 1f* and 2f* with relatively high IC\textsubscript{50} values were unable to resensitize *Mtb* K204 to the action of KAN. These observations, together with the lack of toxicity of these inhibitors when used without KAN for either *Mtb* strains, validate Eis inhibition as the principal mechanism of MIC\textsubscript{KAN} reduction by these compounds. *Mtb* H37Rv, for which MIC\textsubscript{KAN} is virtually unaffected by the inhibitors, serves as an important negative control in this regard. Some of the good Eis inhibitors such as compounds 1c-1e, 1g, 1h, 2d, 2e, 2h, 3a, 3d, 3h, and 3k did not resensitize *Mtb* K204 to the action of KAN despite their nanomolar IC\textsubscript{50} values (MIC\textsubscript{KAN} $\geq$ 10 mg/mL), indicating that these molecules may not go through the cell envelope.
Compounds 1a*-1e*, 1g*, 1h*, 1i, 1j*, 1k*, 2a*-2e*, 2g*, 2i*-2k* partially restored activity of KAN (MIC\textsubscript{KAN} = 2.5-5 µg/mL). Generally, the analogues in series 1 and 2 displayed better potency compared to the analogues in series 3 in \textit{Mtb} culture. While the charged nature of these compounds may contribute adversely to the permeability of the compounds through the greasy mycobacterial cell envelope, their better solubility in aqueous solution when compared to other uncharged Eis inhibitors may offset this potential issue. Therefore, these compounds serve as valuable alternatives to our previously reported uncharged Eis inhibitors of other scaffolds in further preclinical development of Eis inhibitors as KAN adjuvants in TB therapy. Indeed, two compounds, 1i* and 2h*, are highly promising for future development, as they completely restore the potency of KAN, fully overcoming Eis upregulation.

2.3.2.3. Mammalian cytotoxicity

Cytotoxicity to three different mammalian cell lines was tested for five representative potent Eis inhibitors (Figure 2.2) in the absence and presence of KAN at the concentration of 50 µg/mL (equivalent to 86 µM) greatly exceeding the MIC\textsubscript{KAN} of \textit{Mtb}. The negative control corresponded to no inhibitor treatment and was standardized as 100% cell survival. The positive control was a treatment with Triton 100-X®, where we observed most of the cells killed. We observed that at sub-IC\textsubscript{50} concentrations, our Eis inhibitors induced cell proliferation, thereby displaying >100% cell survival. With the exception of compound 1e*, which at 50 µM exhibited significant cytotoxicity against one of the three cell lines, none of the compounds were cytotoxic against any of the three cell lines up to 50 µM. Three compounds (1i*, 2c*, 2h*) had no significant cytotoxicity up to 100 µM, without or with KAN. The lack of cytotoxicity indicates the absence of toxic off-target effects in the
mammalian cells, strengthening the promise of these compounds as candidates for animal and clinical studies. Eis inhibitors appear to be less toxic when co-administering with KAN. Upon assessing the cytotoxicity of KAN alone (Figure 2.3) and Eis inhibitors alone (Figure 2.2), one can observe that exposure to KAN or to Eis inhibitors alone at sub-IC$_{50}$ concentrations promotes cell growth. This phenomenon of increased growth in the presence of small quantities of xenobiotics has been previously observed.$^{110-114}$ Due to this effect, co-treatment with KAN and Eis inhibitors may result in a faster cell growth than that for KAN alone.

**Figure 2.2.** Mammalian cytotoxicity of selected compounds (1e*, 1i*, 2c*, 2h*, and 2i*) alone (represented as dark color columns) or in the presence of 50 mg/mL (equivalent to 86 mM) KAN (represented as light color columns immediately to the right of the dark color
column of the corresponding compound in the absence of KAN) against A. A549, B. HEK-293, and C. J774A.1 cells. Note: The data collected at lower concentrations (0.78, 1.56, 3.13, and 6.26 mM) did not indicate any toxicity and are therefore omitted for clarity.

![Graph](image)

**Figure 2.3.** Cytotoxicity of KAN against A549 (pale yellow), HEK-293 (light orange), and J774A.1 (red) mammalian cell lines.

### 2.3.3. Structural biology studies

To rationalize our SAR study and understand the binding mode of our inhibitors to Eis, we determined a crystal structure of Eis in complex with CoA and inhibitor 2k* (one of our best inhibitors; IC$_{50}$ = 0.08 ± 0.03 µM) at the resolution of 2.4 Å (Figure 2.4 and Table 2.2). The crystal structure demonstrates that inhibitor 2k* is indeed bound in the AG binding site (established by our reported Eis-CoA-TOB crystal structure, Figure 2.5) and is stabilized in the bound state by numerous hydrophobic interactions with Eis. The pyrazine ring is stacked between the side chain of Glu401 of Eis and its C-terminal carboxyl group, with mutually orthogonal π-π interactions of the pyrazine ring with the indole ring of Trp36. This
observation supports our initial hypothesis that the aromaticity of the pyrrolo[1,5-\(a\)]pyrazine core is crucial for activity and explains why aromatized compounds resulted in better activities during SAR analysis. Attached to the pyrrolo[1,5-\(a\)]pyrazine core, the acetophenone ring displayed parallel \(\pi-\pi\) interaction with Phe84 and showed that our prediction about the importance of the aromatic acetophenone was correct. Additionally, the \(R_2\)-substituted acetophenone fits in a hydrophobic environment of Leu63, Trp36, and Arg37. Hence, adding a polar hydroxyl group at \(R_2\), such as in compounds \(1f, 1f^*, 2f, \) and \(2f^*\), destabilizes binding within the hydrophobic environment and results in weaker Eis inhibitory activity. Furthermore, the \(para\) position of the acetophenone ring is sterically restrained on all sides, being sandwiched between Phe84 and Trp36 and abutting Trp13 and Met65, explaining why the larger groups in \(para\) position, such as in compounds \(1i/i^*, 1j/j^*, 2i/i^*, \) and \(2j/j^*\) resulted in decreased Eis inhibitory activities. This \(R_2\) binding pocket of Eis accommodates structurally similar substitutions of our previously published inhibitors with different core scaffolds (Figure 2.5). Otherwise, these previously reported inhibitors are bound in distinct orientations in the large AG binding cavity. As shown in Figure 2.4B and in our SAR analysis, near the two \(meta\) positions of the acetophenone ring, there are spacious pockets allowing incorporation of larger substituents at the \(meta\) position without compromising activity. Lastly, the phenyl ring containing \(R_1\) is located in a spacious binding pocket lined by the terminus of the phosphopantetheiny1 tail of the CoA molecule, Asp26, the C-terminal carboxyl group, Ser83, and Phe24, explaining why a phenyl group is preferred over an ethyl group at the \(R_1\) position. In summary, the crystal structure of the EisC204A-CoA-inhibitor \(2k^*\) complex allowed us to explain our biological data and provides a basis for future additional structure-based development of Eis inhibitors.
Figure 2.4. A. Crystal structure of EisC204A-CoA-inhibitor 2k* complex (PDB ID 5TVJ). CoA is colored yellow. Compound 2k* is colored in green. B. A zoom-in view of the binding pocket of compound 2k*. Amino acid residues that are interactive with compound 2k* are highlighted in red. The strong omit F_o-F_c electron density map contoured at 3σ generated without the inhibitor is shown by the mesh, demonstrating that the inhibitor molecule is unambiguously defined by the X-ray diffraction data.
Figure 2.5. A zoom-in view of the inhibitor/aminoglycoside binding site showing the superimposition of Eis inhibitors and TOB from several crystal structures. Inhibitor $2k^*$ (PDB ID 5TVJ) from this study is depicted as green sticks. The previously published structure of bound TOB (PDB ID 4JD6\textsuperscript{86}) is in blue, that of an isothiazole $S,S$-dioxide heterocyclic core (labeled $13g$ in ref\textsuperscript{105}, PDB ID 5EC4\textsuperscript{105}) is in red, and that of a sulfonamide-based inhibitor (labeled $39$ in ref\textsuperscript{106}, PDB ID 5IV0\textsuperscript{106}) is in orange. Bound CoA from the structure described in the current study is depicted as yellow sticks. The chemical structures of inhibitors $2k^*$, $13g$, and $39$, as well as of TOB are shown below the crystal structure.
2.4. CONCLUSION

In conclusion, via a SAR study, we tailor-fitted Eis inhibitors possessing the pyrrolo[1,5-\(a\)]pyrazine core to its Eis binding pocket and identified multiple novel nanomolar potency inhibitors. We validated our hypothesis that the aromaticity of the pyrrolo[1,5-\(a\)]pyrazine core was important for activity and that aromatic analogues were overall better inhibitors than their non-aromatic counterparts. For the aromatic analogues, our study indicated that the SAR strongly correlates with the size of the halogen substituent(s). At the meta position of the acetophenone, bigger halogens such as Cl and Br were generally well tolerated. On the other hand, at the para position, substitutions of a smaller F atom and a methyl group produced analogues with substantially improved activities. The SAR analysis also revealed that the substitution of a polar functional group such as the hydroxyl group greatly perturbed the hydrophobic environment leading to decreased activity. These SAR observations were explained by the crystal structure analysis, which will greatly facilitate future medicinal chemistry studies. Most significantly, by in vitro \(Mtb\) culture assays, we confirmed that our Eis inhibitors were capable of penetrating the \(Mtb\) cell wall and cancelling the KAN-resistance of \(Mtb\) K204, which overexpresses Eis. As exemplified by a clinically used combination of a \(\beta\)-lactamase inhibitor, clavulanic acid, and penicillin, these Eis inhibitors may become similarly significant as adjuvant molecules in a combination therapy with KAN to prevent emergence of and combat KAN resistance in MDR- and XDR-TB.
2.5. MATERIALS AND INSTRUMENTATION

All reagents were purchased from commercial sources and used without any further purification. Deuterated solvents were purchased from Cambridge Isotope Laboratories Inc. (Tewksbury, MA). TLC analyses were performed on silica gel plates (pre-coated on glass; 0.25 mm thickness with fluorescent indicator UV254) and were visualized by UV or charring in KMnO4 stains. 1H and 13C NMR spectra were recorded on 400 and 500 MHz NMR spectrometers (VARIAN INOVA) using CDCl3 or (CD3)2SO. Chemical shifts are reported in parts per million (ppm) and are referenced to residual solvent peaks (7.24 ppm for CDCl3 and 2.50 ppm for (CD3)2SO in 1H NMR; 77.00 ppm for CDCl3 and 39.50 ppm for (CD3)2SO in 13C NMR). Abbreviations used in NMR spectra: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublets, dt = doublet of triplets, td = triplet of doublets, tt = triplet of triplets, and ddd = doublet of doublet of doublets. All reactions were carried out under nitrogen atmosphere and all yields reported represent isolated yields. Known compounds were characterized by 1H NMR and are in complete agreement with samples reported in the literature. All new compounds were characterized by 1H and 13C NMR as well as mass spectrometry. Low-resolution electrospray mass spectra (LRMS) were recorded on a liquid chromatography-mass spectrometry using an Agilent 1200 series Quaternary LC system equipped with a diode array detector, and Eclipse XDB-C18 column (250 mm x 4.6 mm, 5 mm), and an Agilent 6120 Quadrupole MSD mass spectrometer. All final molecules 1a-3k and 1a*-2k* that were tested against purified Eis and in Mtb cells are >95% pure according to NMR spectra. Further confirmation of purity for these final molecules was obtained by RP-HPLC, which was performed on an Agilent Technologies 1260 Infinity HPLC system by using the following general method 1: Flow rate = 1 mL/min; λ = 254 nm; column =
Vydac 201SP™ C18, 250 × 4.6 mm, 90Å 5 mm; Eluents: A = H₂O + 0.1% TFA, B = MeCN; gradient profile: starting from 5% B, increasing from 5% B to 100% B over 20 min, holding at 100% B from 20-27 min, decreasing from 100% B to 5% B from 27-30 min. Prior to each injection, the HPLC column was equilibrated for 15 min with 5% B.

2.6. METHODS

2.6.1. Chemical methods

2.6.1.1. Synthesis of compound 4

Compound 4 was prepared following a previously published protocol. Sodium hydroxide (14.41 g, 360.35 mmol) and tetrabutylammonium hydrogen sulfate (TBAS) (1.22 g, 3.60 mmol) were added to a solution of pyrrole (5.0 mL, 72.07 mmol) in MeCN (220 mL). After stirring for 30 min at rt, 2-chloroethylamine hydrochloride (10.03 g, 86.48 mmol) was added. The reaction mixture was then refluxed for 24 h. After cooling to rt, the solvent was removed under reduced pressure to afford compound 4 (7.9 g, quantitative yield) as a yellow oil, which was used without any further purification: ¹H NMR (400 MHz, CDCl₃, Figure A1, which matches lit.¹¹⁵) δ 6.66 (t, J = 2.0 Hz, 2H), 6.14 (t, J = 2.0 Hz, 2H), 3.92 (t, J = 6.4 Hz, 2H), 3.00 (t, J = 6.4 Hz 2H), 1.36 (br s, 2H).

2.6.1.2. General procedure for benzoylation (e.g., Preparation of compound 5)

Benzoylation was performed following a previously reported protocol.¹¹⁶ Pyridine (3.22 mL, 39.94 mmol) and benzoyl chloride (4.22 mL, 36.31 mmol)
were successively added to a solution of compound 4 (4.00 g, 36.31 mmol) in 1,4-dioxane (144 mL). The reaction mixture was refluxed for 12 h prior to removing the solvent under reduced pressure. The residue was triturated with H$_2$O (100 mL) and extracted with Et$_2$O (2 x 80 mL). The organic layers were collected, washed with aq. NaHCO$_3$ (100 mL) then with H$_2$O (100 mL), dried over MgSO$_4$, and evaporated to dryness to afford compound 5 (5.3 g, 69%) as a light brown solid, which was used without any further purification: $^1$H NMR (400 MHz, CDCl$_3$, Figure A2, which matches lit.$^{117}$) δ 7.67 (d, $J = 6.8$ Hz, 2H), 7.59 (t, $J = 7.2$ Hz, 1H), 7.59-7.37 (m, 2H), 6.67 (t, $J = 2.0$ Hz, 2H), 6.20 (br s, 1H), 6.18 (t, $J = 2.0$ Hz, 2H), 4.12 (t, $J = 6.0$ Hz, 2H), 3.73 (q, $J = 6.0$ Hz, 2H).

Preparation of compound 6. As described for the synthesis of compound 5, pyridine (3.14 mL, 35.40 mmol), 4-fluorobenzoyl chloride (4.19 mL, 35.40 mmol), compound 4 (3.90 g, 35.40 mmol), and 1,4-dioxane (140 mL) were used to afford compound 6 (5.4 g, 66%) as a gray solid: $^1$H NMR (400 MHz, CDCl$_3$, Figure A3) δ 7.69-7.65 (m, 2H), 7.14-7.04 (m, 2H), 6.65 (t, $J = 2.0$ Hz, 2H), 6.17 (t, $J = 2.0$ Hz, 2H), 6.15 (br s, 1H), 4.11 (t, $J = 5.6$ Hz, 2H), 3.71 (q, $J = 5.6$ Hz, 2H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure A4) δ 166.6, 166.1, 163.6, 132.8, 129.3, 129.2, 120.7, 115.8, 115.58, 115.55, 109.0, 48.8, 41.5; LRMS m/z calcd for C$_{13}$H$_{13}$FN$_2$O [M+H]$^+$: 232.1; found 232.8.

Preparation of compound 7. As described for the synthesis of compound 5, pyridine (1.61 mL, 19.97 mmol), 3,4-difluorobenzoyl chloride (2.29 mL, 18.15 mmol), compound 4 (2.0 g, 18.15 mmol), and CH$_2$Cl$_2$ (72 mL) were
used to afford compound 7 (3.20 g, 71%) as a white solid: $^1$H NMR (400 MHz, CDCl$_3$, Figure A5) δ 7.55 (m, 1H), 7.36 (m, 1H), 7.17 (q, $J = 8.4$ Hz, 1H), 6.65 (m, 2H), 6.18 (m, 2H), 6.02 (br s, 1H), 4.12 (t, $J = 5.6$ Hz, 2H), 3.72 (q, $J = 5.6$ Hz, 2H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure A6) δ 165.4, 123.29, 123.26, 123.23, 123.19, 120.7, 117.6, 117.4, 116.9, 116.73, 116.72, 109.0, 48.7, 41.6; LRMS $m/z$ calcd for C$_{13}$H$_{12}$F$_2$N$_2$O [M+H]$^+$: 250.1; found 250.8.

2.6.1.3. General procedure for cyclization (e.g., Preparation of compound 8)

Cyclization was performed following a previously reported protocol.$^{116}$ A solution of compound 5 (5.00 g, 23.33 mmol) and phosphorous(V) oxychloride (10.9 mL, 116.67 mmol) in toluene (117 mL) was heated under reflux for 12 h. After cooling, the precipitate was filtered and dissolved in H$_2$O (200 mL). The solution was then made alkaline with NaHCO$_3$ and extracted with CH$_2$Cl$_2$ (200 mL). The organic layer was dried over MgSO$_4$ and evaporated to dryness under reduced pressure to give a residue, which was purified by flash column chromatography (SiO$_2$, MeOH:CH$_2$Cl$_2$/5:95, $R_f$ 0.23) to yield compound 8 (1.10 g, 24%) as a brown solid: $^1$H NMR (400 MHz, CDCl$_3$, Figure A7, which matches lit.$^{118}$) δ 7.77 (m, 2H), 7.45-7.40 (m, 3H), 6.83 (m, 1H), 6.41 (m, 1H), 6.22 (t, $J = 2.8$ Hz, 1H), 4.02 (m, 4H).

Preparation of compound 9. As described for the synthesis of compound 8, compound 6 (5.40 g, 23.25 mmol), phosphorous(V) oxychloride (10.9 mL, 116.25 mmol), and toluene (117 mL) were used to afford compound 9 (1.0 g, 18%, $R_f$ 0.13 in MeOH:CH$_2$Cl$_2$/5:95) as a brown solid: $^1$H NMR (400 MHz, CDCl$_3$, Figure
A8, which matches lit.\textsuperscript{119} δ 7.77-7.81 (m, 2H), 7.11 (t, \( J = 8.0 \) Hz, 2H), 6.88 (m, 1H), 6.43 (d, \( J = 3.2 \) Hz, 1H), 6.26 (t, \( J = 3.2 \) Hz, 1H), 4.08-3.99 (m, 4H).

**Preparation of compound 10.** As described for the synthesis of compound 8, compound 7 (3.20 g, 12.79 mmol), phosphorous(V) oxychloride (5.98 mL, 63.94 mmol), and toluene (65 mL) were used to afford compound 10 (0.47 g, 16%, \( R_f 0.50 \) in MeOH:CH\textsubscript{2}Cl\textsubscript{2}/1:9) as a yellow solid: \( ^1\text{H} \) NMR (400 MHz, CDCl\textsubscript{3}, Figure A9) δ 7.61 (ddd, \( J = 10.8, 7.6, 2.0 \) Hz, 1H), 7.54-7.51 (m, 1H), 7.19 (q, \( J = 8.4 \) Hz 1H), 6.82 (m, 1H), 6.36 (dd, \( J = 3.6, 1.6 \) Hz, 1H), 6.21 (dd, \( J = 3.6, 1.6 \) Hz, 1H), 4.02-3.96 (m, 4H); \( ^{13}\text{C} \) NMR (100 MHz, CDCl\textsubscript{3}, Figure A10) δ 158.3, 152.7, 152.6, 151.3, 151.2, 150.2, 150.1, 148.8, 148.7, 135.0, 124.69, 124.65, 124.62, 124.59, 123.9, 123.6, 117.6, 117.4, 117.0, 116.8, 112.1, 108.7, 48.0, 42.0; LRMS \( m/z \) calcd for C\textsubscript{13}H\textsubscript{10}F\textsubscript{2}N\textsubscript{2}[M+H]\textsuperscript{+}: 232.1; found 232.8.

### 2.6.1.4. General procedure for \textit{N}-alkylation (e.g., Preparation of compound 1a)

A solution of compound 8 (0.10 g, 0.51 mmol) and 2-bromoacetophenone (0.10 g, 0.51 mmol) in EtOH (2.5 mL) was heated under reflux for 12 h. After cooling to rt, the solvent was removed and the residue was purified by column chromatography (SiO\textsubscript{2}, MeOH:CH\textsubscript{2}Cl\textsubscript{2}/5:95, \( R_f 0.05 \)) to yield compound 1a (0.10 g, 50%) as a light brown solid: \( ^1\text{H} \) NMR (400 MHz, CDCl\textsubscript{3}, Figure A11) δ 7.91 (dd, \( J = 8.4, 1.2 \) Hz, 2H), 7.59-7.41 (m, 9H), 6.58 (dd, \( J = 4.0, 1.6 \) Hz, 1H), 6.45 (dd, \( J = 4.0, 2.0 \) Hz, 1H), 5.90 (br s, 2H), 4.86 (t, \( J = 6.0 \) Hz, 2H), 4.60 (t, \( J = 6.0 \) Hz, 2H); \( ^{13}\text{C} \) NMR (100 MHz, CDCl\textsubscript{3}, Figure A12) δ 192.4, 163.7, 135.22, 135.16, 134.9, 133.3, 132.7, 129.14, 129.07, 128.8, 128.5,
Purity of the compound was further confirmed by RP-HPLC by using method 1: \( R_t = 9.94 \text{ min (98\% pure; Figure A13).} \)

**Preparation of compound 1b.** A solution of compound 8 (0.05 g, 0.28 mmol) and 2-bromo-2'-fluoroacetophenone (0.06 g, 0.28 mmol) in anhydrous THF (1.25 mL) was stirred at rt for 12 h. The product precipitated, was filtered and washed with THF (2 x 10 mL) to yield compound 1b (0.05 g, 40\%) as a yellow solid: \(^1\)H NMR (500 MHz, (CD\(_3\))\(_2\)SO, Figure A14) \( \delta \) 7.93-7.89 (m, 2H), 7.78-7.73 (m, 1H), 7.68 (tt, \( J = 7.5, 1.0 \text{ Hz, 1H} \)), 7.61 (t, \( J = 7.0 \text{ Hz, 2H} \)), 7.51 (d, \( J = 7.5 \text{ Hz, 2H} \)), 7.39 (t, \( J = 7.0 \text{ Hz, 1H} \)), 7.36 (d, \( J = 8.5 \text{ Hz, 1H} \)) \( J = 1.0 \text{ Hz, 1H} \)), 6.70 (dd, \( J = 4.0, 1.0 \text{ Hz, 1H} \)), 6.61 (dd, \( J = 4.5, 2.0 \text{ Hz, 1H} \)), 5.31 (d, \( J = 1.5 \text{ Hz, 2H} \)), 4.62 (t, \( J = 6.0 \text{ Hz, 2H} \)), 4.37 (t, \( J = 6.0 \text{ Hz, 2H} \)); \(^{13}\)C NMR (125 MHz, (CD\(_3\))\(_2\)SO, Figure A15) \( \delta \) 189.87, 189.83, 162.9, 162.6, 160.5, 136.94, 136.87, 136.3, 132.5, 130.4, 129.1, 128.6, 128.3, 127.4, 125.3, 125.2, 124.3, 121.9, 121.8, 117.1, 116.9, 115.1, 64.2, 64.1, 50.8, 42.6; LRMS \( m/z \) calcd for C\(_{21}\)H\(_{19}\)N\(_2\)O: 333.1; found 332.8 \([\text{M}^+\] \). Purity of the compound was further confirmed by RP-HPLC by using method 1: \( R_t = 8.75 \text{ min (100\% pure; Figure A16).} \)

**Preparation of compound 1c.** As described for the synthesis of compound 1a, compound 8 (0.05 g, 0.28 mmol), 2-bromo-3'-fluoroacetophenone (0.06 g, 0.28 mmol), and EtOH (1.25 mL) were used to afford compound 1c (0.07 g, 66\%, \( R_f \) 0.12 in MeOH:CH\(_2\)Cl\(_2\)/1:9) as a yellow hydroscopic wax: \(^1\)H NMR (400
Preparation of compound 1d. As described for the synthesis of compound 1a, compound 8 (0.05 g, 0.28 mmol), 2-bromo-3'-chloroacetophenone (0.06 g, 0.28 mmol), and EtOH (1.25 mL) were used to afford compound 1d (0.01 g, 10%, Rf 0.10 in MeOH:CH₂Cl₂/1:9) as a yellow hydrosopic wax: ¹H NMR (400 MHz, CDCl₃, Figure A20) δ 7.87-7.85 (m, 2H), 7.60-7.46 (m, 7H), 7.39 (t, J = 7.2 Hz, 2H), 6.61 (dd, J = 4.4, 1.2 Hz, 1H), 6.47 (dd, J = 4.4, 2.4 Hz, 1H), 5.90 (br s, 2H), 4.85 (t, J = 6.4 Hz, 2H), 4.61 (t, J = 6.4 Hz, 2H); ¹³C NMR (100 MHz, CDCl₃, Figure A21) δ 191.8, 164.0, 135.7, 135.5, 135.1, 135.0, 133.1, 130.8, 129.5, 129.0, 128.5, 128.4, 128.3, 127.3, 125.0, 115.6, 63.6, 51.8, 44.1; LRMS m/z calcd for C₂₁H₁₈FN₂O: 349.1; found 348.8 [M⁺]. Purity of the compound was further confirmed by RP-HPLC by using method 1: Rₜ = 8.23 min (95% pure; Figure A22).

Preparation of compound 1e. As described for the synthesis of compound 1b, compound 8 (0.05 g, 0.28 mmol), 2,3'-
dibromoacetophenone (0.07 g, 0.28 mmol), and anhydrous THF (1.25 mL) were used to afford compound 1e (0.04 g, 35%) as a yellow solid: $^1$H NMR (500 MHz, (CD$_3$)$_2$SO, Figure A23) $\delta$ 8.09 (s, 1H), 7.93-7.91 (m, 2H), 7.68 (t, $J = 7.5$ Hz, 1H), 7.60 (t, $J = 7.5$ Hz, 2H), 7.53 (t, $J = 8.0$ Hz, 1H), 7.49 (d, $J = 8.0$ Hz, 2H), 6.68 (d, $J = 4.0$ Hz, 1H), 6.61 (dd, $J = 3.5$, 2.0 Hz, 1H), 5.56 (s, 2H), 4.61 (t, $J = 6.0$ Hz, 2H), 4.35 (t, $J = 6.0$ Hz, 2H); $^{13}$C NMR (125 MHz, (CD$_3$)$_2$SO, Figure A24) $\delta$ 191.4, 163.1, 137.1, 136.2, 135.4, 132.3, 131.1, 130.9, 129.0, 128.6, 128.3, 127.3, 127.2, 124.4, 122.1, 115.0, 61.7, 50.9, 42.5; LRMS $m/z$ calcd for C$_{21}$H$_{18}$BrN$_2$O: 393.1; found 392.7 [M]$^+$. Purity of the compound was further confirmed by RP-HPLC by using method 1: $R_t$ = 9.66 min (95% pure; Figure A25).

**Preparation of compound 1f.** As described for the synthesis of compound 1b, compound 8 (0.05 g, 0.28 mmol), 2-bromo-3'-hydroxyacetophenone (0.06 g, 0.28 mmol), and anhydrous THF (1.25 mL) were used to afford compound 1f (0.05 g, 43%) as a yellow solid: $^1$H NMR (500 MHz, (CD$_3$)$_2$SO, Figure A26) $\delta$ 9.93 (s, 1H), 7.91 (m, 1H), 7.67 (t, $J = 7.5$ Hz, 1H), 7.59 (t, $J = 8.0$ Hz, 1H), 7.49 (d, $J = 7.5$ Hz, 2H), 7.33 (d, $J = 4.5$ Hz, 2H), 7.26 (br s, 1H), 7.11-7.09 (m, 1H), 6.67 (dd, $J = 4.5$, 1.5 Hz, 1H), 6.60 (dd, $J = 4.5$, 2.0 Hz, 2H), 5.46 (s, 2H), 4.62 (t, $J = 6.0$ Hz, 2H), 4.36 (t, $J = 6.0$ Hz, 2H); $^{13}$C NMR (125 MHz, (CD$_3$)$_2$SO, Figure A27) $\delta$ 192.2, 163.2, 157.9, 136.3, 134.8, 132.5, 130.3, 129.2, 128.7, 128.5, 127.4, 124.6, 122.0, 119.3, 115.2, 114.4, 61.9, 51.2, 42.8; LRMS $m/z$ calcd for C$_{21}$H$_{19}$N$_2$O$_2$: 331.1; found 330.8 [M]$^+$. Purity of the compound was further confirmed by RP-HPLC by using method 1: $R_t$ = 9.25 min (97% pure; Figure A28).
Preparation of compound 1g. As described for the synthesis of compound 1b, compound 8 (0.05 g, 0.28 mmol), 2-bromo-3'-methoxyacetophenone (0.06 g, 0.28 mmol), and anhydrous THF (1.25 mL) were used to afford compound 1g (0.09 g, 87%) as a yellow solid: ^1H NMR (500 MHz, (CD$_3$)$_2$SO, Figure A29) δ 7.93 (s, 1H), 7.67 (t, $J = 7.5$ Hz, 1H), 7.59 (t, $J = 7.5$ Hz, 2H), 7.51-7.49 (m, 3H), 7.46 (t, $J = 7.5$ Hz, 1H), 7.42 (m, 1H), 7.28 (dd, $J = 8.0$, 1.5 Hz, 1H), 6.67 (dd, $J = 4.0$, 1.5 Hz, 1H), 6.60 (dd, $J = 4.5$, 2.0 Hz, 1H), 5.56 (s, 2H), 4.64 (t, $J = 6.0$ Hz, 2H), 4.39 (t, $J = 6.0$ Hz, 2H). ^13C NMR (125 MHz, (CD$_3$)$_2$SO, Figure A30) δ 192.1, 163.0, 159.4, 136.1, 134.6, 132.3, 130.2, 129.0, 128.5, 128.3, 127.2, 124.4, 120.64, 120.59, 114.9, 112.9, 61.8, 55.49, 55.47, 51.0, 42.6; LRMS m/z calcd for C$_{21}$H$_{21}$N$_2$O$_2$: 345.2; found 344.8 [M$^+$]. Purity of the compound was further confirmed by RP-HPLC by using method 1: $R_t = 8.87$ min (98% pure; Figure A31).

Preparation of compound 1h. As described for the synthesis of compound 1b, compound 8 (0.05 g, 0.28 mmol), 2-bromo-4'-fluoroacetophenone (0.06 g, 0.28 mmol), and anhydrous THF (1.25 mL) were used to afford compound 1h (0.07 g, 60%) as a yellow solid: ^1H NMR (500 MHz, (CD$_3$)$_2$SO, Figure A32) δ 8.02 (dd, $J = 8.0$, 5.5 Hz, 2H), 7.93 (m, 1H), 7.67 (t, $J = 7.5$ Hz, 1H), 7.59 (t, $J = 7.5$ Hz, 2H), 7.50 (d, $J = 7.5$ Hz, 2H), 7.39 (t, $J = 8.5$ Hz, 2H), 6.67 (d, $J = 3.5$ Hz, 1H), 6.61-6.60 (m, 1H), 5.54 (s, 2H), 4.63 (t, $J = 6.0$ Hz, 2H), 4.38 (t, $J = 6.0$ Hz, 2H); ^13C NMR (125 MHz, (CD$_3$)$_2$SO, Figure A33) δ 190.9, 163.0, 136.2, 132.4, 131.6, 131.5, 129.0,
128.6, 128.3, 127.3, 124.4, 116.2, 116.0, 115.0, 61.6, 51.0, 42.6; LRMS m/z calcd for C$_{21}$H$_{18}$FN$_2$O: 333.1; found 332.9 [M$^+$]. Purity of the compound was further confirmed by RP-HPLC by using method 1: $R_t$ = 8.66 min (96% pure; Figure A34).

Preparation of compound 1i. As described for the synthesis of compound 1b, compound 8 (0.05 g, 0.28 mmol), 2-bromo-4'-chloroacetophenone (0.06 g, 0.28 mmol), and anhydrous THF (1.25 mL) were used to afford compound 1i (0.08 g, 89%) as a yellow solid: $^1$H NMR (500 MHz, (CD$_3$)$_2$SO, Figure A35) $\delta$ 7.94 (d, $J$ = 8.5 Hz, 2H), 7.91 (s, 1H), 7.67 (t, $J$ = 7.5 Hz, 1H), 7.64 (d, $J$ = 8.5 Hz, 1H), 7.59 (t, $J$ = 8.0 Hz, 2H), 7.49 (d, $J$ = 7.5 Hz, 2H), 6.69 (d, $J$ = 4.0 Hz, 1H), 6.61 (dd, $J$ = 4.0, 2.0 Hz, 1H), 5.51 (s, 2H), 4.61 (t, $J$ = 6.5 Hz, 2H), 4.35 (t, $J$ = 6.5 Hz, 2H); $^{13}$C NMR (125 MHz, (CD$_3$)$_2$SO, Figure A36) $\delta$ 191.5, 163.1, 139.6, 136.2, 132.4, 132.1, 130.3, 129.12, 129.06, 128.6, 128.3, 127.3, 124.4, 115.0, 61.7, 51.0, 42.6; LRMS m/z calcd for C$_{21}$H$_{18}$ClN$_2$O: 349.1; found 348.8 [M$^+$]. Purity of the compound was further confirmed by RP-HPLC by using method 1: $R_t$ = 8.99 min (96% pure; Figure A37).

Preparation of compound 1j. As described for the synthesis of compound 1a, compound 8 (0.05 g, 0.28 mmol), 2,4'-dibromoacetophenone (0.07 g, 0.28 mmol), and EtOH (1.25 mL) were used to afford compound 1j (0.11 g, 79%, $R_f$ 0.12 in MeOH:CH$_2$Cl$_2$/1:9) as a yellow solid: $^1$H NMR (500 MHz, (CD$_3$)$_2$SO, Figure A38) $\delta$ 7.90 (s, 1H), 7.85 (d, $J$ = 8.5 Hz, 2H), 7.78 (d, $J$ = 8.5 Hz, 2H), 7.67 (t, $J$ = 8.0 Hz, 1H), 7.59 (t, $J$ = 8.0 Hz, 2H), 7.48 (d, $J$ = 8.0 Hz, 2H), 6.68 (d,
Preparation of compound 1k. As described for the synthesis of compound 1a, compound 8 (0.10 g, 0.51 mmol), 2-bromo-4'-methylacetophenone (0.11 g, 0.51 mmol), and EtOH (2.5 mL) were used to afford compound 1k (0.19 g, 91%, R\textsubscript{f} 0.38 in MeOH:CH\textsubscript{2}Cl\textsubscript{2}/1:9) as a light brown solid: \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}, Figure A41) \(\delta\) 7.81 (d, \(J = 8.0\) Hz, 2H), 7.59-7.45 (m, 6H), 7.23 (d, \(J = 8.0\) Hz, 2H), 6.59 (dd, \(J = 4.4, 0.8\) Hz, 1H), 6.46 (dd, \(J = 4.4, 2.0\) Hz, 1H), 5.85 (br s, 2H), 4.84 (t, \(J = 5.6\) Hz, 2H), 4.60 (t, \(J = 5.6\) Hz, 2H), 2.37 (s, 3H); \textsuperscript{13}C NMR (100 MHz, CDCl\textsubscript{3}, Figure A42) \(\delta\) 191.9, 163.8, 146.3, 134.8, 134.7, 132.7, 130.8, 129.8, 129.2, 128.9, 128.7, 128.4, 127.9, 124.7, 115.1, 63.4, 51.6, 43.9, 21.8; LRMS m/z calcd for C\textsubscript{22}H\textsubscript{21}N\textsubscript{2}O: 329.2; found 329.8 [M]+. Purity of the compound was further confirmed by RP-HPLC by using method 1: \(R_t = 10.52\) min (98% pure; Figure A43).

Preparation of compound 2a. As described for the synthesis of compound 1a, compound 9 (0.10 g, 0.47 mmol), 2-bromoacetophenone (0.08 g, 0.47 mmol), and EtOH (2.0 mL) were used to afford compound 2a (0.12 g, 71%, \(J = 4.0\) Hz, 1H), 6.61-6.60 (m, 1H), 5.50 (s, 2H), 4.60 (t, \(J = 6.5\) Hz, 2H), 4.35 (t, \(J = 6.5\) Hz, 2H); \textsuperscript{13}C NMR (100 MHz, CDCl\textsubscript{3}, Figure A39) \(\delta\) 191.4, 163.4, 136.1, 132.7, 132.4, 132.3, 132.0, 130.1, 129.9, 129.3, 129.1, 128.8, 128.7, 128.6, 128.0, 124.5, 115.3, 62.9, 51.4, 43.5; LRMS m/z calcd for C\textsubscript{21}H\textsubscript{18}BrN\textsubscript{2}O: 393.1; found 392.7 [M]+. Purity of the compound was further confirmed by RP-HPLC by using method 1: \(R_t = 8.96\) min (95% pure; Figure A40).
Rf 0.30 in MeOH:CH₂Cl₂/1:9) as a brown solid: ¹H NMR (400 MHz, CDCl₃, Figure A44) δ 7.94 (dd, J = 8.4, 1.2 Hz, 2H), 7.65-7.59 (m, 3H), 7.49-7.44 (m, 3H), 7.18 (t, J = 8.8 Hz, 2H), 6.60 (dd, J = 4.4, 1.2 Hz, 1H), 6.48 (dd, J = 4.0, 2.4 Hz, 1H), 5.89 (br s, 2H), 4.86 (t, J = 6.0 Hz, 2H), 4.58 (t, J = 6.0 Hz, 2H); ¹³C NMR (100 MHz, CDCl₃, Figure A45) δ 192.1, 166.2, 163.6, 162.5, 135.8, 135.7, 134.9, 133.1, 131.5, 129.0, 128.4, 127.81, 127.76, 127.7, 124.6, 124.3, 124.2, 116.7, 116.5, 115.34, 115.25, 63.2, 51.6, 43.6; LRMS m/z calcd for C₂₁H₁₈FN₂O: 333.1; found 333.8 [M]+. Purity of the compound was further confirmed by RP-HPLC by using method 1: Rₜ = 9.85 min (97% pure; Figure A46).

Preparation of compound 2b. As described for the synthesis of compound 1a, compound 9 (0.10 g, 0.47 mmol), 2-bromo-2'-fluoroacetophenone (0.10 g, 0.47 mmol), and EtOH (2.0 mL) were used to afford compound 2b (0.12 g, 60%, Rf 0.21 in MeOH:CH₂Cl₂/1:9) as a brown solid: ¹H NMR (400 MHz, CDCl₃, Figure A47) δ 7.85 (t, J = 7.2 Hz, 1H), 7.68 (m, 1H), 7.56-7.50 (m, 3H), 7.18 (t, J = 7.2 Hz, 1H), 7.13 (t, J = 7.6 Hz, 2H), 7.03 (dd, J = 10.8, 8.8 Hz, 1H), 6.53 (dt, J = 4.4, 1.6 Hz, 1H), 6.41 (p, J = 1.6 Hz, 1H), 5.50 (br s, 2H), 4.84 (t, J = 6.0 Hz, 2H), 4.51 (t, J = 6.0 Hz, 2H); ¹³C NMR (100 MHz, CDCl₃, Figure A48) δ 190.1, 190.0, 166.1, 163.5, 163.3, 162.3, 160.8, 136.7, 136.6, 136.2, 131.4, 131.3, 130.52, 130.50, 127.9, 124.9, 124.8, 124.6, 124.28, 124.25, 121.7, 121.6, 117.0, 116.7, 116.6, 116.5, 116.4, 115.4, 65.5, 51.6, 43.5; LRMS m/z calcd for C₂₁H₁₇F₂N₂O: 351.1; found 351.8 [M]+. Purity of the compound was further confirmed by RP-HPLC by using method 1: Rₜ = 9.22 min (96% pure; Figure A49).
Preparation of compound 2c. As described for the synthesis of compound 1a, compound 9 (0.05 g, 0.23 mmol), 2-bromo-3'-fluoroacetophenone (0.05 g, 0.23 mmol), and EtOH (1.0 mL) were used to afford compound 2c (0.06 g, 60%, R_f 0.21 in MeOH:CH_2Cl_2/1:9) as a yellow solid: ^1H NMR (400 MHz, (CD_3)_2SO, Figure A50) δ 7.91 (m, 1H), 7.77 (m, 2H), 7.63-7.55 (m, 4H), 7.46 (t, J = 8.4 Hz, 2H), 6.74 (dd, J = 4.0, 1.2, 1H), 6.61 (dd, J = 4.4, 2.4 Hz, 1H), 5.56 (s, 2H), 4.60 (t, J = 6.8 Hz, 2H), 4.34 (t, J = 6.8 Hz, 2H); ^13C NMR (100 MHz, (CD_3)_2SO, Figure A51) δ 191.54, 191.52, 165.5, 163.2, 163.0, 162.3, 160.8, 136.3, 135.53, 135.47, 131.7, 131.6, 131.25, 131.16, 127.5, 124.6, 124.5, 121.4, 116.5, 116.3, 115.2, 115.1, 114.9, 61.7, 51.0, 42.6; LRMS m/z calcd for C_{21}H_{17}F_2N_2O: 351.1; found 351.8 [M]^+. Purity of the compound was further confirmed by RP-HPLC by using method 1: R_t = 9.07 min (98% pure; Figure A52).

Preparation of compound 2d. As described for the synthesis of compound 1a, compound 9 (0.05 g, 0.23 mmol), 2-bromo-3'-chloroacetophenone (0.05 g, 0.23 mmol), and EtOH (1.0 mL) were used to afford compound 2d (0.07 g, 70%, R_f 0.22 in MeOH:CH_2Cl_2/1:9) as a yellow solid: ^1H NMR (400 MHz, (CD_3)_2SO, Figure A53) δ 7.98 (t, J = 1.6 Hz, 1H), 7.91 (t, J = 1.6 Hz, 1H), 7.89 (d, J = 8.0 Hz, 1H), 7.79 (dd, J = 8.4, 1.6 Hz, 1H), 7.61 (t, J = 8.0 Hz, 1H), 7.59-7.55 (dd, J = 8.4, 5.6 Hz, 2H), 7.46 (t, J = 8.4 Hz, 1H), 6.73 (dd, J = 4.0, 1.2, 1H), 6.61 (dd, J = 4.4, 2.4 Hz, 1H), 5.58 (s, 2H), 4.60 (t, J = 6.8 Hz, 2H), 4.33 (t, J = 6.8 Hz, 2H); ^13C NMR (100 MHz, (CD_3)_2SO, Figure A54) δ 191.6, 165.5, 163.0, 162.3, 136.3, 135.2, 134.2, 133.7, 131.7, 131.6, 130.9, 128.2, 127.5, 127.0, 124.70, 124.67, 124.5, 116.5, 116.3, 115.1, 61.7, 50.9, 42.6; LRMS m/z calcd for C_{21}H_{17}ClFN_2O: 367.1; found 367.8 [M]^+. Purity of the compound was further
confirmed by RP-HPLC by using method 1: $R_t = 8.39$ min (95% pure; Figure A55).

### Preparation of compound 2e.
As described for the synthesis of compound 1a, compound 9 (0.05 g, 0.23 mmol), 2-bromo-3'-bromoacetophenone (0.06 g, 0.23 mmol), and EtOH (1.0 mL) were used to afford compound 2e (0.06 g, 55%, $R_f$ 0.31 in MeOH:CH$_2$Cl$_2$/1:9) as an orange solid: $^1$H NMR (400 MHz, (CD$_3$)$_2$SO, Figure A56) δ 8.09 (d, $J = 1.2$ Hz, 1H), 7.91 (t, $J = 8.0$ Hz, 3H), 7.57-7.52 (m, 3H), 7.45 (td, $J = 8.0$, 1.2 Hz, 2H), 6.74 (d, $J = 4.4$ Hz, 1H), 6.61-6.60 (m, 1H), 5.56 (s, 2H), 4.58 (t, $J = 6.4$ Hz, 2H), 4.31 (t, $J = 6.4$ Hz, 2H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure A57) δ 191.2, 166.3, 163.7, 162.7, 137.7, 135.9, 134.8, 131.5, 131.4, 131.0, 130.7, 128.0, 127.4, 124.7, 124.3, 124.2, 123.3, 116.8, 116.6, 115.4, 63.2, 51.6, 43.7; LRMS $m/z$ calcd for C$_{21}$H$_{17}$BrFNO: 411.1; found 411.8 [M]$^+$. Purity of the compound was further confirmed by RP-HPLC by using method 1: $R_t = 9.44$ min (98% pure; Figure A58).

### Preparation of compound 2f.
As described for the synthesis of compound 1a, compound 9 (0.05 g, 0.23 mmol), 2-bromo-3'-hydroxyacetophenone (0.05 g, 0.23 mmol), and EtOH (1.0 mL) were used to afford compound 2f (0.05 g, 51%, $R_f$ 0.15 in MeOH:CH$_2$Cl$_2$/1:9) as a white solid: $^1$H NMR (400 MHz, (CD$_3$)$_2$SO, Figure A59) δ 10.04 (s, 1H), 7.86 (t, $J = 2.0$ Hz, 1H), 7.56-7.53 (m, 2H), 7.44 (t, $J = 8.4$ Hz, 2H), 7.35-7.32 (m, 2H), 7.25 (m, 1H), 7.11-7.08 (m, 1H), 6.72 (dd, $J = 4.4$, 1.2 Hz, 1H), 6.60 (dd, $J = 4.4$, 2.4 Hz, 1H), 5.45 (s, 2H), 4.57 (t, $J = 6.0$ Hz, 2H), 4.31 (t, $J = 6.0$ Hz, 2H); $^{13}$C NMR (100 MHz, (CD$_3$)$_2$SO, Figure A60) δ 192.2, 163.1, 162.3, 157.8,
136.4, 134.8, 131.8, 131.7, 130.3, 127.6, 124.8, 124.6, 122.0, 119.4, 116.7, 116.5, 115.3, 114.5, 61.8, 51.2, 42.7; LRMS m/z calcd for C_{21}H_{18}FN_{2}O_{2}: 349.1; found 349.8 [M]+. Purity of the compound was further confirmed by RP-HPLC by using method 1: R_f = 8.25 min (99% pure; Figure A61).

**Preparation of compound 2g.** As described for the synthesis of compound 1a, compound 9 (0.05 g, 0.23 mmol), 2-bromo-3′-methoxyacetophenone (0.05 g, 0.23 mmol), and EtOH (1.0 mL) were used to afford compound 2g (0.05 g, 50%, R_f 0.20 in MeOH:CH_{2}Cl_{2}/1:9) as an orange solid: 1H NMR (400 MHz, (CD_{3})_{2}SO, Figure A62) δ 7.62 (br s, 1H), 7.58 (d, J = 8.0 Hz, 1H), 7.57 (d, J = 8.0 Hz, 1H), 7.47 (d, J = 8.0 Hz, 1H), 7.40 (m, 1H), 7.29 (t, J = 8.0 Hz, 1H), 7.13 (t, J = 8.0 Hz, 2H), 7.07 (dd, J = 8.0, 2.8 Hz, 1H), 6.54 (d, J = 4.4 Hz, 1H), 6.42 (dd, J = 4.4, 2.4 Hz, 1H), 5.78 (s, 2H), 4.85 (t, J = 6.4 Hz, 2H), 4.51 (t, J = 6.4 Hz, 2H), 3.78 (s, 3H); 13C NMR (100 MHz, CDCl_{3}, Figure A63) δ 192.1, 166.2, 163.7, 162.6, 159.9, 135.7, 134.5, 131.5, 131.4, 130.1, 127.7, 124.7, 124.34, 124.30, 121.6, 121.1, 116.7, 116.5, 115.3, 112.2, 63.3, 55.6, 51.6, 43.6; LRMS m/z calcd for C_{22}H_{20}FN_{2}O_{2}: 363.1; found 363.8 [M]+. Purity of the compound was further confirmed by RP-HPLC by using method 1: R_f = 8.89 min (99% pure; Figure A64).

**Preparation of compound 2h.** As described for the synthesis of compound 1a, compound 9 (0.10 g, 0.47 mmol), 2-bromo-4′-fluoroacetophenone (0.10 g, 0.47 mmol), and EtOH (2.0 mL) were used
to afford compound 2h (0.08 g, 40%, Rf 0.21 in MeOH:CH$_2$Cl$_2$/1:9) as a green solid: $^1$H NMR (400 MHz, CDCl$_3$, Figure A65) $\delta$ 8.03 (dd, $J = 8.4, 4.8$ Hz, 2H), 7.63 (m, 2H), 7.44 (m, 1H), 7.20 (t, $J = 8.4$ Hz, 2H), 7.14 (t, $J = 8.4$ Hz, 2H), 6.62 (d, $J = 2.4$, 1H), 6.50 (dd, $J = 4.4, 2.4$ Hz, 1H), 5.92 (br s, 2H), 4.82 (m, 2H), 4.59 (m, 2H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure A66) $\delta$ 190.9, 168.1, 166.4, 165.5, 163.9, 162.9, 135.4, 131.64, 131.57, 131.5, 129.7, 129.71, 128.0, 124.8, 124.3, 124.27, 116.9, 116.7, 116.6, 116.4, 115.4, 63.4, 51.6, 43.9; LRMS m/z calcd for C$_{21}$H$_{17}$F$_2$N$_2$O: 351.1; found 351.8 [M$^+$]. Purity of the compound was further confirmed by RP-HPLC by using method 1: $R_t = 8.97$ min (100% pure; Figure A67).

![Preparation of compound 2i.](image)

**Preparation of compound 2i.** As described for the synthesis of compound 1a, compound 9 (0.10 g, 0.47 mmol), 2-bromo-4'-chloroacetophenone (0.11 g, 0.47 mmol), and EtOH (2.0 mL) were used to afford compound 2i (0.11 g, 52%, Rf 0.34 in MeOH:CH$_2$Cl$_2$/1:9) as a brown solid: $^1$H NMR (400 MHz, (CD$_3$)$_2$SO, Figure A68) $\delta$ 7.94 (d, $J = 8.4$, 2H), 7.90 (m, 1H), 7.65 (dd, $J = 8.4, 1.2$, 2H), 7.56 (app. t, $J = 7.2$, 2H), 7.45 (app. t, $J = 8.4$ Hz, 2H), 6.74 (m, 1H), 6.61 (m, 1H), 5.53 (s, 2H), 4.59 (t, $J = 6.0$, 2H), 4.33 (t, $J = 6.0$, 2H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure A69) $\delta$ 191.1, 166.1, 163.6, 162.5, 141.3, 135.9, 131.5, 131.42, 131.37, 129.9, 129.3, 127.8, 124.6, 124.2, 124.1, 116.7, 116.4, 115.3, 63.0, 51.5, 43.5; LRMS m/z calcd for C$_{21}$H$_{17}$ClFN$_2$O: 367.1; found 367.8 [M$^+$]. Purity of the compound was further confirmed by RP-HPLC by using method 1: $R_t = 9.52$ min (95% pure; Figure A70).

![Preparation of compound 2j.](image)

**Preparation of compound 2j.** As described for the synthesis of
compound 1a, compound 9 (0.10 g, 0.47 mmol), 2-bromo-4'-bromoacetophenone (0.13 g, 0.47 mmol), and EtOH (2.0 mL) were used to afford compound 2j (0.11 g, 48%, R_f 0.32 in MeOH:CH₂Cl₂/1:9) as a brown solid: ¹H NMR (400 MHz, (CD₃)₂SO, Figure A71) δ 7.90-7.85 (m, 3H), 7.79 (d, J = 8.4 Hz, 2H), 7.57-7.54 (m, 2H), 7.45 (t, J = 8.4 Hz, 2H), 6.74 (dd, J = 4.0, 1.2 Hz, 1H), 6.61 (dd, J = 4.4, 2.4 Hz, 1H), 5.52 (s, 2H), 4.58 (t, J = 5.6, 2H), 4.32 (t, J = 5.6, 2H); ¹³C NMR (100 MHz, CDCl₃, Figure A72) δ 191.3, 166.1, 163.6, 162.5, 135.9, 132.2, 131.8, 131.4, 131.3, 131.2, 130.2, 129.9, 127.8, 124.5, 124.14, 124.11, 116.7, 116.6, 116.4, 115.3, 63.0, 51.5, 43.5; LRMS m/z calcd for C₂₁H₁₇BrFN₂O: 411.1; found 411.8 [M]+. Purity of the compound was further confirmed by RP-HPLC by using method 1: R_t = 9.53 min (99% pure; Figure A73).

Preparation of compound 2k. As described for the synthesis of compound 1a, compound 9 (0.10 g, 0.47 mmol), 2-bromo-4'-methylacetophenone (0.09 g, 0.47 mmol), and EtOH (2.0 mL) were used to afford compound 2k (0.10 g, 57%, R_f 0.33 in MeOH:CH₂Cl₂/1:9) as a yellow solid: ¹H NMR (400 MHz, CDCl₃, Figure A74) δ 7.82 (d, J = 8.4 Hz, 2H), 7.63 (dd, J = 8.0, 4.4 Hz, 2H), 7.45 (t, J = 1.6 Hz, 1H), 7.25 (d, J = 7.2 Hz, 2H), 7.17 (t, J = 8.4 Hz, 2H), 6.59 (dd, J = 4.8, 1.6 Hz, 1H), 6.47 (dd, J = 4.4, 2.4 Hz, 1H), 5.82 (br s, 2H), 4.84 (t, J = 6.0 Hz, 2H), 4.58 (t, J = 6.0 Hz, 2H), 2.38 (s, 3H); ¹³C NMR (100 MHz, CDCl₃, Figure A75) δ 191.8, 166.4, 163.8, 162.9, 146.5, 134.9, 131.6, 130.7, 129.9, 128.7, 127.8, 124.8, 124.3, 116.8, 116.6, 115.3, 63.5, 51.7, 43.9, 21.9; LRMS m/z calcd for C₂₂H₂₀FN₂O: 347.2; found 347.8 [M]+.
Purity of the compound was further confirmed by RP-HPLC by using method 1: $R_t = 9.98$ min (95% pure; Figure A76).

**Preparation of compound 3a.** As described for the synthesis of compound 1a, compound 10 (0.05 g, 0.22 mmol), 2-bromoacetophenone (0.04 g, 0.22 mmol), and EtOH (1 mL) were used to afford compound 3a (0.07 g, 78%, $R_f$ 0.23 in MeOH:CH$_2$Cl$_2$/1:9) as a yellow solid: $^1$H NMR (400 MHz, CDCl$_3$, Figure A77) $\delta$ 7.92 (dd, $J = 8.8$, 1.2 Hz, 2H), 7.63 (dd, $J = 2.0$, 1.2 Hz, 1H), 7.57 (tt, $J = 7.2$, 1.2 Hz, 1H), 7.53-7.40 (m, 4H), 7.27 (m, 1H), 6.57 (dd, $J = 4.4$, 1.2 Hz, 1H), 6.45 (dd, $J = 4.4$, 2.4 Hz, 1H), 5.76 (q, $J = 18.0$ Hz, 2H), 4.86 (t, $J = 6.4$ Hz, 2H), 4.59-4.45 (m, 2H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure A78) $\delta$ 192.0, 161.2, 136.1, 135.0, 133.1, 129.1, 128.5, 127.8, 126.2, 124.6, 119.0, 118.8, 118.7, 118.6, 115.6, 63.3, 51.8, 43.6; LRMS m/z calcd for C$_{21}$H$_{17}$F$_2$N$_2$O: 351.1; found 351.8 [M$^+$]. Purity of the compound was further confirmed by RP-HPLC by using method 1: $R_t = 8.79$ min (98% pure; Figure A79).

**Preparation of compound 3d.** As described for the synthesis of compound 1a, compound 10 (0.06 g, 0.26 mmol), 2-bromo-3'-chloroacetophenone (0.06 g, 0.26 mmol), and EtOH (2 mL) were used to afford compound 3d (0.08 g, 67%, $R_f$ 0.26 in MeOH:CH$_2$Cl$_2$/1:9) as a brown solid: $^1$H NMR (400 MHz, CDCl$_3$, Figure A80) $\delta$ 7.81-7.79 (m, 2H), 7.73 (m, 1H), 7.49-7.45 (m, 2H), 7.40-7.37 (m, 1H), 7.34 (t, $J = 7.2$ Hz, 1H), 7.28-7.21 (m, 1H), 6.55 (dd, $J = 4.4$, 1.6 Hz, 1H), 6.42 (dd, $J = 4.4$, 2.0 Hz, 1H), 5.75 (q, $J = 7.6$ Hz, 2H), 4.86 (t, $J = 6.4$ Hz, 2H), 4.56-4.45 (m, 2H);
$^{13}$C NMR (100 MHz, CDCl$_3$, Figure A81) δ 190.9, 160.9, 136.6, 135.2, 134.7, 134.4, 130.4, 128.0, 127.9, 126.8, 126.0, 124.4, 118.9, 118.7, 118.5, 115.7, 63.1, 51.6, 43.5; LRMS $m/z$ calcd for C$_{21}$H$_{16}$ClF$_2$N$_2$O: 385.1; found 385.8 [M]$^+$. Purity of the compound was further confirmed by RP-HPLC by using method 1: $R_t = 9.94$ min (95% pure; Figure A82).

**Preparation of compound 3h.** As described for the synthesis of compound 1a, compound 10 (0.05 g, 0.22 mmol), 2-bromo-4'-fluoroacetophenone (0.05 g, 0.22 mmol), and EtOH (1 mL) were used to afford compound 3h (0.08 g, 82%, $R_f$ 0.20 in MeOH:CH$_2$Cl$_2$/1:9) as a yellow solid: $^1$H NMR (400 MHz, CDCl$_3$, Figure A83) δ 7.99 (m, 2H), 7.65 (m, 1H), 7.50-7.42 (m, 2H), 7.29 (q, $J$ = 8.4 Hz, 1H), 7.09 (t, $J$ = 8.4 Hz, 2H), 6.58 (dd, $J$ = 4.8, 1.6 Hz, 1H), 6.46 (dd, $J$ = 4.8, 2.4 Hz, 1H), 5.79 (q, $J$ = 18.0 Hz, 2H), 4.86 (t, $J$ = 6.4 Hz, 2H), 4.59-4.47 (m, 2H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure A84) δ 190.5, 168.0, 165.4, 161.2, 151.6, 151.5, 148.9, 148.8, 136.2, 131.6, 131.5, 129.62, 129.59, 127.9, 126.2, 124.8, 124.5, 119.0, 118.8, 118.6, 116.5, 116.3, 115.7, 63.2, 51.7, 43.6; LRMS $m/z$ calcd for C$_{21}$H$_{16}$F$_3$N$_2$O: 369.1; found 369.8 [M]$^+$. Purity of the compound was further confirmed by RP-HPLC by using method 1: $R_t = 9.02$ min (95% pure; Figure A85).

**Preparation of compound 3k.** As described for the synthesis of compound 1a, compound 10 (0.05 g, 0.22 mmol), 2-bromo-4'-methylacetophenone (0.05 g, 0.22 mmol), and EtOH (1 mL) were used to afford compound 3k (0.05 g, 51%, $R_f$ 0.26 in MeOH:CH$_2$Cl$_2$/1:9) as a yellow solid: $^1$H NMR
2.6.1.5. General procedure for aromatization (e.g., Preparation of compound 11)

Aromatization was prepared following a previously published protocol.\textsuperscript{120} 10% Palladium on activated carbon (0.51 g) was added to a solution of compound 8 (0.75 g, 3.82 mmol) in xylenes (10 mL). The mixture was refluxed for 20 h, then cooled and filtered through Celite® and washed with CH\textsubscript{2}Cl\textsubscript{2}. The solution was concentrated under reduced pressure to yield compound 11 (0.44 g, 60%) as a brown liquid, which was used without further purification: \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}, Figure A89, which matches lit.\textsuperscript{121}) δ 7.97-9.94 (m, 2H), 7.78 (dd, J = 4.8, 1.2 Hz, 1H), 7.61 (d, J = 4.8 Hz, 1H), 7.50-7.46 (m, 4H), 6.93 (dt, J = 4.4, 1.2 Hz, 1H), 6.88 (dd, J = 4.4, 2.4 Hz, 1H).

\textbf{Preparation of compound 12.} As described for the synthesis of compound 11, compound 9 (0.65 g, 3.03 mmol), 10% Pd/C (0.40 g), and xylenes (8 mL) were used to afford compound 12 (0.30 g, 48%) as a brown solid: \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}, Figure A90, which matches lit.\textsuperscript{121}) δ 7.94-7.72 (m, 2H), 7.78 (d, J = 4.4, 1.2 Hz, 1H),
7.61 (dd, J = 6.8, 4.8 Hz, 1H), 7.47 (t, J = 2.0 Hz, 1H), 7.18 (t, J = 8.8 Hz, 2H), 6.89 (d, J = 2.4 Hz, 2H).

**Preparation of compound 1a*.** As described for the synthesis of compound 1a, compound 11 (0.10 g, 0.51 mmol), 2-bromoacetophenone (0.10 g, 0.51 mmol), and EtOH (2.0 mL) were used to afford compound 1a* (0.07 g, 35%, Rf 0.20 in MeOH:CH2Cl2/1:9) as a yellow solid: 1H NMR (400 MHz, CDCl3, Figure A91) δ 9.00 (d, J = 5.6 Hz, 1H), 8.54 (dd, J = 2.4, 1.2 Hz, 1H), 7.98 (d, J = 6.0 Hz, 1H), 7.65 (dd, J = 6.8, 1.2 Hz, 2H), 7.30-7.23 (m, 6H), 7.14 (t, J = 8.0 Hz, 2H), 6.93 (dd, J = 4.8, 2.4 Hz, 1H), 6.66 (d, J = 4.8 Hz, 1H), 6.12 (br s, 2H); 13C NMR (100 MHz, CDCl3, Figure A92) δ 191.3, 151.5, 134.6, 133.2, 132.1, 129.4, 128.9, 128.5, 128.4, 128.3, 127.6, 127.0, 126.2, 121.7, 121.0, 117.5, 62.3; LRMS m/z calcd for C21H17N2O: 313.1; found 313.8 [M]+. Purity of the compound was further confirmed by RP-HPLC by using method 1: Rt = 8.99 min (98% pure; Figure A93).

**Preparation of compound 1b*.** As described for the synthesis of compound 1a, compound 11 (0.010 g, 0.051 mmol), 2-bromo-2'-fluoroacetophenone (0.011 g, 0.051 mmol), and EtOH (0.5 mL) were used to afford compound 1b* (0.006 g, 28%, Rf 0.19 in MeOH:CH2Cl2/1:9) as a yellow solid: 1H NMR (400 MHz, CDCl3, Figure A94) δ 9.12 (d, J = 5.6 Hz, 1H), 8.66 (m, 1H), 8.38 (d, J = 5.2 Hz, 1H), 7.90 (t, J = 7.6 Hz, 1H), 7.60 (d, J = 6.8 Hz, 1H), 7.54 (t, J = 7.6 Hz, 3H), 7.49 (t, J = 8.0 Hz, 2H), 7.23-7.20 (m, 2H), 7.08 (dd, J = 10.8, 8.4 Hz 1H), 6.95 (d, J = 4.8 Hz, 1H),
6.09 (br s, 2H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure A95) δ 189.31, 189.26, 163.4, 160.9, 151.3, 136.5, 136.4, 132.3, 130.89, 130.87, 129.5, 128.5, 128.4, 127.7, 127.0, 126.2, 125.0, 124.9, 122.3, 121.8, 121.1, 117.7, 117.0, 116.8, 64.6, 64.5; LRMS m/z calcd for C$_{21}$H$_{16}$FN$_2$O: 331.1; found 331.8 [M$^+$]. Purity of the compound was further confirmed by RP-HPLC by using method 1: $R_t = 8.69$ min (97% pure; Figure A96).

**Preparation of compound 1c*.** As described for the synthesis of compound 1a, compound 11 (0.010 g, 0.051 mmol), 2-bromo-3'-fluoroacetophenone (0.011 g, 0.051 mmol), and EtOH (0.5 mL) were used to afford compound 1c* (0.004 g, 20%, $R_f$ 0.17 in MeOH:CH$_2$Cl$_2$/1:9) as a brown solid: $^1$H NMR (400 MHz, CDCl$_3$, Figure A97) δ 9.05 (d, $J = 6.0$ Hz, 1H), 8.60 (m, 1H), 8.31 (d, $J = 6.0$ Hz, 1H), 7.81 (d, $J = 7.6$ Hz, 1H), 7.60-7.49 (m, 6H), 7.45-7.40 (m, 1H), 7.27-7.21 (m, 2H), 6.96 (d, $J = 4.4$ Hz, 1H), 6.41 (br s, 2H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure A98) δ 190.5, 164.0, 161.5, 151.7, 135.23, 135.16, 132.3, 131.05, 130.97, 129.5, 128.5, 127.6, 127.1, 125.9, 124.85, 124.82, 122.0, 121.9, 121.8, 121.7, 120.9, 117.8, 115.0, 114.8, 62.4; LRMS m/z calcd for C$_{21}$H$_{16}$FN$_2$O: 331.1; found 331.8 [M$^+$]. Purity of the compound was further confirmed by RP-HPLC by using method 1: $R_t = 9.14$ min (95% pure; Figure A99).

**Preparation of compound 1d*.** As described for the synthesis of compound 1a, compound 11 (0.020 g, 0.103 mmol), 2-bromo-3'-chloroacetophenone (0.024 g, 0.103 mmol), and EtOH (1.0 mL) were used to afford compound 1d* (0.023 g, 52%, $R_f$ 0.17 in MeOH:CH$_2$Cl$_2$/1:9) as a brown solid: $^1$H
NMR (400 MHz, CDCl₃, Figure A100) δ 9.23 (d, J = 6.0 Hz, 1H), 8.74 (m, 1H), 8.32 (d, J = 6.0 Hz, 1H), 7.90 (d, J = 7.6 Hz, 1H), 7.81 (s, 1H), 7.59-7.48 (m, 6H), 7.36 (t, J = 7.6 Hz, 1H), 7.20-7.19 (m, 1H), 6.93 (d, J = 4.8 Hz, 1H), 6.39 (br s, 2H); ¹³C NMR (100 MHz, CDCl₃, Figure A101) δ 190.5, 151.5, 135.2, 134.7, 134.5, 132.2, 130.5, 129.5, 128.5, 128.0, 127.6, 127.1, 127.0, 126.3, 121.8, 121.7, 121.1, 117.7, 62.3; LRMS m/z calcd for C₂₁H₁₆ClN₂O: 347.1; found 347.8 [M⁺]. Purity of the compound was further confirmed by RP-HPLC by using method 1: Rₜ = 8.95 min (100% pure; Figure A102).

**Preparation of compound 1e*.** As described for the synthesis of compound 1a, compound 11 (0.020 g, 0.103 mmol), 2-bromo-3’-bromoacetophenone (0.029 g, 0.103 mmol), and EtOH (1.0 mL) were used to afford compound 1e* (10:1 mixture of product and hydrated product) (0.023 g, 48%, Rᵢ 0.17 in MeOH:CH₂Cl₂/1:9) as a yellow solid: ¹H NMR (400 MHz, CDCl₃, Figure A103) for product δ 9.00 (d, J = 6.0 Hz, 1H), 8.57 (m, 1H), 8.30 (d, J = 6.0 Hz, 1H), 7.98 (m, 1H), 7.67 (m, 1H), 7.59 (dt, J = 6.8, 2.4 Hz, 1H), 7.53 (t, J = 7.6 Hz, 2H), 7.50 (t, J = 6.8 Hz, 3H), 7.32 (t, J = 7.6 Hz, 1H), 7.22 (dd, J = 4.4, 2.4 Hz, 1H), 6.96 (d, J = 4.4 Hz, 1H), 6.40 (br s, 2H); ¹H NMR (400 MHz, CDCl₃, Figure A103) for hydrated product δ 8.96 (d, J = 6.0 Hz, 1H), 8.53 (m, 1H), 8.25 (d, J = 6.0 Hz, 1H), 7.96 (m, 1H), 7.71-7.69 (m, 1H), 7.62 (m, 1H), 7.46-7.44 (m, 5H), 7.26 (m, 1H), 7.18 (dd, J = 4.8, 2.4 Hz, 1H), 6.92 (d, J = 4.4 Hz, 1H), 6.35 (br s, 2H), 1.59 (br s, 3H, 1.5xH₂O); ¹³C NMR (100 MHz, CDCl₃, Figure A104) for the mixture of product and hydrated product δ 190.5, 151.7, 137.5, 134.9, 132.3, 131.0, 130.8, 130.7, 129.5, 128.52, 128.48, 127.6, 127.1, 125.9, 123.3, 122.0, 121.8, 120.9, 117.81, 117.78, 62.2; LRMS m/z calcd for C₂₁H₁₆BrN₂O: 391.1; found 391.8 [M⁺]. Purity of the compound was further
confirmed by RP-HPLC by using method 1: $R_t = 9.24$ min (99% pure; Figure A105).

**Preparation of compound 1f**. As described for the synthesis of compound 1a, compound 11 (0.010 g, 0.051 mmol), 2-bromo-3'-hydroxycetophenone (0.011 g, 0.051 mmol), and EtOH (0.5 mL) were used to afford compound 1f* (0.007 g, 34%, $R_f$ 0.11 in MeOH:CH$_2$Cl$_2$/1:9) as a yellow solid:

$^1$H NMR (400 MHz, CDCl$_3$, Figure A106) δ 9.40 (br s, 1H), 8.80 (d, $J = 6.0$ Hz, 1H), 8.48 (m, 1H), 8.01 (d, $J = 6.0$ Hz, 1H), 7.58 (m, 1H), 7.53 (m, 1H), 7.48 (t, $J = 7.6$ Hz, 2H), 7.43 (m, 2H), 7.20-7.17 (m, 2H), 7.07 (d, $J = 8.8$ Hz, 1H), 7.00 (t, $J = 7.6$ Hz, 1H), 6.91 (d, $J = 4.8$ Hz, 1H), 6.02 (br s, 2H);

$^{13}$C NMR (100 MHz, CDCl$_3$, Figure A107) δ 191.2, 157.7, 151.6, 134.1, 132.3, 129.9, 129.8, 129.5, 128.5, 127.4, 127.0, 125.8, 122.5, 121.7, 120.9, 119.0, 117.7, 115.6, 62.1; LRMS m/z calcld for C$_{21}$H$_{17}$N$_2$O$_2$: 329.1; found 329.8 [M]$^+$. Purity of the compound was further confirmed by RP-HPLC by using method 1: $R_t = 8.00$ min (97% pure; Figure A108).

**Preparation of compound 1g**. As described for the synthesis of compound 1a, compound 11 (0.010 g, 0.051 mmol), 2-bromo-3'-methoxyacetophenone (0.012 g, 0.051 mmol), and EtOH (0.5 mL) were used to afford compound 1g* (0.007 g, 32%, $R_f$ 0.23 in MeOH:CH$_2$Cl$_2$/1:9) as a yellow solid:

$^1$H NMR (400 MHz, CDCl$_3$, Figure A109) δ 9.02 (d, $J = 6.0$ Hz, 1H), 8.58 (m, 1H), 8.22 (d, $J = 6.0$, 1H), 7.57-7.45 (m, 7H), 7.31 (t, $J = 7.6$ Hz, 1H), 7.20 (dd, $J = 4.8$, 2.4 Hz, 1H), 7.08 (d, $J = 8.4$, 1H), 6.94 (d, $J = 4.0$, 1H), 6.36 (s, 2H) 3.82 (s, 3H);

$^{13}$C NMR (100 MHz, CDCl$_3$, Figure A109) δ 191.2, 157.7, 151.6, 134.1, 132.3, 129.9, 129.8, 129.5, 128.5, 127.4, 127.0, 125.8, 122.5, 121.7, 120.9, 119.0, 117.7, 115.6, 62.1; LRMS m/z calcld for C$_{21}$H$_{17}$N$_2$O$_2$: 329.1; found 329.8 [M]$^+$. Purity of the compound was further confirmed by RP-HPLC by using method 1: $R_t = 8.00$ min (97% pure; Figure A108).
Figure A110) δ 191.4, 159.9, 151.8, 132.2, 130.1, 129.4, 129.2, 128.6, 127.7, 127.1, 125.8, 122.1, 121.7, 121.2, 121.0, 117.6, 112.3, 62.3, 55.7; LRMS m/z calcd for C_{22}H_{19}N_{2}O_{2}: 343.1; found 343.8 [M]^+. Purity of the compound was further confirmed by RP-HPLC by using method 1: R_t = 8.86 min (99% pure; Figure A111).

**Preparation of compound 1h**. As described for the synthesis of compound 1a, compound 1l (0.010 g, 0.051 mmol), 2-bromo-4'-fluoroacetophenone (0.011 g, 0.051 mmol), and EtOH (0.5 mL) were used to afford compound 1h (2:1 mixture of product and hydrated product) (0.008 g, 38%, R_f 0.17 in MeOH:CH_2Cl_2/1:9) as a yellow solid: ^1H NMR (400 MHz, CDCl_3, Figure A112) for product δ 9.10 (d, J = 6.0 Hz, 1H), 8.63 (m, 1H), 8.25 (d, J = 6.0 Hz, 1H), 8.03-7.99 (m, 2H), 7.58-7.56 (m, 1H), 7.53-7.50 (m, 4H), 7.22-7.20 (m, 1H), 7.11-7.05 (m, 2H), 6.94 (d, J = 4.0 Hz, 1H), 6.39 (br s, 2H); ^1H NMR (400 MHz, CDCl_3, Figure A112) for hydrated product δ 9.04 (d, J = 6.0 Hz, 1H), 8.59 (m, 1H), 8.27 (d, J = 6.0 Hz, 1H), 8.03-7.99 (m, 2H), 7.58-7.56 (m, 1H), 7.53-7.50 (m, 4H), 7.22-7.20 (m, 1H), 7.11-7.05 (m, 2H), 6.95 (d, J = 4.0 Hz, 1H), 6.41 (br s, 2H), 1.58 (br s, 1H, 0.5xH_2O); ^13C NMR (100 MHz, CDCl_3, Figure A113) for the mixture of product and hydrated product δ 190.0, 167.9, 165.3, 151.7, 132.2, 131.6, 131.5, 129.8, 129.7, 129.4, 128.5, 127.6, 127.1, 125.9, 121.9, 121.7, 121.0, 117.7, 116.4, 116.2, 62.1; LRMS m/z calcd for C_{21}H_{16}FN_{2}O: 331.1; found 331.8 [M]^+. Purity of the compound was further confirmed by RP-HPLC by using method 1: R_t = 8.67 min (99% pure; Figure A114).

**Preparation of compound 1i**. As described for the synthesis of
compound 1a, compound 1i (0.010 g, 0.051 mmol), 2-bromo-4'-chloroacetophenone (0.012 g, 0.051 mmol), and EtOH (0.5 mL) were used to afford compound 1i* (8:2 mixture of product and hydrated product) (0.011 g, 50%, Rf 0.23 in MeOH:CH₂Cl₂/1:9) as a yellow solid: ^1^H NMR (400 MHz, CDCl₃, Figure A115) for product δ 9.14 (d, J = 6.0 Hz, 1H), 8.66 (m, 1H), 8.28 (d, J = 6.0 Hz, 1H), 7.90 (d, J = 8.4 Hz, 2H), 7.58-7.55 (m, 1H), 7.52-7.47 (m, 4H), 7.36 (d, J = 8.4 Hz, 2H), 7.20 (dd, J = 4.4, 2.4 Hz, 1H), 6.94 (d, J = 4.4 Hz, 1H), 6.40 (br s, 2H); ^1^H NMR (400 MHz, CDCl₃, Figure A115) for hydrated product δ 9.18 (d, J = 5.6 Hz, 1H), 8.71 (m, 1H), 8.32 (d, J = 5.6 Hz, 1H), 7.94 (d, J = 8.4 Hz, 2H), 7.61-7.59 (m, 1H), 7.52-7.47 (m, 4H), 7.40 (d, J = 8.4 Hz, 2H), 7.28 (m, 1H), 6.98 (d, J = 4.4 Hz, 1H), 6.44 (br s, 2H), 1.58 (br s, 1H, 0.5xH₂O); ^13^C NMR (100 MHz, CDCl₃, Figure A116) for the mixture of product and hydrated product δ 190.4, 151.7, 141.3, 132.3, 131.6, 130.1, 129.45, 129.36, 128.5, 127.6, 127.1, 126.1, 121.82, 121.77, 121.0, 117.7, 62.2; LRMS m/z calcd for C₂₁H₁₆ClN₂O: 347.1; found 347.8 [M]^+. Purity of the compound was further confirmed by RP-HPLC by using method 1: R_t = 9.25 min (99% pure; Figure A117).

![Preparation of compound 1j*](image)

Preparation of compound 1j*. As described for the synthesis of compound 1a, compound 11 (0.010 g, 0.051 mmol), 2-bromo-4'-bromoacetophenone (0.014 g, 0.051 mmol), and EtOH (0.5 mL) were used to afford compound 1j* (0.011 g, 46%, Rf 0.23 in MeOH:CH₂Cl₂/1:9) as a yellow solid: ^1^H NMR (400 MHz, CDCl₃, Figure A118) δ 9.15 (d, J = 6.0 Hz, 1H), 8.68 (m, 1H), 8.28 (d, J = 4.8 Hz, 1H), 7.82 (d, J = 6.8 Hz, 2H), 7.57-7.49 (m, 7H), 7.21 (dd, J = 4.0, 1.6 Hz, 1H), 6.94 (d, J = 4.0 Hz, 1H), 6.39 (br s, 2H); ^13^C NMR (100 MHz, CDCl₃, Figure A119) δ 190.6, 151.7, 132.4, 132.3, 132.0, 130.2, 129.5, 128.5, 127.6, 127.1, 126.1, 121.83, 121.79, 121.0,
Preparation of compound 1k*. As described for the synthesis of compound 1a, compound 11 (0.10 g, 0.51 mmol), 2-bromo-4'-methylacetophenone (0.11 g, 0.51 mmol), and EtOH (2.0 mL) were used to afford compound 1k* (0.05 g, 25%, \( R_f \) 0.24 in MeOH:CH\(_2\)Cl\(_2\)/1:9) as a dark green solid: \(^1\)H NMR (400 MHz, CDCl\(_3\), Figure A121) \( \delta \) 9.15 (dd, \( J = 6.0, 0.8 \) Hz, 1H), 8.70 (dd, \( J = 2.4, 1.2 \) Hz, 1H), 8.21 (d, \( J = 6.0 \) Hz, 1H), 7.82 (d, \( J = 8.4 \) Hz, 2H), 7.56-7.46 (m, 5H), 7.21-7.18 (m, 3H), 6.92 (d, \( J = 4.8 \) Hz, 1H), 6.35 (br s, 2H), 2.34 (s, 3H); \(^{13}\)C NMR (100 MHz, CDCl\(_3\), Figure A122) \( \delta \) 190.7, 145.9, 132.1, 130.8, 129.7, 129.4, 128.7, 128.6, 128.45, 128.37, 127.7, 127.1, 125.9, 122.0, 121.6, 120.9, 117.5, 62.4, 21.8; LRMS \( m/z \) calcd for C\(_{22}\)H\(_{19}\)N\(_2\)O: 327.1; found 327.8 [M]\(^+\). Purity of the compound was further confirmed by RP-HPLC by using method 1: \( R_t \) = 9.25 min (95% pure; Figure A123).

Preparation of compound 2a*. As described for the synthesis of compound 1a, compound 12 (0.10 g, 0.47 mmol), 2-bromoacetophenone (0.09 g, 0.47 mmol), and EtOH (2.0 mL) were used to afford compound 2a* (3:2 mixture of product and hydrated product) (0.09 g, 47%, \( R_f \) 0.25 in MeOH:CH\(_2\)Cl\(_2\)/1:9) as a yellow solid: \(^1\)H NMR (400 MHz, CDCl\(_3\), Figure A124) for product \( \delta \) 8.99 (d, \( J = 6.0 \) Hz, 1H), 8.57 (dd, \( J = 2.4, 1.2 \) Hz, 1H), 8.23 (d, \( J = 6.0 \) Hz, 1H), 7.98-7.95 (m, 2H), 7.62-7.43 (m,
7H), 7.24-7.20 (m, 1H), 6.95 (t, \(J = 4.8\ \text{Hz},\ 1H\)), 6.43 (br s, 2H); \(^1\)H NMR (400 MHz, CDCl\(_3\), Figure A124) for hydrated product \(\delta\) 8.95 (d, \(J = 5.2\ \text{Hz},\ 1H\)), 8.54 (dd, \(J = 1.6, 0.8\ \text{Hz},\ 1H\)), 8.30 (d, \(J = 6.0\ \text{Hz},\ 1H\)), 7.98-7.95 (m, 2H), 7.62-7.43 (m, 7H), 7.24-7.20 (m, 1H), 6.95 (t, \(J = 4.8\ \text{Hz},\ 1H\)), 6.44 (br s, 2H), 1.64 (br s, 4H, 2xH\(_2\)O); \(^{13}\)C NMR (100 MHz, CDCl\(_3\), Figure A125) for the mixture of product and hydrated product \(\delta\) 191.2, 191.1, 165.7, 163.2, 151.4, 150.5, 134.7, 134.5, 133.1, 133.0, 132.1, 131.3, 131.2, 129.3, 129.0, 128.94, 128.89, 128.5, 128.4, 127.5, 127.1, 126.9, 126.2, 126.1, 123.6, 123.5, 121.8, 121.7, 121.6, 121.1, 121.0, 117.5, 117.3, 116.9, 116.7, 62.5, 62.3; LRMS \(m/z\) calcd for C\(_{21}\)H\(_{16}\)FN\(_2\)O: 331.1; found 331.8 [M\(^+\)]. Purity of the compound was further confirmed by RP-HPLC by using method 1: \(R_t = 9.43\ \text{min}\) (99% pure; Figure A126).

**Preparation of compound 2b**. As described for the synthesis of compound 1a, compound 12 (0.100 g, 0.051 mmol), 2-bromo-2'-fluoroacetophenone (0.011 g, 0.051 mmol), and EtOH (0.5 mL) were used to afford compound 2b* (3:2 mixture of product and hydrated product) (0.103 g, 51%, \(R_f\) 0.27 in MeOH:CH\(_2\)Cl\(_2\)/1:9) as a yellow solid: \(^1\)H NMR (400 MHz, CDCl\(_3\), Figure A127) for product \(\delta\) 9.28 (d, \(J = 6.0\ \text{Hz},\ 1H\)), 8.80 (m, 1H), 8.39 (d, \(J = 6.0\ \text{Hz},\ 1H\)), 7.90 (t, \(J = 7.2\ \text{Hz},\ 1H\)), 7.60-7.48 (m, 4H), 7.25-7.19 (m, 3H), 7.10 (t, \(J = 8.0\ \text{Hz},\ 1H\)), 6.93 (d, \(J = 4.8\ \text{Hz},\ 1H\)), 6.13 (s, 2H); \(^1\)H NMR (400 MHz, CDCl\(_3\), Figure A127) for hydrated product \(\delta\) 9.25 (d, \(J = 5.2\ \text{Hz},\ 1H\)), 8.78 (m, 1H), 8.45 (d, \(J = 6.0\ \text{Hz},\ 1H\)), 7.90 (t, \(J = 7.2\ \text{Hz},\ 1H\)), 7.60-7.48 (m, 4H), 7.25-7.19 (m, 3H), 7.07 (t, \(J = 8.0\ \text{Hz},\ 1H\)), 6.94 (d, \(J = 5.2\ \text{Hz},\ 1H\)), 6.13 (s, 2H), 1.84 (br s, 2H, 1xH\(_2\)O); \(^{13}\)C NMR (100 MHz, CDCl\(_3\), Figure A128) for the mixture of product and hydrated product \(\delta\) 189.5, 189.4, 166.2, 163.64, 163.62, 161.1, 151.5, 150.6, 136.85, 136.75,
Preparation of compound 2c*. As described for the synthesis of compound 1a, compound 12 (0.107 g, 0.506 mmol), 2-bromo-3'-fluoroacetophenone (0.1023 g, 0.471 mmol), and EtOH (5.0 mL) were used to afford compound 2c* (1.3:1 mixture of product and hydrated product) (0.123 g, 61%, R<sub>f</sub> 0.13 in MeOH:CH<sub>2</sub>Cl<sub>2</sub>/1:9) as a yellow solid: <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>, Figure A130) for product δ 8.97 (d, J = 5.6 Hz, 1H), 8.55 (dd, J = 2.4, 1.2 Hz, 1H), 8.37 (d, J = 6.0 Hz, 1H), 7.86 (t, J = 7.6 Hz, 1H), 7.62-7.41 (m, 6H), 7.31-7.22 (m, 2H), 6.97 (d, J = 4.8 Hz, 1H), 6.49 (br s, 2H); <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>, Figure A130) for hydrated product δ 8.93 (d, J = 5.2 Hz, 1H), 8.52 (dd, J = 2.4, 1.2 Hz, 1H), 8.43 (d, J = 5.2 Hz, 1H), 7.86 (t, J = 7.6 Hz, 1H), 7.62-7.41 (m, 6H), 7.31-7.22 (m, 2H), 6.98 (d, J = 4.8 Hz, 1H), 6.49 (br s, 2H), 1.65 (br s, 4H, 2xH<sub>2</sub>O); <sup>13</sup>C NMR (100 MHz, (CD<sub>3</sub>)<sub>2</sub>SO, Figure A131) for the mixture of product and hydrated product δ 190.99, 190.97, 190.92, 190.89, 165.2, 163.27, 163.26, 162.7, 160.8, 151.5, 150.7, 135.24, 135.18, 135.1, 132.1, 131.7, 131.6, 131.39, 131.35, 131.31, 131.27, 129.3, 128.7, 127.5, 126.7, 126.6, 126.1, 124.61, 124.58, 124.51, 124.49, 123.87, 123.84, 122.1, 122.0, 121.9, 121.8, 121.64, 121.62, 121.3, 121.03, 120.98, 117.70, 117.66, 116.8, 116.5, 115.1, 115.0, 114.9, 114.8, 61.7; LRMS m/z calcd for C<sub>21</sub>H<sub>15</sub>F<sub>2</sub>N<sub>2</sub>O: 349.1; found 349.1 [M]<sup>+</sup>. Purity of the compound was further confirmed by RP-HPLC by using method 1: R<sub>t</sub> = 8.59 min.
Preparation of compound 2d*. As described for the synthesis of compound 1a, compound 12 (0.108 g, 0.509 mmol), 2-bromo-3'-chloroacetophenone (0.110 g, 0.471 mmol), and EtOH (5.0 mL) were used to afford compound 2d* (1.1:1 mixture of product and hydrated product) (0.087 g, 42%, $R_f$ 0.30 in MeOH:CH$_2$Cl$_2$/1:9) as a yellow solid: $^1$H NMR (400 MHz, CDCl$_3$, Figure A133) for product δ 9.07 (d, $J = 5.2$ Hz, 1H), 8.61 (m, 1H), 8.31 (d, $J = 6.0$ Hz, 1H), 7.95 (t, $J = 7.2$ Hz, 1H), 7.86 (t, $J = 1.2$ Hz, 1H), 7.62-7.48 (m, 5H), 7.40 (td, $J = 7.6$, 5.2 Hz, 1H), 7.25-7.21 (m, 1H), 6.95 (d, $J = 5.2$ Hz, 1H), 6.43 (br s, 2H); $^1$H NMR (400 MHz, CDCl$_3$, Figure A133) for hydrated product δ 9.04 (d, $J = 6.0$ Hz, 1H), 8.59 (m, 1H), 8.36 (d, $J = 6.0$ Hz, 1H), 7.95 (t, $J = 7.2$ Hz, 1H), 7.85 (t, $J = 1.6$ Hz, 1H), 7.62-7.48 (m, 5H), 7.40 (td, $J = 7.6$, 5.2 Hz, 1H), 7.25-7.21 (m, 1H), 6.95 (d, $J = 4.8$ Hz, 1H), 6.43 (br s, 2H), 1.76 (br s, 4H, 2xH$_2$O); $^{13}$C NMR (100 MHz, (CD$_3$)$_2$SO, Figure A134) for the mixture of product and hydrated product δ 191.5, 191.4, 165.6, 163.2, 152.0, 151.2, 135.41, 135.35, 134.91, 134.89, 134.32, 134.27, 132.5, 132.2, 132.1, 131.49, 131.45, 129.7, 129.2, 128.7, 128.5, 127.9, 127.4, 127.3, 127.2, 127.1, 126.5, 124.32, 124.29, 122.53, 122.49, 121.7, 121.51, 121.46, 118.2, 118.1, 117.2, 117.0, 62.1; LRMS $m/z$ calcd for C$_{21}$H$_{15}$ClF$_7$NO: 365.1; found 365.1 [M$^+$]. Purity of the compound was further confirmed by RP-HPLC by using method 1: $R_t = 9.26$ min (100% pure; Figure A135).

Preparation of compound 2e*. As described for the synthesis of compound 1a, compound 12 (0.107 g, 0.504 mmol), 2-bromo-3'-
bromoacetophenone (0.138 g, 0.497 mmol), and EtOH (5.0 mL) were used to afford compound 2e* (1:1 mixture of product and hydrated product) (0.051 g, 22%, Rf 0.31 in MeOH:CH₂Cl₂/1:9) as a yellow solid: ¹H NMR (400 MHz, CDCl₃, Figure A136) for product δ 8.72 (d, J = 5.6 Hz, 1H), 8.44 (d, J = 5.6 Hz, 1H), 8.40-8.36 (m, 1H), 8.06-8.01 (m, 2H), 7.73-7.69 (m, 1H), 7.64-7.50 (m, 4H), 7.36 (t, J = 8.0 Hz, 1H), 7.29-7.24 (m, 1H), 7.00 (d, J = 4.4 Hz, 1H), 6.51 (br s, 2H); ¹H NMR (400 MHz, CDCl₃, Figure A136) for hydrated product δ 8.75 (d, J = 5.2 Hz, 1H), 8.40-8.36 (m, 1H), 8.37 (d, J = 5.6 Hz, 1H), 8.06-8.01 (m, 2H), 7.73-7.69 (m, 1H), 7.64-7.50 (m, 4H), 7.35 (t, J = 8.0 Hz, 1H), 7.29-7.24 (m, 1H), 6.99 (d, J = 4.0 Hz, 1H), 6.51 (br s, 2H), 1.58 (br s, 7H, 3.5xH₂O); ¹³C NMR (100 MHz, (CD₃)₂SO, Figure A137) for the mixture of product and hydrated product δ 190.94, 190.86, 165.2, 162.7, 151.5, 150.8, 137.32, 137.30, 135.2, 135.1, 132.0, 131.7, 131.6, 131.22, 131.18, 131.0, 130.9, 129.3, 128.7, 127.5, 127.3, 127.2, 126.7, 126.6, 126.1, 125.9, 123.9, 123.8, 122.3, 122.2, 122.1, 122.0, 121.2, 117.72, 117.67, 116.7, 116.5, 61.7, 61.6; LRMS m/z calcd for C₂₁H₁₅BrFN₂O: 409.0 found 409.0 [M]+. Purity of the compound was further confirmed by RP-HPLC by using method 1: Rₜ = 8.89 min (100% pure; Figure A138).

Preparation of compound 2f*. As described for the synthesis of compound 1a, compound 12 (0.101 g, 0.476 mmol), 2-bromo-3'-hydroxycetophenone (0.108 g, 0.501 mmol), and EtOH (0.5 mL) were used to afford compound 2f* (1.3:1 mixture of product and hydrated product) (0.146 g, 73%, Rf 0.13 in MeOH:CH₂Cl₂/1:9) as a yellow solid: ¹H NMR (400 MHz, CDCl₃, Figure A139) for product δ 9.24 (br s, 1H), 8.84 (d, J = 6.4 Hz, 1H), 8.51 (m, 1H), 8.08 (t, J = 5.2 Hz, 1H), 7.63 (br t, 1H), 7.57-7.43 (m, 4H), 7.21-7.09 (m, 2H), 6.99 (d, J = 8.0 Hz, 2H), 6.90 (d, J =
4.4 Hz, 2H), 6.05 (br s, 2H); $^1$H NMR (400 MHz, CDCl$_3$, Figure A139) for hydrated product δ 9.24 (br s, 1H), 8.83 (d, $J = 6.4$ Hz, 1H), 8.52 (m, 1H), 8.08 (t, $J = 5.2$ Hz, 1H), 7.60 (br t 1H), 7.57-7.43 (m, 4H), 7.21-7.09 (m, 2H), 6.97 (d, $J = 8.0$ Hz, 2H), 6.91 (d, $J = 4.8$ Hz, 2H), 6.03 (br s, 2H), 1.85 (br s, 3H, 1.5xH$_2$O); $^{13}$C NMR (100 MHz, (CD$_3$)$_2$SO, Figure A140) for the mixture of product and hydrated product δ 191.5, 191.4, 165.1, 162.6, 157.8, 151.4, 150.6, 134.4, 134.3, 132.0, 131.7, 131.6, 130.2, 131.1, 129.2, 128.7, 127.5, 126.7, 126.6, 125.95, 125.93, 123.90, 123.87, 122.04, 121.98, 121.93, 121.4, 121.0, 120.9, 119.2, 119.1, 117.6, 117.5, 116.7, 116.5, 114.3, 114.2, 61.6; LRMS m/z calcd for C$_{21}$H$_{17}$FN$_2$O$_2$: 347.1; found 347.1 [M$^+$]. Purity of the compound was further confirmed by RP-HPLC by using method 1: $R_t = 7.66$ min (99% pure; Figure A141).

**Preparation of compound 2g*.** As described for the synthesis of compound 1a, compound 12 (0.1071 g, 0.505 mmol), 2-bromo-3'-methoxyacetophenone (0.1089 g, 0.475 mmol), and EtOH (5.0 mL) were used to afford compound 2g* (1.7:1 mixture of product and hydrated product) (0.136 g, 66%, R$_f$ 0.37 in MeOH:CH$_2$Cl$_2$/1:9) as a yellow solid: $^1$H NMR (400 MHz, CDCl$_3$, Figure A142) for product δ 9.09 (d, $J = 6.0$ Hz, 1H), 8.65 (m, 1H), 8.24 (d, $J = 6.0$ Hz, 1H), 7.61-7.47 (m, 5H), 7.34 (t, $J = 8.0$ Hz, 2H), 7.24-7.20 (m, 2H), 7.13-7.09 (m, 1H), 6.94 (d, $J = 4.0$ Hz, 1H), 6.41 (s, 2H), 3.84 (s, 3H); $^1$H NMR (400 MHz, CDCl$_3$, Figure A142) for hydrated product δ 9.05 (d, $J = 5.6$ Hz, 1H), 8.62 (m, 1H), 8.30 (d, $J = 5.6$ Hz, 1H), 7.61-7.47 (m, 5H), 7.32 (t, $J = 5.2$ Hz, 2H), 7.24-7.22 (m, 2H), 7.13-7.09 (m, 1H), 6.95 (d, $J = 4.8$ Hz, 1H), 6.43 (s, 2H), 3.84 (s, 3H), 1.71 (br s, 2H, 1xH$_2$O); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure A143) for the mixture of product and hydrated product δ 191.5, 191.4, 166.2, 163.6, 160.3, 160.2, 152.1,
151.2, 134.8, 134.7, 132.5, 131.5, 131.4, 130.43, 130.37, 129.7, 128.8, 127.9, 127.5, 127.4, 126.2, 126.0, 123.88, 123.85, 122.33, 122.30, 122.13, 122.10, 122.0, 121.9, 121.50, 121.48, 121.2, 121.0, 117.9, 117.8, 117.3, 117.1, 112.60, 112.56, 77.4, 63.0, 62.8, 56.0; LRMS m/z calcd for C_{22}H_{18}FN_2O_2: 361.1; found 361.1 [M]^+. Purity of the compound was further confirmed by RP-HPLC by using method 1: \( R_t = 9.45 \) min (100% pure; Figure A144).

**Preparation of compound 2h**. As described for the synthesis of compound 1a, compound 12 (0.1064 g, 0.501 mmol), 2-bromo-4'-fluoroacetophenone (0.1065 g, 0.491 mmol), and EtOH (5.0 mL) were used to afford compound 2h* (3:2 mixture of product and hydrated product) (0.078 g, 39%, \( R_f \) 0.29 in MeOH:CH_2Cl_2/1:9) as a yellow solid: ^1H NMR (400 MHz, CDCl_3, Figure A145) for product \( \delta \) 8.83 (d, \( J = 5.6 \) Hz, 1H), 8.45 (m, 1H), 8.32 (d, \( J = 6.0 \) Hz, 1H), 8.09-8.04 (m, 2H), 7.61-7.51 (m, 3H), 7.26-7.22 (m, 2H), 7.13 (t, \( J = 8.8 \) Hz, 2H), 6.98 (d, \( J = 4.8 \) Hz, 1H), 6.49 (br s, 2H); ^1H NMR (400 MHz, CDCl_3, Figure A145) for hydrated product \( \delta \) 8.79 (d, \( J = 5.6 \) Hz, 1H), 8.42 (m, 1H), 8.37 (d, \( J = 5.6 \) Hz, 1H), 8.09-8.04 (m, 2H), 7.61-7.51 (m, 3H), 7.26-7.22 (m, 2H), 7.11 (t, \( J = 8.4 \) Hz, 2H), 6.99 (d, \( J = 3.6 \) Hz, 1H), 6.49 (br s, 2H), 1.58 (br s, 4H, 2xH_2O); ^13C NMR (100 MHz, CDCl_3, Figure A146) for the mixture of product and hydrated product \( \delta \) 190.1, 190.0, 169.1, 168.3, 168.2, 166.2, 165.7, 165.6, 163.7, 152.2, 151.3, 132.6, 131.99, 131.94, 131.9, 131.5, 131.4, 129.99, 129.97, 129.86, 129.83, 129.7, 128.8, 127.8, 127.5, 127.4, 126.1, 125.9, 123.82, 123.78, 122.37, 122.36, 122.2, 122.0, 121.1, 121.0, 118.0, 117.9, 117.4, 117.2, 116.8, 116.7, 116.6, 116.5, 77.4, 62.8, 62.6; LRMS m/z calcd for C_{21}H_{15}F_2N_2O: 349.1; found 349.1 [M]^+. Purity of the compound was further confirmed by RP-HPLC by using method 1: \( R_t = 8.31 \) min (100% pure; Figure A147).
Preparation of compound 2i*. As described for the synthesis of compound 1a, compound 12 (0.1065 g, 0.502 mmol), 2-bromo-4'-chloroacetophenone (0.114 g, 0.510 mmol), and EtOH (5.0 mL) were used to afford compound 2i* (5:4 mixture of product and hydrated product) (0.128 g, 61%, R_f 0.24 in MeOH:CH₂Cl₂/1:9) as a yellow solid: ¹H NMR (400 MHz, CDCl₃, Figure A148) for product δ 9.12 (d, J = 5.6 Hz, 1H), 8.65 (dd, J = 2.4, 0.8 Hz, 1H), 8.32 (d, J = 5.6 Hz, 1H), 7.96-7.92 (m, 2H), 7.60-7.48 (m, 4H), 7.41-7.38 (m, 2H), 7.25-7.20 (m, 1H), 6.95 (d, J = 4.8 Hz, 1H), 6.46 (br s, 2H); ¹H NMR (400 MHz, CDCl₃, Figure A148) for hydrated product δ 9.08 (d, J = 6.0 Hz, 1H), 8.63 (dd, J = 2.4, 0.8 Hz, 1H), 8.36 (d, J = 6.0 Hz, 1H), 7.96-7.92 (m, 2H), 7.59-7.48 (m, 4H), 7.41-7.38 (m, 2H), 7.25-7.2 (m, 1H), 6.96 (d, J = 5.2 Hz, 1H), 6.46 (br s, 2H), 1.78 (br s, 2H, 1xH₂O); ¹³C NMR (100 MHz, CDCl₃, Figure A149) for the mixture of product and hydrated product δ 190.6, 190.5, 166.2, 163.7, 152.1, 151.1, 141.8, 141.6, 132.6, 131.8, 131.7, 131.5, 131.4, 130.40, 130.36, 129.72, 129.65, 128.8, 127.8, 127.46, 127.36, 126.3, 126.1, 123.81, 123.77, 122.2, 122.0, 121.2, 121.1, 118.0, 117.8, 117.4, 117.2, 77.4, 62.7, 62.6; LRMS m/z calcd for C₂₁H₁₅ClF₂N₂O: 365.1; found 365.1 [M⁺]. Purity of the compound was further confirmed by RP-HPLC by using method 1: R_t = 9.13 min (100% pure; Figure A150).

Preparation of compound 2j*. As described for the synthesis of compound 1b, compound 12 (0.052 g, 0.245 mmol), 2-bromo-4'-bromoacetophenone (0.062 g, 0.224 mmol), and THF (1.25 mL) were
used to afford compound 2j* (0.052 g, 46%, Rf 0.10 in MeOH:CH2Cl2/1:9) as a white solid: 1H NMR (400 MHz, CDCl3, Figure A151) δ 9.06 (d, J = 6.0 Hz, 1H), 8.61-8.60 (m, 1H), 8.32 (d, J = 5.2 Hz, 1H), 7.87 (d, J = 8.4 Hz, 2H), 7.59-7.56 (m, 4H), 7.25-7.21 (m, 3H), 6.96 (d, J = 4.4 Hz, 1H), 6.46 (s, 2H); 13C NMR (100 MHz, CDCl3, Figure A152) δ 190.7, 166.2, 163.7, 151.2, 132.7, 132.1, 131.5, 131.4, 130.8, 130.4, 127.5, 126.2, 123.8, 123.7, 122.3, 121.1, 117.9, 117.4, 117.2, 77.4, 62.8; LRMS m/z calcd for C21H15BrFN2O: 409.0; found 409.0 [M]+. Purity of the compound was further confirmed by RP-HPLC by using method 1: Rf = 9.30 min (100% pure; Figure A153).

Preparation of compound 2k*. As described for the synthesis of compound 1a, compound 12 (0.10 g, 0.47 mmol), 2-bromo-4'-methylacetophenone (0.10 g, 0.47 mmol), and EtOH (2.0 mL) were used to afford compound 2k* (3:2 mixture of product and hydrated product) (0.10 g, 50%, Rf 0.26 in MeOH:CH2Cl2/1:9) as a yellow solid: 1H NMR (400 MHz, CDCl3, Figure A154) for product δ 8.91 (m, 1H), 8.51 (m, 1H), 8.17 (m, 1H), 7.85 (m, 2H), 7.60-7.52 (m, 4H), 7.25-7.23 (m, 3H), 6.95 (d, J = 3.6 Hz, 1H), 6.36 (br s, 2H), 2.37 (s, 3H); 1H NMR (400 MHz, CDCl3, Figure A154) for hydrated product δ 8.88 (m, 1H), 8.49 (m, 1H), 8.25 (m, 1H), 7.85 (m, 2H), 7.60-7.52 (m, 4H), 7.25-7.23 (m, 3H), 6.95 (d, J = 3.6 Hz, 1H), 6.38 (br s, 2H), 2.37 (s, 3H), 1.63 (br s, 4H, 2xH2O); 13C NMR (100 MHz, CDCl3, Figure A155) for the mixture of product and hydrated product δ 190.6, 190.2, 165.7, 163.2, 151.5, 150.6, 146.0, 145.8, 132.0, 131.3, 131.2, 130.6, 130.5, 129.64, 129.59, 129.3, 128.61, 128.58, 128.5, 127.6, 127.1, 127.0, 126.1, 126.0, 123.63, 123.59, 121.7, 121.6, 121.1, 121.0, 117.4, 117.3, 116.9, 116.6, 62.4, 62.2, 21.7; LRMS m/z calcd for C22H18FN2O: 345.1; found 345.8 [M]+. Purity of the
compound was further confirmed by RP-HPLC by using method 1: $R_t = 9.62$ min (99% pure; Figure A156).

2.6.2. Biochemical and biological methods

2.6.2.1. Protein, reagents, and small-molecule libraries

The Eis protein from *Mycobacterium tuberculosis* (Eis-*Mtb*) was expressed and purified as reported previously. All chemicals for biochemical/biological assays, including 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB), Tween® 80, neomycin B (NEO), kanamycin A (KAN), acetyl-CoA (AcCoA), and chlorhexidine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Albumin-dextrose-catalase (ADC) was from BD Biosciences (San Jose, CA, USA). The high-throughput screening (HTS) that identified compound 1a* as capable of inhibiting the acetyltransferase activity of purified Eis-*Mtb* was performed at the Center for Chemical Genomics (CCG, University of Michigan) against 23,000 compounds from three different libraries: (i) a ChemDiv library (20,000), (ii) the BioFocus NCC library, and (iii) the MicroSource MS2000 library. The immediate follow-up hit validation assay to confirm the inhibition of Eis-*Mtb* by compound 1a* was performed by using fresh powder purchased from ChemDiv (San Diego, CA, USA). Compound 1a* was freshly synthesized for all biochemical and structural studies described in sections 2.4-3.2 below. All small molecules synthesized (1a-3k and 1a*-2k*) in this study were dissolved in DMSO (10 mM stock) prior to testing. Note: All concentrations noted below are the final concentrations in the assays. The pH of all buffers was adjusted at rt.
2.6.2.2. Eis chemical library screening

For HTS, as previously described,\textsuperscript{108} the absorbance signal from the reaction of the enzymatically released CoASH with Ellman’s reagent (DTNB) at 412 nm ($e_{412} = 14150 \text{ M}^{-1}\text{cm}^{-1}$) was used to monitor the acetylation by Eis\textsubscript{Mtb}. The reactions (40 mL) contained Tris-HCl (50 mM, pH 8.0), Eis (0.25 mM), NEO (100 mM), AcCoA (40 mM), DTNB (0.5 mM), and library compounds (20 mM). Chlorhexidine (5 mM) and DMSO (0.5%) served as positive and negative controls, respectively. Plates were incubated at room temperature and read on a PHERAstar plate reader 5 min after initiation of the reaction. The average $Z'$ score for the HTS assay was 0.60.

2.6.2.3. Hit validation

The hit compound 1a* was defined as displaying $\geq 3$-fold inhibition than the standard deviation (calculated for the inert compounds) in the HTS. This compound (purchased from ChemDiv) was tested in triplicate. As it exhibited reproducible inhibitory activity in all three trials, we then tested it in dose-response assays in the concentration range from 20 mM to 78 nM (generated by 2-fold dilutions of the compound stock). As this lead compound 1a* showed promise, we synthesized it along with 47 of its analogues and determined their IC\textsubscript{50} values.

2.6.2.4. Inhibition kinetics

For compounds 1a-3k and 1a*-2k* freshly synthesized in this study, the absorbance at 412 nm was measured on a SpectraMax M5 plate reader for the reaction mixtures in 96-well plates.
(Thermo Fisher Scientific). Measurements were taken every 30 s for 10 min. Compounds 1a-3k and 1a*-2k* were first serially dissolved in Tris-HCl (50 mM, pH 8.0, containing 10% v/v DMSO). A mixture (50 mL) of Eis (1 mM), KAN (400 mM), and Tris-HCl (50 mM, pH 8.0) was added to compounds 1a-3k and 1a*-2k* (100 mL) and incubated for 10 min. Reactions were initiated by the addition of a mixture (50 mL) of DTNB (8 mM), AcCoA (2 mM), and Tris-HCl (50 mM, pH 8.0). All assays were performed in triplicate. IC$_{50}$ values were calculated by curved fitting to a Hill plot with KaleidaGraph 3.6 software (Table 2.1 and Figure 2.6).

Figure 2.6. Representative IC$_{50}$ curves.
2.6.2.5. Mycobacterial MIC determination by alamarBlue® assay

*Mtb* strains H37Rv and K204 were inoculated from frozen stocks into Middlebrook 7H9 broth supplemented with ADC (10%), Tween® 80 (0.05%), and glycerol (0.4%), and incubated at 37 °C until turbidity appeared. The cultures were diluted with fresh 7H9 medium to OD<sub>600</sub> of 0.2, further diluted 1:25 in fresh 7H9 medium in 50 mL polypropylene tubes containing glass beads and vortexed for 30 s. The cultures were kept still for 10 min, and then 90 mL aliquots were distributed into the wells of a clear 96-well culture plate. Compounds 1a-3k and 1a*-2k* were initially tested at concentrations that were either 100-fold higher than their IC<sub>50</sub> values or at 100 mM if their IC<sub>50</sub> values was >1 mM, while keeping the DMSO concentration ≤ 1% in test wells. Working stocks for each compound were prepared at concentrations twice that of the desired final concentration in fresh 7H9 medium, and 100 mL of each of these working stocks was added to the 90 mL of bacterial cultures in the wells. The plates were incubated at 37 °C for 24 h in a humid environment before the addition of KAN (10 mL). Growth of H37Rv was evaluated at 2.5, and 1.25 mg/mL KAN while growth of K204 was evaluated at 10, 5, 2.5, and 1.25 mg/mL KAN. The plates were incubated at 37 °C for 6 days after the addition of KAN. Then, 40 mL of alamarBlue® diluted 1:2 in 10% Tween® 80 was added to each well and the plates continued to be incubated 37 °C. The color of each well was preliminarily evaluated 24 h after the addition of alamarBlue®, with a final evaluation after 48 h. AlamarBlue® changes from indigo blue to pink as a result of bacterial growth. The lowest concentration of KAN that resulted in no change in color was defined as the MIC for each concentration of an inhibitory compound. Compound screening was carried out on biological replicates in duplicate. Several controls were included with every compound: uninoculated 7H9, compound and inoculated 7H9 only, inoculated 7H9 + DMSO only, and
inoculated 7H9 only. For each plate, 200 mL of sterile water was added to all perimeter wells to minimize evaporation.

2.6.2.6. Mammalian cytotoxicity assays

Mammalian cytotoxicity assays were performed as previously described with minor modifications. The human embryonic kidney cells HEK-293 (ATCC CRL-1573) were purchased from ATCC (Manassas, VA). The human lung carcinoma epithelial cells A549 (ATCC CCL-185) and the murine macrophage cells J774A.1 (ATCC TIB-67) were generous gifts from Dr. David K. Orren and Dr. David J. Feola (University of Kentucky, Lexington, KY), respectively. The A549 and HEK-293 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (catalog # 11965-092, ThermoFisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS, ATCC, Manassas, VA) and 1% penicillin/streptomycin (ATCC, Manassas, VA) at 37 °C with 5% CO₂. J774A.1 cells were cultured in a different DMEM (catalog # 30-2002, ATCC, Manassas, VA) with the same supplements under the same conditions as those described for the A549 and HEK-293 cells. A549 and HEK-293 cells were dislodged from culturing dish by treating with 0.05% trypsin-0.53 mM EDTA (ATCC, Manassas, VA) when sub-culturing, whereas J774A.1 cells were dislodged from culturing dish by mechanical scraping. For the assessment of the toxicity of the selected compounds (1e*, 1i*, 2c*, 2h*, and 2i*) (Figure 2.2) or KAN alone (Figure 2.6), assays were performed in 96-well tissue culture plates in quadruplicate where HEK-293 and J774A.1 cells were plated at 10,000 cells/well density in 100 mL of the appropriate media and A549 cells were plated at 3,000 cells/well density in the same amount of DMEM. After overnight incubation at 37 °C with 5% CO₂, the old medium was replaced with fresh medium.
containing serially diluted compounds (concentration ranging from 0.78-200 mM with 0.5% DMSO) or KAN (concentration ranging from 2-500 mM). Cells for negative and positive controls were treated with the appropriate medium containing 0.5% DMSO (no DMSO for KAN treated cells) or 0.5% DMSO and 1% v/v triton-X 100® (no DMSO for KAN treated cells), respectively. After 24 h of incubation at 37 °C with 5% CO₂, rezasurin (10 mL of 2 mM solution, Sigma Aldrich, Milwaukee, WI) was added to each well and incubated for 6 h in order to assess the amount of cells viable, as rezasurin can be converted to resorufin, which has excitation and emission wavelengths at λ_{560} and λ_{590} nm, respectively. Fluorescence was measured with a SpectraMax M5 plate reader. The percent cell survival in the presence of each compound alone or KAN alone is summarized in Figure 2.2 and 2.3, respectively. The cytotoxicity assays for these compounds in the presence of 50 mg/mL KAN were performed with the same protocol as described above, except that the DMEM was further supplemented with 50 mg/mL (equivalent to 86 mM) KAN in order to assess the combined toxicity of these compounds in the presence of KAN (Figure 2.2).

2.6.3. Structural biological methods

2.6.3.1. Purification of EisC204A

The active point mutant EisC204A that is less prone to oxidation than the wild-type Eis_Mtb was overexpressed in E. coli BL21 (DE3) and purified as previously reported, with only minor modifications, as follows. The EisC204A-pET28a plasmid was transformed into E. coli BL21 (DE3) chemically competent cells and plated onto LB agar containing KAN (50 mg/mL). After overnight incubation, a single colony from the plate was inoculated into LB
broth (5 mL) containing KAN (50 mg/mL) (LB/KAN). This culture was grown at 37 °C until an OD\textsubscript{600} of 0.5, then the culture was inoculated into 4 L of LB/KAN and grown at 37 °C. At an OD\textsubscript{600} of ~0.1, the culture was transferred to 16 °C for 1.5 h, and then IPTG was added at the final concentration of 0.5 mM. The induced culture was grown for an additional 16-18 h at 16 °C with shaking (200 rpm). All following purification steps were done at 4 °C. The cells were pelleted by centrifugation at 5,000 rpm for 10 min. The cell pellets were resuspended in chilled lysis buffer (NaCl (300 mM), Tris-HCl pH 8.0 (40 mM), glycerol (10% v/v), and b-mercaptoethanol (2 mM)). The cells were then disrupted by sonication and the insoluble material was removed by centrifugation at 35,000×g for 45 min at 4 °C. The supernatant was passed through a 0.45 mm Millex-HV PVDF filter (Millipore, Billerica, MA, USA) and then loaded onto a 5 mL Ni-IMAC HisTrap FF column (GE Healthcare) pre-equilibrated in the lysis buffer. The column was washed with 100 mL of lysis buffer containing 20 mM imidazole and then the protein was eluted in 10 mL of lysis buffer containing 200 mM imidazole. The eluate was concentrated using an Amicon Ultra-15 (10,000 MWCO) centrifugal filter device (Millipore) to the volume of 5 mL. The protein was then purified on a size-exclusion S-200 column (GE Healthcare) equilibrated in gel filtration buffer (Tris-HCl pH 8.0 (40 mM), NaCl (100 mM), and b-mercaptoethanol (2 mM)). The EisC204A-containing fractions were pooled and concentrated using an Amicon Ultra (10,000 MWCO) centrifugal filter device (Millipore) to 4 mg/mL. The pure EisC204A protein was stored on ice at 4 °C.
2.6.3.2. Crystallization, diffraction data collection, and structure determination and refinement of EisC204A-CoA-inhibitor 2k* complex

Crystals of EisC204A-CoA complex were grown by vapor diffusion in hanging drops. The drops were set by mixing 1 mL of concentrated (4 mg/mL) EisC204A protein containing KAN (10 mM), and CoA (8 mM) with 1 μL of the reservoir solution (100 mM Tris-HCl pH 8.5 adjusted at room temperature, 10-15% w/v PEG 8,000, and 0.4 M (NH₄)₂SO₄). The drops were incubated against 1 mL of the reservoir solution at 22 °C. Crystals were grown in 2-3 weeks. The crystals were first transferred into the reservoir solution. Then the (NH₄)₂SO₄ and KAN were exchanged out by a gradual transfer into the reservoir solution lacking (NH₄)₂SO₄: (100 mM Tris-HCl pH 8.5 and 15% w/v PEG 8,000) and incubated for 30 min. Then the crystals were transferred into the cryoprotectant solution (100 mM Tris-HCl pH 8.5, 15% w/v PEG 8,000, and 20% v/v glycerol) and incubated in this solution for 10 min. After the incubation, the crystals were transferred into the cryoprotectant solution containing 0.5 mM 2k*, incubated for 30 min, and then frozen in liquid nitrogen by quick immersion. All crystal transfer and incubation steps were carried out at 22 °C.

The X-ray diffraction data were collected at synchrotron beamline 22-ID of the Advanced Photon Source at the Argonne National Laboratory (Argonne, IL) at 100 K. The data were processed with HKL2000. The crystal form in space group R32 was the same as that of wild-type Eis-CoA-acetamide complex determined previously by our group (PDB code 3R1K). The crystals contained one Eis monomer per asymmetric unit, with the Eis hexamer generated by crystal symmetry operations. For this structure, we performed rigid body refinement by Refmac, with the entire Eis monomer as a single rigid-body domain. After
the rigid body refinement, strong difference $F_o-F_c$ electron density for a bound inhibitor $2k^*$ and a CoA molecule was observed. The conformations of the inhibitor and the CoA were clearly defined by the difference density. We built these molecules into this difference electron density by using Coot. The structure of EisC204A-CoA-inhibitor $2k^*$ complex was then adjusted and refined iteratively using programs Coot and Refmac, respectively. The data collection and structure refinement statistics are given in Table 2.2. The crystal structure coordinates and structure factor amplitudes for EisC204A-CoA-$2k^*$ complex were deposited in the Protein Data Bank with the PDB accession number 5TVJ.

<table>
<thead>
<tr>
<th>Data collection</th>
<th>R32</th>
</tr>
</thead>
<tbody>
<tr>
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<td>R32</td>
</tr>
<tr>
<td>Number of monomers per asymmetric unit</td>
<td>1</td>
</tr>
<tr>
<td>Unit cell dimensions</td>
<td></td>
</tr>
<tr>
<td>$a, b, c$ (Å)</td>
<td>175.5, 175.5, 120.7</td>
</tr>
<tr>
<td>$a, b, g$ (°)</td>
<td>90, 90, 120</td>
</tr>
<tr>
<td>cResolution (Å)</td>
<td>50.0-2.3 (2.34-2.30)$^a$</td>
</tr>
<tr>
<td>$I/s$</td>
<td>24 (2.1)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>99.7 (96.5)</td>
</tr>
<tr>
<td>Redundancy</td>
<td>7.3 (6.9)</td>
</tr>
<tr>
<td>$R_{merge}$</td>
<td>0.13 (0.69)</td>
</tr>
<tr>
<td>Number of unique reflections</td>
<td>30,515</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Structure refinement statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolution (Å)</td>
</tr>
<tr>
<td>$R$ (%)</td>
</tr>
<tr>
<td>$R_{free}$ (%)</td>
</tr>
<tr>
<td>Bond length deviation (rmsd) from ideal (Å)</td>
</tr>
<tr>
<td>Bond angle deviation (rmsd) from ideal (°)</td>
</tr>
</tbody>
</table>
Ramachandran plot statistics\textsuperscript{b}
\begin{itemize}
\item % of residues in most allowed regions 93.5
\item % of residues in additional allowed regions 6.5
\item % of residues in generously allowed regions 0.0 (0 residues)
\item % of residues in disallowed regions 0.0 (0 residues)
\end{itemize}
\textsuperscript{a} Numbers in parentheses indicate the values in the highest-resolution shell. \textsuperscript{b} Indicates Procheck statistics.\textsuperscript{127}

2.7. ACKNOWLEDGMENTS

We thank Martha Larsen, Steve Vander Roest, and Paul Kirchhoff (from the CCG) for their help with HTS.

This study was funded by a National Institutes of Health (NIH) Grant AI090048 (S.G.-T.), a grant from the Firland Foundation (S.G.-T.), a grant from the Center for Chemical Genomics (CCG) at the University of Michigan (S.G.-T), and by startup funds from the College of Pharmacy at the University of Kentucky (S.G.-T. and O.V.T.). S.Y.L.H. is partially supported by a University of Kentucky Presidential Fellowship.

2.8. AUTHORS’ CONTRIBUTIONS

AG and HXN synthesized all compounds.

MJW, KDG, and SYLH performed all biochemical and biological assays.

CSG and CH performed structural biological studies.

HXN, AG, JEP, OVT, and SGT analyzed data and wrote the manuscript.
Potent 1,2,4-triazino[5,6b]indole-3-thioether inhibitors of the kanamycin resistance enzyme Eis from *Mycobacterium tuberculosis*

3.1. ABSTRACT

A common cause of resistance to kanamycin (KAN) in tuberculosis is overexpression of the enhanced intracellular survival (Eis) protein. Eis is an acetyltransferase that multiacetylates KAN and other aminoglycosides, rendering them unable to bind the bacterial ribosome. By high-throughput screening, a series of substituted 1,2,4-triazino[5,6b]indole-3-thioether molecules were identified as effective Eis inhibitors. Herein, we purchased 18 and synthesized 22 new compounds, evaluated their potency, and characterized their steady-state kinetics. Four inhibitors were found not only to inhibit Eis *in vitro*, but also to act as adjuvants of KAN and partially restore KAN sensitivity in a *Mycobacterium tuberculosis* KAN-resistant strain in which Eis is upregulated. A crystal structure of Eis in complex with a potent inhibitor and CoA shows that the inhibitors bind in the aminoglycoside binding site snugly inserted into a hydrophobic cavity. These inhibitors will undergo preclinical development as novel KAN adjuvant therapies to treat KAN-resistant tuberculosis.
3.2. INTRODUCTION

*Mycobacterium tuberculosis* (*Mtb*), the etiological agent of tuberculosis (TB), is a dominant worldwide threat to human health.\textsuperscript{128-129} The evolution of resistance of this bacterium to conventional anti-tubercular drugs has exacerbated the problem.\textsuperscript{130-131} This is particularly concerning as in the last 50 years only two new anti-tubercular agents, bedaquiline (Food and Drug Administration (FDA), USA) and delamanid (European Medicines Agency, EU) were approved by these agencies for treatment of multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB), respectively.\textsuperscript{132} The need to combat drug-resistant TB remains a pressing issue, and work towards developing new anti-tubercular agents continues to be at the forefront of TB research. Several directions are being pursued in TB drug development: (i) investigation of chemical modifications of current TB treatments (*e.g.*, isoniazid and rifampin), (ii) development of agents inhibiting already exploited targets, but capable of avoiding the resistance mechanisms (*e.g.*, KatG and topoisomerase mutations), (iii) development of inhibitors of novel pathways or enzymes that are specific to *Mtb* or bacteria in general (*e.g.*, mycolic acid pathway, amino acid biosynthesis, PanK, and dihydrofolate reductase), and (iv) development of inhibitors of resistance enzymes (*e.g.*, BlaC and Eis) as adjuvants of currently approved anti-tubercular drugs.\textsuperscript{62,133}

Aminoglycosides (AGs) have been used extensively to treat a broad spectrum of bacterial infections since the discovery of streptomycin in the 1950s. The injectable AGs, kanamycin (KAN) and amikacin (AMK), have been efficacious therapeutic agents against *Mtb* strains for decades. However, KAN- and AMK-resistant *Mtb* strains have emerged. *Mtb* has three main mechanisms to overcome the action of KAN and AMK: (i) increased expression of the
enhanced intracellular survival (eis) gene as a result of a mutation in its promoter, increased expression of Eis and the efflux pump, Tap, due to upregulation of transcriptional activator WhiB7, and (iii) ribosome mutations that interfere with AG binding. Upregulation of the eis gene by either mechanism (i) or (ii) confers resistance to KAN in one-third of KAN-resistant clinical isolates. Eis is a unique AG acetyltransferase (AAC) due to its distinct structure and ability to multi-acetylate AGs at several different positions including the 3'-amine of 4,6-disubstituted 2-deoxystreptamine AGs and 4'"-amine of AGs containing an (S)-4-amino-2-hydroxybutyryl group that are not modified by other AACs. It is also of note that one AG modification may not completely abolish the antibacterial activity of an AG, whereas two or more modifications fully eliminate this activity. Eis has a larger and more complex active site than that of other AACs, which results in its broader substrate profile and multi-acetylating activity. The kinetics of AG acetylation by Eis have been studied and this bisubstrate enzyme was found to operate by a random sequential mechanism.

Two potential strategies that may be envisioned to overcome the resistance caused by Eis are: (i) identification of new AGs that are not susceptible to inactivation by Eis, and (ii) discovery of structurally unrelated inhibitors of Eis, which would sensitize the KAN-resistant bacteria to KAN when administered as a combination therapy. Because Eis can acetylate multiple amines on clinically relevant AGs of different structures, the first strategy is not likely to be productive. We recently demonstrated that the second strategy is a useful approach by discovering several structural classes of potent Eis inhibitors and demonstrating that they indeed target Eis in Mtb cells. Diversifying a drug pipeline with different
structural classes early on in the drug development process increases the chances of one of the compounds progressing to clinical applications. Development of drugs that need to act in the *Mtb* cytoplasm is especially complicated by the requirement for these molecules to cross the extremely waxy *Mtb* envelope as well as the membrane of the macrophage, which harbors persistent *Mtb* infections. Therefore, we sought a structural class of Eis inhibitors that would be distinct from those that we discovered previously.

In this study, we present novel 1,2,4-triazino[5,6b]indole-3-thioether-based Eis inhibitors. The structures of these inhibitors are unique as they contain a tricyclic core and a long flexible linker, which allows access to novel binding surfaces of Eis that were unexplored in our previous Eis inhibitor studies. We also introduce a simple and easily scalable synthetic route to derivatize the 1,2,4-triazino[5,6b]indole-3-thioether scaffold. We present kinetic and crystallographic analysis of these inhibitors as well as describe their biological activity as KAN adjuvants. We also perform a thorough comparison of the interactions of this scaffold with those of four previously published divergent Eis inhibitor classes.

### 3.3. RESULTS AND DISCUSSION

#### 3.3.1. Identification of lead compounds and synthetic optimization
Inhibitors of Eis were sought by a high-throughput screening (HTS) assay against a collection of ~23,000 compounds from three different libraries: the BioFocus NCC library (~1,000 compounds), the ChemDiv library (~20,000 compounds), and the MicroSource MS2000 library (~2,000 compounds). The average Z’ score for the HTS was 0.65, indicating assay robustness. Forty-six compounds with a 1,2,4-triazino[5,6b]indole-3-thioether core substituted at three different positions were present in the HTS campaign (Figure 3.1 and 3.2). Out of these 46 compounds, seven molecules (14a, 23a, 23c, 37b, 37d, 39b, and 40b; Figure 3.2), some of which displayed Eis inhibition, were selected for additional investigation (Table 3.1).
Four (14a, 37b, 37d, and 39b) of these seven molecules were confirmed to be Eis inhibitors by dose-response assays. Seventeen additional compounds were purchased to further explore the utility of this scaffold. Based on IC$_{50}$ data obtained for the commercially available compounds against purified Eis (Table 3.1), 22 new derivatives (Figure 3.3) were synthesized. As neomycin B (NEO) was found to be the one of the best substrates for Eis in a previous study, the HTS was performed with NEO as a substrate for optimal signal-to-noise ratio relative to the background hydrolysis of acetyl coenzyme A (AcCoA). However, as KAN is the clinically relevant substrate for inactivation by Eis in Mtb, we determined IC$_{50}$ using KAN (IC$_{50,KAN}$) for all of our synthesized compounds to validate their potential clinical use.
Figure 3.2. Structures of all molecules used in this study along with their origin. *Note:* At the bottom of the figure, the compound numbers listed in orange indicate that the compounds were tested in the HTS only. Those listed in pink were tested in the HTS and repurchased for validation. Those listed in gray were compounds that were not present in the HTS, but were purchased to expand the preliminary SAR study. Those in green were new derivatives.
synthesized to further expand our SAR study. Compound 37b is in purple as it was present in the HTS, repurchased, and re-synthesized to confirm the values obtained for purchased material. Compound 39b is in blue as, not only was it present in the HTS, repurchased, and re-synthesized, but was additionally crystallized with Eis. All compounds (except those listed with orange numbers) had an IC$_{50}$ or/and an MIC$_{KAN}$ value determined as presented in Table 3.2.

The synthesis of all compounds started with N-alkylation of the commercially available compounds 54 and 55 with alkyl halides, to afford compounds 56 and 57, respectively, in quantitative yields. The resulting products were mixed with thiosemicarbazide in the presence of potassium carbonate or cesium carbonate, followed by acidification with concentrated hydrochloric acid to generate the cyclized or uncyclized products 58-59 in 22-52% yields. Subsequently, intermediates 58-59 were reacted with various organo halides to obtain the desired S-substituted products (32 and 34-49) with fluoro or methyl substituents at the C8-position (indicated as X in Figure 3.3) in 14-82% yields. The free hydroxyl group of 32b was methylated in the presence of iodomethane and sodium hydride to generate 33b in 47% yield. Compound 58b was reacted with bromoacetonitrile to afford the desired molecule 47b in 63% yield. Compounds 52-53 were prepared in three steps from 58b. S-Methylation of compound 58b with iodomethane afforded 60b (91% yield), which was oxidized with m-CPBA to give sulfone 61b (45% yield). Compound 61b was reacted with different N,N-dialkylaminoalkylamines to generate the desired compounds 52 and 53.
Figure 3.3. Synthetic schemes used for the synthesis of 24 compounds generated in this study.

3.3.2. Structure-activity-relationship (SAR) study
Table 3.1. IC₅₀ values of synthetic Eis inhibitors and compounds purchased and/or from HTS with activity under 28 µM and MICₖₐ₅ values for *Mtb* H37Rv and *Mtb* K204 when treated in combination with the inhibitor at the listed concentration.

<table>
<thead>
<tr>
<th>Cpd</th>
<th>IC₅₀,KAN (µM)</th>
<th>IC₅₀,NEO (µM)</th>
<th>H₃₇Rv MICₖₐ₅ (µg/mL)</th>
<th>K204 MICₖₐ₅ (µg/mL)</th>
<th>Concentration tested (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>≤1.25</td>
<td>&gt;10</td>
<td></td>
</tr>
<tr>
<td>9a</td>
<td>5.8 ± 0.8</td>
<td>≤1.25</td>
<td>5</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>10k</td>
<td>28 ± 4</td>
<td>≤1.25</td>
<td>10</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>11b</td>
<td>0.23 ± 0.02</td>
<td>0.64 ± 0.15</td>
<td>≤1.25</td>
<td>5</td>
<td>23</td>
</tr>
<tr>
<td>11c</td>
<td>0.27 ± 0.05</td>
<td>1.1 ± 0.2</td>
<td>≤1.25</td>
<td>5-10</td>
<td>27</td>
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<tr>
<td>11d</td>
<td>2.4 ± 0.5</td>
<td>≤1.25</td>
<td>10</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>11e</td>
<td>4.4 ± 0.4</td>
<td>≤1.25</td>
<td>5-10</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>14a</td>
<td>1.5 ± 0.1</td>
<td>5.3 ± 1.0</td>
<td>≤1.25</td>
<td>5</td>
<td>100</td>
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<tr>
<td>16a</td>
<td>3.9 ± 0.9</td>
<td>≤1.25</td>
<td>10</td>
<td>100</td>
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<tr>
<td>23a</td>
<td>&gt;200</td>
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<tr>
<td>24a</td>
<td>&gt;200</td>
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<tr>
<td>25a</td>
<td>13 ± 2</td>
<td>≤1.25, ≤1.25</td>
<td>10</td>
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<tr>
<td>30b</td>
<td>&gt;200</td>
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<tr>
<td>31a</td>
<td>&gt;200</td>
<td></td>
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<td></td>
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<td>33b</td>
<td>&gt;200</td>
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<tr>
<td>34b</td>
<td>0.056 ± 0.006</td>
<td>--</td>
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<td>100</td>
</tr>
<tr>
<td>35b</td>
<td>&gt;200</td>
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<td></td>
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<td></td>
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<tr>
<td>36b</td>
<td>0.43 ± 0.04</td>
<td>0.26 ± 0.07</td>
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<td>2.5-5</td>
<td>43</td>
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<tr>
<td>37b</td>
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<td>0.39 ± 0.14</td>
<td>≤1.25</td>
<td>2.5</td>
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<tr>
<td>37d</td>
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<td>0.21 ± 0.03</td>
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<td>80</td>
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<tr>
<td>37g</td>
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<td>0.5 ± 0.1</td>
<td>≤1.25</td>
<td>5-10</td>
<td>43</td>
</tr>
<tr>
<td>37h</td>
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<td></td>
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<tr>
<td>37i</td>
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<td>1.7 ± 0.5</td>
<td>≤1.25</td>
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<td>56</td>
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<td>38b</td>
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<td>≤1.25-2.5</td>
<td>5-10</td>
<td>7</td>
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<tr>
<td>39b</td>
<td>0.030 ± 0.005</td>
<td>0.15 ± 0.02</td>
<td>≤1.25</td>
<td>2.5-5</td>
<td>13</td>
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</table>
Inhibitor potency (IC$_{50}$) against purified Eis (Tables 3.1 and 3.2, Figure 3.4-3.10) was determined for a total of 46 compounds. Examination of the activity of this series of derivatives of the 1,2,4-triazino[5,6b]indole-3-thioether scaffold (Tables 3.1 and 3.2, Figure 3.2), revealed several SAR insights. Overall, our chemical library can be divided into four chemical series (I, II, III, and IV; Figure 3.2). Series I contains the 1,2,4-triazino[5,6b]indole-3-thioether core with a hydrogen atom at the C8-position, while series II and III contain a
fluoro and a methyl substituent at this position, respectively. In series IV, we replaced the thioether at the C3-position of the core by secondary amines in an effort to understand the biological importance of a sulfur atom at this position.

Table 3.2. IC$_{50}$ values and MIC values of tested compounds.

<table>
<thead>
<tr>
<th>Cp</th>
<th>Status</th>
<th>IC$_{50,KAN}$ (µM)</th>
<th>IC$_{50,NEO}$ (µM)</th>
<th>H37Rv MIC$_{KAN}$ (µg/mL)</th>
<th>K204 MIC$_{KAN}$ (µg/mL)</th>
<th>Concentration tested (µM)</th>
</tr>
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</tr>
<tr>
<td>1b</td>
<td>x</td>
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<td>x</td>
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<td>9a</td>
<td>√√</td>
<td>5.8 ± 0.8</td>
<td></td>
<td>≤1.25</td>
<td>5</td>
<td>100</td>
</tr>
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<td>x</td>
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<td>≤1.25</td>
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<td>≤1.25-2.5</td>
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<td>0.15 ± 0.04</td>
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<td>≤1.25</td>
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<td>46b</td>
<td>S/</td>
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<td>47b</td>
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<td>0.51 ± 0.03</td>
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<td>0.6 ± 0.1</td>
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<td>≤1.25-2.5</td>
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<td>49b</td>
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<td>0.19 ± 0.07</td>
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<td>52</td>
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<td>0.30 ± 0.04</td>
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<td>2.27 ± 0.17</td>
<td>≤1.25</td>
<td>5-10</td>
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</table>

**Symbol legend:**

- Indicates that the compound did not inhibit purified Eis enzyme in the initial HTS.
- Indicates that the compound did not inhibit purified Eis enzyme in the initial HTS, but the compound was purchased as a control and displayed an IC$_{50}$ value >200 µM and was not further pursued for determination of MIC values in *Mtb* H37Rv and *Mtb* K204.
- Indicates that we purchased this compound, not initially in the HTS, which displayed an IC$_{50}$ value >200 µM and was not further pursued for determination of MIC values in *Mtb* H37Rv and *Mtb* K204.
- Indicates that we purchased this compound, which was found to be a


| ✓ | good inhibitor of the purified Eis enzyme, but when tested in Mtb K204 was found to not restore the activity of KAN. |
| Sx-- | Indicates that we synthesized this compound, which displayed an IC_{50} value >200 μM and was not further pursued for determination of MIC values in Mtb H37Rv and Mtb K204. |
| S✓ | Indicates that we synthesized this compound, which was found to be a good inhibitor of the purified Eis enzyme, and when tested in Mtb K204 was found to not restore the activity of KAN. |
| √✓ | Indicates that we purchased this compound, which was found to be a good inhibitor of the purified Eis enzyme, and when tested in Mtb K204 was found to restore the activity of KAN. |
| S✓ | Indicates that we synthesized this compound, which was found to be a good inhibitor of the purified Eis enzyme, and when tested in Mtb K204 was found to restore the activity of KAN. |

Figure 3.4. IC_{50} curves for purchased compounds A. 9a, B. 10k, C. 11b, D. 11c, E. 11d, F. 11e, G. 14a, H. 16a, and I. 25a against Eis by using NEO as the substrate.
Figure 3.5. IC₅₀ curves for purchased compounds A. 37b, B. 37d, C. 37g, D. 37i, E. 39b, and F. 39c against Eis by using NEO as the substrate.

Figure 3.6. IC₅₀ curves for purchased compounds A. 39i and B. 50d, against Eis by using NEO as the substrate. Insets show the double reciprocal plots for the fitted Kᵢ values.
Figure 3.7. IC$_{50}$ curves for purchased compounds that displayed an IC$_{50}$ value ≥1 μM with NEO. A. 11b, B. 11c, C. 14a, D. 37b, E. 37d, F. 37g, G. 37i, H. 39b, I. 39c, J. 39i, and K. 50d against Eis by using KAN as the substrate.
Figure 3.8. IC$_{50}$ curves for synthesized compounds **A. 36b, B. 37b, C. 38b, D. 39b, E. 43b, F. 48b, G. 52, and H. 53** against E_{is} by using NEO as the substrate.
**Figure 3.9.** IC$_{50}$ curves for synthesized compounds A. 34b, B. 36b, C. 37b, D. 37h, E. 38b, F. 39b, G. 43b, H. 43d, and I. 45b against Eis by using KAN as the substrate.
We first assessed the importance of the substituent at the C8-position (H “series I” vs F “series II” vs methyl “series III”). We compared the Eis inhibitory activity of compounds from series I, II, and III, with matching R₁ and R₂ groups when using KAN as the AG substrate (IC₅₀,KAN). To understand whether the fluoro substitution was beneficial, we compared compounds from series I and II. We looked at compound 11b (series I, X = H, R₁ = methyl, and R₂ = 2-(piperidin-1-yl)ethyl; IC₅₀,KAN = 0.23 ± 0.02 µM) and its C8-fluorinated counterpart 39b from series II (IC₅₀,KAN = 0.030 ± 0.005 µM) and noticed that compound 39b was much more potent. Similarly, we observed that compound 11c (series I, X = H, R₁ = ethyl, and R₂ = 2-
(piperidin-1-yl)ethyl; IC$_{50,\text{KAN}}$ = 0.27 ± 0.05 µM) and its C8-fluorinated counterpart 39c from series II (IC$_{50,\text{KAN}}$ = 0.14 ± 0.01 µM) displayed a similar trend, with the fluoro-substituted compound 39c being about twice as potent. These observations suggested that adding a fluorine atom at the C8-position increased the potency of Eas inhibition. We concluded that the strong electronegativity of the fluorine atom may play a critical role in inhibition. Next, we explored the effect of replacing the C8-hydrogen of series I or the C8-fluoro of series II by a methyl group (series III). Compounds 48d (series III, X = methyl, R$_1$ = n-propyl, and R$_2$ = 2-(N,N-diethylamino)ethyl; IC$_{50,\text{KAN}}$ = 0.15 ± 0.02 µM) and its R$_1$ = N5-i-pentyl counterpart 48h (IC$_{50,\text{KAN}}$ = 0.54 ± 0.13 µM) both with a methyl at their C8-position, were slightly more potent than their direct respective counterparts with a fluorine at the C8-position, 37d (IC$_{50,\text{KAN}}$ = 0.8 ± 0.1 µM) and 37h (IC$_{50,\text{KAN}}$ = 1.3 ± 0.1 µM). On the other hand, in the case of compounds 37b (series II, X = F, R$_1$ = methyl, R$_2$ = 2-(N,N-diethylamino)ethyl; IC$_{50,\text{KAN}}$ = 0.13 ± 0.03 µM) and its C8-methylated counterpart 48b (IC$_{50,\text{KAN}}$ = 0.17 ± 0.04 µM) from series III, the IC$_{50,\text{KAN}}$ values were virtually the same. Overall, we observed that compounds with a methyl substituent at their C8-position displayed the same or more potent activity when compared to those with a fluoro substituent, which in turn were more potent than the C8-unsubstituted compounds.

Having analyzed the effect of three substituents at the C8-position of the 1,2,4-triazino[5,6b]indole-3-thioether scaffold, we next explored the effect of modifying the N5-position with eleven different chemical moieties (indicated by the letters a-k following the compound numbers in Figure 3.2). We varied the diversity at N5 by not substituting the amine (a) or by introducing various linear alkyl chains (b-e and g), branched alkyl chains (f and h),
alkylaryl groups (i and j), and an amido group (k). To establish the favorable N5-substituents, as we did during our analysis of the C8-substituent, we compared pairs of compounds that only differed in their N5-substituent. We first looked at the N5-unsubstituted compound 11a (Table 3.2, series I, X = H, R₁ = H, and R₂ = 2-(piperidin-1-yl)ethyl), which was found to not be an inhibitor in our HTS. We next investigated the effect of adding linear alkyl chains at the N5-position. For compounds 11b-d, systematically increasing the size of the R₁ substituent from methyl (b) to ethyl (c) and n-propyl (d) led to 2-fold incremental decrease in Eis inhibitory activity (IC₅₀,NEO = 0.64 ± 0.15 µM, 1.1 ± 0.2 µM, and 2.4 ± 0.5 µM, respectively). This relationship between the size of R₁ and Eis inhibitory activity did not perfectly translate to compounds 37b-d (X = F, R₂ = 2-(N,N-diethylamino)ethyl), 39b-c (X = F, R₂ = 2-(piperidin-1-yl)ethyl), or 48b-d (X = Me, R₂ = 2-(N,N-diethylamino)ethyl). However, we noticed by looking at IC₅₀,KAN values that, generally, a methyl group (R₁ = b) at the N5-position of most compounds resulted in the best inhibitors as demonstrated with compounds 11b, 37b, 39b, 43b, and 48b. When examining the effect of branched alkyl chains (f and h) at the N5-position, we observed that these moieties typically decreased or abolished inhibition of Eis. For the alkyaryl (i and j) and amido (k) functionalities, a concrete conclusion could not be drawn from our data due to a small sample size. Overall, we found that the methyl substituent (R₁ = b) was the most favored out of all N5-modifications tested.

Next, we studied the effect of modifications at the C3-position of the 1,2,4-triazino[5,6b]indole-3-thioether scaffold. Compared to the substituents at the C8- and N5-positions, the groups added at the C3-position contained the highest diversity as a result of various heteroatoms in their structures. Examination of compounds 1-6, 41d, and 42b led us
to conclude that any C3-side chain containing only carbon and hydrogen atoms (e.g., aromatic, unsaturated, or aliphatic chains) resulted in compounds with no Eis inhibitory activity. Likewise, substituents at the C3-position containing oxygen atoms (e.g., carbonyl (compounds 13 and 17-30), hydroxyl (compound 32b), ether (compounds 8a and 33b), morpholine (compounds 12d, 12e, 12j, 15a, 40b, 40c, 40d, and 51d)) or a cyano group (compounds 31a and 47b) were not tolerated. However, we observed that, with the exception of the morpholino and cyano groups, C3-side chains containing a tertiary non-aromatic nitrogen atom located 2-3 carbons away from the sulfur atom yielded potent Eis inhibitors (Figure 3.2). We then closely examined the (i) spatial and (ii) steric effects of these tertiary amines on Eis inhibition. For spatial effects, through comparisons of pairs of compounds that differ by a 2- versus 3-carbon linker, such as compounds 11a and 14a, 36b and 43b, as well as 39b and 45b, we found that, with the exception of 11a, the length of the linker did not greatly influence the IC₅₀ values against purified Eis. To evaluate the steric effects on Eis inhibition, compounds 36b (R₂ = 2-(N,N-dimethylamino)ethyl; IC₅₀,KAN = 0.43 ± 0.04 µM), 37b (R₂ = 2-(N,N-diethylamino)ethyl; IC₅₀,KAN = 0.17 ± 0.04 µM), 38b (R₂ = 2-(pyrrol-1-yl)ethyl; IC₅₀,KAN = 0.07 ± 0.01 µM), and 39b (R₂ = 2-(piperidin-1-yl)ethyl; IC₅₀,KAN = 0.030 ± 0.005 µM) were assessed. In these instances, we recognized that increasing the number of carbons attached to the nitrogen atom from the dimethyl- to the diethylamino functionality correlated with increased Eis inhibitory activity as evidenced by comparison of 36b and 37b. Further cyclization of the diethylamino to the pyrrolyl moiety led to more potent Eis inhibitors as seen when comparing 37b and 38b. Expectedly, this observation was also consistent when comparing the diethylamino-containing analogue 48d (IC₅₀,KAN = 0.15 ± 0.02 µM) with the piperidinyl-containing inhibitor 50d (IC₅₀,KAN = 0.08 ± 0.02 µM). Size expansion of the
pyrrolyl ring in 38b (IC$_{50,KAN}$ = 0.07 ± 0.01 µM) to the piperidinyl ring in 39b (IC$_{50,KAN}$ = 0.030 ± 0.005 µM) additionally suggested that the larger six-membered heterocycle occupied the chemical space more efficiently, which resulted in an improved inhibitor (2-fold improvement). Comparison of compounds 43b (IC$_{50,KAN}$ = 0.10 ± 0.02 µM) and 44b (IC$_{50,KAN}$ = 3.3 ± 0.7 µM) showed that the chemical space around the tertiary amino group has a limit, and the addition of the phenyl group was not well-tolerated. The rigidity of the tertiary amine was explored by assessing compounds 34b (IC$_{50,KAN}$ = 0.056 ± 0.006 µM), 43b (IC$_{50,KAN}$ = 0.10 ± 0.02 µM), and 45b (IC$_{50,KAN}$ = 0.17 ± 0.03 µM). We observed that additional rigidity in the side chain as in 43b led to improved Eis inhibitory activity compared to 43b and 45b. Further substitution of the piperidinyl group of 45b (IC$_{50,KAN}$ = 0.17 ± 0.03 µM), as in 46b (IC$_{50,KAN}$ = 0.14 ± 0.04 µM), was well tolerated. Overall, our study of the R$_2$ side chains demonstrated that side chains containing tertiary amino groups were favorable for Eis inhibition. We suspected that this phenomenon was the result of these tertiary amino groups possessing positive charges, which mimicked the positively charged nature of the AG substrates. Additionally, we found that tertiary amines with higher degree of bulkiness and rigidity were typically more potent Eis inhibitors.

Finally, we explored the importance of the thioether at C3 (series II) by replacing it with an amine (series IV). When comparing series IV compounds 52 (IC$_{50,KAN}$ = 0.23 ± 0.03 µM) and 53 (IC$_{50,KAN}$ = 0.71 ± 0.08 µM) to their counterparts from series II 37b (IC$_{50,KAN}$ = 0.17 ± 0.04 µM) and 43b (IC$_{50,KAN}$ = 0.10 ± 0.02 µM), respectively, we found that the thioether afforded compounds with slightly better (1.4 to 7.1-fold) Eis inhibitory activity.
3.3.3. Inhibition kinetics

Once we completed the analysis of the IC\textsubscript{50} measurements, we aimed to understand the mechanism by which these Eis inhibitors function. For this analysis, we selected seven inhibitors, all with an IC\textsubscript{50} < 1 \mu M when using NEO as the substrate. Data analysis of kinetics as a function of the concentrations of the inhibitors and the substrate indicated that the inhibitors are competitive with NEO and yielded the inhibition constants (\(K_i\), Table 3.3) for compounds 36b, 37b, 37d, 37g, 39b, 39c, 39i, and 50d. Representative plots for 39i and 50d as insets in the IC\textsubscript{50} curves are presented in Figure 3.7. All compounds, with the exception of 39i, showed a \(K_i\) < 1 \mu M. From this data, the best inhibitor is 37b, followed by 39b, 39c, 37g, 36b, 37d = 50d, and 39i. Interestingly, based on the IC\textsubscript{50} values 39i is the best followed by 50d, 39b, 37b = 36b, 39c, 37g, and 37d. These observations indicate that the most effective Eis inhibitors (lowest IC\textsubscript{50} values) may not bind the tightest to the enzyme (lowest \(K_i\) values).

<table>
<thead>
<tr>
<th>Compound</th>
<th>(K_i) (\mu M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>36b</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>37b</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>37d</td>
<td>0.82 ± 0.48</td>
</tr>
<tr>
<td>37g</td>
<td>0.45 ± 0.05</td>
</tr>
<tr>
<td>39b</td>
<td>0.16 ± 0.03</td>
</tr>
<tr>
<td>39c</td>
<td>0.32 ± 0.13</td>
</tr>
<tr>
<td>39i</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>50d</td>
<td>0.89 ± 0.23</td>
</tr>
</tbody>
</table>

3.3.4. Selectivity of inhibitors towards Eis over other AACs

\textit{Mtb} contains another AAC chromosomally encoded in its genome, AAC(2')-Ic.\textsuperscript{83} To
determine the selectivity of the 1,2,4-triazino[5,6b]indole-3-thioether-based Eis inhibitors towards Eis, we also tested the potential ability of these compounds to inhibit other AACs. Previously, only one Eis inhibitor, from a completely unrelated scaffold, displayed inhibitory activity against AAC(2')-Ic. Compounds 36b and 37b were tested for inhibitory activity with AAC(2')-Ic, AAC(3)-IV from *E. coli*, and AAC(6')-Ie from *S. aureus*. No inhibition of these three AACs was observed with these two compounds at concentrations up to 200 µM, indicating that the compounds presented here are highly selective to Eis.

3.3.5. Effect of inhibitors on KAN MIC for *M. tuberculosis*

To determine if the identified Eis inhibitors could restore the activity of KAN in KAN-resistant *Mtb*, the MIC of KAN (MIC<sub>KAN</sub>) was determined in KAN-sensitive *Mtb* H37Rv and KAN-resistant *Mtb* strain K204 for all compounds displaying Eis inhibition (<28 µM). *Mtb* K204 is genetically identical to H37Rv with the exception of one clinically important *eis* promoter mutation upregulating Eis expression resulting in resistance to KAN; therefore, *Mtb* K204 is also an excellent tool for validation of Eis inhibition as a mode of action of these compounds. Inhibitors were tested at concentrations of 100-fold of their respective IC<sub>50,KAN</sub> or at 100 µM when 100× IC<sub>50</sub> was unknown or unachievable. As expected, the Eis inhibitors did not affect the MIC<sub>KAN</sub> in the KAN-sensitive *Mtb* H37Rv strain. Four compounds (36b, 37b, 39b, and 43d) lowered the MIC<sub>KAN</sub> value for K204 from >10 µg/mL in the absence of inhibitors to 2.5-5 µg/mL. Interestingly, compound 43d, while not the most potent Eis inhibitor when tested against the purified Eis, reduced the MIC<sub>KAN</sub> for *Mtb* K204 to 2.5-5 µg/mL. Conversely, two of the most potent inhibitors, 38b and 39i, did not efficiently
restore the activity of KAN in *Mtb* K204. Thus, while many Eis compounds aid in the restoration of KAN activity, there is not always a direct correlation between Eis inhibition and MIC$_{\text{KAN}}$. There may be off-target proteins that bind the compounds and possibly a portion gets caught up in the complex cell wall of *Mtb*, accounting for the large excess required to restore MIC$_{\text{KAN}}$.

Several factors validate Eis inhibition as the likely mechanism of action of these molecules. Compounds increased the sensitivity of *Mtb* K204, but not H37Rv to KAN while not affecting *Mtb* viability in general. Furthermore, we performed MIC$_{\text{KAN}}$ dose-dependence experiments with compounds 36b and 39b using a double-dilution protocol and a concentration range of Eis inhibitors of 0-32 µM (Table 3.4). We observed an inversely proportional relationship between the MIC$_{\text{KAN}}$ for *Mtb* K204 and the concentration of compound implying increasing Eis inhibition as the concentration of compound increased.

<table>
<thead>
<tr>
<th>Concentration tested (µM)</th>
<th>36b</th>
<th>39b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H37Rv MIC$_{\text{KAN}}$ (µg/mL)</td>
<td>K204 MIC$_{\text{KAN}}$ (µg/mL)</td>
</tr>
<tr>
<td>0</td>
<td>1.25, 2.5</td>
<td>20, 20</td>
</tr>
<tr>
<td>0.5</td>
<td>1.25, 1.25</td>
<td>10, 20</td>
</tr>
<tr>
<td>1</td>
<td>1.25, 1.25</td>
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</tr>
<tr>
<td>2</td>
<td>1.25, 1.25</td>
<td>10, 20</td>
</tr>
<tr>
<td>4</td>
<td>0.625, 1.25</td>
<td>10, 10</td>
</tr>
<tr>
<td>8</td>
<td>0.625, 1.25</td>
<td>5, 5</td>
</tr>
</tbody>
</table>

Table 3.4. MIC$_{\text{KAN}}$ values for *Mtb* H37Rv and K204 with varying concentrations of cpds 36b and 39b.
3.3.6. A crystal structure of Eis-CoA-inhibitor 39b complex confirms mechanism of inhibition

To establish the structural basis for Eis inhibition by compounds with a 1,2,4-triazino[5,6b]indole-3-thioether core, we determined the crystal structure of the enzyme in complex with CoA and one of the potent inhibitors, 39b (Figure 3.11). Compound 39b (Figure 3.11A,B) binds at a site that partially overlaps the site occupied by the AG tobramycin (TOB) as well as other Eis inhibitors in the literature (Figure 3.11C). This binding site supports the mode of action of inhibitor 39b as an AG competitive inhibitor. The crystal structure shows that the aromatic 1,2,4-triazino[5,6b]indole core of compound 39b is stabilized by interactions with two neighboring aromatic residues, Trp36 and Phe84 (highlighted in orange) via sandwich π–π stacking interactions (Figure 3.11B). We have previously observed similar π–π interactions with Trp36 and Phe84 during studies with Eis inhibitors of other structural classes (inhibitors a, b, and c; Figure 3.11B,D-F). Additionally, the fluorine atom at the C8-position of the 1,2,4-triazino[5,6b]indole-3-thioether core projects towards a hydrophobic pocket and interacts with the Cγ of Arg37 (~3.3 Å away from the fluorine atom) and the Cδ atoms of Leu63 (~3.6 Å away from the fluorine atom) by hydrophobic interactions (Figure 3.11B). Hydrophobic interactions with Arg37 and Leu63 were also seen with other Eis inhibitors (Figure 3.11B,D-F).
While sharing some interactions with other Eis inhibitor classes, this structural scaffold enabled us to explore a novel inhibitor-Eis interface. The unique tricyclic core of 39b allows the flexible C3-linker to extend towards a large opening in the AG-binding pocket, such that the piperidinyl group on the end of the linker comes into a hydrophobic contact with the aliphatic stem of Glu401. In this conformation, the linker and the piperidinyl group at the end are juxtaposed against the Asp26-Ser32 loop (Figure 3.11B,C). Interactions with Asp26-Ser32 loop appear to be essential for Eis binding of this inhibitor class, because analogues 1b, 32b and 33b, which all have shorter C3 substituents, do not inhibit Eis. A water molecule located 2.8 Å away from the N2-nitrogen of the tricyclic core could form a hydrogen bond with either the hydroxyl of Ser32 or the backbone carbonyl oxygen of Gly29. Furthermore, the sulfur atom of 39b is in a hydrophobic contact with the Cε of Ile28, and the linker makes extensive van der Waals contacts with the backbone of residues 26-28. Two carbons away from the sulfur, the nitrogen atom of the piperidinyl moiety bearing a positive charge at the physiological pH forms a salt bridge with the negatively charged carboxylate of Asp26 (located 3.4 Å away). Even though none of the previous Eis inhibitor classes exploit interactions with the Asp26-Ser32 as extensively as this class, among the previously discovered inhibitor classes, inhibitor b interacts with this region by forming hydrogen bonds with the carboxyl group of Asp26 and the backbone N of Ile28 (Figure 3.11E).

This crystal structure and the SAR will guide future rational fine-tuning of the inhibitors with this new scaffold. First, other C3 substitutions bearing a positive charge could favorably interact with another negatively charged residue (e.g., Glu401 or the terminal carboxyl group). These electrostatic interactions support our previous SAR observations that positively charged
tertiary amine functionalities such as those in compounds 37b or 36b are more tolerated compared to the neutral or partially negatively charged C3 substituents. In the R1 substitution of the tricyclic core, the carbon of the N5-methyl is in close proximity to the terminal carboxyl group of Eis (located 3.4 Å away) and the hydroxyl group of Ser83 (located 3.1 Å away), which explains why N5 alkylation with bulkier groups is not well tolerated. For instance, the IC50,NEO values for compounds 11b, 11c, and 11d increase almost 2 folds for every carbon atom added to the N5-alkyl chain. Additionally, there are a variety of ways to install different hydrogen bond donors at the N5 to take advantage of the polar protein C-terminus and side chain of Ser83. In summary, the crystal structure of the Eis-CoA-inhibitor 39b complex allowed us to compare and contrast the current class of inhibitors with previous inhibitors in the literature, explain some of the observed SAR trends, and gain insight into further optimization of this class of inhibitors. Even though these 1,2,4-triazino[5,6b]indole-3-thioether-based inhibitors are competitive KAN inhibitors as are the other previously published Eis inhibitors, the new inhibitors interact extensively with an underexplored surface of the substrate binding cavity of Eis. This property may help combat resistance to other Eis inhibitor classes that could arise due to point mutations in other inhibitor interacting surfaces.
Figure 3.11. A. Crystal structure of one of the six monomers of the EisC204A-CoA-inhibitor 39b (shown as yellow sticks in a box) complex. Note: For the sake of simplicity, the CoA is omitted in this figure, but is shown in panel C. B. Zoom-in view of the binding pocket of compound 39b. The strong omit $F_o-F_c$ electron density map contoured at 3$\sigma$ generated without compound 39b is shown by a magenta mesh. The amino acid residues interacting with compound 39b are depicted in dark turquoise. The conserved residues that interact with 39b and previously published Eis inhibitors are depicted as orange sticks. The Asp26-Ser32 loop is shown as dark turquoise sticks. The C-terminus is labeled as C-ter. A water molecule is shown as a green sphere. The distances between atoms of the inhibitor 39b and those of amino acid residues of the Eis protein are shown by a dark blue dashed line and are in Å. For compound 39b, the carbon, oxygen, nitrogen, fluorine, and sulfur atoms are colored pale green, red, blue, dark green, and orange, respectively. C. Compound 39b is overlapped with previously published Eis inhibitors. The previously published structure of bound tobramycin (TOB) (PDB ID 4JD6$^{86}$) is in blue. Bound CoA is depicted as red sticks. D. Inhibitor “a”, a pyrrolo[1,5-a]pyrazine-based Eis inhibitor (labeled 2k* in ref$^{141}$, PDB ID 5TVJ), is depicted as green sticks. E. Inhibitor “b”, a sulfonamide-based inhibitor (labeled 39 in ref$^{106}$, PDB ID 5IV0), is depicted as gray sticks. F. Inhibitor “c”, an isothiazole S,S-dioxide heterocyclic core (labeled 11c in ref$^{105}$, PDB ID 5EBV) is depicted as purple sticks. Note: In panels D-F, amino acid residues that interact specifically with the inhibitors presented are depicted in lilac. G. The chemical structures of TOB as well as inhibitors 39b and “a-c”.
3.3.7. Mammalian cytotoxicity of Eis inhibitors

The ability of some molecules bearing the 1,2,4-triazino[5,6b]indole-3-thioether scaffold to restore the activity of KAN in Mtb K204 is highly encouraging. However, to understand the utility of these compounds as potential therapeutic agents, the mammalian cell cytotoxicity needed to be examined. Four of the best compounds (34b, 37b, 39b, and 43d) were tested against three mammalian cell lines (A549 (lung), HEK-293 (kidney), and J774A.1 (macrophage)) alone and in the presence of KAN (50 µg/mL = 86 µM). Compounds were evaluated for mammalian cytotoxicity at concentrations ranging from 0 to 100 µM. Cells displayed 100% survival rate from 0 to 12.5 µM; therefore, we showed the cytotoxicity data only in the range from 12.5 to 100 µM (Figure 3.12). The negative and positive controls used for these experiments were 0.5% DMSO (100% cell survival) and 1% v/v Triton-X 100® in 0.5% DMSO (>5% cell survival), respectively. Overall, we found that the compounds did not display significant toxicity until concentrations of 50 µM with and without KAN (Figure 3.13). Because compounds 34b, 37b and 39b sensitize KAN-resistant Mtb cells to KAN when used at concentrations between 5.6 to 17 µM, where they are not cytotoxic, these molecules are good candidates for further development into therapeutically useful KAN adjuvants for treatment of KAN-resistant Mtb.
Figure 3.12. Mammalian cytotoxicity of selected compounds (34b, 37b, 39b, and 43d) alone (depicted as dark color columns) or in the presence of 50 µg/mL (86 µM) KAN (depicted as light color column directly to the right of the dark color column for the compound alone) against A. A549, B. HEK-293, and C. J774A.1 cells. Note: No cytotoxicity was observed from 0-12.5 µM.
Figure 3.13. Mammalian cell cytotoxicity of KAN in a concentration range of 2.0-500 µM against A. A549, B. HEK-293, and C. J774A.1 cell lines showing that KAN is not toxic at least up to 500 µM.

3.4. CONCLUSION

In summary, by chemical, biochemical, biological, and structural studies, we discovered, optimized, and extensively characterized Eis inhibitors with the 1,2,4-triazinyl-indole scaffold. This scaffold enables inhibitor interactions with a previously underexploited region of the Eis active site. These compounds act as KAN adjuvants against KAN-resistant *Mtb*. This study lays the groundwork for the further optimization of these compounds for clinical applications.

3.5. MATERIALS AND INSTRUMENTATION

3.5.1. Materials and instrumentation for chemistry experiments

All chemicals were bought from Sigma-Aldrich (St. Louis, MO, USA) and AK Scientific (Union City, CA, USA) and used without further purification. Deuterated solvents were obtained from Cambridge Isotope Laboratories Inc. (Tewksbury, MA, USA). TLC (Merck, Silica gel 60 F254) was used to monitor reaction progress. Silica gel (SiO2, Dynamic
Adsorbents Inc. Flash silica gel 32-63u) was used to perform flash column chromatography. Visualization of reaction progress was achieved using one or more of the following methods: UV absorption and a cerium-molybdate stain \((\text{NH}_4)_2\text{Ce(NO}_3)_6\) (5 g), \((\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot 4\text{H}_2\text{O}\) (120 g), \(\text{H}_2\text{SO}_4\) (80 mL), and \(\text{H}_2\text{O}\) (720 mL)). NMR spectra were measured in \(\delta\) (ppm) using the \(^1\text{H}\) NMR CDCl\(_3\) (\(\delta 7.24\) ppm), \(^{13}\text{C}\) NMR CDCl\(_3\) (\(\delta 77.23\) ppm), \(^1\text{H}\) NMR (CD\(_3\))\(_2\)SO (\(\delta 2.50\) ppm), and \(^{13}\text{C}\) NMR (CD\(_3\))\(_2\)SO (\(\delta 39.51\) ppm) as internal standards. Abbreviations used: \(J\) = coupling constants in Hz, app. = apparent, br = broad, d = doublet, dd = doublet of doublets, dt = doublet of triplets, m = multiplet, p = pentet, q = quartet, s = singlet, t = triplet, td = triplet of doublets. \(^1\text{H}\) NMR and \(^{13}\text{C}\) NMR spectra were recorded on a Varian 400 MHz instrument (Palo Alto, CA, USA). Low-resolution electrospray mass spectra (LRMS) were recorded on a liquid chromatography-mass spectrometry using an Agilent 1200 series Quaternary LC system (Santa Clara, CA, USA) equipped with a diode array detector, and Eclipse XDB-C\(_{18}\) column (250 mm \(\times\) 4.6 mm, 5 \(\mu\)m), and an Agilent 6120 Quadrupole MSD mass spectrometer. Further confirmation of purity for these final molecules was obtained by reversed-phase high-performance liquid chromatography (HPLC), which was performed on an Agilent Technologies 1260 Infinity HPLC system (Santa Clara, CA, USA), by using the following general method 1: Flow rate = 1 mL/min; \(\lambda\) = 254 nm; column = Vydac 201SP\textsuperscript{TM} C18, 250 \(\times\) 4.6 mm, 90A 5 \(\mu\)m; Eluents: A = H\(_2\)O + 0.1\% TFA, B = MeCN; gradient profile: starting from 5\% B, increasing from 5\% B to 100\% B over 20 min, holding at 100\% B from 20-27 min, decreasing from 100\% B to 5\% B from 27-30 min. Prior to each injection, the HPLC column was equilibrated for 15 min with 5\% B. All compounds were \(\geq\)95\% pure. All reactions were conducted under nitrogen atmosphere, unless otherwise specified. All yields reported refer to isolated yields.
3.5.2. Materials and instrumentation for biochemistry and biology experiments

UV-Vis assays were performed using a multimode SpectraMax M5 plate reader from Molecular Devices (Sunnyvale, CA) and 96-well plates from Fisher Scientific (Hampton, NH, USA). Reagents for Eis inhibition assays such as 5',5-dithiobis-(2-nitrobenzoic acid) DTNB, Tween® 80, neomycin B (NEO), kanamycin A (KAN), acetyl-CoA (AcCoA), and chlorhexidine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Albumin-dextrose-catalase (ADC) was purchased from BD Biosciences (San Jose, CA, USA). Eis was screened at the Center for Chemical Genomics (CCG, University of Michigan) against ~23,000 compounds from a ChemDiv library (~20,000), a BioFocus NCC library (~1,000 compounds), and a MicroSource MS2000 library (~2,000 compounds). Stock compounds for HTS were dissolved in DMSO. The human embryonic kidney cell line HEK-293 (ATCC CRL-1573) was purchased from ATCC (Manassas, VA, USA). The human lung carcinoma epithelial cell line A549 (ATCC CCL-185) and the murine macrophage cell line J774A.1 (ATCC TIB-67) were kindly provided by Dr. David K. Orren and Dr. David J. Feola (University of Kentucky, Lexington, KY, USA), respectively. The A549 and HEK-293 cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) (ATCC, Manassas, VA, USA) with 10% fetal bovine serum (FBS) (ATCC, Manassas, VA, USA) and 1% penicillin/streptomycin (ATCC, Manassas, VA, USA) at 37 °C with 5% CO₂. The J774A.1 cell line was grown under the same conditions, except that the medium used was a different type of DMEM (catalog # 30-2002, ATCC, Manassas, VA, USA). The A549 and HEK-293 cell lines were dislodged from the tissue culture Petri dish by trypsinization with 0.05%-trypsin-0.53 mM EDTA (ATCC, Manassas, VA, USA) for subculturing. The J774A.1 cell line was dislodged from the tissue
culture Petri dish by mechanical scraping for subculturing. The cells’ confluence was observed by using a Nikon Eclipse TS100 microscope (Minato, Tokyo, Japan). Resazurin was purchased from Sigma Aldrich (Milwaukee, WI, USA). Fluorescence of the resazurin product, resorufin, was detected with a SpectraMax M5 plate reader.

3.6. METHODS

3.6.1. Chemical methods

3.6.1.1. General procedure A for the preparation of the $N$-alkylisatin compounds $56b$, $56d$, $56h$, $57b$, $57c$, $57d$, $57f$, and $57h$

5-Fluoroisatin (54) (2.5 g, 15.2 mmol) or 5-methylisatin (55) (2.45 g, 15.2 mmol), and cesium carbonate (5 g, 15.5 mmol) were added to MeCN (100 mL). The reaction mixture was stirred at 30 min at rt, and then an alkyl halide (30 mmol) was added dropwise over a period of 1-2 h. After complete addition of the alkyl halide, the reaction mixture was stirred at rt for an additional 5-24 h. Upon completion of the reaction (as determined by monitoring by TLC) the solvent was removed under reduced pressure and the solid residue was dissolved in EtOAc (100 mL) and washed with H$_2$O (100 mL). The organic layer was washed with 1 M NH$_4$Cl (10 mL), dried over anhydrous MgSO$_4$, and evaporated to afford the desired products. The syntheses of 5-fluoro-$N$-methylisatin (56b)$^{142}$, 5-fluoro-$N$-propylisatin (56d)$^{143}$, 5-methyl-$N$-ethylisatin (57c)$^{144}$, 5-methyl-$N$-$n$-propylisatin (57d)$^{145}$, 5-methyl-$N$-isopropylisatin (57f)$^{146}$ have been previously reported. Compounds from groups 56 and 57 were used directly in the next step of the synthesis without any further purification. Confirmation of the identity of these intermediates (56b, 56d, 56h, 57b, 57c, 57d, 57f, and 57h) was accomplished by $^1$H
NMR spectroscopy for known compounds and by $^1$H NMR and $^{13}$C NMR, as well as mass spectrometry for compounds 56h, 57b, and 57h.

Preparation of 5-fluoro-N-methylisatin (56b). The known compound 56b was prepared by using general procedure A with methyl iodide (4.3 g, 30 mmol). Red solid (2.7 g, quantitative); R$_f$ 0.10 (Hexanes:EtOAc/4:1); $^1$H NMR (400 MHz, CDCl$_3$, which matches the lit.$^{142}$) $\delta$ 7.34-7.26 (m, 2H), 6.84 (dd, $J_1 = 8.0$ Hz, $J_2 = 3.2$ Hz, 1H), 3.23 (s, 3H).

Preparation of 5-fluoro-N-propylisatin (56d). The known compound 56d was prepared by using general procedure A with propyl bromide (3.7 g, 30 mmol). Red solid (3.1 g, quantitative); R$_f$ 0.20 (Hexanes:EtOAc/4:1); $^1$H NMR (400 MHz, CDCl$_3$, which matches the lit.$^{143}$) $\delta$ 7.30-7.26 (m, 2H), 6.84 (dd, $J_1 = 7.6$ Hz, $J_2 = 3.2$ Hz, 1H), 3.67 (t, $J = 7.2$ Hz, 2H), 1.71 (sextet, $J = 7.2$ Hz, 2H), 0.98 (t, $J = 7.2$ Hz, 3H).

Preparation of 5-fluoro-N-isopentylisatin (56h). Compound 56h was prepared by using general procedure A with 1-bromo-3-methyl butane (4.5 g, 30 mmol). Red solid (3.6 g, quantitative); R$_f$ 0.41 (Hexanes:EtOAc/4:1); $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.31-7.4 (m, 2H), 6.83 (dd, $J_1 = 8.0$ Hz, $J_2 = 3.6$ Hz, 1H), 3.69 (t, $J = 7.6$ Hz, 2H), 1.63 (nonet, $J = 6.4$ Hz, 1H), 1.53 (q, $J = 7.2$ Hz, 2H), 0.95 (d, $J = 6.8$ Hz, 6H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 183.3, 160.6, 158.2, 158.0, 147.2, 124.9, 124.7, 118.5, 118.4,
Preparation of 5-methyl-N-methylisatin (57b). Compound 57b was prepared by using general procedure A with methyl iodide (4.3 g, 30 mmol). Red solid (2.7 g, quantitative); R_f 0.10 (Hexanes:EtOAc/4:1); ^1H NMR (400 MHz, CDCl_3) δ 7.39 (s, 1H), 7.38 (d, J = 6.8 Hz, 1H), 6.76 (d, J = 8.4 Hz, 1H), 3.21 (s, 3H), 2.32 (s, 3H); ^13C NMR (100 MHz, CDCl_3) δ 183.6, 158.3, 149.3, 138.7, 133.6, 125.6, 117.5, 109.7, 26.2, 20.6; LRMS m/z calcd for C_{13}H_{14}FNO_2: 235.1; found 236.1 [M+H]^+. 

Preparation of 5-methyl-N-ethylisatin (57c). The known compound 57c was prepared by using general procedure A with ethyl bromide (3.3 g, 30 mmol). Red solid (2.9 g, quantitative); R_f 0.16 (Hexanes:EtOAc/4:1); ^1H NMR (400 MHz, CDCl_3, which matches the lit.\textsuperscript{144}) δ 7.39 (s, 1H), 7.36 (d, J = 8.4 Hz, 1H), 6.77 (d, J = 8.0 Hz, 1H), 3.74 (q, J = 7.2 Hz, 2H), 2.31 (s, 3H), 1.28 (t, J = 7.2 Hz, 3H).

Preparation of 5-methyl-N-propylisatin (57d). The known compound 57d was prepared by using general procedure A with propyl bromide (3.7 g, 30 mmol). Red solid (3.1 g, quantitative); R_f 0.28 (Hexanes:EtOAc/4:1); ^1H NMR (400 MHz, CDCl_3, which matches the lit.\textsuperscript{145}) δ 7.38 (s, 1H), 7.36 (d, J = 7.6 Hz, 1H), 6.77 (d, J = 7.6 Hz,
Preparation of 5-methyl-N-isopropylisatin (57f). Compound 57f was prepared by using general procedure A with isopropyl bromide (3.7 g, 30 mmol). Red solid (3.1 g, quantitative); Rf 0.25 (Hexanes:EtOAc/4:1); $^1$H NMR (400 MHz, CDCl$_3$, which matches the lit.$^{146}$ δ 7.40 (s, 1H), 7.33 (d, $J = 8.8$ Hz, 1H), 6.89 (d, $J = 8.0$ Hz, 1H), 4.50 (septet, $J = 6.8$ Hz, 1H), 2.30 (s, 3H), 1.48 (d, $J = 6.8$ Hz, 6H).

Preparation of 5-methyl-N-isopentylisatin (57h). Compound 57h was prepared by using general procedure A with 1-bromo-3-methyl butane (4.5 g, 30 mmol). Red solid (3.5 g, quantitative); Rf 0.38 (Hexanes:EtOAc/4:1); $^1$H NMR (400 MHz, CDCl$_3$) δ 7.38 (s, 1H), 7.36 (d, $J = 8.4$ Hz, 1H), 6.75 (d, $J = 7.6$ Hz, 1H), 3.68 (t, $J = 7.6$ Hz, 2H), 2.31 (s, 3H), 1.64 (nonet, $J = 6.8$ Hz, 1H), 1.55 (q, $J = 6.8$ Hz, 2H), 0.96 (d, $J = 6.8$ Hz, 6H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 184.1, 158.3, 148.9, 138.8, 133.6, 125.9, 117.8, 110.1, 38.8, 36.0, 26.1, 22.6, 20.8; LRMS m/z calcd for C$_{14}$H$_{17}$NO$_2$: 231.1; found 232.1 [M+H]$^+$. 

1H), 3.64 (t, $J = 7.6$ Hz, 2H), 2.31 (s, 3H), 1.70 (sextet, $J = 7.6$ Hz, 2H), 0.96 (t, $J = 7.6$ Hz, 3H).
3.6.1.2. General procedure B for the preparation of compounds 58b, 58d, 58h, 59b, 59c,
59d, 59f, and 59h

N-Alkylisatin (1 eq.), thiosemicarbazide (1.2 eq.), and K$_2$CO$_3$ (1.2 eq.) were added to dH$_2$O (50-250 mL) for compounds 58b, 58d, 58h, and 59b. The same protocol was used for compounds 59c, 59d, 59f, and 59h, but the base was changed to Cs$_2$CO$_3$ (1.2 eq.) and the solvent was changed to 30% aq. 1,4-dioxane (5-10 mL) to assist in solubilizing the isatin starting materials. The reaction mixture was stirred at 100 °C for 12-24 h. After cooling to rt, the solid by-products were filtered-off and the filtrate was acidified with conc. HCl to a pH of 1.5-2.5. The precipitated solid was collected by filtration, washed with copious amount of dH$_2$O and dried under vacuum. If no purification methods specified, it indicates that the dried solid was used directly in the next step of the synthesis without any further purification. Note: In some instances (e.g., compounds 58b, 58d, 58h, 59b, and 59f) the cyclized version of the product was obtained, whereas in others, the uncyclized version of the product (e.g., 59c, 59d, and 59h) was obtained. It is important to note that both the cyclized and uncyclized versions for the products can be used in the next synthetic step to generate the desired products.

Preparation of 8-fluoro-2,5-dihydro-5-methyl-3H-1,2,4-triazino[5,6-
b]indole-3-thione (58b). Compound 58b was prepared by using general procedure B with compound 56b (2.0 g, 11.2 mmol), thiosemicarbazide (1.22 g, 13.4 mmol), and K$_2$CO$_3$ (3.09 g, 22.4 mmol). Yellow solid (2.20 g, 42%); $^1$H NMR (400 MHz, (CD$_3$)$_2$SO) $\delta$ 7.92 (dd, $J_1 = 8.0$ Hz, $J_2 = 2.4$ Hz, 1H), 7.68 (dd, $J_1 = 9.2$ Hz, $J_2 = 4.0$ Hz, 1H), 7.57 (app. td, $J_1 = 9.2$ Hz, $J_2 = 2.4$ Hz, 1H), 3.65 (s, 3H); $^{13}$C NMR (100 MHz, (CD$_3$)$_2$SO) $\delta$ 179.1, 160.0,
157.6, 148.6, 140.6, 134.9, 118.9, 118.7, 118.5, 118.4, 113.0, 112.9, 108.5, 108.3, 27.6; LRMS m/z calcd for C\textsubscript{10}H\textsubscript{7}FN\textsubscript{4}S: 234.0; found 235.0 [M+H].

**Preparation of 8-fluoro-2,5-dihydro-5-propyl-3H-1,2,4-triazino[5,6-b]indole-3-thione (58d).** Compound 58d was prepared by using general procedure B with compound 56d (60 mg, 0.48 mmol), thiosemicarbazide (32 mg, 0.35 mmol), and K\textsubscript{2}CO\textsubscript{3} (80 mg, 0.58 mmol). Yellow solid (53 mg, 42%); \(^1\)H NMR (400 MHz, (CD\textsubscript{3})\textsubscript{2}SO) \(\delta 7.85\) (d, \(J = 6.4\) Hz, 1H), 7.70 (dd, \(J_1 = 8.4\) Hz, \(J_2 = 3.6\) Hz, 1H), 7.54-7.47 (m, 1H), 4.12 (t, \(J = 7.2\) Hz, 2H), 1.75 (sextet, \(J = 7.6\) Hz, 2H), 0.89 (t, \(J = 7.2\) Hz, 3H); \(^{13}\)C NMR (100 MHz, (CD\textsubscript{3})\textsubscript{2}SO) \(\delta 179.2, 159.9, 157.5, 148.5, 140.0, 134.9, 119.0, 118.8, 118.7, 118.6, 113.3, 113.2, 108.6, 108.4, 42.6, 20.9, 11.2; LRMS m/z calcd for C\textsubscript{12}H\textsubscript{11}FN\textsubscript{4}S: 262.1; found 262.9 [M+H].

**Preparation of 8-fluoro-2,5-dihydro-5-isopentyl-3H-1,2,4-triazino[5,6-b]indole-3-thione (58h).** Compound 58h was prepared by using general procedure B with compound 56h (100 mg, 0.43 mmol), thiosemicarbazide (48 mg, 0.52 mmol), and K\textsubscript{2}CO\textsubscript{3} (48 mg, 0.86 mmol). Yellow solid (35 mg, 28%); \(^1\)H NMR (400 MHz, (CD\textsubscript{3})\textsubscript{2}SO) \(\delta 7.89\) (d, \(J = 7.6\) Hz, 1H), 7.66 (dd, \(J_1 = 8.8\) Hz, \(J_2 = 3.6\) Hz, 1H), 7.57-7.52 (m, 1H), 4.14 (t, \(J = 6.8\) Hz, 2H), 1.63-1.60 (m, 3H), 0.95 (d, \(J = 5.2\) Hz, 6H); \(^{13}\)C NMR (100 MHz, (CD\textsubscript{3})\textsubscript{2}SO) \(\delta 179.5, 160.3, 157.9, 148.7, 140.1, 135.3, 119.4, 119.2, 119.0, 113.5, 109.1, 108.8, 36.4, 25.8, 27.7, 21.9; LRMS m/z calcd for C\textsubscript{14}H\textsubscript{13}FN\textsubscript{4}S: 290.1; found 291.0 [M+H].
Preparation of 8-methyl-2,5-dihydro-5-methyl-3H-1,2,4-triazino[5,6-b]indole-3-thione (59b). Compound 59b was prepared by using general procedure B with compound 57b (108 mg, 0.62 mmol), thiosemicarbazide (68 mg, 0.74 mmol), and K$_2$CO$_3$ (171 mg, 1.24 mmol). Red solid (104 mg, 45%); $^1$H NMR (400 MHz, (CD$_3$)$_2$SO) δ 7.40-7.70 (m, 1H), 7.49-7.46 (m, 2H), 3.60 (s, 3H), 2.41 (s, 3H); $^{13}$C NMR (100 MHz, (CD$_3$)$_2$SO) δ 178.8, 148.0, 142.3, 135.2, 133.0, 132.6, 121.6, 117.3, 111.2, 27.4, 20.7; LRMS m/z calcd for C$_{11}$H$_{10}$N$_4$S: 230.1; found 231.0 \([M+H]^+\).

Preparation of 2-(1-ethyl-1,2-dihydro-5-methyl-2-oxo-3H-indol-3-ylidene)-hydrazinecarbothiamide (59c). Compound 59c was prepared by using general procedure B with compound 57c (65 mg, 0.34 mmol), thiosemicarbazide (38 mg, 0.41 mmol), and Cs$_2$CO$_3$ (113 mg, 0.82 mmol) in 30% aq. 1,4-dioxane (5 mL). The crude product was purified via flash column chromatography (SiO$_2$, 100% CH$_2$Cl$_2$) to afford compound 59c as an orange solid (22 mg, 25%; $R_f$ 0.39 (CH$_2$Cl$_2$:MeOH/49:1); $^1$H NMR (400 MHz, (CD$_3$)$_2$SO) δ 12.37 (s, 1H), 9.00 (s, 1H), 8.65 (s, 1H), 7.51 (s, 1H), 7.21 (d, $J$ = 8.4 Hz, 1H), 7.05 (d, $J$ = 7.6 Hz, 1H), 3.72 (q, $J$ = 7.2 Hz, 2H), 2.28 (s, 3H), 1.15 (t, $J$ = 7.2 Hz, 3H); $^{13}$C NMR (100 MHz, (CD$_3$)$_2$SO) δ 178.7, 160.4, 140.4, 131.9, 131.5, 131.3, 121.3, 119.4, 109.6, 34.0, 20.6, 12.6; LRMS m/z calcd for C$_{13}$H$_6$N$_4$OS: 262.1; found 263.1 \([M+H]^+\).

Preparation of 2-(1-propyl-1,2-dihydro-5-methyl-2-oxo-3H-indol-3-ylidene)-hydrazinecarbothiamide (59d). Compound 59d was prepared by
using general procedure B with compound 57d (200 mg, 0.98 mmol), thiosemicarbazide (108 mg, 1.18 mmol), and Cs2CO3 (637 mg, 1.96 mmol) in 30% aq. 1,4-dioxane (10 mL). The crude product was purified via recrystallization from EtOH/DMF and then flash column chromatography (SiO2, 100% CH2Cl2, CH2Cl2:MeOH/49:1, CH2Cl2:MeOH/19:1) to afford compound 59d as an orange solid (169 mg, 52%; Rf 0.39 in CH2Cl2:MeOH/49:1); 1H NMR (400 MHz, CDCl3) δ 12.89 (s, 1H), 7.49 (br s, 1H), 7.38 (s, 1H), 7.16 (d, J = 8.0 Hz, 1H), 6.76 (d, J = 8.0 Hz, 1H), 6.52 (br s, 1H), 3.67 (t, J = 6.8 Hz, 2H), 2.34 (s, 3H), 1.70 (sextet, J = 6.8 Hz, 2H), 0.94 (t, J = 6.8 Hz, 3H); 13C NMR (100 MHz, CDCl3) δ 180.2, 141.5, 133.0, 132.8, 132.2, 124.6, 121.7, 119.5, 109.5, 41.6, 21.2, 21.1, 11.6; LRMS m/z calcd for C13H6N4OS: 276.1; found 277.0 [M+H]+.

Preparation of 8-methyl-2,5-dihydro-5-isopropyl-3H-1,2,4-triazino[5,6-b]indole-3-thione (59f). Compound 59f was prepared by using general procedure B with compound 57f (80 mg, 0.393 mmol), thiosemicarbazide (43 mg, 0.471 mmol), and Cs2CO3 (255 mg, 0.786 mmol) in 30% aq. 1,4-dioxane (10 mL). The crude product was purified via recrystallization from EtOH/DMF and then flash column chromatography (SiO2, 100% CH2Cl2, CH2Cl2:MeOH/49:1, CH2Cl2:MeOH/19:1) to afford compound 59f as a yellow solid (22 mg, 22%; Rf 0.35 in CH2Cl2:MeOH/49:1); 1H NMR (400 MHz, CDCl3) δ 7.85 (s, 1H), 7.69 (d, J = 8.4 Hz, 1H), 7.49 (d, J = 8.4 Hz, 1H), 5.03 (septet, J = 6.8 Hz, 1H), 2.43 (s, 3H), 1.56 (d, J = 6.8 Hz, 6H); 13C NMR (100 MHz, (CD3)2SO) δ 178.4, 147.5, 140.6, 135.2, 132.7, 132.5, 121.8, 118.0, 112.7, 45.7, 20.6, 19.5; LRMS m/z calcd for C13H14N4S: 258.1; found 259.0 [M+H]+.
Preparation of 2-(1-isopentyl-1,2-dihydro-5-methyl-2-oxo-3H-indol-3-ylidene)-hydrazinecarbothiamide (59h). Compound 59h was prepared by using general procedure B with compound 57h (110 mg, 0.48 mmol), thiosemicarbazide (52 mg, 0.57 mmol), and Cs$_2$CO$_3$ (313 mg, 0.96 mmol) in 30% aq. 1,4-dioxane (10 mL). The crude product was further purified via recrystallization from EtOH/DMF to afford compound 59h as an orange solid (40 mg, 29%; R$_f$ 0.42 in CH$_2$Cl$_2$:MeOH/49:1); $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 12.91 (s, 1H), 7.49 (br s, 1H), 7.38 (s, 1H), 7.16 (dd, $J_1$ = 8.0 Hz, $J_2$ = 2.0 Hz, 1H), 6.75 (d, $J$ = 8.0 Hz, 1H), 6.43 (br s, 1H), 3.72 (t, $J$ = 7.6 Hz, 2H), 2.34 (s, 3H), 1.65-1.52 (m, 3H), 0.96 (d, $J$ = 6.4 Hz, 6H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 180.2, 161.2, 141.4, 133.0, 132.8, 132.3, 121.7, 119.6, 109.4, 38.4, 36.3, 26.1, 22.6, 21.2; LRMS m/z calcd for C$_{15}$H$_{20}$N$_4$OS: 304.1; found 305.0 [M+H]$^+$.  

3.6.1.3 General procedure C for the preparation of compounds 32b, 34b, 35b, 36b, 37b, 37h, 38b, 39b, 42b, 43b, 43d, 44b, 45b, 46b, 48b, 48c, 48d, 48f, 48h, and 49b

Compound 58 or 59 (1 eq.), an organo halide (1.1 eq.), KI (3 eq.), and K$_2$CO$_3$ (1.2 eq.) were added to DMF (5-25 mL). The reaction mixture was stirred at 100 °C for 6-12 h, and then allowed to cool to rt prior to quenching by addition of H$_2$O. The precipitate formed during quenching of the reaction was filtered and purified by column chromatography (SiO$_2$, CH$_2$Cl$_2$:MeOH/95:5 or 97:3) to afford the desired products.
Preparation of 3-{(8-fluoro-5-methyl-5H-[1,2,4]triazino[5,6-b]indol-3-yl)thio}ethan-1-ol (32b). Compound 32b was prepared by using general procedure C with compound 58b (300 g, 1.28 mmol), 2-bromoethanol (176 mg, 1.41 mmol), KI (640 mg, 3.84 mmol), and K$_2$CO$_3$ (210 mg, 1.54 mmol). Yellow solid (130 mg, 36%); $R_f$ 0.38 (CH$_2$Cl$_2$:MeOH/9:1); $^1$H NMR (400 MHz, (CD$_3$)$_2$SO, Figure B1) δ 7.78 (d, $J = 8.0$ Hz, 1H), 7.54 (dd, $J_1 = 8.8$ Hz, $J_2 = 4.0$ Hz, 1H), 7.46 (t, $J = 8.8$ Hz, 1H), 3.73 (t, $J = 7.2$ Hz, 2H), 3.56 (br s, 1H), 3.54 (s, 3H), 3.20 (t, $J = 7.2$ Hz, 2H); $^{13}$C NMR (100 MHz, (CD$_3$)$_2$SO, Figure B2) δ 1557.3, 154.6, 154.4, 140.78, 140.77, 118.9, 118.8, 117.6, 117.3, 112.2, 112.1, 107.8, 107.5, 34.4, 31.5, 27.1; LRMS m/z calcd for C$_{12}$H$_{11}$FN$_4$OS: 278.1; found 279.1 [M+H]$^+$. Purity of the compound was further confirmed by RP-HPLC by using method 1: $R_t = 5.02$ min (97% pure; Figure B3).

Preparation of 8-fluoro-5-methyl-3-{{(1-methylpiperidin-3-yl)methyl}thio}-5H-[1,2,4]triazino[5,6-b]indole (34b). Compound 34b was prepared by using general procedure C with compound 58b (40 mg, 0.17 mmol), 2-(chloromethyl)-1-methylpiperidine hydrochloride (35 mg, 0.19 mmol), KI (85 mg, 0.51 mmol), and K$_2$CO$_3$ (28 mg, 0.20 mmol). Dark yellow solid (23 mg, 39%); $R_f$ 0.55 (CH$_2$Cl$_2$:MeOH/97:3); $^1$H NMR (400 MHz, CDCl$_3$, Figure B4) δ 8.03 (d, $J = 8.0$ Hz, 1H), 7.45-7.30 (m, 2H), 3.80 (s, 3H), 3.36 (dd, $J_1 = 13.6$ Hz, 6.4 Hz, 1H), 3.23 (dd, $J_1 = 13.6$ Hz, 7.6 Hz, 1H), 3.03 (d, $J = 10.8$ Hz, 1H), 2.75 (d, $J = 11.2$ Hz, 1H), 2.27 (s, 3H), 2.15-2.05 (m, 1H), 2.00-1.80 (m, 3H), 1.75-1.55 (m, 2H), 1.18-1.05 (m, 1H); $^{13}$C NMR (400 MHz, CDCl$_3$, Figure B5) δ 169.0, 160.5, 158.0, 147.1, 140.8, 137.8, 119.3, 118.4, 118.2, 111.0, 110.9, 108.7,
108.4, 61.4, 56.2, 46.8, 36.2, 35.1, 30.1, 27.5, 25.2; LRMS \( m/z \) calcd for \( \text{C}_{17}\text{H}_{20}\text{FN}_{5}\text{S} \): 345.1; found 346.0 [M+H]\(^+\). Purity of the compound was further confirmed by RP-HPLC by using method 1: \( R_t = 7.90 \) min (99% pure; Figure B6).

**Preparation of 8-fluoro-5-methyl-3-[(pyridin-3-ylmethyl)thio]-5\(H\)-[1,2,4]triazino[5,6-b]indole (35b).** Compound 35b was prepared by using general procedure C with compound 58b (49 mg, 0.21 mmol), 3-(chloromethyl)pyridine hydrochloride (38 mg, 0.23 mmol), KI (104 mg, 0.63 mmol), and K\(_2\)CO\(_3\) (34 mg, 0.24 mmol). Brownish solid (46 mg, 68%); \( R_f \) 0.55 (CH\(_2\)Cl\(_2\):MeOH/97:3); \(^1\)H NMR (400 MHz, CDCl\(_3\), Figure B7) \( \delta \) 8.67 (s, 1H), 8.35 (d, \( J = 3.6 \) Hz, 1H), 7.88 (d, \( J = 7.2 \) Hz, 1H), 7.76 (d, \( J = 7.6 \) Hz, 1H), 7.28-7.20 (m, 2H), 7.16-7.12 (m, 1H), 4.44 (s, 2H), 3.67 (s, 3H); \(^{13}\)C NMR (100 MHz, CDCl\(_3\), Figure B8) \( \delta \) 167.6, 160.5, 158.1, 150.4, 148.6, 147.0, 141.2, 141.1, 137.7, 136.9, 133.8, 123.6, 119.1, 119.0, 118.7, 118.5, 111.1, 111.0, 108.7, 108.5, 32.5, 27.6; LRMS \( m/z \) calcd for \( \text{C}_{16}\text{H}_{12}\text{FN}_5\text{S} \): 325.1; found 326.0 [M+H]\(^+\). Purity of the compound was further confirmed by RP-HPLC by using method 1: \( R_t = 7.38 \) min (99% pure; Figure B9).

**Preparation of 2-[(8-fluoro-5-methyl-5\(H\)-[1,2,4]triazino[5,6-b]indol-3-yl)thio]-\(N,N\)-dimethylethan-amine (36b).** Compound 36b was prepared by using general procedure C with compound 58b (23 mg, 0.10 mmol), 2-chloro-\(N,N\)-dimethylethylamine hydrochloride (16 mg, 0.11 mmol), KI (49 mg, 0.30 mmol), and K\(_2\)CO\(_3\) (16 mg, 0.12 mmol). Yellow solid (12 mg, 40%); \( R_f \) 0.60 (CH\(_2\)Cl\(_2\):MeOH/97:3);
\(^1\)H NMR (400 MHz, CDCl\(_3\), Figure B10) \(\delta\) 8.07-8.03 (m, 1H), 7.43-7.35 (m, 2H), 3.80 (s, 3H), 3.49 (t, \(J = 7.2\) Hz, 2H), 2.79 (t, \(J = 7.2\) Hz, 2H), 2.35 (s, 6H); \(^{13}\)C NMR (100 MHz, CDCl\(_3\), Figure B11) \(\delta\) 169.2, 160.8, 158.4, 147.6, 138.2, 119.6, 118.9, 118.6, 111.4, 111.3, 109.2, 108.9, 58.9, 45.8, 29.0, 27.9; LRMS \(m/z\) calcd for C\(_{14}\)H\(_{16}\)FN\(_5\)S: 305.1; found 306.0 [M+H]\(^+\).

Purity of the compound was further confirmed by RP-HPLC by using method 1: \(R_t = 7.02\) min (98% pure; Figure B12).

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**Preparation of \(N,N\)-diethyl-2-{(8-fluoro-5-methyl-5H-[1,2,4]triazino[5,6-b]indol-3-yl)thio ethanamine (37b).** Compound 37b was prepared by using general procedure C with compound 58b (25 mg, 0.10 mmol), 2-chloro-\(N,N\)-diethylethylamine hydrochloride (20 mg, 0.11 mmol), KI (49 mg, 0.30 mmol), and K\(_2\)CO\(_3\) (16 mg, 0.12 mmol). Yellow solid (15 mg, 43%); \(R_f\) 0.39 (CH\(_2\)Cl\(_2\):MeOH/97:3); \(^1\)H NMR (400 MHz, CDCl\(_3\), Figure B13) \(\delta\) 8.02 (dd, \(J_1 = 7.2\) Hz, \(J_2 = 2.0\) Hz, 1H), 7.42-7.34 (m, 2H), 3.80 (s, 3H), 3.49 (t, \(J = 7.6\) Hz, 2H), 3.01 (t, \(J = 7.6\) Hz, 2H), 2.77 (q, \(J = 7.6\) Hz, 4H), 1.16 (t, \(J = 7.6\) Hz, 6H); \(^{13}\)C NMR (100 MHz, CDCl\(_3\), Figure B14) \(\delta\) 168.6, 160.5, 158.1, 147.3, 141.13, 141.08, 137.9, 119.3, 119.2, 118.6, 118.4, 111.1, 111.0, 108.8, 108.6, 52.1, 47.3, 27.8, 27.6, 11.6; LRMS \(m/z\) calcd for C\(_{16}\)H\(_{20}\)FN\(_5\)S: 333.1; found 334.0 [M+H]\(^+\). Purity of the compound was further confirmed by RP-HPLC by using method 1: \(R_t = 7.71\) min (97% pure; Figure B15).

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**Preparation of \(N,N\)-diethyl-2-{(8-fluoro-5-isopentyl-5H-[1,2,4]triazino[5,6-b]indol-3-yl)thio}ethan-amine (37h).** Compound
37h was prepared by using general procedure C with compound 58h (29 mg, 0.10 mmol), 2-chloro-N,N-diethylethylamine hydrochloride (20 mg, 0.11 mmol), KI (49 mg, 0.30 mmol), and K₂CO₃ (16 mg, 0.12 mmol). Yellow solid (12 mg, 31%); Rᵣ 0.35 (CH₂Cl₂:MeOH/9:1); ¹H NMR (400 MHz, CDCl₃, Figure B16) δ 8.06 (d, J = 7.6 Hz, 1H), 7.37 (d, J = 6.4 Hz, 2H), 4.31 (t, J = 7.2 Hz, 2H), 3.47 (t, J = 7.6 Hz, 2H), 3.01 (t, J = 7.6 Hz, 2H), 2.75 (q, J = 7.2 Hz, 4H), 1.72 (q, J = 7.2 Hz, 2H), 1.60 (nonet, J = 6.4 Hz, 1H), 1.14 (t, J = 7.2, 6H), 0.99 (d, J = 6.4 Hz, 6H); ¹³C NMR (100 MHz, CDCl₃, Figure B17) δ 168.5, 160.2, 157.8, 146.8, 136.9, 119.4, 119.3, 118.2, 118.0, 111.1, 111.0, 110.0, 108.7, 108.4, 52.0, 47.0, 39.9, 36.9, 27.8, 25.9, 22.4, 11.5; LRMS m/z calcd for C₂₀H₂₈FN₅S: 389.2; found 390.2 [M+H]⁺. Purity of the compound was further confirmed by RP-HPLC by using method 1: Rᵣ = 9.49 min (98% pure; Figure B18).

Preparation of 8-fluoro-5-methyl-3-{(2-(pyrrolidin-1-yl)ethyl)thio}-5H-[1,2,4]triazino[5,6-b]indole (38b). Compound 38b was prepared by using general procedure C with compound 58b (25 mg, 0.11 mmol), 1-(2-chloroethyl)pyrrolidine hydrochloride (20 mg, 0.12 mmol), KI (52 mg, 0.33 mmol), and K₂CO₃ (17 mg, 0.13 mmol). Yellow solid (16 mg, 18%); Rᵣ 0.41 (CH₂Cl₂:MeOH/97:3); ¹H NMR (400 MHz, CDCl₃, Figure B19) δ 8.00 (dd, J₁ = 7.6 Hz, J₂ = 2.0 Hz, 1H), 7.42-7.34 (m, 2H), 3.80 (s, 3H), 3.57 (t, J = 7.6 Hz, 2H), 3.09 (t, J = 7.6 Hz, 2H), 2.86 (br t, 4H), 1.90 (p, J = 3.2 Hz, 4H); ¹³C NMR (100 MHz, CDCl₃, Figure B20) δ 168.4, 160.5, 158.1, 147.2, 141.14, 141.10, 137.9, 119.3, 119.2, 118.7, 118.4, 111.1, 111.0, 108.8, 108.5, 55.3, 54.2, 28.8, 27.6, 23.7; LRMS m/z calcd for C₁₆H₁₈FN₅S: 331.1; found 332.0 [M+H]⁺. Purity of the compound
was further confirmed by RP-HPLC by using method 1: $R_t = 7.73$ min (100% pure; Figure B21).

![Chemical structure of 39b](image)

**Preparation of 8-fluoro-5-methyl-3-\{(2-(piperidin-1-yl)ethyl)thio\}-5H-[1,2,4]triazino[5,6-b]indole (39b).** Compound 39b was prepared by using general procedure C with compound 58b (23 mg, 0.10 mmol), 1-(2-chloroethyl)piperidine hydrochloride (20 mg, 0.11 mmol), KI (50 mg, 0.30 mmol), and K$_2$CO$_3$ (17 mg, 0.12 mmol). Yellow solid (22 mg, 64%); $R_f 0.32$ (CH$_2$Cl$_2$:MeOH/97:3); $^1$H NMR (400 MHz, CDCl$_3$, Figure B22) $\delta$ 8.06 (d, $J = 7.6$ Hz, 1H), 7.44-7.36 (m, 2H), 3.82 (s, 3H), 3.54 (t, $J = 7.2$ Hz, 2H), 2.88 (t, $J = 7.2$ Hz, 2H), 2.70-2.60 (m, 4H), 1.67 (p, $J = 5.6$ Hz, 4H), 1.45 (m, 2H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure B23) $\delta$ 169.0, 160.6, 158.1, 147.3, 140.7, 137.8, 119.4, 118.5, 118.3, 111.1, 111.0, 108.9, 108.6, 58.3, 54.6, 28.0, 27.6, 26.0, 24.4; LRMS $m/z$ calcd for C$_{17}$H$_{20}$FN$_5$S: 345.1; found 346.0 [M+H]$^+$. Purity of the compound was further confirmed by RP-HPLC by using method 1: $R_t = 8.18$ min (99% pure; Figure B24).

![Chemical structure of 42b](image)

**Preparation of 8-fluoro-5-methyl-3-\{(3-phenylpropyl)thio\}-5H-[1,2,4]triazino[5,6-b]indole (42b).** Compound 42b was prepared by using general procedure C with compound 58b (118 mg, 0.50 mmol), 1-chloro-3-phenylpropane (85 mg, 0.55 mmol), KI (250 mg, 1.50 mmol), and K$_2$CO$_3$ (83 mg, 0.60 mmol). Pale yellow solid (92 mg, 52%); $R_f 0.83$ (CH$_2$Cl$_2$:MeOH/97:3); $^1$H NMR (400 MHz, CDCl$_3$, Figure B25) $\delta$ 8.06 (dd, $J_1 = 7.6$ Hz, $J_2 = 2.0$ Hz, 1H), 7.44-7.34 (m, 2H), 7.32-7.16 (m, 5H), 3.78 (s, 3H), 3.38 (t, $J = 7.6$ Hz, 2H), 2.85 (t, $J = 7.6$ Hz, 2H), 2.17 (p, $J = 7.6$ Hz, 2H); $^{13}$C
NMR (100 MHz, CDCl₃, Figure B26) δ 169.1, 160.5, 158.1, 147.3, 141.5, 140.93, 140.88, 137.8, 128.8, 128.7, 128.6, 126.2, 119.4, 119.3, 118.5, 118.2, 111.0, 110.9, 108.8, 108.5, 35.1, 31.0, 30.6, 27.5; LRMS m/z calcd for C₁₉H₁₇FN₄S: 352.1; found 353.1 [M+H]⁺. Purity of the compound was further confirmed by RP-HPLC by using method 1: R_t = 10.36 min (98% pure; Figure B27).

Preparation of 3-[(8-fluoro-5-methyl-5H-[1,2,4]triazino[5,6-b]indol-3-yl)thio]-N,N-dimethylpropan-1-amine (43b). Compound 43b was prepared by using general procedure C with compound 58b (30 mg, 0.13 mmol), 3-dimethylamino-1-propylchloride hydrochloride (22 mg, 0.14 mmol), KI (64 mg, 0.39 mmol), and K₂CO₃ (21 mg, 0.16 mmol). Dark yellow solid (21 mg, 51%); R_f 0.11 (CH₂Cl₂:MeOH/97:3); ¹H NMR (400 MHz, CDCl₃, Figure B28) δ 8.06 (d, J = 7.2 Hz, 1H), 7.42-7.36 (m, 2H), 3.81 (s, 3H), 3.38 (t, J = 7.2 Hz, 2H), 2.47 (t, J = 7.2 Hz, 2H), 2.26 (s, 6H), 2.01 (p, J = 7.2 Hz, 2H); ¹³C NMR (100 MHz, CDCl₃, Figure B29) δ 168.7, 160.3, 157.9, 140.8, 137.6, 118.3, 118.1, 110.8, 110.7, 108.6, 108.4, 58.3, 45.2, 28.8, 27.4, 26.9; LRMS m/z calcd for C₁₅H₁₈FN₅S: 319.1; found 320.0 [M+H]⁺. Purity of the compound was further confirmed by RP-HPLC by using method 1: R_t = 7.41 min (100% pure; Figure B30).

Preparation of 3-[(8-fluoro-5-propyl-5H-[1,2,4]triazino[5,6-b]indol-3-yl)thio]-N,N-dimethylpropan-1-amine (43d). Compound 43d was prepared by using general procedure C with compound 58d (25 mg, 0.09 mmol), 3-dimethylamino-1-propylchloride hydrochloride (16 mg, 0.10 mmol), KI (47 mg, 0.27 mmol),
and K$_2$CO$_3$ (16 mg, 0.11 mmol). Yellow solid (13 mg, 40%); R$_f$ 0.58 (CH$_2$Cl$_2$:MeOH/97:3); $^1$H NMR (400 MHz, CDCl$_3$, Figure B31) δ 8.24 (d, $J$ = 8.0 Hz, 1H), 7.60-7.54 (m, 2H), 4.46 (t, $J$ = 7.2 Hz, 2H), 3.56 (t, $J$ = 7.2 Hz, 2H), 2.68 (t, $J$ = 7.2 Hz, 2H), 2.46 (s, 6H), 2.22 (p, $J$ = 7.2 Hz, 2H), 2.09 (sextet, $J$ = 7.2 Hz, 2H), 1.14 (t, $J$ = 7.6 Hz, 3H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure B32) δ 169.0, 160.4, 157.9, 147.2, 140.8, 140.7, 137.2, 119.6, 119.5, 118.4, 118.1, 111.4, 111.3, 108.8, 108.6, 58.7, 45.6, 43.2, 29.1, 27.5, 21.9, 11.7; LRMS m/z calcd for C$_{17}$H$_{22}$FN$_5$S: 347.2; found 348.0 [M+H]$^+$. Purity of the compound was further confirmed by RP-HPLC by using method 1: $R_t$ = 6.29 min (99% pure; Figure B33).

Preparation of N-[(3-[8-fluoro-5-methyl-5H-1,2,4]triazino[5,6-b]indol-3-yl)thio]propyl]-N-methyl-aniline (44b). Compound 44b was prepared by using general procedure C with compound 58b (109 mg, 0.46 mmol), N-methyl-N-(3-chloropropyl)aniline (93 mg, 0.51 mmol) (Note: this reagent was prepared by reacting N-methylaniline (1 eq.) with 1-bromo-3-chloropropane (5 eq.) in DMF at 70 °C for 24 h), KI (231 mg, 1.38 mmol), and K$_2$CO$_3$ (77 mg, 0.55 mmol). Beige solid (74 mg, 39%); R$_f$ 0.77 (CH$_2$Cl$_2$:MeOH/97:3); $^1$H NMR (400 MHz, CDCl$_3$, Figure B34) δ 8.04 (dd, $J_1$ = 8.0 Hz, $J_2$ = 2.0 Hz, 1H), 7.41-7.34 (m, 2H), 7.19 (t, $J$ = 7.2 Hz, 2H), 6.73 (d, $J$ = 8.0 Hz, 2H), 6.66 (t, $J$ = 7.2 Hz, 1H), 3.75 (s, 3H), 3.53 (t, $J$ = 7.2 Hz, 2H), 3.37 (t, $J$ = 7.2 Hz, 2H), 2.96 (s, 3H), 2.13 (p, $J$ = 7.2 Hz, 2H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure B35) δ 168.6, 160.3, 157.9, 147.0, 140.8, 140.7, 137.6, 129.2, 119.1, 119.0, 118.3, 118.1, 116.2, 112.2, 110.8, 110.7, 108.6, 108.4, 51.6, 38.6, 28.5, 27.3, 26.6; LRMS m/z calcd for C$_{20}$H$_{20}$FN$_5$S: 381.1; found 382.1 [M+H]$^+$. Purity of the compound was further confirmed by RP-HPLC by using method 1: $R_t$ = 8.58 min (95% pure; Figure B36).
Preparation of 8-fluoro-5-methyl-3-{[3-(piperidin-1-yl)propyl]thio}-5H-[1,2,4]triazino[5,6-b]indole (45b).

Compound 45b was prepared by using general procedure C with compound 58b (94 mg, 0.40 mmol), 1-(3-chloropropyl)piperidine hydrochloride (88 mg, 0.44 mmol), KI (201 mg, 1.20 mmol), and K$_2$CO$_3$ (67 mg, 0.48 mmol). Yellow solid (42 mg, 29%); R$_f$ 0.51 (CH$_2$Cl$_2$:MeOH/97:3); $^1$H NMR (400 MHz, CDCl$_3$, Figure B37) δ 8.02 (dd, $J_1 = 7.6$ Hz, $J_2 = 1.6$ Hz, 1H), 7.40-7.34 (m, 2H), 3.81 (s, 3H), 3.37 (t, $J = 7.2$ Hz, 2H), 2.74 (t, $J = 7.2$ Hz, 2H), 2.54-2.52 (m, 2H), 2.19 (p, $J = 7.2$ Hz, 2H), 1.73 (p, $J = 5.2$ Hz, 4H), 1.48 (br p, 2H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure B38) δ 168.9, 160.4, 158.0, 147.1, 140.7, 137.6, 131.0, 118.4, 118.1, 111.0, 110.9, 108.5, 108.2, 58.0, 54.5, 29.1, 27.5, 26.1, 25.5, 24.1; LRMS m/z calcd for C$_{18}$H$_{22}$FN$_5$S: 359.2; found 360.0 [M+H]$^+$. Purity of the compound was further confirmed by RP-HPLC by using method 1: R$_t$ = 8.11 min (95% pure; Figure B39).

Preparation of 3-{[3-(3,5-dimethylpiperidin-1-yl)propyl]thio}-8-fluoro-5-methyl-5H-[1,2,4]triazino[5,6-b]indole (46b).

Compound 46b was prepared by using general procedure C with compound 58b (52 mg, 0.22 mmol), 1-(3-chloropropyl)-3,5-dimethylpiperidine hydrochloride (47 mg, 0.24 mmol), KI (111 mg, 0.66 mmol), and K$_2$CO$_3$ (37 mg, 0.26 mmol). Yellow solid (32 mg, 37%); R$_f$ 0.49 (CH$_2$Cl$_2$:MeOH/97:3); $^1$H NMR (400 MHz, CDCl$_3$, Figure B40) δ 7.98 (dd, $J_1 = 8.4$ Hz, $J_2 = 2.0$ Hz, 1H), 7.38-7.28 (m, 2H), 3.77 (s, 3H), 3.32 (t, $J = 7.2$ Hz, 2H), 2.85-2.79 (m, 2H), 2.48 (t, $J = 7.2$ Hz, 2H), 2.02 (p, $J = 7.2$ Hz, 2H), 1.67-1.61 (m, 2H), 1.41 (t, $J = 10.8$ Hz, 2H), 0.91
(m, 1H), 0.81 (d, J = 6.8 Hz, 6H), 0.55-0.40 (m, 1H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure B41) δ 169.1, 160.4, 158.0, 147.2, 140.74, 140.69, 137.7, 119.3, 119.2, 118.4, 118.1, 111.0, 110.9, 108.6, 108.4, 61.8, 57.9, 53.9, 42.3, 32.3, 31.2, 29.4, 27.5, 26.7, 19.8, 19.7; LRMS m/z calcd for C$_{20}$H$_{26}$FN$_{5}$S: 387.2; found 388.1 [M+H]$^+$. Purity of the compound was further confirmed by RP-HPLC by using method 1: $R_t$ = 8.84 min (98% pure; Figure B42).

Preparation of 2-\{(5,8-dimethyl-5H-[1,2,4]triazino[5,6-b]indol-3-yl)thio\}-N,N-diethylethanamine (48b). Compound 48b was prepared by using general procedure C with compound 59b (50 mg, 0.22 mmol), 2-chloro-N,N-diethylethylamine hydrochloride (41 mg, 0.24 mmol), KI (111 mg, 0.66 mmol), and K$_2$CO$_3$ (37 mg, 0.26 mmol). Orange solid (31 mg, 43%); R$_f$ 0.40 (CH$_2$Cl$_2$:MeOH/9:1); $^1$H NMR (400 MHz, CDCl$_3$, Figure B43) δ 8.16 (s, 1H), 7.47 (d, J = 8.8 Hz, 1H), 7.30 (d, J = 8.4 Hz, 1H), 3.78 (s, 3H), 3.55 (m, 2H), 3.12 (m, 2H), 2.87 (q, J = 7.2 Hz, 4H), 2.52 (s, 3H), 1.23 (t, J = 7.2 Hz, 6H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure B44) δ 146.9, 141.6, 140.0, 133.0, 132.2, 122.4, 118.4, 109.8, 52.0, 47.3, 27.5, 27.2, 21.6, 11.1; LRMS m/z calcd for C$_{17}$H$_{23}$N$_{5}$S: 329.2; found 330.1 [M+H]$^+$. Purity of the compound was further confirmed by RP-HPLC by using method 1: $R_t$ = 8.26 min (98% pure; Figure B45).

Preparation of N,N-diethyl-2-\{(5-ethyl-8-methyl-5H-[1,2,4]triazino[5,6-b]indol-3-yl)thio\}ethan-amine (48c). Compound 48c was prepared by using general procedure C with compound 59c (21 mg, 0.09 mmol), 2-chloro-N,N-diethylethylamine hydrochloride (17 mg, 0.10 mmol), KI (44 mg, 0.27 mmol),
and K$_2$CO$_3$ (14 mg, 0.11 mmol). Yellow solid (12 mg, 40%; R$_f$ 0.40 in CH$_2$Cl$_2$:MeOH/9:1); $^1$H NMR (400 MHz, CDCl$_3$, Figure B46) δ 8.19 (s, 1H), 7.49 (d, $J$ = 8.0 Hz, 1H), 7.35 (d, $J$ = 8.4 Hz, 1H), 4.36 (q, $J$ = 7.2 Hz, 2H), 3.67 (t, $J$ = 7.2 Hz, 2H), 3.40-3.20 (m, 2H), 3.10-3.00 (m, 4H), 2.53 (s, 3H), 1.44 (t, $J$ = 6.8 Hz, 3H), 1.36 (t, $J$ = 6.8 Hz, 6H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure B47) δ 166.8, 146.1, 141.5, 138.9, 132.7, 131.9, 122.4, 118.3, 109.7, 51.7, 47.14, 47.07, 36.2, 21.3, 13.6, 12.8, 10.7, 10.5; LRMS m/z calcd for C$_{18}$H$_{25}$N$_5$S: 343.2; found 344.1 [M+H]$^+$. Purity of the compound was further confirmed by RP-HPLC by using method 1: $R_t$ = 8.55 min (95% pure; Figure B48).

Preparation of N,N-diethyl-2-[(5-propyl-8-methyl-5H-[1,2,4]triazino[5,6-b]indol-3-yl)thio]ethan-amine (48d). Compound 48d was prepared by using general procedure C with compound 59d (26 mg, 0.10 mmol), 2-chloro-N,N-diethylethylamine hydrochloride (19 mg, 0.11 mmol), KI (51 mg, 0.30 mmol), and K$_2$CO$_3$ (17 mg, 0.12 mmol). Yellow solid (16 mg, 44%); R$_f$ 0.42 (CH$_2$Cl$_2$:MeOH/9:1); $^1$H NMR (400 MHz, CDCl$_3$, Figure B49) δ 8.18 (s, 1H), 7.47 (dd, $J_1$ = 8.4 Hz, $J_2$ = 1.2 Hz, 1H), 7.33 (d, $J$ = 8.8 Hz, 1H), 4.25 (t, $J$ = 7.6 Hz, 2H), 3.63 (t, $J$ = 7.6 Hz, 2H), 3.23 (br t, 2H), 3.10-2.90 (m, 4H), 2.52 (s, 3H), 2.20 (sextet, $J$ = 7.6 Hz, 2H), 1.32 (t, $J$ = 7.2 Hz, 6H), 0.94 (t, $J$ = 7.6 Hz, 3H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure B50) δ 166.5, 146.5, 141.5, 139.4, 132.7, 132.0, 122.3, 118.2, 110.0, 51.6, 47.0, 43.0, 26.0, 21.7, 21.3, 11.4, 10.0; LRMS m/z calcd for C$_{19}$H$_{27}$N$_5$S: 357.2; found 358.1 [M+H]$^+$. Purity of the compound was further confirmed by RP-HPLC by using method 1: $R_t$ = 8.96 min (100% pure; Figure B51).
Preparation of \( N,N\)-diethyl-2-\{(5-isopropyl-8-methyl-5H-[1,2,4]triazino[5,6-b]indol-3-yl)thio\}ethanamine (48f). Compound 48f was prepared by using general procedure C with compound 59f (41 mg, 0.16 mmol), 2-chloro-\( N,N \)-diethylethylamine hydrochloride (30 mg, 0.18 mmol), KI (80 mg, 0.48 mmol), and K\(_2\)CO\(_3\) (27 mg, 0.19 mmol). Yellow solid (8 mg, 14%); R\(_f\) 0.43 (CH\(_2\)Cl\(_2\):MeOH/97:3); \(^1\)H NMR (400 MHz, CDCl\(_3\), Figure B52) \( \delta \) 8.18 (s, 1H), 7.41 (m, 2H), 5.12 (septet, \( J = 6.8 \) Hz, 1H), 3.46 (t, \( J = 6.8 \) Hz, 2H), 2.99 (t, \( J = 7.2 \) Hz, 2H), 2.74 (q, \( J = 7.2 \) Hz, 4H), 2.50 (s, 3H), 1.65 (d, \( J = 7.2 \) Hz, 6H), 1.13 (t, \( J = 7.2 \) Hz, 6H); \(^{13}\)C NMR (100 MHz, CDCl\(_3\), Figure B53) \( \delta \) 167.3, 146.3, 141.3, 138.4, 132.4, 131.7, 122.5, 119.0, 111.4, 52.3, 47.3, 46.3, 27.9, 21.4, 20.6, 11.7; LRMS \( m/z \) calcd for \( C_{19}H_{27}N_5S \): 357.2; found 358.1 [M+H]\(^+\). Purity of the compound was further confirmed by RP-HPLC by using method 1: \( R_t = 9.13 \) min (95% pure; Figure B54).

Preparation of \( N,N\)-diethyl-2-\{(8-methyl-5-isopentyl-5H-[1,2,4]triazino[5,6-b]indol-3-yl)thio\}ethanamine (48h). Compound 48h was prepared by using general procedure C with compound 59h (34 mg, 0.12 mmol), 2-chloro-\( N,N \)-diethylethylamine hydrochloride (22 mg, 0.13 mmol), KI (59 mg, 0.36 mmol), and K\(_2\)CO\(_3\) (20 mg, 0.14 mmol). Yellow solid (10 mg, 22%); R\(_f\) 0.39 (CH\(_2\)Cl\(_2\):MeOH/9:1); \(^1\)H NMR (400 MHz, CDCl\(_3\), Figure B55) \( \delta \) 8.18 (s, 1H), 7.48 (dd, \( J_1 = 8.4 \) Hz, \( J_2 = 2.4 \) Hz, 1H) (m, 4H), 7.32 (d, \( J = 8.4 \) Hz, 1H), 4.29 (t, \( J = 7.6 \) Hz, 2H), 3.68 (t, \( J = 7.6 \) Hz, 2H), 3.33 (br t, 2H), 3.11-3.00 (m, 4H), 2.53 (s, 3H), 1.71 (q, \( J = 7.2 \) Hz, 2H), 1.60 (nonet, \( J = 6.8 \) Hz, 1H), 1.36 (t, \( J = 7.2 \) Hz, 6H), 0.98 (d, \( J = 6.8 \) Hz, 6H); \(^{13}\)C NMR (100 MHz, CDCl\(_3\), Figure B56) \( \delta \) 166.6, 146.3, 141.5, 139.2, 132.7, 132.0, 122.3, 118.3, 109.9,
51.6, 47.0 (2 carbons), 39.8, 36.9, 25.9, 22.4 (2 carbons), 21.3; LRMS m/z calcd for C_{21}H_{31}N_{5}S: 385.2; found 386.1 [M+H]^+. Purity of the compound was further confirmed by RP-HPLC by using method 1: R_t = 9.35 min (95% pure; Figure B57).

Preparation of 5,8-dimethyl-3-[(2-(pyrrolidin-1-yl)ethyl)thio]-5H-[1,2,4]triazino[5,6-b]indole (49b). Compound 49b was prepared by using general procedure C with compound 59b (26 mg, 0.11 mmol), 1-(2-chloroethyl)pyrrolidine hydrochloride (21 mg, 0.12 mmol), KI (56 mg, 0.33 mmol), and K_2CO_3 (19 mg, 0.13 mmol). Pale yellow solid (30 mg, 82%); R_f 0.40 (CH_2Cl_2:MeOH/9:1); ^1H NMR (400 MHz, CDCl_3, Figure B58) δ 8.15 (s, 1H), 7.48 (d, J = 8.4 Hz, 1H), 7.31 (d, J = 8.0 Hz, 1H), 3.78 (s, 3H), 3.65 (t, J = 7.6 Hz, 2H), 3.26 (t, J = 7.2 Hz, 2H), 3.12-3.02 (m, 4H), 2.55 (s, 3H), 2.04-1.94 (m, 4H); ^13C NMR (100 MHz, CDCl_3, Figure B59) δ 166.9, 146.8, 141.6, 140.1, 133.1, 132.3, 122.4, 118.2, 109.8, 55.2, 54.1, 27.8, 27.5, 23.7, 21.5; LRMS m/z calcd for C_{17}H_{21}N_{5}S: 327.2; found 328.0 [M+H]^+. Purity of the compound was further confirmed by RP-HPLC by using method 1: R_t = 8.13 min (96% pure; Figure B60).

Preparation of 8-fluoro-3-[(3-methoxyethyl)thio]-5-methyl-5H-[1,2,4]triazino[5,6-b]indole (32b). Compound 32b (110 mg, 0.40 mmol) was dissolved in anhydrous CH_2Cl_2 (12 mL), under nitrogen gas, at 0 °C and NaH (64 mg, 60%, 1.60 mmol) was added. The reaction mixture was stirred at 0 °C for 15 min at which point methyl iodide (0.25 mL, 4.00 mmol) was added dropwise. After complete addition of the methyl iodide the reaction mixture was stirred for an additional 1 h at 0 °C and
allowed to warm up to rt prior to quenching with H$_2$O (10 mL). CH$_2$Cl$_2$ (10 mL) was then added and the organic layer was separated, dried over anhydrous MgSO$_4$, and concentrated under reduced pressure. The brown solid obtained was purified using column chromatography (SiO$_2$, CH$_2$Cl$_2$:MeOH/99:1) to afford compound 33b (55 mg, 47%) as a white solid. R$_f$ 0.45 (CH$_2$Cl$_2$:MeOH/99:1); $^1$H NMR (400 MHz, CDCl$_3$, Figure B61) δ 8.04 (app. dt, $J_1 = 7.6$ Hz, $J_2 = 1.2$ Hz, 1H), 7.37 (d, $J = 7.6$ Hz, 1H), 7.36 (d, $J = 6.4$ Hz, 1H), 4.81 (t, $J = 6.8$ Hz, 2H), 3.80 (s, 3H), 3.01 (t, $J = 6.8$ Hz, 2H), 2.23 (s, 3H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure B62) δ 162.9, 160.4, 158.0, 140.8, 138.3, 119.3, 117.8, 117.5, 110.8, 108.5, 108.3, 67.2, 32.6, 27.4, 15.9; LRMS m/z calcd for C$_{13}$H$_{13}$FN$_4$OS: 292.1; found 293.0 [M+H]$^+$. Purity of the compound was further confirmed by RP-HPLC by using method 1: $R_t = 8.05$ min (95% pure; Figure B63).

Preparation of 2-[(8-fluoro-5-methyl-5H-[1,2,4]triazino[5,6-b]indol-3-yl)thio]acetonitrile (47b). Compound 58b (300 mg, 1.28 mmol) and K$_2$CO$_3$ (190 mg, 1.41 mmol) were added to a stirred solution of 2-bromoacetonitrile (0.45 mL, 6.4 mmol) in DMF (13 mL). The reaction mixture was heated to reflux and stirred for 12 h. After cooling to rt, the reaction mixture was quenched with H$_2$O (20 mL). The flocculant solid was collected by filtration, washed 3 times with MeOH (5 mL), and recrystallized from an EtOAc:DMF mixture (1:6) to afford the desired compound 47b (220 mg, 63%) a beige solid. R$_f$ 0.63 (CH$_2$Cl$_2$:MeOH/95:5); $^1$H NMR (400 MHz, (CD$_3$)$_2$SO, Figure B64) δ 8.19 (dd, $J = 8.8$, 2.8 Hz, 1H), 7.84 (dd, $J_1 = 8.8$ Hz, $J_2 = 4.0$ Hz, 1H), 7.68 (td, $J_1 = 8.8$ Hz, $J_2 = 2.8$ Hz, 1H), 4.46 (s, 2H), 3.85 (s, 3H); $^{13}$C NMR (100 MHz, (CD$_3$)$_2$SO, Figure B65) δ 164.6, 159.7, 157.4, 146.5, 141.42, 141.38, 138.3, 118.9, 118.6, 118.1, 117.9, 112.94, 112.85, 107.9, 107.7, 27.6, 146.5, 141.42, 141.38, 138.3, 118.9, 118.6, 118.1, 117.9, 112.94, 112.85, 107.9, 107.7, 27.6,
16.3; LRMS \textit{m}/\textit{z} calcld for C$_{12}$H$_8$FN$_5$S: 273.1; found 274.0 [M+H]$^+$. Purity of the compound was further confirmed by RP-HPLC by using method 1: $R_t = 7.92$ min (100% pure; Figure B66).

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**Preparation of 8-fluoro-5-methyl-3-(methylthio)-5\textit{H}-[1,2,4]triazino[5,6-\textit{b}]indole (60b).** Compound 58b (235 mg, 1.0 mmol) was dissolved in MeCN (20 mL). Cs$_2$CO$_3$ (350 mg, 1.1 mmol) was added and the reaction mixture was allowed to stir for 30 min at rt. Methyl iodide (0.2 mL, 3.1 mmol) was added dropwise over 1 h. After complete addition of the methyl iodide, the reaction mixture was stirred for an addition 1 h more prior to filtration. The organic compounds were concentrated and purified by column chromatography (SiO$_2$, CH$_2$Cl$_2$:MeOH/97:3, $R_f$ 0.6) to yield compound 60b (225 mg, 91%) as a brown solid. $^1$H NMR (400 MHz, CDCl$_3$, Figure B67) $\delta$ 8.07 (dd, $J_1 = 8.0$ Hz, $J_2 = 2.8$ Hz, 1H), 7.44-7.36 (m, 2H), 3.80 (s, 3H), 2.75 (s, 3H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure B68) $\delta$ 169.4, 160.6, 158.2, 137.9, 119.5, 118.6, 118.3, 111.05, 110.96, 108.9, 108.7, 27.6, 14.4; LRMS \textit{m}/\textit{z} calcld for C$_{11}$H$_9$FN$_4$S: 248.1; found 249.0 [M+H]$^+$. Purity of the compound was further confirmed by RP-HPLC by using method 1: $R_t = 7.92$ min (100% pure; Figure B69).

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**Preparation of 8-fluoro-5-methyl-3-(methylsulfonyl)-5\textit{H}-[1,2,4]triazino[5,6-\textit{b}]indole (61b).** Compound 61b (250 mg, 1.0 mmol) was suspended in anhydrous CH$_2$Cl$_2$ (10 mL) under nitrogen. \textit{m}-Chloroperbenzoic acid (430 mg, 2.5 mmol) was carefully added and the reaction mixture was stirred at rt for 24 h. The
reaction mixture was partitioned between a sodium thiosulfate solution (5% in H₂O, 50 mL) and EtOAc (50 mL). The organic layer was separated, washed with brine (20 mL), dried over anhydrous MgSO₄, and evaporated under reduced pressure. The brown solid obtained was purified by column chromatography (SiO₂, CH₂Cl₂:MeOH/97:3, Rf 0.34) to yield compound 61b (126 mg, 45%) as a brown solid. ¹H NMR (400 MHz, CDCl₃, Figure B70) δ 8.25 (dd, J₁ = 8.0 Hz, J₂ = 2.4 Hz, 1H), 7.64-7.54 (m, 2H), 4.01 (s, 3H), 3.59 (s, 3H); ¹³C NMR (100 MHz, CDCl₃, Figure B71) δ 162.3, 161.0, 158.5, 147.0, 145.2, 139.8, 121.4, 121.2, 118.4, 118.3, 112.1, 112.0, 110.1, 109.9, 40.4, 28.5; LRMS m/z calcd for C₁₁H₉FN₄O₂S: 280.0; found 280.9 [M+H]⁺. Purity of the compound was further confirmed by RP-HPLC by using method 1: Rᵣ = 5.99 min (100% pure; Figure B72).

Preparation of N¹,N¹-diethyl-N²-(8-fluoro-5-methyl-5H-[1,2,4]triazino[5,6-b]indol-3-yl)ethane-1,2-diamine (52).  N¹,N¹-Diethylethane-1,3-diamine (15 mg, 0.13 mmol) was added to a solution of compound 62b (30 mg, 1.0 mmol) in anhydrous THF (5 mL). The reaction mixture was stirred under reflux for 6 h. After cooling the reaction mixture to rt, the solvent was evaporated under reduced pressure. The residual material was purified by column chromatography (SiO₂, CH₂Cl₂:MeOH/97:3, Rf 0.35) to yield compound 52 (13 mg, 42%) as a yellow solid. ¹H NMR (400 MHz, CDCl₃, Figure B73) δ 7.87 (d, J = 8.0 Hz, 1H), 7.22-7.18 (m, 2H), 6.10 (br s, 1H), 3.67 (s, 3H), 3.60 (q, J = 5.2 Hz, 2H), 2.72 (t, J = 6.4 Hz, 2H), 2.60 (q, J = 7.2 Hz, 4H), 1.04 (t, J = 7.2 Hz, 6H); ¹³C NMR (100 MHz, CDCl₃, Figure B74) δ 160.4, 160.0, 149.4, 137.1, 120.7, 120.6, 115.7, 115.5, 110.2, 110.1, 107.4, 107.2, 51.7, 46.9, 39.2, 27.0, 11.9; LRMS m/z calcd for
C_{16}H_{21}FN_{6}S: 316.4; found 317.1 [M+H]^+. Purity of the compound was further confirmed by RP-HPLC by using method 1: $R_t = 3.30$ min (98% pure; Figure B75).

Preparation of $N^1$-(8-fluoro-5-methyl-5H-[1,2,4]triazino[5,6-b]indol-3-yl)-$N^3$-$N^3$-dimethylpropane-1,3-diamine (53). $N^1,N^1$-Dimethylpropane-1,3-diamine (110 mg, 1.1 mmol) was added to a solution of compound 62b (280 mg, 1.0 mmol) in anhydrous THF (10 mL). The reaction mixture was stirred under reflux for 2 h. After cooling the reaction mixture to rt, the solvent was evaporated under reduced pressure. The residual material was purified by column chromatography (SiO$_2$, CH$_2$Cl$_2$:MeOH/97:3, $R_f$ 0.27) to yield compound 53 (24 mg, 8%) as a yellow solid. $^1$H NMR (400 MHz, CDCl$_3$, Figure B76) δ 7.87 (dd, $J_1 = 8.0$ Hz, $J_2 = 1.2$ Hz, 1H), 7.20-7.16 (m, 2H), 6.39 (br s, 1H), 3.66 (s, 3H), 3.61 (q, $J = 6.0$ Hz, 2H), 2.45 (t, $J = 6.8$ Hz, 2H), 2.26 (s, 6H), 1.85 (p, $J = 6.8$ Hz, 2H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure B77) δ 160.3, 157.9, 149.4, 137.1, 120.7, 120.6, 115.6, 115.4, 110.14, 110.05, 107.3, 107.1, 58.2, 45.7, 40.7, 27.0; LRMS m/z calcd for C$_{15}$H$_{15}$FN$_6$: 302.2; found 303.1 [M+H]^+. Purity of the compound was further confirmed by RP-HPLC by using method 1: $R_t = 4.97$ min 98% pure; Figure B78).

3.6.2. Biochemical, biological, and biophysical methods

3.6.2.1. Eis chemical library screening

Eis activity suppression was determined by monitoring the increase in absorbance at 412 nm resulting from a secondary reaction of DTNB with the CoA-SH released upon acetylation of NEO. Reactions (40 µL) contained a mixture of Eis (0.25 µM), NEO (100 µM), Tris-HCl (50
mM, pH 8.0 adjusted at rt), AcCoA (40 µM), DTNB (0.5 mM), and the potential inhibitors (20 µM). Control experiments were performed using a known inhibitor, chlorhexidine (5 µM) (positive) and DMSO (0.5% v/v, negative), instead of the potential inhibitors. Briefly, a solution (30 µL) of Eis (0.33 µM) and NEO (133.33 µM) in Tris-HCl (50 mM, pH 8.0 adjusted at rt) was added to 384-well non-binding-surface plates (Thermo Fisher Scientific, Waltham, MA) using a Multidrop dispenser (Thermo Fisher Scientific). The potential inhibitors (0.2 µL of a 4 mM stock), chlorhexidine (0.2 µL of a 1 mM stock), or DMSO (0.2 µL) were then added to each well by Biomek HDR (Beckman, Fullerton, CA). After incubation at rt (10 min), reactions were initiated by adding a mixture (10 µL) containing AcCoA (160 µM), DTNB (2 mM), and Tris-HCl (50 mM, pH 8.0 adjusted at rt). After an additional 5 min of reaction at rt, the absorbance was measured at 412 nm using a PHERAstar plate reader (BMG Labtech, Cary, NC). The average Z' score for the entire high-throughput screening assay was 0.65.

3.6.2.2 Hit validation

With the aforementioned conditions, all compounds with > 3σ, as a statistical hit threshold from the mean negative control, were characterized as an inhibitory compound and tested in triplicate. Compounds that displayed inhibition at least 2 of the 3 independent assays were then tested for a dose-response using 2-fold dilutions from 20 µM to 78 nM. IC₅₀ values were determined for all select compounds displaying a dose-dependent activity.
3.6.2.3. Inhibition kinetics

IC\textsubscript{50} values were determined on a multimode SpectraMax M5 plate reader using 96-well plates (Thermo Fisher Scientific) by using the UV-Vis assay as above taking measurements every 30 s for 20 min. Compounds were dissolved in Tris (50 mM, pH 8.0 adjusted at rt containing 10% \textit{v/v} DMSO) (100 \textmu L) and a 5-fold dilution was performed. To the solution of inhibitors, a mixture (50 \textmu L) of Eis (1 mM), NEO or KAN (400 \textmu M), and Tris (50 mM, pH 8.0 adjusted at rt) was added and incubated for 10 min, to allow for competitive binding. Reactions were initiated by addition of a mixture (50 \textmu L) containing AcCoA (2 mM), DTNB (2 mM), and Tris-HCl (50 mM, pH 8.0 adjusted at rt). All assays were performed at least in triplicate. Initial rates were calculated from the first 2-5 min of reaction, where linear product accumulation is observed, to ensure the steady-state regime. Data was fit to a Hill-plot fit using KaleidaGraph 4.1 software and IC\textsubscript{50} values were calculated. All IC\textsubscript{50} values are listed in Tables 3.1 and 3.4 and IC\textsubscript{50} curves are presented in Figures 3.5-3.11.

3.6.2.4. Mode of inhibition

By using the conditions described for inhibition kinetics with varying concentrations of NEO (50, 75, 100, 125, 150, and 200 \textmu M) and inhibitory compounds that displayed an IC\textsubscript{50} value <1 \textmu M using NEO as the substrate (compounds \textbf{36b}, \textbf{37b}, \textbf{37d}, \textbf{37g}, \textbf{39b}, \textbf{39c}, \textbf{39i}, and \textbf{50d}) (0, 0.13, 0.25, 0.50 and 1.0 \textmu M), inhibitory constants (\textit{K}_\textit{i}) were determined (Table 3.3). Resulting reaction rates (\textit{v}), substrate concentrations ([\textit{S}]) and inhibitor concentrations ([\textit{I}]) were analyzed by nonlinear regression with SigmaPlot (SysStat) using Equation 1 to determine the \textit{K}_\textit{i} values. Representative IC\textsubscript{50} curves and Lineweaver-Burk double reciprocal
plots (insets; to simplify visualization of the competitive mode of inhibition) are presented in Figure 3.7.

Equation 1: \[ v = \frac{v_{\max}[S]}{[S]+K_m(1+\frac{I}{K_I})} \]

3.6.2.5. Selectivity of inhibitors towards Eis over other AACs

To determine if the inhibitors were Eis specific or pan-AAC, we verified the specificity of two of our inhibitors (36b and 37b) with three regiospecific AACs: AAC(6')-Ie from the bifunctional AAC(6')-Ie/APH(2")-Ia,\textsuperscript{139} AAC(3)-IV,\textsuperscript{139} and AAC(2')-Ic.\textsuperscript{85} Similar conditions to those described above were used. AAC(6')-Ie (0.5 µM) and AAC(3)-IV (0.125 µM) were tested in MES buffer (50 mM, pH 6.6), and AAC(2')-Ic (0.125 µM) was tested in phosphate buffer (50 mM, pH 7.0) using neomycin B (100 µM) as the substrate. AcCoA (150 µM) was used to initiate the reactions. Both AAC(2')-Ic and AAC(3)-IV were incubated at 25 °C, while AAC(6')-Ie was incubated at 37 °C.

3.6.2.6. Mycobacterium tuberculosis MIC values determination

Compounds were tested at 100-fold IC\textsubscript{50,KAN} or 100 µM (if IC\textsubscript{50,KAN} was unknown or could not be achieved while keeping the DMSO concentration \(\leq 1\%\) in test wells). Experiments were conducted in 96-well dishes as previously reported.\textsuperscript{141} Briefly, M\textit{tb} strains were grown in Middlebrook 7H9 supplemented with ADC (10%), Tween® 80 (0.05%), and glycerol (0.4%) at 37 °C until slightly turbid, diluted in fresh 7H9 to attenuation at 600 nM of 0.2, diluted
again 1:25 in fresh 7H9 in a 50 mL polypropylene tube containing glass beads, vortexed for
30 s, and allowed to rest for 10 min. Compounds were diluted to 2× the desired final
concentrations in 7H9 and 100 µL was added to test wells along with 90 µL of prepared
bacteria cultures. Plates were incubated at 37 °C in a humid environment for 24 h. KAN was
diluted to 20× the desired final concentration in H2O and 10 µL was added to test wells.
H37Rv was evaluated at 2.5 and 1.25 µg/mL KAN and K204 was evaluated at 10, 5, 2.5, and
1.25 µg/mL KAN. The plates were then incubated for 6 days at 37 °C. To assess growth
inhibition, AlamarBlue® was diluted 1:1 in 10% Tween® 80 and (40 µL) added to each test
well, plates were further incubated at 37 °C and the color of the wells was observed at 24 h
and 48 h. AlamarBlue® is converted from indigo blue to pink in the presence of bacterial
growth. The MIC was defined as the lowest concentration of KAN which resulted in no
change in color. Compounds were tested at least twice in duplicate. Controls for this study
included: uninoculated 7H9, inoculated 7H9 only, inoculated 7H9 + DMSO only, and
compound and inoculated 7H9 only. To prevent drying out of the plates, 200 µL of sterile
H2O was added to all perimeter wells. Dose-dependent MICKAN values were determined
similarly for compounds 36b and 39b with Eis inhibitor concentrations of 0, 0.5, 1, 2, 4, 8, 16,
32 µM.

3.6.2.7. Crystallization, diffraction data collection, and structure determination and
refinement of EisC204A-CoA-inhibitor 39b complex
Crystals were grown, soaked in the inhibitor solution, and frozen using a previously published
protocol with the exception that the inhibitor used was compound 39b from the current
The diffraction data was collected at the synchrotron beamline 22-ID of the Advanced Photon Source at the Argonne National Laboratory (Argonne, IL) and processed as previously reported. The X-ray diffraction data collection and refinement statistics are summarized in Table 3.5. The EisC204A-CoA-inhibitor 39b structure was deposited in the Protein Data Bank (PDB) with accession number 6B3T. Note: An overlay of inhibitor 39b, TOB, and three other Eis inhibitors of different scaffolds is presented in Figure 3.12.

### Table 3.5. X-ray diffraction data collection and refinement statistics for the EisC204A-CoA-inhibitor 39b ternary complex structure.

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<tr>
<td><strong>Unit cell dimensions</strong></td>
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<td>$a, b, c$ (Å)</td>
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<tr>
<td>$a, b, g$ (º)</td>
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<tr>
<td><strong>Resolution (Å)</strong></td>
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<td>$I/s$</td>
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<tr>
<td><strong>Completeness (%)</strong></td>
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<td><strong>Redundancy</strong></td>
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<table>
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</tr>
<tr>
<td>% of residues in disallowed regions</td>
<td>0.0 (0 residues)</td>
</tr>
</tbody>
</table>

*a Numbers in parentheses indicate the values in the highest-
3.6.2.8. Mammalian cytotoxicity assays

In order to assess the potential cytotoxicity of our inhibitors to mammalian cells, we selected our best Eis inhibitors and tested their cytotoxicity alone or in the presence of KAN against A549, HEK-293, and J774A.1 cells. Experiments were performed as previously described with slight modifications. HEK-293 and J774A.1 cells were plated at 10,000 cells/well density in 100 µL/well of the appropriate media in 96-well tissue culture plates (see Materials and instrumentations section). A549 cells were plated at 3,000 cells/well density in the same amount of medium overnight. For the cytotoxicity of Eis inhibitors alone, after overnight culturing at 37 °C with 5% CO₂, the old medium was replaced with 200 µL of fresh medium supplemented with serially diluted Eis inhibitors with final concentrations ranging from 0.34 to 100 µM and 0.5% DMSO. Cells for negative control were treated with the appropriate DMEM medium supplemented with 0.5% DMSO. Cells for positive control were treated with the appropriate medium with 0.5% DMSO and 1% v/v Triton-X 100. The cells were then incubated at 37 °C with 5% CO₂ for 24 h. Resazurin (10 µL of 2 mM sterile solution in 1× PBS) was then added to each well in order to detect the amount of live cells, which convert resazurin into highly fluorescent resorufin. After incubation at 37 °C with 5% CO₂ for 6 h, fluorescence was measured with plate reader with an excitation wavelength of λ₅60 nm and an emission wavelength of λ₅90 nm. The percent cell survival was summarized and presented in Figure 3.12. Experiments were done in quadruplicate.
By using the same protocol, the toxicity of KAN alone against the above three cell lines was determined with the final concentration of KAN ranging from 2 to 500 µM (without DMSO since KAN is water soluble). After seeing that KAN displayed no toxicity up to 500 µM (Figure 3.13), we decided to perform the above toxicity experiments with Eis inhibitors in the presence of 50 µg/mL KAN (equivalent of 86 µM), which is 10–40× MIC of KAN against \textit{Mtb} H37Rv in order to better assess the combined toxicity of Eis inhibitors and KAN (Figure 3.13). Experiments were done in quadruplicate.

3.7. ACKNOWLEDGEMENTS

This study was funded by a National Institutes of Health (NIH) grant AI090048 (to S.G.-T.), a grant from the Firland Foundation (to S.G.-T.), a grant from the Center for Chemical Genomics (CCG) at the University of Michigan (to S.G.-T.), as well as by startup funds from the College of Pharmacy at the University of Kentucky (to S.G.-T. and O.V.T). H.X.N. was in part supported by a Pharmaceutical Sciences Excellence in Graduate Achievement Fellowship from the College of Pharmacy at the University of Kentucky. We thank S. Vander Roest, M. Larsen, and P. Kirchhoff from the CCG at the University of Michigan for their help with HTS. We thank the staff of sector 22 (SER-CAT) of the Advanced Photon Source at the Argonne National Laboratories for their assistance with the remote X-ray diffraction data collection. The beamline use was supported, in part, by the Center for Structural Biology at the University of Kentucky. We thank Dr. Wenjing Chen for preliminary work on IC$_{50}$ value determination. We thank Helen V. Waldschmidt and Matthew D. Demars for the help in the preliminary synthesis of a few molecules. Use of trade names is for identification only and does not constitute endorsement by the U.S. Department of Health and Human Services, the
U.S. Public Health Service, or the CDC. The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the funding agencies.


3.8. AUTHORS’ CONTRIBUTIONS

HXN, AG, and ASM synthesized all compounds.

KDG, MJW, and SYLH performed all biochemical and biological assays.

CSG and CH performed structural biological studies.

HXN, JEP, OVT, and SGT analyzed data and wrote the manuscript.
Chapter 4

Eis inhibitors with potential synergistic bactericidal activity with Kanamycin against

*Mycobacterium tuberculosis*

4.1. INTRODUCTION

Since the discovery of the penicillin, antibiotics have arguably been the most successful technological advancement in healthcare as they are responsible for the rapid reduction of mortality and morbidity associated with a large number of previously fatal diseases.\(^{149}\)

Unfortunately, in the last few decades, resistance to antibiotics has emerged to be a global crisis. Unlike other classes of drugs, there is a constant need for discovery of new molecules and strategies to combat antibiotic resistance.\(^{150}\) The antibiotic crisis has been attributed to the lack of new drug development by the pharmaceutical industry as well as inappropriate usage of these drugs in the clinical setting.\(^{151}\) Unlike anticancer or cardiovascular drugs, typical antibiotic courses normally only last 7 to 10 days, which severely limits the potential for antibiotics as blockbuster drugs.\(^{101}\) The lack of potential significant profit return, hence, discourages investment by major pharmaceutical companies.

Amongst the most common bacterial infections, tuberculosis (TB) has become a major health problem, which was responsible for approximately 710,000 deaths in 2015.\(^9\) To make matters worse, ineffective TB treatment potentially led to over 200,000 cases of drug-resistant TB
(DR-TB) in 2015. Treating DR-TB can cost as much as $5000 per case. More importantly, among communicable diseases, TB is the most deadly disease from the age of 15 to 49, which is considered to be the most productive time in a human lifespan. Thus, there is a clear need for new anti-tubercular drugs. Many new promising anti-tubercular drug scaffolds currently being developed in the literature include: the selective 1,3,5-triazaspiro[5.5]undeca-2,4-diene-based inhibitors of dihydrofolate reductase,\textsuperscript{152} a β-lactone inhibitor that can knock out Pks13 and Ag85, which halts mycolic acid synthesis,\textsuperscript{153} and the natural products Kuwanon G and H, which are inhibitors of PtpA and PtpB of Mycobacterium tuberculosis (\textit{Mtb}).\textsuperscript{154}

Previously, we proposed Eis inhibitors as adjuvants to reactivate the anti-tubercular activity of kanamycin (KAN) in KAN-resistant \textit{Mtb} strains. These inhibitors were found to specifically inhibit Eis and thus, did not kill \textit{Mtb} strains by themselves. However, during our search for Eis inhibitors, we were able to identify a novel class of thieno[2,3-\textit{d}]pyrimidine-based inhibitors that do not only inhibit Eis to restore the activity of KAN, but also possessed their own anti-tubercular activity. Basal-level expression of Eis is hypothesized to decrease the efficacy of KAN and limit the MIC of KAN to 1.25 µg/mL. Thus, compounds that can inhibit Eis and display their own activities may be synergistic with KAN against \textit{Mtb} cells. To validate this hypothesis, we will perform synergy checkerboard assays with these compounds. Additionally, we accessed the therapeutic indices of these analogues \textit{via} mammalian cytotoxicity.
4.2. RESULTS AND DISCUSSION

4.2.1. High-throughput screen

To identify potential Eis inhibitors, we performed a high-throughput screen (HTS) against purified Eis enzyme using ~23,000 compounds from three commercially available chemical libraries: the BioFocus NCC library (~1,000 compounds), the ChemDiv library (~20,000 compounds), and the MicroSource MS2000 library (~2,000 compounds). We achieved high robustness in our HTS with a Z’ score of 0.65. Sixty-six compounds with thieno[2,3-d]pyrimidine core structure were identified as potential inhibitors and compound 2i was validated as an Eis inhibitor. We then re-synthesized 2i and 71 other analogues and determined the IC50 values for these inhibitors. More interestingly, we found that some of these inhibitors are also active against Mtb cells on their own. Thus, we determined the MIC values for all of our compounds and found 11 analogues with MIC of 4 µM against Mtb MC26020, a fast-growing attenuated double lysine and pantothenate auxotroph of Mtb.

4.2.2. Chemistry

The library is composed of 72 molecules shown in Figure 4.1. Overall, the library can be divided into 7 series of compounds where various modifications are introduced in the aliphatic ring that is annulated and adjacent to the thiophene ring. For instance, the annulated ring can be a cyclopentane ring (series 1), cyclohexane (series 2), cycloheptane (series 8). Additionally, we also introduced substitutions at the R2 and R3 positions. These substitutions could be unsubstituted (H) (series 2) or substituted with methyl (series 3), ethyl (series 5), tert-butyl (series 6), or phenyl groups (series 7). Furthermore, within these 7 major series, we
also installed aliphatic tertiary amino side chains at the R_1 position (Figure 4.1). These aliphatic tertiary amino side chains were selected based on our previous observations that inhibitors with a tertiary amino groups tend to have superior IC_{50} compared to the ones that do not. This phenomenon can perhaps be explained by the fact that these tertiary amino compounds are positively charged at physiology pH allowing them to better mimic the positively charged natural AG substrates. To access the thieno[2,3-d]pyrimidine core, we initially prepared a series of 2-aminothiophenes (17-24) as building blocks via the classic Gewald reaction starting from the commercially available malononitrile, ethyl cyanoacetate, elemental sulfur, and various cyclic ketones (9-15). The building blocks 17-24 were reacted with benzoyl isothiocyanate in 1,2-dioxane to afford the thioureas (25-32). The intermediates 25-32 were condensed in 2N NaOH and EtOH to afford the thieno[2,3-d]pyrimide core scaffolds (33-40). Compounds 33-40 precipitated after neutralization with 10% acetic acid. Side chains were introduced to compounds 33-40 via thiol alkylations to afford the final products 1-8(a-i). Cesium carbonate was found to be superior to potassium carbonate in terms of reaction times and yields. Please note that the alkyl halides 41-44 were not commercially available and were prepared by reacting commercially primary amines with bromoacetyl chloride. The alkyl halides 41-44 were used in alkylation reactions with intermediates 33-40 to afford final products with side chains: g, h, and i.
Figure 4.1. Structures of the synthesized thieno[2,3-d]pyrimidine compounds
Figure 4.2. Synthetic scheme to generate the thieno[2,3-d]pyrimidine library. A. Reactions to prepare the thieno[2,3-d]pyrimidine. B. Reactions to prepare the amido side chains.

4.2.3. Eis inhibitory activity

The SAR study was analyzed based on IC$\text{_{50}}$ values of inhibitors in the presence of purified Eis enzyme, acetyl coenzyme A (AcCoA), and the AG substrate (KAN). From our HTS, we confirmed that our hit compound, 2i is an Eis inhibitor with IC$\text{_{50}}$ value of 1.61 ± 0.16 µM.
(Table 4.1). From the hit 2i, we started to derivitize and synthesize a library of 72 analogues. Our library contains analogues that are modified at 2 sites: (1) the annulated ring at the western side of the thieno[2,3-\(d\)]pyrimidine core and (2) the side chain \(R_1\) (Figure 4.1 and Table 4.1). By comparing the IC\(_{50}\) values of analogues with the same side chains (\(R_1\)), we realized that some annulated rings that are more likely to generate potent Eis inhibitors. In 7 out of the 9 side chains, the cyclohexane (series 2) was most tolerated with IC\(_{50}\) values ranging from 0.154 ± 0.022 to 1.61 ± 0.16 \(\mu\)M. Other than the cyclohexane ring, the next two annulated rings that most likely yield potent compounds were the cyclopentane (series 1) and the 4-methyl cyclohexane (series 4) rings. On the other hand, all of the bulkier annulated rings such as 4-tertbutyl cyclohexane (series 6), 4-phenyl cyclohexane (series 7), and cycloheptane (series 8) tend to lead to analogues with diminished or no Eis inhibitory activity. This observation indicates that the Eis active site likely has spatial limitation in the area surrounding the western side of the thieno[2,3-\(d\)]pyrimidine core.

To assess the side chain (\(R_1\)), we compared compounds with the same annulated rings and varying \(R_1\) side chains. The \(R_1\) side chains are greatly diverse in terms of chemical structures. Despite the diversity, they all share the same characteristics, which is that they all contain tertiary aliphatic nitrogen-containing rings. In terms of the connection to the core, the linker may be methylene, ethylene, or propylene. Because the hit compound 2i contains an amide bond, we initially tried to replicate this feature in our study. Side chains g, h, and i generated a few potent inhibitors such as compounds 2g (IC\(_{50}\) = 0.249 ± 0.019 \(\mu\)M), 4g (0.197 ± 0.032 \(\mu\)M), 2h (0.460 ± 0.061 \(\mu\)M), 5h (0.676 ± 0.0896 \(\mu\)M), and 4i (0.466 ± 0.080 \(\mu\)M). However, overall, adding amide bonds to these molecules typically decreased inhibitory activity.
Table 4.1. IC\textsubscript{50} and MIC values of compounds.

<table>
<thead>
<tr>
<th>Cpd</th>
<th>IC\textsubscript{50} ((\mu)M)</th>
<th>MC\textsuperscript{2}6020 MIC ((\mu)M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>2.19 ± 0.30</td>
<td>8</td>
</tr>
<tr>
<td>2a</td>
<td>0.986 ± 0.055</td>
<td>4</td>
</tr>
<tr>
<td>3a</td>
<td>6.68 ± 0.69</td>
<td>4</td>
</tr>
<tr>
<td>4a</td>
<td>2.37 ± 0.16</td>
<td>4</td>
</tr>
<tr>
<td>5a</td>
<td>&gt;200</td>
<td></td>
</tr>
<tr>
<td>6a</td>
<td>22.6 ± 5.4</td>
<td>4</td>
</tr>
<tr>
<td>7a</td>
<td>4.02 ± 1.12</td>
<td>4</td>
</tr>
<tr>
<td>8a</td>
<td>6.64 ± 0.58</td>
<td>4</td>
</tr>
<tr>
<td>1b</td>
<td>1.86 ± 0.16</td>
<td>64</td>
</tr>
<tr>
<td>2b</td>
<td>1.14 ± 0.08</td>
<td>32</td>
</tr>
<tr>
<td>3b</td>
<td>13.8 ± 1.3</td>
<td>32</td>
</tr>
<tr>
<td>4b</td>
<td>1.97 ± 0.30</td>
<td>8</td>
</tr>
<tr>
<td>5b</td>
<td>5.42 ± 0.52</td>
<td>4</td>
</tr>
<tr>
<td>6b</td>
<td>15.8 ± 1.8</td>
<td>4</td>
</tr>
<tr>
<td>7b</td>
<td>15.5 ± 2.1</td>
<td>16</td>
</tr>
<tr>
<td>8b</td>
<td>7.55 ± 1.06</td>
<td>32</td>
</tr>
<tr>
<td>1c</td>
<td>0.748 ± 0.064</td>
<td>32-64</td>
</tr>
<tr>
<td>2c</td>
<td>0.172 ± 0.037</td>
<td>64</td>
</tr>
<tr>
<td>3c</td>
<td>3.76 ± 0.47</td>
<td>16</td>
</tr>
<tr>
<td>4c</td>
<td>0.612 ± 0.079</td>
<td>16</td>
</tr>
<tr>
<td>5c</td>
<td>0.912 ± 0.134</td>
<td>16</td>
</tr>
<tr>
<td>6c</td>
<td>6.62 ± 1.19</td>
<td></td>
</tr>
<tr>
<td>7c</td>
<td>0.230 ± 0.078</td>
<td>4</td>
</tr>
<tr>
<td>8c</td>
<td>1.22 ± 0.12</td>
<td>16</td>
</tr>
<tr>
<td>1d</td>
<td>1.81 ± 0.12</td>
<td>16</td>
</tr>
<tr>
<td>2d</td>
<td>0.450 ± 0.029</td>
<td>16</td>
</tr>
<tr>
<td>3d</td>
<td>3.58 ± 0.16</td>
<td>16</td>
</tr>
<tr>
<td>4d</td>
<td>0.810 ± 0.132</td>
<td>8</td>
</tr>
<tr>
<td>5d</td>
<td>&gt;200</td>
<td>&gt;100</td>
</tr>
<tr>
<td>6d</td>
<td>6.33 ± 1.46</td>
<td>4</td>
</tr>
<tr>
<td>7d</td>
<td>0.533 ± 0.038</td>
<td></td>
</tr>
<tr>
<td>8d</td>
<td>2.25 ± 0.18</td>
<td>16</td>
</tr>
<tr>
<td>-----</td>
<td>------------</td>
<td>----</td>
</tr>
<tr>
<td>1e</td>
<td>0.796 ± 0.101</td>
<td>128</td>
</tr>
<tr>
<td>2e</td>
<td>0.154 ± 0.022</td>
<td>64</td>
</tr>
<tr>
<td>3e</td>
<td>1.98 ± 0.11</td>
<td>64</td>
</tr>
<tr>
<td>4e</td>
<td>0.358 ± 0.053</td>
<td>8</td>
</tr>
<tr>
<td>5e</td>
<td>6.95 ± 0.72</td>
<td>&gt;100</td>
</tr>
<tr>
<td>6e</td>
<td>4.64 ± 0.63</td>
<td>4</td>
</tr>
<tr>
<td>7e</td>
<td>0.463 ± 0.174</td>
<td>8</td>
</tr>
</tbody>
</table>

| 8e  | 2.52 ± 0.07 | 32 |
| 1f  | 11.4 ± 0.8 | >100 |
| 2f  | 1.48 ± 0.10 | >100 |
| 3f  | 51.6 ± 6.5 | >100 |
| 4f  | 3.56 ± 0.35 | |
| 5f  | 1.05 ± 0.07 | |
| 6f  | ~200 | |
| 7f  | 2.96 ± 0.58 | 16 |
| 8f  | 8.74 ± 1.23 | >100 |
| 1g  | 1.86 ± 0.59 | |
| 2g  | 0.249 ± 0.019 | >100 |
| 3g  | ~2.4 | |
| 4g  | 0.197 ± 0.032 | 64 |

| 5g  | 1.07 ± 0.24 | 16 |
| 6g  | >200 | |
| 7g  | 0.484 ± 0.058 | |

| 8g  | 3.10 ± 0.31 | 8 |
| 1h  | 2.46 ± 0.37 | |
| 2h  | 0.460 ± 0.061 | >100 |
| 3h  | 6.79 ± 0.59 | 128 |
| 4h  | 3.74 ± 0.650 | 64 |
| 5h  | 0.676 ± 0.896 | 32 |
| 6h  | 10.0 ± 4.1 | 16 |
| 7h  | 0.925 ± 0.291 | 8 |
| 8h  | 0.297 ± 0.061 | |

<p>| 1i  | 17.4 ± 1.3 | &gt;100 |
| 2i  | 1.61 ± 0.16 | &gt;100 |</p>
<table>
<thead>
<tr>
<th></th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>3i</td>
<td>55.6 ± 5.5</td>
</tr>
<tr>
<td>4i</td>
<td>0.466 ±</td>
</tr>
<tr>
<td></td>
<td>&gt;100</td>
</tr>
<tr>
<td></td>
<td>0.080</td>
</tr>
<tr>
<td>5i</td>
<td>18.5 ± 3.4</td>
</tr>
<tr>
<td>6i</td>
<td>&gt;200</td>
</tr>
<tr>
<td>7i</td>
<td>15.7 ± 3.3</td>
</tr>
<tr>
<td>8i</td>
<td>8.53 ± 1.31</td>
</tr>
</tbody>
</table>

Subsequently, we analyzed the IC$_{50}$ values for each series of compounds separately and recognized that side chain e (Figure 4.1) tends to be associated with the most potent Eis inhibitors. For instance, within the compound series 2a-e, compound 2e (IC$_{50}$ = 0.154 ± 0.022) was the most potent inhibitor. Additionally, except in the case of compounds 5e (IC$_{50}$ = 6.95 ± 0.72), all molecules with side chain e, such as compounds 1e (IC$_{50}$ = 0.796 ± 0.101), 3e (IC$_{50}$ = 1.98 ± 0.11), 4e (IC$_{50}$ = 0.358 ± 0.053), and 6e (IC$_{50}$ = 4.64 ± 0.63), were always part of the top 2 inhibitors in their respective compound series.

Other than fragment e, we found that fragment c is also well representative within the best inhibitors. Compounds 1c (IC$_{50}$ = 0.748 ± 0.064), 2c (IC$_{50}$ = 0.172 ± 0.037), 4c (IC$_{50}$ = 0.612 ± 0.079), 5c (IC$_{50}$ = 0.912 ± 0.134), 7c (IC$_{50}$ = 0.230 ± 0.078), and 8c (IC$_{50}$ = 1.22 ± 0.12) were amongst the top two inhibitors in their respective series.

While fragments e and c are highly associated with the most inhibitors, fragment i significantly diminished the activity of inhibitors. In the cases of compounds 1i, 2i, 4i, 5i, 6i, 7i, and 8i, we observed that they were the worst inhibitors in their respective compound series.
4.2.4. Activity of thieno[2,3-d]pyrimidine Eis inhibitors against *Mtb* K204 cells

With the help of our collaborators at the CDC, we were also able to co-administer some of these Eis inhibitors along with KAN against *Mtb* K204 cells to verify whether these inhibitors could restore the activity of KAN (Table 4.2). We observed that the most potent Eis inhibitors (1c, 2c, and 2e) were able to restore the activity of KAN at 2.5 µg/mL. On the other hand, the bad Eis inhibitors (3i and 8f) were not able to restore the activity of KAN. More interestingly, we also found that some compounds such as compounds 5g and 7g were displayed toxicity effects against *Mtb* K204 cells independently of KAN. This finding was intriguing and prompted us to evaluate all of our inhibitors against *Mtb* MC²6020, an engineered fast-growing *Mtb* strain. Since *Mtb* MC²6020 is also a BSL 2 cell line, we were able to conveniently perform these MIC assays in the laboratory.

<table>
<thead>
<tr>
<th>Cpd</th>
<th>IC₅₀ (µM)</th>
<th>MICKAN (K204) treated with 100 µM inhibitor (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1c</td>
<td>0.748 ± 0.064</td>
<td>2.5</td>
</tr>
<tr>
<td>2c</td>
<td>0.172 ± 0.037</td>
<td>2.5</td>
</tr>
<tr>
<td>2e</td>
<td>0.154 ± 0.022</td>
<td>2.5</td>
</tr>
<tr>
<td>3i</td>
<td>55.6 ± 5.5</td>
<td>10</td>
</tr>
<tr>
<td>8f</td>
<td>8.74 ± 1.23</td>
<td>10</td>
</tr>
<tr>
<td>5g</td>
<td>1.07 ± 0.24</td>
<td>Toxic without KAN</td>
</tr>
<tr>
<td>7g</td>
<td>0.484 ± 0.058</td>
<td>Toxic without KAN</td>
</tr>
</tbody>
</table>

**Table 4.2. Activity of compounds on *Mtb* K204 cells.**

4.2.5. Antitubercular activity against *Mtb* MC²6020

The best compounds displayed MIC as low as 4 µM (Table 4.1). Comparing IC₅₀ with MIC values revealed that there is definitely no correlation between Eis inhibition and toxicity against *Mtb* cells. This finding suggested the mechanism of toxicity of these thieno[2,3-
pyrimidine compounds is likely independent of Eis inhibition. We found that a large numbers of the compounds with potent MIC of 4 \( \mu \)M contain the side chain a \( (2a, 3a, 4a, 6a, 7a, \) and \( 8a) \). Additionally, many molecules with the tert-butyl moiety (series 6) \( (6a, 6b, \) and 6d) also have potent activity against \( \textit{Mtb} \) cells. Compounds 2a and 7c are interesting because in addition to low MIC values, they are also low micromolar Eis inhibitors making them interesting molecules to evaluate for synergism with aminoglycosides. Efforts to elucidate the mechanism of action and synergism with aminoglycosides are ongoing.

4.3. CONCLUSION
From HTS, we were able to identify a novel class of Eis inhibitors. We also discovered that this class of compounds also displays cytotoxicity to \( \textit{Mtb} \) cells, which is most likely unrelated to their abilities to inhibit Eis enzyme. However, this finding presents an intriguing application for these molecules. While capable of restoring the activity of KAN, these thieno[2,3-\(d\)]pyrimidine compounds are also likely to complement the bactericidal effect of KAN with their own mechanism of action. Future studies are ongoing to elucidate the unknown mechanism of action and explore the potential synergism between thieno[2,3-\(d\)]pyrimidine compounds and aminoglycosides.

4.4. MATERIALS AND INSTRUMENTATION
All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and AK Scientific (Union City, CA, USA) and used without further purification. Deuterated solvents ((\( \text{CD}_3 \))\textsubscript{2}SO, CDCl\textsubscript{3}, and CD\textsubscript{3}OD) were bought from Cambridge Isotope Laboratories Inc. (Tewksbury,
MA, USA). TLC (Merck, Silica gel 60 F254) was used to monitor reaction progress. Visualization of reaction progress was achieved using one or more of the following methods: UV absorption and a cerium-molybdate stain \(((\text{NH}_4)_2\text{Ce(NO}_3)_6\) (5 g), \((\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot 4\text{H}_2\text{O}\) (120 g), \(\text{H}_2\text{SO}_4\) (80 mL), and \(\text{H}_2\text{O}\) (720 mL)). Silica gel (SiO2, Dynamic Adsorbents Inc. Flash silica gel 32-63u) was used to perform flash column chromatography. NMR spectra were measured in \(\delta\) (ppm) using the \(^1\text{H NMR CDCl}_3\) (\(\delta\) 7.24 ppm), \(^{13}\text{C NMR CDCl}_3\) (\(\delta\) 77.23 ppm), \(^1\text{H NMR (CD}_3)_2\text{SO}\) (\(\delta\) 2.50 ppm), \(^{13}\text{C NMR (CD}_3)_2\text{SO}\) (\(\delta\) 39.51 ppm), \(^1\text{H NMR CD}_3\text{OD}\) (\(\delta\) 3.31 ppm), and \(^{13}\text{C NMR CD}_3\text{OD}\) (\(\delta\) 49.15 ppm) as internal standards. Abbreviations used: \(J\) = coupling constants in Hz, app. = apparent, br = broad, d = doublet, dd = doublet of doublets, dt = doublet of triplets, m = multiplet, p = pentet, q = quartet, s = singlet, t = triplet, qd = quartet of doublets, qt = quartet of triplets, td = triplet of doublets, tt = triplet of triplets; ddd = doublet of doublets of doublets, ddt = doublet of doublets of triplets, tdd = triplet of doublets of doublets. \(^1\text{H NMR and }^{13}\text{C NMR spectra were recorded on a Varian 400 MHz instrument (Palo Alto, CA, USA). Low-resolution electrospray mass spectra (LRMS) were recorded on a liquid chromatography-mass spectrometry using an Agilent 1200 series Quaternary LC system (Santa Clara, CA, USA) equipped with a diode array detector, and Eclipse XDB-C18 column (250 mm \(\times\) 4.6 mm, 5 \(\mu\)m), and an Agilent 6120 Quadrupole MSD mass spectrometer. Further confirmation of purity for these final molecules was performed on an Agilent Technologies 1260 Infinity HPLC system (Santa Clara, CA, USA), by using the following general method 1 and 2. Method 1: Flow rate = 0.5 mL/min; \(\lambda\) = 254 nm; column = Apollo C18 column, 250 \(\times\) 4.6 mm, 100 Å, 5 \(\mu\)m; Eluents: A = \(\text{H}_2\text{O} + 0.1%\) formic acid, B = \(\text{MeCN} + 0.1%\) formic acid; gradient profile: starting from 5% B, increasing from 5% B to 95% B over 16 min, hold 100% B from 16-20 min, decreasing from 100% B to
5% B from 20-30 min. Method 2: Flow rate = 0.5 mL/min; λ = 254 nm; column = Vydac DENALI C18 column, 150 x 4.6 mm, 120 Å, 5 μm; Eluents: A = H₂O + 0.1% TFA, B = MeCN; gradient profile: starting from 5% B to 100% B over 20 min, hold 100% B from 20-25 min, decreasing from 100% B to 5% B from 25-27 min. Prior to each injection, the HPLC column was equilibrated for 15 min with 5% B. All compounds were ≥95% pure. All reactions were conducted under nitrogen atmosphere, unless otherwise specified. All yields reported refer to isolated yields.

4.5. METHODS

4.5.1. Chemical methods

4.5.1.1. General procedure A for the preparation of compounds 17-24

Synthesis of compounds 17-24 was done following a modified published protocol.¹⁵⁵ Malononitrile (1 eq) and sulfur (1 eq) were added to a solution of a cycloalkyl ketone (1 eq) in EtOH (30 mL). Morpholine (5 mL) was added to the mixture. After stirring at rt for 1-15 h, the reaction was quenched by the addition of ddH₂O. The product was filtered, washed with ddH₂O, and dried to afford the desired product. Note: Although we did not purify these compounds via SiO₂ column chromatography, we report R_f values as an additional mean to verify that the correct compound is obtained.

Preparation of compound 17. The known compound 17 was prepared by using the general procedure A. Malononitrile (3.97 g, 60.0 mmol), sulfur (1.91 g, 59.5 mmol), cyclopentanone (9) (5.3 mL, 59.9 mmol), and morpholine (5 mL) in EtOH (30 mL)
were used to afford compound 17 (9.18 g, 94%; Rf 0.39 in 5:1/Hexanes:EtOAc) as a yellow solid after reacting for 15 h. This product was without any further purification: $^1$H NMR (400 MHz, (CD$_3$)$_2$SO) δ 7.03 (s, 2H), 2.65 (m, 2H), 2.56 (m, 2H), 2.26 (m, 2H); $^{13}$C NMR (100 MHz, (CD$_3$)$_2$SO) δ 168.6, 141.0, 121.3, 116.5, 78.8, 28.9, 28.0, 26.8; LRMS m/z calcd for C$_8$H$_8$N$_2$S: 164.0; found 165.0 [M+H]$^+$. 

Preparation of compound 18. The known compound 18 was prepared by using the general procedure A. Malononitrile (3.40 g, 50.1 mmol), sulfur (1.64 g, 50.1 mmol), cyclohexanone (10) (4.7 mL, 50.1 mmol), and morpholine (5 mL) in EtOH (30 mL) were used to afford compound 18 (7.60 g, 84%) as a yellow solid after reacting for 5 h and purification using flash column chromatography (SiO$_2$, 5:1/Hexanes:EtOAc, Rf 0.59): $^1$H NMR (400 MHz, CDCl$_3$, which matches lit.$^{155}$) δ 4.55 (s, 2H), 2.49-2.46 (m, 4H), 1.80-1.74 (m, 4H).

Preparation of compound 19. Compound 19 was prepared by using the general procedure A. Malononitrile (2.94 g, 44.58 mmol), sulfur (1.47 g, 51.40 mmol), 2-methylcyclohexanone (11) (5.40 mL, 44.58 mmol), and morpholine (5 mL) in EtOH (30 mL) were used to afford compound 19 (1.43 g, 17%; Rf 0.25 in 4:1/Hexanes:EtOAc) as a brown crystal after reacting for 5 h: $^1$H NMR (400 MHz, CDCl$_3$) δ 4.16 (br s, 2H), 2.80-2.75 (m, 1H), 2.50-2.39 (m, 2H), 1.84-1.70 (m, 3H), 1.58-1.50 (m, 1H), 1.25 (d, J = 6.8 Hz, 3H); $^{13}$C NMR (100 MHz, (CD$_3$)$_2$SO) δ 163.2, 135.9, 116.8, 116.5, 82.7, 29.7, 28.6, 23.8, 20.4, 19.3; LRMS m/z calcd for C$_{10}$H$_{12}$N$_2$S: 192.1; found 193.1 [M+H]$^+$. 

172
Preparation of compound 20. Compound 20 was prepared by using the general procedure A. Malononitrile (2.95 g, 44.60 mmol), sulfur (1.43 g, 44.60 mmol), 4-methylcyclohexanone (12) (5.47 mL, 44.60 mmol), and morpholine (5 mL) in EtOH (30 mL) were used to afford compound 20 (6.29 g, 73%; R_f 0.88 in 9:1/CH_2Cl_2:MeOH) as a yellow solid after reacting for 5 h: ^1^H NMR (400 MHz, (CD_3)_2SO) δ 6.93 (s, 2H), 2.46 (d, J = 5.2 Hz, 1H), 2.41-2.29 (m, 2H), 2.02 (ddt, J = 16.0, 9.6, 2.8 Hz, 1H), 1.86-1.75 (m, 2H), 1.35-1.25 (m, 1H), 0.99 (d, J = 6.4 Hz, 3H); ^1^C NMR (100 MHz, (CD_3)_2SO) δ 162.9, 130.8, 116.4, 116.3, 83.0, 31.6, 30.0, 29.3, 23.9, 21.2; LRMS m/z calcd for C_{10}H_{12}N_2S: 192.1; found 193.1 [M+H]^+.

Preparation of compound 21. Compound 21 was prepared by using the general procedure A. Malononitrile (2.65 g, 39.62 mmol), sulfur (1.26 g, 39.62 mmol), 4-ethylcyclohexanone (13) (5.6 mL, 39.62 mmol), and morpholine (5 mL) in EtOH (30 mL) were used to afford compound 21 (2.1 g, 26%; R_f 0.52 in 4:1/Hexanes:EtOAc) as a yellow solid after reacting for 5 h: ^1^H NMR (400 MHz, CDCl_3, which matches the lit.\(^{156}\)) δ 4.66 (br s, 2H), 2.56 (m, 2H), 2.42 (m, 1H), 2.14-2.07 (m, 1H), 1.92-1.86 (m, 1H), 1.67-1.59 (m, 1H), 1.43-1.29 (m, 3H), 0.92 (t, J = 7.6 Hz, 3H).

Preparation of compound 22. Compound 22 was prepared by using the general procedure A. Malononitrile (2.16 g, 32.41 mmol), sulfur (1.06 g, 32.41 mmol), 4-tert-butylcyclohexanone (5.07, 32.41 mmol), and morpholine (5 mL) in EtOH
(30 mL) were used to afford compound 22 (7.32 g, 97%; Rf 0.42 in 4:1/Hexanes:EtOAc) as a yellow solid after reacting for 5 h: $^1$H NMR (400 MHz, CDCl$_3$, which matches the lit.$^{156}$) $\delta$ 2.65-2.60 (m, 1H), 2.54-2.49 (m, 1H), 2.44-2.35 (m, 1H), 2.31-2.22 (m, 1H), 2.00-1.95 (m, 1H), 1.48 (tdd, $J$ = 12.0, 5.2, 2.0 Hz, 1H), 1.29 (qd, $J$ = 12.0, 5.2 Hz, 1H), 0.92 (s, 9H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 163.0, 131.1, 117.3, 116.3, 82.8, 44.9, 32.2, 27.1, 25.1, 25.0, 23.4.

**Preparation of compound 23.** Compound 23 was prepared by using the general procedure A. Malononitrile (1.18 g, 17.21 mmol), sulfur (0.58 g, 17.21 mmol), 4-phenylcyclohexanone (3.06, 17.21 mmol), and morpholine (3 mL) in EtOH (20 mL) were used to afford compound 23 (3.22 g, 74%; Rf 0.22 in 4:1/Hexanes:EtOAc) as a yellow solid after reacting for 5 h: $^1$H NMR (400 MHz, CDCl$_3$, which matches the lit.$^{157}$) $\delta$ 7.35-7.31 (m, 2H), 7.26-7.22 (m, 3H), 4.27 (br s, 2H), 3.06-2.98 (m, 1H), 2.82-2.77 (m, 1H), 2.73-2.58 (m, 3H), 2.15-2.10 (m, 1H), 2.00-1.89 (m, 1H); $^{13}$C NMR (100 MHz, (CD$_3$)$_2$SO) $\delta$ 163.1, 145.7, 130.9, 128.4, 126.9, 126.3, 116.4, 116.2, 83.0, 40.4, 31.4, 29.1, 24.4.

**Preparation of compound 24.** Compound 24 was prepared by using the general procedure A. Malononitrile (2.95 g, 44.60 mmol), sulfur (1.43 g, 44.60 mmol), cycloheptanone (5.26 mL, 44.60 mmol), and morpholine (5 mL) in EtOH (30 mL) to afford compound 24 (5.36 g, 63%; Rf 0.37 in 4:1/Hexanes:EtOAc) as a dark red solid after reacting for 5 h: $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 4.18 (br s, 2H), 2.62-2.55 (m, 4H), 1.85-1.75 (m, 2H), 1.68-1.58 (m, 4H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 158.2, 136.8, 123.6, 116.0, 91.6, 31.8, 29.4, 29.1, 28.0, 27.2; LRMS m/z calcd for C$_{10}$H$_{12}$N$_2$S: 192.1; found 193.1 [M+H]$^+$. 

174
4.5.1.2. General procedure B for the preparation of compounds 25-32

Synthesis of compounds 25-32 was done following a published protocol. Benzoyl isothiocyanate (1 eq) was added to a solution of a thiophene derivative (1 eq) in anhydrous 1,4-dioxane (20 mL). After stirring at rt for 1-3 h, the product was filtered and washed with a 4:1/Hexanes:EtOAc mixture to afford the desired product, which was used without any further purification. Note: Although we did not purify these compounds via SiO$_2$ column chromatography, we report R$_f$ values as an additional mean to verify that the correct compound is obtained.

**Preparation of compound 25.** The known compound 25 was prepared by using general procedure B. Benzoyl isothiocyanate (7.15 mL, 53.2 mmol) and compound 17 (8.73 g, 53.2 mmol) in anhydrous 1,4-dioxane (20 mL) were used to afford compound 25 (12.54 g, 72%; R$_f$ 0.66 in 10:1/Hexanes:EtOAc) as a yellow solid after reacting for 2 h: $^1$H NMR (400 MHz, (CD$_3$)$_2$SO) δ 12.23 (s, 1H), 8.01 (app. d, $J$ = 7.6 Hz, 2H), 7.69 (app. t, $J$ = 7.6 Hz, 1H), 7.56 (t, $J$ = 7.6 Hz, 2H), 2.89 (t, $J$ = 7.2 Hz, 2H), 2.80 (t, $J$ = 7.2 Hz, 2H), 2.39 (p, $J$ = 7.2 Hz, 2H); $^{13}$C NMR (100 MHz, (CD$_3$)$_2$SO) δ 175.1, 169.4, 151.6, 140.9, 135.6, 133.5, 131.5, 128.9, 128.5, 113.9, 109.5, 29.1, 27.6, 27.5; LRMS m/z calcd for C$_{16}$H$_{13}$N$_3$OS$_2$: 327.1; found 328.0 [M+H]$^+$. 
**Preparation of compound 26.** The known compound 26 was prepared using general procedure B. Benzoyl isothiocyanate (1.45 mL, 10.78 mmol) and compound 18 (2.01 g, 10.78 mmol) in anhydrous 1,4-dioxane (25 mL) were used to afford compound 26 (3.36 g, 90%; Rf 0.76 in 49:1/CH2Cl2:MeOH) as a yellow solid after reacting for 1 h: $^1$H NMR (400 MHz, (CD$_3$)$_2$SO, which matches lit.$^{155}$) δ 12.26 (s, 1H), 8.01 (d, $J$ = 7.6 Hz, 2H), 7.69 (t, $J$ = 8.0 Hz, 1H), 7.56 (t, $J$ = 8.0 Hz, 2H), 2.66 (m, 2H), 2.57 (m, 2H), 1.78 (m, 4H); LRMS m/z calcld for C$_{17}$H$_{15}$N$_3$OS$_2$: 341.1; found 342.7 [M+H]$^+$. 

**Preparation of compound 27.** Compound 27 was prepared using general procedure B. Benzoyl isothiocyanate (3.90 mL, 29.01 mmol) and compound 19 (5.54 g, 28.83 mmol) in anhydrous 1,4-dioxane (67 mL) were used to afford compound 27 (5.39 g, 53%; Rf 0.50 in 1:2/Hexanes:EtOAc) as a yellow solid after reacting for 1 h: $^1$H NMR (400 MHz, CDCl$_3$) δ 9.09 (s, 1H), 7.91 (dd, $J$ = 8.8, 1.2 Hz, 2H), 7.64 (tt, $J$ = 6.8, 1.2 Hz, 1H), 7.53 (t, $J$ = 8.0 Hz, 2H), 2.98-2.95 (m, 1H), 2.69-2.55 (m, 2H), 2.14-1.75 (m, 3H), 1.63-1.54 (m, 1H), 1.31 (d, $J$ = 7.2 Hz, 3H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 174.3, 167.3, 147.7, 136.9, 134.3, 131.0, 129.8, 129.5, 128.0, 114.4, 98.4, 30.3, 29.0, 24.7, 21.2, 19.8; LRMS m/z calcld for C$_{18}$H$_{17}$N$_3$OS$_2$: 355.1; found 356.1 [M+H]$^+$. 

**Preparation of compound 28.** Compound 28 was prepared using general procedure B. Benzoyl isothiocyanate (4.40 mL, 32.7 mmol) and compound 20 (6.29 g, 32.7 mmol) in anhydrous 1,4-dioxane (60 mL) were used to afford compound 28 (9.00 g, 78%; Rf 0.55 in 1:2/Hexanes:EtOAc) as a yellow solid after reacting for
Preparation of compound 29. Compound 29 was prepared using general procedure B. Benzoyl isothiocyanate (2.70 mL, 20.36 mmol) and compound 21 (4.20 g, 20.36 mmol) in anhydrous 1,4-dioxane (30 mL) were used to afford compound 29 (6.04 g, 80%, Rf 0.62 in 1:2/Hexanes:EtOAc) as a yellow solid after reacting for 1 h: $^1$H NMR (400 MHz, (CD$_3$)$_2$SO) $\delta$ 12.24 (s, 1H), 8.01 (app. d, $J = 8.0$ Hz, 2H), 7.69 (app. t, $J = 7.2$ Hz, 1H), 7.56 (t, $J = 8.0$ Hz, 2H), 2.79 (dd, $J = 16.9$, 4.8 Hz, 1H), 2.67-2.62 (m, 1H), 2.57-2.54 (m, 1H), 2.25 (dd, $J = 15.6$, 10.4 Hz, 1H), 1.94-1.91 (m, 1H), 1.64 (m, 1H), 1.44-1.30 (m, 3H), 0.93 (t, $J = 7.6$ Hz, 3H); $^{13}$C NMR (100 MHz, (CD$_3$)$_2$SO) $\delta$ 175.3, 169.4, 147.0, 133.5, 131.5, 130.7, 129.0, 128.6, 128.5, 113.6, 96.8, 31.3, 29.7, 29.0, 23.1, 21.1; LRMS m/z calcd for C$_{18}$H$_{17}$N$_3$OS$_2$: 355.1; found 356.1 [M+H]$^+$. 

Preparation of compound 30. Compound 30 was prepared using general procedure B. Benzoyl isothiocyanate (4.40 mL, 32.7 mmol) and compound 22 (3.04 g, 13.0 mmol) in anhydrous 1,4-dioxane (30 mL) were used to afford compound 30 (5.09 g, 100%; Rf 0.49 in 4:1/Hexanes:EtOAc) as a yellow solid after reacting
for 3 h: \(^1\)H NMR (400 MHz, (CD\(_3\))\(_2\)SO) \(\delta\) 12.23 (s, 1H), 8.03-8.00 (dd, \(J = 8.8, 1.2\) Hz, 2H), 7.69 (tt, \(J = 7.6, 1.2\) Hz, 1H), 7.56 (t, \(J = 7.6\) Hz, 2H), 2.71 (td, \(J = 16.4, 4.4\) Hz, 2H), 2.53-2.46 (m, 2H), 2.38 (m, 1H), 2.03-2.00 (m, 1H), 1.51-1.44 (m, 1H), 1.28 (qd, \(J = 12.4, 5.2\) Hz, 1H), 0.92 (s, 9H); \(^{13}\)C NMR (100 MHz, (CD\(_3\))\(_2\)SO) \(\delta\) 175.7, 169.9, 147.5, 133.9, 131.9, 131.4, 130.0, 129.4, 128.9, 114.1, 97.0, 45.0, 32.7, 27.5, 25.5, 24.8, 23.8; LRMS \(m/z\) calcd for C\(_{21}\)H\(_{23}\)N\(_3\)OS\(_2\): 397.1; found 398.1 [M+H]\(^+\).

**Preparation of compound 31.** Compound 31 was prepared using general procedure B. Benzoyl isothiocyanate (1.58 mL, 11.8 mmol) and compound 23 (3.03 g, 11.8 mmol) in anhydrous 1,4-dioxane (30 mL) were used to afford compound 31 (3.64 g, 74%; \(R_f\) 0.50 in 4:1/Hexanes:EtOAc) as a yellow solid after reacting for 1 h: \(^1\)H NMR (400 MHz, (CD\(_3\))\(_2\)SO) \(\delta\) 12.22 (s, 1H), 8.02 (d, \(J = 7.2\) Hz, 2H), 7.69 (t, \(J = 7.2\) Hz, 1H), 7.55 (t, \(J = 7.2\) Hz, 2H), 7.33-7.30 (m, 4H), 7.23-7.20 (m, 1H), 2.99-2.90 (m, 2H), 2.76-2.70 (m, 3H), 2.04-1.87 (m, 2H); \(^{13}\)C NMR (100 MHz, (CD\(_3\))\(_2\)SO) \(\delta\) 175.2, 169.4, 147.2, 145.3, 133.5, 131.5, 130.7, 128.9, 128.5, 128.4, 126.9, 126.4, 113.6, 96.6, 66.3, 40.0, 31.1, 28.9, 23.8; LRMS \(m/z\) calcd for C\(_{23}\)H\(_{19}\)N\(_3\)OS\(_2\): 417.1; found 418.0 [M+H]\(^+\).

**Preparation of compound 32.** Compound 32 was prepared using general procedure B. Benzoyl isothiocyanate (3.80 mL, 27.8 mmol) and compound 24 (5.36 g, 27.8 mmol) in anhydrous 1,4-dioxane (50 mL) were used to afford compound 32 (8.06 g, 82%; \(R_f\) 0.35 in 4:1/Hexanes:EtOAc) as a yellow solid after reacting for 1 h: \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 9.12 (s, 1H), 7.93 (s, 1H), 7.91 (app. d, \(J = 1.1\) Hz, 1H),
7.65 (app. t, $J = 7.9$ Hz, 1H), 7.53 (t, $J = 8.1$ Hz, 2H), 7.30-2.72 (m, 4H), 1.90-1.83 (m, 2H), 1.73-1.63 (m, 4H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 173.8, 167.1, 144.6, 136.7, 134.1, 133.3, 130.8, 129.2, 127.7, 114.2, 101.0, 32.0, 29.4, 29.1, 27.9, 27.3; LRMS m/z calcd for C$_{18}$H$_{17}$N$_3$O$_2$: 355.1; found 356.1 [M+H]$^+$.  

4.5.1.3. General procedure C for the preparation of compounds 33-40  
Synthesis of compounds 33-40 was done following a published protocol.$^{155}$ 2 N NaOH (50 mL) was added to a mixture of benzamides (38.3 mmol) in EtOH (70 mL). After refluxing at 100 °C for 30 min to 2 h, the solution was cooled to rt and then neutralized in an ice bath by adding 10% AcOH until the pH reached 7. The resultant precipitate was filtered and washed with a 4:1/H$_2$O:EtOH mixture to afford the desired product, which was used without any further purification. Note: Although we did not purify these compounds via SiO$_2$ column chromatography, we report R$_f$ values as an additional mean to verify that the correct compound is obtained.  

Preparation of compound 33. Compound 33 was prepared using general procedure C. 2 N NaOH (50 mL) and compound 25 (12.54 g, 38.3 mmol) in EtOH (70 mL) were used to afford compound 33 (7.55 g, 88%; R$_f$ 0.16 in 9:1/CH$_2$Cl$_2$:MeOH) as a brown solid after refluxing for 2 h: $^1$H NMR (400 MHz, (CD$_3$)$_2$SO) $\delta$ 12.58 (br s, 1H), 7.42 (br s, 2H), 2.91 (app. t, $J = 7.2$ Hz, 2H), 2.82 (app. t, $J = 7.2$ Hz, 2H), 2.37 (app. p, $J = 7.2$ Hz, 2H); LRMS m/z calcd for C$_9$H$_9$N$_3$S$_2$: 223.0; found 224.0 [M+H]$^+$.  

179
Preparation of compound 34. Compound 34 was prepared using general procedure C. 2 N NaOH (30 mL) and compound 26 (8.39 g, 24.6 mmol) in EtOH (45 mL) were used to afford compound 34 (5.80 g, 98%; $R_f$ 0.22 in 49:1/CH$_2$Cl$_2$:MeOH) as a yellow solid after refluxing for 30 min: $^1$H NMR (400 MHz, (CD$_3$)$_2$SO, which matches lit.$^{155}$) $\delta$ 12.73 (br s, 1H), 7.23 (br s, 2H), 2.76 (m, 2H), 2.64 (m, 2H), 1.77 (m, 4H); LRMS m/z calcd for C$_{10}$H$_{11}$N$_3$S$_2$: 237.0; found 238.8 [M+H]$^+$. 

Preparation of compound 35. Compound 35 was prepared using general procedure C. 2 N NaOH (19 mL) and compound 27 (5.34 g, 15.0 mmol) in EtOH (28 mL) were used to afford compound 35 (2.88 g, 76%; $R_f$ 0.27 in 49:1/CH$_2$Cl$_2$:MeOH) as a yellow solid after refluxing for 45 min: $^1$H NMR (400 MHz, (CD$_3$)$_2$SO) $\delta$ 12.60 (br s, 1H), 7.34 (br s, 1H), 2.70-2.50 (m, 2H), 1.90-1.70 (m, 3H), 1.54-1.50 (m, 1H), 1.08 (d, $J$ = 4.4 Hz, 3H); LRMS m/z calcd for C$_{11}$H$_{13}$N$_3$S$_2$: 251.1; found 252.0 [M+H]$^+$. 

Preparation of compound 36. Compound 36 was prepared using general procedure C. 2 N NaOH (38 mL) and compound 28 (8.91 g, 25.3 mmol) in EtOH (50 mL) were used to afford compound 36 (6.10 g, 96%; $R_f$ 0.26 in 1:2/Hexanes:EtOAc) as a yellow solid after refluxing for 30 min: $^1$H NMR (400 MHz, (CD$_3$)$_2$SO) $\delta$ 13.04 (br s, 1H), 7.18 (br s, 2H), 2.90-2.60 (m, 1H), 2.73 (dd, $J$ = 16.4, 5.1 Hz, 1H), 2.25 (ddt, $J$ = 16.5, 9.6, 2.5 Hz, 1H), 1.88-1.83 (m, 2H), 1.44-1.34 (m, 1H), 1.03 (d, $J$ = 6.5 Hz, 3H); LRMS m/z calcd for C$_{11}$H$_{13}$N$_3$S$_2$: 251.1; found 252.0 [M+H]$^+$. 

180
Preparation of compound 37. Compound 37 was prepared using general procedure C. 2 N NaOH (4 mL) and compound 29 (1.09 g, 5.24 mmol) in EtOH (4 mL) were used to afford compound 37 (0.79 g, 57%; $R_f$ 0.32 in 9:1/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7 mL/L)) as a yellow solid after refluxing for 30 min: $^1$H NMR (400 MHz, (CD$_3$)$_2$SO) $\delta$ 12.53 (very br s, 1H), 7.50 (very br s, 2H), 2.88-2.84 (m, 1H), 2.77 (dd, $J$ = 16.4, 4.8 Hz, 1H), 2.74-2.64 (m, 1H), 2.27 (dd, $J$ = 16.4, 9.6 Hz, 1H), 1.91 (m, 1H), 1.64 (m, 1H), 1.37 (m, 3H), 0.93 (t, $J$ = 7.6 Hz, 3H); LRMS m/z calcd for C$_{12}$H$_{15}$N$_3$S$_2$: 265.1; found 266.1 [M+H]$^+$.  

Preparation of compound 38. Compound 38 was prepared using general procedure C. 2 N NaOH (38 mL) and compound 30 (8.91 g, 25.3 mmol) in EtOH (50 mL) were used to afford compound 38 (6.10 g, 96%; $R_f$ 0.26 in 1:2/Hexanes:EtOAc) as a white solid after refluxing for 30 min: $^1$H NMR (400 MHz, (CD$_3$)$_2$SO) $\delta$ 12.70 (very br s, 1H), 7.42 (very br s, 2H), 2.91-2.88 (m, 1H), 2.67-2.63 (m, 2H), 2.37 (t, $J$ = 13.2 Hz, 1H), 1.96-1.93 (m, 1H), 1.44 (td, $J$ = 12.8, 4.4 Hz, 1H), 1.26 (qd, $J$ = 12.8, 4.8 Hz, 1H), 0.88 (s, 9H); $^{13}$C NMR (100 MHz, (CD$_3$)$_2$SO) $\delta$ 168.2, 131.8, 129.2, 128.3, 128.1, 127.1, 43.9, 32.1, 27.0, 25.93, 25.87, 23.4; LRMS m/z calcd for C$_{14}$H$_{19}$N$_3$S$_2$: 293.1; found 294.1 [M+H]$^+$.  

181
Preparation of compound 39. Compound 39 was prepared using general procedure C. 2 N NaOH (13.2 mL) and compound 31 (4.50 g, 10.8 mmol) in EtOH (20 mL) were used to afford compound 39 (3.30 g, 98%; Rf 0.25 in 9:1/CH₂Cl₂:MeOH) as a yellow solid after refluxing for 30 min: ¹H NMR (400 MHz, (CD₃)₂SO) δ 12.64 (br s, 1H), 7.29 (m, 4H), 7.19 (m, 1H), 3.28 (br s, 2H), 2.94 (m, 1H), 2.88 (m, 3H), 2.76 (m, 1H), 2.02-1.86 (m, 2H); LRMS m/z calcd for C₁₆H₁₅N₃S₂: 313.1; found 314.1 [M+H]^+.

Preparation of compound 40. Compound 40 was prepared using general procedure C. 2 N NaOH (26 mL) and compound 32 (8.06 g, 22.6 mmol) in EtOH (45 mL) were used to afford compound 40 (4.64 g, 82%; Rf 0.19 in 9:1/CH₂Cl₂:MeOH with NH₄OH (7 mL/L)) as a yellow solid after reacting for 30 min: ¹H NMR (400 MHz, (CD₃)₂SO) δ 12.82 (br s 1H), 7.42 (br s, 2H), 2.83 (m, 2H), 2.71 (m, 2H), 1.79-1.73 (m, 2H), 1.64-1.56 (m, 4H); LRMS m/z calcd for C₁₁H₁₃N₃S₂: 251.1; found 252.0 [M+H]^+.

4.5.1.4. General procedure for S-alkylation of compounds 33-40

4.5.1.4.1. General procedure D for the preparation of compounds 1a, 2a, 7a, 8a, 1b, 2b, 4b, 5b, 6b, 7b, 8b, 1c, 2c, 3c, 4c, 7c, 8c, 1d, 2d, 4d, 7d, 8d, 1e, 2e, 4e, 5e, 6e, 7e, 8e, 2f, 4f, 5f, 6f, 7f, 8f, 1g, 2g, 4g, 7g, 8g, 1h, 2h, 4h, 7h, 8h, 1i, 2i, 3i, 4i, 7i, and 8i

K₂CO₃ (2 eq) was added to a solution of thieno[2,3-d]pyrimidin-4amines (1 eq) in anhydrous DMF and stirred at 80 °C for about 20 min. Alkyl halide (1 eq) was added to the mixture, which was stirred for an additional 3-4 h at 80 °C. The reaction mixture was cooled to rt and
quenched by the addition of aq. NaHCO₃ or ddH₂O. The product was either extracted with EtOAc or CH₂Cl₂ three times and then washed with brine three times or filtered. The organic layer was then dried over MgSO₄ and evaporated to dryness under reduced pressure to give a residue, which was purified by column chromatography to afford the desired product.

4.5.1.4.2. General procedure E for the preparation of compounds 3a, 4a, 5a, 6a, 3b, 5c, 6c, 3d, 5d, 6d, 3e, 1f, 3f, 3g, 5g, 6g, 3h, 5h, 6h, 3i, 5i, and 6i.

The synthesis of these compounds was done following a published thiol alkylation protocol. Cs₂CO₃ (1.1 eq) and TBAI (1 eq) were added to a solution of thieno[2,3-d]pyrimidin-4amines (1 eq) in anhydrous DMF and stirred at 80 °C for 30 min. Alkyl halide (1.5 eq) was added, and the mixture was heated at 80 °C for an additional 1-4 h. The reaction was cooled to rt and quenched by addition of aq. NaHCO₃, extracted with EtOAc three times, and washed with brine three times. The organic layer was then dried over MgSO₄ and evaporated to dryness under reduced pressure to give a residue, which was purified by column chromatography to afford the desired product.

**Preparation of compound 1a.** Compound 1a was synthesized using general procedure D. Compound 33 (0.20 g, 0.91 mmol), K₂CO₃ (0.28 g, 2.00 mmol), and 1-(2-chloroethyl)pyrrolidine hydrochloride (0.15 g, 0.91 mmol) in anhydrous DMF (7.4 mL) were used to afford compound 1a (103 mg, 35%, Rf 0.22 in 9:1/CH₂Cl₂:MeOH with NH₄OH (7 mL/L)) as a tan solid after purification by column chromatography (SiO₂, 9:1/CH₂Cl₂:MeOH with NH₄OH (7 mL/L)): ¹H NMR (400 MHz,
CD3OD, Figure C7) δ 3.38 (t, J = 6.8 Hz, 2H), 3.20 (t, J = 6.4 Hz, 2H), 3.08 (br t, J = 6.0 Hz, 4H), 3.02 (t, J = 7.2 Hz, 2H), 2.94 (t, J = 7.2 Hz, 2H), 2.50 (p, J = 7.2 Hz, 2H), 1.98 (p, J = 3.2 Hz, 4H); 13C NMR (100 MHz, (CD3)2SO, Figure C8) δ 171.5, 163.3, 157.3, 135.9, 134.3, 109.3, 54.2, 53.2, 29.0, 28.7, 27.2, 27.0, 22.9; LRMS m/z calcd for C18H20N4S2: 320.1; found 321.1 [M+H]+ (Figure C1A); Purity of the compound was further confirmed by RP-HPLC by using method 2: Rf = 19.29 min (96% pure, Figure C9).

**Preparation of compound 2a HXN-1-98.** Compound 2a was synthesized using general procedure D. Compound 34 (0.24 g, 0.78 mmol), K2CO3 (0.24 g, 1.72 mmol), and 1-(2-chloroethyl)pyrrolidine hydrochloride (0.13 g, 0.78 mmol) in anhydrous DMF (4 mL) were used to afford compound 2a (145 mg, 56%, Rf 0.36 in 9:1/CH2Cl2:MeOH with NH4OH (7 mL/L)) as a white solid after purification by column chromatography (SiO2, 9:1/CH2Cl2:MeOH with NH4OH (7 mL/L)): 1H NMR (400 MHz, (CD3)2SO, Figure C10) δ 6.86 (br s, 2H), 3.21 (t, J = 7.4 Hz, 2H), 2.84 (m, 2H), 2.77 (t, J = 7.4 Hz, 2H), 2.69 (m, 2H), 2.64-2.55 (m, 4H), 1.79-1.77 (m, 4H), 1.73-1.64 (m, 4H); 13C NMR (100 MHz, (CD3)2SO, Figure C11) δ 166.6, 164.4, 158.0, 129.4, 127.2, 112.6, 55.4, 53.8, 28.8, 25.8, 25.1, 23.5, 22.7, 22.4; LRMS m/z calcd for C16H22N4S2: 334.1; found 335.1 [M+H]+ (Figure C1B); Purity of the compound was further confirmed by RP-HPLC by using method 1: Rf = 19.96 min (96% pure, Figure C12).

**Preparation of compound 3a.** Compound 3a was synthesized using general procedure E. Compound 35 (0.20 g, 0.78 mmol), Cs2CO3 (0.29 g,
0.88 mmol), TBAI (0.30 g, 0.80 mmol), and (1-(2-chloroethyl)pyrrolidine hydrochloride (0.16 g, 1.21 mmol) in anhydrous DMF (4 mL) were used to afford compound 3a (144 mg, 54%, Rf 0.39 in 9:1/CH₂Cl₂:MeOH with NH₄OH (7 mL/L)) as a white solid after purification by column chromatography (SiO₂, 9:1/CH₂Cl₂:MeOH with NH₄OH (7 mL/L)): ¹H NMR (400 MHz, CD₃OD, Figure C13) δ 3.30-3.26 (m, 2H), 3.24-3.19 (m, 1H), 2.84-2.79 (m, 2H), 2.78-2.70 (m, 2H), 2.66 (br t, J = 5.6 Hz, 4H), 2.02-1.94 (m, 2H), 1.94-1.78 (m, 2H), 1.84 (app. p, J = 3.6 Hz, 4H), 1.30 (d, J = 6.4 Hz, 3H); ¹³C NMR (100 MHz, CDCl₃, Figure C14) δ 168.1, 164.3, 156.5, 131.1, 130.9, 112.3, 55.9, 54.0, 29.7, 29.5, 29.1, 25.3, 23.4, 21.7, 17.7; LRMS m/z calcd for C₁₇H₂₄N₄S₂: 348.1; found 349.0 [M+H]+ (Figure C1C); Purity of the compound was further confirmed by RP-HPLC by using method 2: Rᵣ = 20.82 min (95% pure, Figure C15).

**Preparation of compound 4a.** Compound 4a was synthesized using general procedure E. Compound 36 (0.31 g, 1.00 mmol), Cs₂CO₃ (0.14 g, 0.42 mmol), TBAI (0.16 g, 0.42 mmol), and 1-(2-chloroethyl)-pyrrolidine hydrochloride (0.10 g, 0.60 mmol) in anhydrous DMF (2 mL) at 90 °C were used to afford compound 4a (28 mg, 20%, Rf 0.35 in 9:1/CH₂Cl₂:MeOH with NH₄OH (7 mL/L)) as a white solid after purification by column chromatography (SiO₂, 9:1/CH₂Cl₂:MeOH with NH₄OH (7 mL/L)): ¹H NMR (400 MHz, CD₃OD, Figure C16) δ 3.26 (m, 2H), 3.02-2.93 (m, 1H), 2.90-2.84 (m, 1H), 2.83-2.76 (m, 3H), 2.64 (br t, J = 5.6 Hz, 4H), 2.38-2.30 (m, 1H), 2.02-1.88 (m, 2H), 1.83 (p, J = 3.6 Hz, 4H), 1.54-1.44 (m, 1H), 1.11 (d, J = 6.8 Hz, 3H); ¹³C NMR (100 MHz, CD₃OD, Figure C17) δ 168.4, 165.8, 159.5, 131.6, 127.6, 113.9, 57.5, 55.1, 34.3, 31.9, 30.5, 29.9, 26.7, 24.4, 21.7; LRMS m/z calcd for C₁₇H₂₄N₄S₂: 348.1; found 349.0
[M+H]$^+$ (Figure C1D); Purity of the compound was further confirmed by RP-HPLC by using method 1: $R_t = 11.72$ min (96% pure, Figure C18).

**Preparation of compound 5a.** Compound 5a was synthesized using general procedure E. Compound 37 (0.20 g, 0.75 mmol), Cs$_2$CO$_3$ (0.24 g, 0.75 mmol), TBAI (0.28 g, 0.75 mmol), and 1-(2-chloroethyl)-pyrrolidine hydrochloride (0.14 g, 0.83 mmol) in anhydrous DMF (4 mL) were used to afford compound 5a (30 mg, 11%, $R_f$ 0.43 in 9:1/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7 mL/L)) as a white solid after purification by column chromatography (SiO$_2$, 9:1/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7 mL/L)): $^1$H NMR (400 MHz, CD$_3$OD, Figure C19) $\delta$ 3.36-3.26 (m, 2H), 3.10 (t, $J = 8.0$ Hz, 2H), 3.00 (m, 4H), 2.91 (m, 1H), 2.79 (dd, $J = 16.0$, 4.0 Hz, 2H), 2.34-2.26 (m, 1H), 2.04-1.96 (m, 1H), 1.93 (m, 4H), 1.70-1.60 (m, 1H), 1.48-1.34 (m, 3H), 0.97 (t, $J = 7.2$ Hz, 3H); $^{13}$C NMR (100 MHz, (CD$_3$)$_2$SO, Figure C20) $\delta$ 166.3, 164.3, 157.5, 128.6, 126.7, 111.9, 35.2, 31.2, 30.6, 29.4, 28.0, 27.9, 25.1, 21.4, 13.6, 11.3; LRMS m/z calcd for C$_{18}$H$_{26}$N$_4$S$_2$: 362.2; found 363.0 [M+H]$^+$ (Figure C1E); Purity of the compound was further confirmed by RP-HPLC by using method 2: $R_t = 22.41$ min (98% pure, Figure C21).

**Preparation of compound 6a.** Compound 6a was synthesized using general procedure E. Compound 38 (0.31 g, 1.00 mmol) Cs$_2$CO$_3$ (0.33 g, 1.00 mmol), TBAI (0.37 g, 1.00 mmol), and 1-(2-chloroethyl)-pyrrolidine hydrochloride (0.19 g, 1.10 mmol) in anhydrous DMF (2 mL) were
used to afford compound 6a (15 mg, 10%, Rf 0.40 in 9:1/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7 mL/L)) as a white solid after purification by column chromatography (SiO$_2$, 9:1/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7 mL/L)): $^1$H NMR (400 MHz, CDCl$_3$, Figure C22) δ 5.33 (br s, 2H), 3.31 (t, $J$ = 7.6 Hz, 2H), 2.81 (dd, $J$ = 15.2, 5.2 Hz, 1H), 2.84 (t, $J$ = 7.2 Hz, 2H), 2.81-2.76 (m, 2H), 2.67 (br t, 4H), 2.55-2.45 (m, 1H), 2.09 (dd, $J$ = 11.6, 5.2 Hz, 1H), 1.89 (app. p, $J$ = 3.2 Hz, 4H), 1.70-1.62 (m, 1H), 1.54 (td, $J$ = 11.6, 3.2 Hz, 2H), 1.48-1.38 (m, 1H), 0.94 (s, 9H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure C23) δ 168.1, 164.7, 157.4, 132.5, 125.6, 112.8, 56.2, 54.3, 44.9, 29.4, 27.5, 27.3, 27.1, 24.3, 23.7; LRMS m/z calcd for C$_{20}$H$_{30}$N$_4$S$_2$: 390.2; found 391.1 [M+H]$^+$ (Figure C1F); Purity of the compound was further confirmed by RP-HPLC by using method 1: $R_t$ = 15.69 min (96% pure, Figure C24).

**Preparation of compound 7a.** Compound 7a was synthesized using general procedure D. Compound 39 (0.2 g, 0.65 mmol), K$_2$CO$_3$ (0.19 g, 1.41 mmol), and 1-(2-chloroethyl)-pyrrolidine hydrochloride (0.11 g, 0.64 mmol) in anhydrous DMF (4 mL) were used to afford compound 7a (124 mg, 47%, Rf 0.26 in 9:1/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7 mL/L)) as a tan solid after purification by column chromatography (SiO$_2$, 9:1/CH$_2$Cl$_2$:MeOH): $^1$H NMR (400 MHz, CDCl$_3$, Figure C25) δ 7.35-7.30 (m, 2H), 7.26-7.20 (m, 3H), 5.38 (br s, 2H), 3.32 (m, 2H), 3.13-2.88 (m, 5H), 2.85 (m, 2H), 2.67 (br p, 4H), 2.30-2.18 (m, 1H), 2.10-2.00 (m, 1H), 1.82 (p, $J$ = 3.2 Hz, 4H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure C26) δ 167.8, 164.1, 157.4, 144.9, 130.9, 128.6, 126.8, 126.6, 125.3, 112.6, 55.6, 53.9, 40.1, 32.9, 29.7, 28.4, 26.2, 23.4; LRMS m/z calcd for C$_{22}$H$_{26}$N$_4$S$_2$: 410.2; found 411.0 [M+H]$^+$ (Figure C1G); Purity of the compound
was further confirmed by RP-HPLC by using method 1: $R_t = 14.85\text{ min (97\% pure, Figure C27).}$

**Preparation of compound 8a.** Compound 8a was synthesized using general procedure D. Compound 40 (0.21 g, 0.82 mmol), $K_2CO_3$ (0.21 g, 1.47 mmol), and 1-(2-chloroethyl)-pyrrolidine hydrochloride (0.14 g, 0.81 mmol) in anhydrous DMF (4 mL) were used to afford compound 8a (117 mg, 41\%, $R_f$ 0.35 in 9:1/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7 mL/L)) as a tan solid after purification by column chromatography (SiO$_2$, 9:1/CH$_2$Cl$_2$:MeOH): $^1$H NMR (400 MHz, (CD$_3$)$_2$SO, Figure C28) $\delta$ 6.92 (s, 2H), 3.14 (t, $J = 7.2\text{ Hz, 2H}$), 2.93-2.91 (m, 2H), 2.77-2.74 (m, 2H), 2.62 (t, $J = 7.2\text{ Hz, 2H}$), 2.45-2.41 (m, 4H), 1.80-1.75 (m, 2H), 1.67-1.57 (m, 8H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure C29) $\delta$ 166.4, 163.9, 157.3, 135.2, 130.3, 114.2, 56.0, 54.0, 30.6, 29.9, 29.3, 28.9, 27.1, 26.4, 23.5; LRMS $m/z$ calcd for C$_{17}$H$_{24}$N$_4$S$_2$: 348.1; found 349.1 [M+H]$^+$ (Figure C1H); Purity of the compound was further confirmed by RP-HPLC by using method 1: $R_t = 13.24\text{ min (97\% pure, Figure C30).}$

**Preparation of compound 1b.** Compound 1b was synthesized using general procedure D. Compound 33 (0.11 g, 0.50 mmol), $K_2CO_3$ (0.15 g, 1.11 mmol), and 1-(2-chloroethyl)piperidine hydrochloride (0.10 g, 0.53 mmol) in anhydrous DMF (4 mL) were used to afford compound 1b (65 mg, 55\%, $R_f$ 0.16 in CH$_2$Cl$_2$:MeOH/9:1 with NH$_4$OH (7mL/L)) as a tan solid after purification by column chromatography (SiO$_2$, 19:1/CH$_2$Cl$_2$:MeOH): $^1$H NMR (400 MHz, CDCl$_3$, Figure C31) $\delta$ 5.13
(s, 2H), 3.29 (t, J = 7.6 Hz, 2H), 2.96 (m, 4H), 2.70 (t, J = 7.6 Hz, 2H), 2.50 (m, 6H), 1.61 (p, J = 5.6 Hz, 4H), 1.44 (p, J = 4.8 Hz, 2H); 13C NMR (100 MHz, (CD3)2SO, Figure C32) δ 171.5, 163.9, 157.2, 135.9, 134.2, 109.3, 57.7, 53.5, 29.0, 28.7, 27.2, 26.7, 25.0, 23.6; LRMS m/z calcd for C16H22N4S2: 334.1; found 335.1 [M+H]+ (Figure C11); Purity of the compound was further confirmed by RP-HPLC by using method 2: Rt = 20.59 min (95% pure, Figure C33).

**Preparation of compound 2b.** Compound 2b was synthesized using general procedure D. Compound 34 (0.20 g, 0.78 mmol), K2CO3 (0.24 g, 1.72 mmol), and 1-(2-chloroethyl)piperidine hydrochloride (0.15 g, 0.78 mmol) in anhydrous DMF (4 mL) were used to afford compound 2b (145 mg, 53%, Rf 0.25 in 9:1/CH2Cl2:MeOH with NH4OH (7 mL/L)) as a light yellow solid after purification by column chromatography (SiO2, 9:1/CH2Cl2:MeOH with NH4OH (7 mL/L)). 1H NMR (400 MHz, CDCl3, Figure C34) δ 5.33 (br s, 2H), 3.31 (m, 2H), 2.84 (m, 2H), 2.74 (m, 4H), 2.58 (br s, 4H), 1.89-1.85 (m, 4H), 1.67 (br s, 4H), 1.46 (br s, 2H); 13C NMR (100 MHz, (CD3)2SO, Figure C35) δ 166.1, 163.9, 157.6, 129.0, 126.8, 112.1, 57.8, 53.6, 26.7, 25.3, 25.1, 24.7, 23.7, 22.2, 21.9; LRMS m/z calcd for C17H24N4S2: 348.1; found 349.1 [M+H]+ (Figure C11); Purity of the compound was further confirmed by RP-HPLC by using method 2: Rt = 20.21 min (97% pure, Figure C36).

**Preparation of compound 3b.** Compound 3b was synthesized using general procedure E. Compound 35 (0.20 g, 0.80 mmol), Cs2CO3 (0.29 g,
0.88 mmol), TBAI (0.29 g, 0.80 mmol), and 1-(2-chloroethyl)piperidine hydrochloride (0.18 g, 1.20 mmol) in anhydrous DMF (4 mL) were used to afford compound 3b (141 mg, 49%, Rf 0.42 in 9:1/CH₂Cl₂:MeOH with NH₄OH (7 mL/L)) as a white solid after purification by column chromatography (SiO₂, 9:1/CH₂Cl₂:MeOH with NH₄OH (7 mL/L)): ¹H NMR (400 MHz, CD₃OD, Figure C37) δ 3.28-3.18 (m, 2H), 3.10-3.00 (m, 2H), 2.82-2.66 (m, 2H), 2.34 (s, 3H), 2.32-2.22 (m, 2H), 2.18-2.08 (m, 2H), 2.22-1.56 (m, 8H), 2.18 (d, J = 7.2 Hz, 3H); ¹³C NMR (100 MHz, CDCl₃, Figure C38) δ 168.1, 164.5, 146.4, 131.0, 130.9, 112.2, 58.8, 54.4, 29.7, 29.5, 27.6, 25.9, 25.31, 24.3, 21.7, 17.7; LRMS m/z calcd for C₁₈H₂₆N₄S₂: 362.2; found 363.0 [M+H]+ (Figure C1K); Purity of the compound was further confirmed by RP-HPLC by using method 1: Rₜ = 13.68 min (96% pure, Figure C39).

Preparation of compound 4b. Compound 4b was synthesized using general procedure D. Compound 36 (0.10 g, 0.39 mmol), K₂CO₃ (0.07 g, 0.43 mmol), and 1-(2-chloroethyl)piperidine hydrochloride (0.07 g, 0.39 mmol) in anhydrous DMF (4 mL) were used to afford compound 4b (78 mg, 55%, Rf 0.44 in 9:1/CH₂Cl₂:MeOH with NH₄OH (7 mL/L)) as a light yellow solid after purification by column chromatography (SiO₂, 9:1/CH₂Cl₂:MeOH with NH₄OH (7 mL/L)): ¹H NMR (400 MHz, (CD₃)₂SO, Figure C40) δ 6.84 (br s, 2H), 3.17 (t, J = 6.8 Hz, 2H), 2.96-2.76 (m, 4H), 2.55 (m, 1H), 2.41 (m, 3H), 2.35-2.28 (m, 2H), 1.90-1.86 (m, 2H), 1.51-1.37 (m, 7H), 1.05 (d, J = 6.5 Hz, 3H); ¹³C NMR (100 MHz, (CD₃)₂SO, Figure C41) δ 166.3, 164.1, 157.6, 128.5, 126.4, 112.0, 58.1, 53.7, 32.7, 30.0, 28.5, 27.1, 25.4, 25.0, 23.9, 21.1; LRMS m/z calcd for C₁₈H₂₆N₄S₂: 362.2; found 363.1 [M+H]+ (Figure C1L); Purity of the compound was further confirmed by RP-HPLC by using method 2: Rₜ = 20.74 min (96% pure, Figure C42).
Preparation of compound 5b. Compound 5b was synthesized using general procedure D. Compound 37 (0.10 g, 0.42 mmol), Cs₂CO₃ (0.14 g, 0.42 mmol), TBAI (0.16 g, 0.42 mmol), and 1-(2-chloroethyl)piperidine hydrochloride (0.11 g, 0.62 mmol) in anhydrous DMF were used to afford compound 5b (56 mg, 35%, Rₐ 0.52 in 9:1/CH₂Cl₂:MeOH with NH₄OH (7 mL/L)) as a white solid after purification by recrystallization from Hexanes:EtOAc: \(^1\)H NMR (400 MHz, CD₃OD, Figure C43) δ 3.28-3.24 (m, 2H), 3.02-2.96 (m, 1H), 2.90-2.80 (m, 2H), 2.69-2.65 (m, 2H), 2.55 (br t, 4H), 2.42-2.33 (m, 1H), 2.11-2.03 (m, 1H), 1.89-1.68 (m, 1H), 1.64 (p, J = 5.2 Hz, 4H), 1.55-1.43 (m, 5H), 1.02 (t, J = 7.6 Hz, 3H); \(^1\)C NMR (100 MHz, CDCl₃, Figure C44) δ 168.0, 164.9, 157.4, 131.6, 125.5, 112.9, 59.0, 54.6, 36.0, 31.4, 28.74, 28.67, 27.9, 26.10, 26.06, 24.5, 11.7; LRMS m/z calcd for C₁₉H₂₈N₄S₂: 376.2; found 377.0 [M+H]^⁺ (Figure C2A); Purity of the compound was further confirmed by RP-HPLC by using method 1: Rᵣ = 14.82 min (95% pure, Figure C45).

Preparation of compound 6b. Compound 6b was synthesized using general procedure D. Compound 38 (0.31 g, 1.02 mmol), K₂CO₃ (0.225, 1.63 mmol), and 1-(2-chloroethyl)piperidine hydrochloride (0.21 g, 1.14 mmol) in anhydrous DMF (6 mL) were used to afford compound 6b (10 mg, 3%, Rₐ 0.42 in 9:1/CH₂Cl₂:MeOH with NH₄OH (7 mL/L)) as a white solid after purification by recrystallization from CH₂Cl₂:Hexanes: \(^1\)H NMR (400 MHz, CDCl₃, Figure C46) δ 5.30 (s, 2H), 3.28 (t, J = 7.2 Hz, 2H), 2.95 (dd, J = 14.8, 4.8 Hz, 1H), 2.78 (dd, J = 16.0, 4.0 Hz, 2H),
2.69 (t, J = 7.6 Hz, 2H), 2.58-2.44 (m, 5H), 2.10-2.08 (m, 1H), 1.61 (p, J = 5.2 Hz, 4H), 1.53 (dd, J = 15.2, 4.0 Hz, 2H), 1.44 (p, J = 5.2 Hz, 2H), 0.94 (s, 9H); 13C NMR (100 MHz, CDCl3, Figure C47) δ 167.8, 164.6, 157.1, 132.2, 125.3, 112.6, 58.7, 54.3, 44.6, 32.5, 27.6, 27.2, 27.1, 26.8, 25.7, 24.2, 24.1; LRMS m/z calcd for C21H32N4S2: 404.2; found 405.0 [M+H]+ (Figure C2B); Purity of the compound was further confirmed by RP-HPLC by using method 1: Rt = 16.03 min (96% pure, Figure C48).

Preparation of compound 7b. Compound 7b was synthesized using general procedure D. Compound 38 (0.20 g, 0.65 mmol), K2CO3 (0.20 g, 1.42 mmol), and 1-(2-chloroethyl)piperidine hydrochloride (0.12 g, 0.64 mmol) in anhydrous DMF (4 mL) were used to afford compound 7b (34 mg, 12%, Rf 0.34 in 9:1/CH2Cl2:MeOH with NH4OH (7 mL/L)) as a tan solid after purification by column chromatography (SiO2, 9:1/CH2Cl2:MeOH with NH4OH (7 mL/L)): 1H NMR (400 MHz, CDCl3, Figure C49) δ 7.35-7.30 (m, 2H), 7.28-7.20 (m, 3H), 5.47 (s, 2H), 3.31 (m, 2H), 3.13-2.85 (m, 5H), 2.75 (m, 2H), 2.57 (br p, 4H), 2.25-2.15 (m, 1H), 2.10-1.98 (m, 1H), 1.65 (p, J = 5.6 Hz, 4H), 1.45 (p, J = 5.6 Hz, 2H); 13C NMR (100 MHz, CDCl3, Figure C50) δ 167.8, 164.6, 157.3, 145.0, 130.9, 128.6, 126.8, 126.6, 125.3, 112.6, 58.5, 54.2, 40.1, 32.9, 29.7, 27.4, 26.2, 25.4, 24.0; LRMS m/z calcd for C23H28N4S2: 424.2; found 425.0 [M+H]+ (Figure C2C); Purity of the compound was further confirmed by RP-HPLC by using method 2: Rt = 22.54 min (96% pure, Figure C51).
Preparation of compound 8b. Compound 8b was synthesized using general procedure D. Compound 39 (0.10 g, 0.39 mmol), K$_2$CO$_3$ (0.12 g, 0.86 mmol), and 1-(2-chloroethyl)piperidine hydrochloride (0.07 g, 0.39 mmol) in anhydrous DMF (4 mL) were used to afford compound 8b (80 mg, 57%, $R_f$ 0.45 in 9:1/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7 mL/L)) as a brown solid after purification by column chromatography (SiO$_2$, 19:1/CH$_2$Cl$_2$:MeOH): $^1$H NMR (400 MHz, CDCl$_3$, Figure C52) δ 5.28 (s, 2H), 3.28-3.24 (m, 2H), 2.95-2.92 (m, 2H), 2.83-2.81 (m, 2H), 2.68-2.64 (m, 2H), 2.48 (t, $J$ = 5.4 Hz, 4H), 1.90-1.84 (m, 2H), 1.81-1.74 (m, 4H), 1.59 (p, $J$ = 5.4 Hz, 4Hz), 1.42 (p, $J$ = 5.4 Hz, 2H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure C53) δ 166.4, 157.4, 135.2, 130.4, 114.2, 58.2, 54.1, 30.6, 29.9, 29.2, 28.9, 27.1, 26.9, 26.4, 24.9, 23.8; LRMS $m/z$ calcd for C$_{18}$H$_{26}$N$_4$S$_2$: 362.2; found 363.1 [M+H]$^+$ (Figure C2D); Purity of the compound was further confirmed by RP-HPLC by using method 2: $R_t$ = 23.15 min (100% pure, Figure C54).

Preparation of compound 1c. Compound 1c was synthesized using general procedure D. Compound 33 (0.20 g, 0.90 mmol), K$_2$CO$_3$ (0.28 g, 2.00 mmol), and 1-(3-chloropropyl)piperidine monohydrochloride (0.18 g, 0.91 mmol) in anhydrous DMF (4 mL) were used to afford compound 1c (120 mg, 39%, $R_f$ 0.16 in 9:1/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7mL/L)) as a brown solid after purification by column chromatography (SiO$_2$, 9:1/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7 mL/L)): $^1$H NMR (400 MHz, (CD$_3$)$_2$SO, Figure C55) δ 6.90 (br s, 2H), 3.03 (t, $J$ = 8.0 Hz, 2H), 2.94 (t, $J$ = 8.0 Hz, 2H), 2.83 (t, $J$ = 8.0 Hz, 2H), 2.34 (p, $J$ = 8.0 Hz, 2H), 1.82 (p, $J$ = 8.0 Hz, 2H), 1.51 (p, $J$ = 4.0 Hz, 4H), 1.36 (p, $J$ = 4.0 Hz, 2H); $^{13}$C NMR (100 MHz, (CD$_3$)$_2$SO, Figure C56) δ 171.5, 164.1, 157.1, 135.9, 134.1, 109.2, 60.1, 55.2, 45.7, 35.5, 33.6,
29.0, 28.8, 28.7, 27.2, 24.0; LRMS \( m/z \) calcld for \( \text{C}_{17}\text{H}_{24}\text{N}_{4}\text{S}_{2} \): 348.1; found 349.1 \([\text{M}+\text{H}]^{+}\) (Figure C2E); Purity of the compound was further confirmed by RP-HPLC by using method 2: \( R_t = 24.10 \) min (99% pure, Figure C57).

**Preparation of compound 2c.** Compound 2c was synthesized using general procedure D. Compound 34 (0.21 g, 0.78 mmol), \( \text{K}_2\text{CO}_3 \) (0.15 g, 0.78 mmol), and 1-(3-chloropropyl)piperidine monohydrochloride (0.15 g, 0.78 mmol) in anhydrous DMF (4 mL) were used to afford compound 2c (120 mg, 42%, \( R_f \) 0.32 in 9:1/\( \text{CH}_2\text{Cl}_2\):MeOH with \( \text{NH}_4\text{OH} \) (7 mL/L)) as a light yellow solid after purification by column chromatography (SiO\(_2\), 9:1/\( \text{CH}_2\text{Cl}_2\):MeOH with \( \text{NH}_4\text{OH} \) (7 mL/L)): \(^1\text{H}\) NMR (400 MHz, \( \text{CD}_3\text{OD} \), Figure C58) \( \delta \) 3.21 (t, \( J = 7.2 \) Hz, 2H), 3.24-3.06 (m, 6H), 2.94-2.86 (m, 2H), 2.80-2.74 (m, 2H), 2.18 (m, 2H), 1.91 (m, 4H), 1.83 (br p, 4H), 1.72-1.60 (m, 2H); \(^{13}\text{C}\) NMR (100 MHz, (CD\(_3\))\text{SO}, Figure C59) \( \delta \) 166.1, 163.7, 157.6, 129.1, 126.8, 112.2, 54.9, 51.9, 27.1, 25.3, 24.7, 23.8, 22.4, 22.2, 21.9, 21.4; LRMS \( m/z \) calcld for \( \text{C}_{18}\text{H}_{26}\text{N}_{4}\text{S}_{2} \): 362.2; found 363.1 \([\text{M}+\text{H}]^{+}\) (Figure C2F); Purity of the compound was further confirmed by RP-HPLC by using method 2: \( R_t = 18.58 \) min (99% pure, Figure C60).

**Preparation of compound 3c.** Compound 3c was synthesized using general procedure D. Compound 35 (0.20 g, 0.80 mmol), \( \text{Cs}_2\text{CO}_3 \) (0.30 g, 0.90 mmol), TBAI (0.29 g, 0.78 mmol), and 1-(3-chloropropyl)piperidine monohydrochloride (0.19 g, 1.19 mmol) in anhydrous DMF (4 mL) were used to afford compound 3c (183 mg, 60%, \( R_f \) 0.26 in 9:1/\( \text{CH}_2\text{Cl}_2\):MeOH with \( \text{NH}_4\text{OH} \))...
(7 mL/L)) as a white solid after purification by column chromatography (SiO₂, 9:1/CH₂Cl₂:MeOH with NH₄OH (7 mL/L)); ¹H NMR (400 MHz, CD₃OD, Figure C61) δ 3.28-3.18 (m, 1H), 3.15 (t, J = 6.8 Hz, 2H), 2.84-2.64 (m, 2H), 2.58-2.40 (m, 6H), 1.97 (t, J = 6.8 Hz, 2H), 2.02-1.92 (m, 2H), 1.92-1.74 (m, 2H), 1.62 (p, J = 5.2 Hz, 4H), 1.48 (m, 2H), 1.29 (d, J = 6.8 Hz, 3H); ¹³C NMR (100 MHz, CDCl₃, Figure C62) δ 168.0 164.8, 156.7, 131.2, 130.9, 112.3, 58.3, 29.7, 29.5, 29.0, 26.9, 26.0, 25.4, 24.5, 21.8, 17.8; LRMS m/z calcd for C₁₉H₂₈N₄S₂: 376.2; found 377.0 [M+H]⁺ (Figure C2G); Purity of the compound was further confirmed by RP-HPLC by using method 1: Rt = 11.81 min (97% pure, Figure C63).

Preparation of compound 4c. Compound 4c was synthesized using general procedure D. Compound 36 (0.21 g, 0.78 mmol), K₂CO₃ (0.21 g, 0.78 mmol), and 1-(3-chloropropyl)piperidine monohydrochloride (0.15 g, 0.78 mmol) in anhydrous DMF (4 mL) were used to afford compound 4c (215 mg, 79%, Rf 0.29 in 9:1/CH₂Cl₂:MeOH with NH₄OH (7 mL/L)) as a white solid after purification by column chromatography (SiO₂, 9:1/CH₂Cl₂:MeOH with NH₄OH (7 mL/L)); ¹H NMR (400 MHz, CD₃OD, Figure C64) δ 3.11 (t, J = 7.2 Hz, 2H), 3.01-2.92 (m, 1H), 2.90-2.83 (m, 1H), 2.81 (dd, J = 17.2 Hz, 4.0 Hz, 1H), 2.46 (t, J = 7.6 Hz, 2H), 2.46-2.37 (m, 4H), 2.36-2.30 (m, 1H), 2.00 (m, 2H), 1.93 (p, J = 7.2 Hz, 2H), 1.57 (p, J = 5.6 Hz, 4H), 1.54-1.48 (m, 1H), 1.44 (p, J = 5.2 Hz, 2H), 1.10 (d, J = 6.4 Hz, 3H); ¹³C NMR (100 MHz, CDCl₃, Figure C65) δ 167.7, 164.8, 157.1, 131.1, 125.0, 112.7, 57.9, 54.3, 33.3, 30.5, 29.0, 28.9, 26.4, 25.8, 25.3, 24.0, 21.2; LRMS m/z calcd for C₁₉H₂₈N₄S₂: 376.2; found 377.1 [M+H]⁺ (Figure C2H); Purity of the compound was further confirmed by RP-HPLC by using method 1: Rt = 13.58 min (97% pure, Figure C66).
Preparation of compound 5c. Compound 5c was synthesized using general procedure E. Compound 37 (0.10 g, 0.42 mmol), Cs$_2$CO$_3$ (0.14 g, 0.42 mmol), TBAI (0.16 g, 0.42 mmol), and 1-(3-chloropropyl)piperidine monohydrochloride (0.12 g, 0.62 mmol) in anhydrous DMF (8 mL) were used to afford compound 5c (44 mg, 27%, $R_f$ 0.33 in 9:1/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7 mL/L)) as a white solid after purification by recrystallization from Hexanes:EtOAc and washed with cold 1:8/Hexanes:EtOAc: $^1$H NMR (400 MHz, CD$_3$OD, Figure C67) δ 3.13 (t, $J$ = 7.2 Hz, 2H), 3.02-2.94 (m, 1H), 2.90-2.78 (m, 2H), 2.48 (t, $J$ = 7.2 Hz, 2H), 2.52-2.42 (m, 4H), 2.42-2.32 (m, 1H), 2.10-2.02 (m, 1H), 1.95 (p, $J$ = 7.6 Hz, 2H), 1.78-1.68 (m, 1H), 1.61 (p, $J$ = 5.6 Hz, 4H), 1.54-1.40 (m, 5H), 1.01 (t, $J$ = 7.6 Hz, 3H); $^{13}$C NMR (100 MHz, CD$_3$OD, Figure C68) δ 168.4, 166.3, 159.4, 131.7, 127.9, 113.8, 59.5, 55.7, 37.3, 32.3, 29.83, 29.79, 29.7, 27.9, 26.7, 26.6, 25.4, 11.9; LRMS $m/z$ calcd for C$_{20}$H$_{30}$N$_4$S$_2$: 390.2; found 391.0 [M+H]$^+$ (Figure C2I); Purity of the compound was further confirmed by RP-HPLC by using method 1: $R_t$ = 14.47 min (96% pure, Figure C69).

Preparation of compound 6c. Compound 6c was synthesized using general procedure E. Compound 38 (0.10 g, 0.42 mmol), Cs$_2$CO$_3$ (0.14 g, 0.42 mmol), TBAI (0.16 g, 0.42 mmol), and 1-(3-chloropropyl)piperidine monohydrochloride (0.12 g, 0.62 mmol) in anhydrous DMF (8 mL) were used to afford compound 6c (80 mg, 45%, $R_f$ 0.33 in 9:1/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7 mL/L)) as a white solid after purification by column chromatography (SiO$_2$, 196
CH$_2$Cl$_2$, 49:1/CH$_2$Cl$_2$:MeOH, 19:1/CH$_2$Cl$_2$:MeOH, 9:1/CH$_2$Cl$_2$:MeOH with 7 mL/L NH$_4$OH, then 7:3/CH$_2$Cl$_2$:MeOH with 7 mL/L NH$_4$OH): $^1$H NMR (400 MHz, CD$_3$OD, Figure C70) δ 3.14 (t, $J = 7.2$ Hz, 2H), 3.09-3.02 (m, 1H), 2.85-2.75 (m, 2H), 2.54 (t, $J = 7.2$ Hz, 2H), 2.59-2.45 (m, 5H), 2.20-2.12 (m, 1H), 1.97 (p, $J = 7.2$ Hz, 2H), 1.63 (p, $J = 5.6$ Hz, 4H), 1.59-1.54 (m, 1H), 1.48 (p, $J = 5.6$ Hz, 2H), 1.52-1.39 (m, 1H), 0.99 (s, 9H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure C71) δ 168.0, 165.2, 157.3, 132.4, 125.6, 112.8, 58.3, 54.6, 44.8, 32.7, 29.6, 27.5, 27.3, 27.1, 26.8, 25.8, 24.4, 24.3; LRMS m/z calcd for C$_{22}$H$_{34}$N$_4$S$_2$: 418.2; found 419.1 [M+H]$^+$ (Figure C2J); Purity of the compound was further confirmed by RP-HPLC by using method 1: $R_t = 15.83$ min (97% pure, Figure C72).

**Preparation of compound 7c.** Compound 7c was synthesized using general procedure D. Compound 39 (0.20 g, 0.65 mmol), K$_2$CO$_3$ (0.20 g, 1.41 mmol), and 1-(2-chloroethyl)piperidine hydrochloride (0.11 g, 0.66 mmol) in anhydrous DMF (4 mL) were used to afford compound 7c (84 mg, 30%, $R_f$ 0.26 in 9:1/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7 mL/L)) as a tan solid after purification by column chromatography (SiO$_2$, 9:1/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7 mL/L)): $^1$H NMR (400 MHz, CD$_3$OD, Figure C73) δ 7.32-7.28 (m, 4H), 7.23-7.19 (m, 1H), 3.15 (t, $J = 7.2$ Hz, 2H), 3.11-2.94 (m, 4H), 2.89 (m, 1H), 2.62 (t, $J = 7.2$ Hz, 2H), 2.65-2.54 (m, 4H), 2.20-2.14 (m, 1H), 2.10-1.96 (m, 3H), 1.65 (p, $J = 5.6$ Hz, 4H), 1.05 (app. p, $J = 5.6$ Hz, 2H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure C74) δ 167.7, 165.1, 157.3, 145.0, 130.7, 128.6, 126.8, 126.6, 125.3, 112.5, 58.0, 54.4, 40.1, 32.9, 29.6, 28.9, 26.6, 26.2, 25.6, 24.2; LRMS m/z calcd for C$_{24}$H$_{30}$N$_4$S$_2$: 438.2; found 439.2 [M+H]$^+$ (Figure C2K); Purity of the compound was further confirmed by RP-HPLC by using method 1: $R_t = 15.61$ min (97% pure, Figure C75).
Preparation of compound 8c. Compound 8c was synthesized using general procedure D. Compound 40 (0.21 g, 0.82 mmol), K$_2$CO$_3$ (0.21 g, 1.47 mmol), and 1-(3-chloropropyl)-piperidine hydrochloride (0.13 g, 0.80 mmol) in anhydrous DMF (4 mL) were used to afford compound 8c (158 mg, 52%, $R_f$ 0.39 in 9:1/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7 mL/L)) as a yellow solid after purification by column chromatography (SiO$_2$, 9:1/CH$_2$Cl$_2$:MeOH): $^1$H NMR (400 MHz, CDCl$_3$, Figure C76) $\delta$ 5.43 (s, 2H), 3.12 (t, $J = 7.3$ Hz, 2H), 2.93-2.90 (m, 2H), 2.81-2.79 (m, 2H), 2.48 (t, $J = 7.2$ Hz, 2H), 2.43 (br t, $J = 5.5$ Hz, 4H), 1.96 (p, $J = 7.3$ Hz, 2H), 1.88-1.82 (m, 2H), 1.79-1.72 (m, 4H), 1.59 (p, $J = 5.7$ Hz, 4H), 1.44-1.38 (p, $J = 5.7$ Hz, 2H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure C77) $\delta$ 166.4, 164.4, 157.2, 135.1, 130.3, 114.1, 58.2, 54.4, 30.6, 29.9, 28.92, 28.89, 27.1, 26.7, 26.4, 25.7, 24.3; LRMS $m/z$ calcd for C$_{19}$H$_{28}$N$_4$S$_2$: 376.2; found 377.1 [M+H]$^+$ (Figure C2L); Purity of the compound was further confirmed by RP-HPLC by using method 2: $R_t$ = 20.59 min (100% pure, Figure C78).

Preparation of compound 1d. Compound 1d was synthesized using general procedure D. Compound 33 (0.21 g, 0.92 mmol), K$_2$CO$_3$ (0.27 g, 1.98 mmol), and 2-(2-chloroethyl)-1-methylpyrrolidine hydrochloride (0.17 g, 0.90 mmol) in DMF (4 mL) were used to afford compound 1d (89 mg, 30%, $R_f$ 0.11 in CH$_2$Cl$_2$:MeOH/9:1 with NH$_4$OH (7mL/L)) as a tan solid after purification by column chromatography (SiO$_2$, 9:1/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7 mL/L)): $^1$H NMR (400 MHz, CD$_3$OD, Figure C79) $\delta$ 3.25 (ddd, $J = 14.8, 8.6, 5.2$ Hz, 2H), 3.15-3.09 (m, 1H), 3.08-3.06 (m,
1H), 3.02 (t, J = 7.6 Hz, 2H), 2.94 (t, J = 7.2 Hz, 2H), 2.50 (p, J = 7.2 Hz, 2H), 2.46-2.39 (m, 1H), 2.41 (s, 3H), 2.36 (q, J = 8.8 Hz, 1H), 2.2-2.13 (m, 2H), 1.83 (p, J = 8.0 Hz, 2H), 1.77-1.60 (m, 2H); 13C NMR (100 MHz, (CD3)2SO, Figure C80) δ 171.5, 164.2, 157.2, 135.9, 134.0, 109.2, 64.9, 56.5, 39.3, 32.5, 29.6, 29.0, 28.7, 27.2, 26.5, 21.6; LRMS m/z calcd for C16H22N4S2: 334.1; found 335.1 [M+H]+ (Figure C3A); Purity of the compound was further confirmed by RP-HPLC by using method 2: Rf = 23.38 min (97% pure, Figure C81).

**Preparation of compound 2d.** Compound 2d was synthesized using general procedure D. Compound 34 (0.20 g, 0.78 mmol), K2CO3 (0.23 g, 1.72 mmol), and 2-(2-chloroethyl)-1-methylpyrrolidine hydrochloride (0.14 g, 0.78 mmol) in anhydrous DMF (4 mL) were used to afford compound 2d (103 mg, 38%, Rf 0.26 in 9:1/CH2Cl2:MeOH with NH4OH (7 mL/L)) as a white solid after purification by column chromatography (SiO2, 9:1/CH2Cl2:MeOH with NH4OH (7 mL/L)): 1H NMR (400 MHz, CD3OD, Figure C82) δ 3.21 (ddd, J = 11.6, 9.2, 5.2 Hz, 2H), 3.04 (ddd, J = 13.6, 9.2, 6.8 Hz, 1H), 2.84-2.78 (m, 2H), 2.72-2.66 (m, 2H), 2.62 (qd, J = 8.8, 3.6 Hz, 1H), 2.50 (q, J = 8.8 Hz, 1H), 2.49 (s, 3H), 2.23-2.14 (m, 2H), 1.90-1.82 (m, 6H), 1.81-1.73 (m, 1H), 1.73-1.63 (m, 1H); 13C NMR (100 MHz, CD3OD, Figure C83) δ 168.1, 165.8, 159.2, 132.0, 127.8, 114.0, 67.8, 57.8, 40.5, 34.0, 31.2, 28.7, 26.9, 26.2, 23.9, 23.7, 22.7; LRMS m/z calcd for C17H24N4S2: 348.1; found 349.1 [M+H]+ (Figure C3B); Purity of the compound was further confirmed by RP-HPLC by using method 1: Rf = 12.45 min (97% pure, Figure C84).
**Preparation of compound 3d.** Compound 3d was synthesized using general procedure E. Compound 35 (0.20 g, 0.80 mmol), Cs₂CO₃ (0.30 g, 0.91 mmol), TBAI (0.29 g, 0.77 mmol), and 2-(2-chloroethyl)-1-methylpyrrolidine hydrochloride (0.18 g, 1.20 mmol) in DMF (4 mL) were used to afford compound 3d (157 mg, 54%, R_f 0.23 in 9:1/CH₂Cl₂:MeOH with NH₄OH (7 mL/L)) as a white solid: ^1^H NMR (400 MHz, CD₃OD, Figure C85) δ 3.28-3.18 (m, 2H), 2.10-2.99 (m, 2H), 2.82-2.66 (m, 2H), 2.35 (s, 3H), 2.33-2.10 (m, 2H), 2.18-2.08 (m, 2H), 2.03-1.56 (m, 8H), 1.29 (d, J = 6.8 Hz, 3H); ^13^C NMR (100 MHz, CDCl₃, Figure C86) δ 168.1, 164.8, 156.7, 131.1, 131.0, 112.3, 65.6, 57.3, 40.51, 33.5, 30.5, 29.7, 29.6, 27.8, 25.4, 22.0, 21.9, 17.8; LRMS m/z calcd for C₁₈H₂₆N₄S₂: 362.2; found 363.2 [M+H]^+ (Figure C3C); Purity of the compound was further confirmed by RP-HPLC by using method 2: R_t = 20.18 min (98% pure, Figure C87).

**Preparation of compound 4d.** Compound 4d was prepared using procedure D. Compound 36 (0.10 g, 0.39 mmol), K₂CO₃ (0.06 g, 0.43 mmol), and 2-(2-chloroethyl)-1-methylpyrrolidine hydrochloride (0.07 g, 0.39 mmol) in anhydrous DMF (4 mL) were used to afford compound 4d (92 mg, 65%, R_f 0.20 in 9:1/CH₂Cl₂:MeOH with NH₄OH (7 mL/L)) as a yellow solid after purification by column chromatography (SiO₂, 19:1/CH₂Cl₂:MeOH): ^1^H NMR (400 MHz, CD₃OD, Figure C88) δ 3.25-3.18 (ddd, J = 13.2, 8.8, 4.4 Hz, 1H), 3.07-2.98 (m, 2H), 2.97-2.93 (m, 1H), 2.91-2.83 (m, 1H), 2.81 (dd, J = 16.8, 4.4 Hz, 1H), 2.40-2.27 (m, 2H), 2.32 (s, 3H), 2.23 (q, J = 9.2 Hz, 1H), 2.16-2.06 (m, 2H), 2.02-1.90 (m, 2H), 1.82-1.72 (m, 2H), 1.67 (ddd, J = 14.4, 9.6, 5.2 Hz, 1H), 1.63-1.44 (m, 2H), 1.10 (d, J = 6.4 Hz, 3H); ^13^C NMR (100 MHz, CDCl₃, Figure
C89) δ 167.7, 165.0, 157.1, 131.1, 125.0, 112.7, 65.5, 57.2, 40.4, 33.4, 33.3, 30.5, 30.4, 29.0, 27.8, 25.8, 21.9, 21.2; LRMS m/z calcd for C₈H₂₆N₄S₂: 362.2; found 363.1 [M+H]+ (Figure C3D); Purity of the compound was further confirmed by RP-HPLC by using method 2: Rₜ = 24.85 min (95% pure, Figure C90).

**Preparation of compound 5d.** Compound 5d was prepared using general procedure E. Compound 37 (0.10 g, 0.75 mmol), Cs₂CO₃ (0.25 g, 0.75 mmol), TBAI (0.28 g, 0.75 mmol), and 2-(2-chloroethyl)-1-methylpyrrolidine hydrochloride (0.11 g, 0.83 mmol) in anhydrous DMF (8 mL were added to afford compound 5d (6 mg, 2%, Rᵣ 0.27 in 9:1/CH₂Cl₂:MeOH with NH₄OH (7 mL/L)) as a white solid after purification by recrystallization from Hexanes:EtOAc: ¹H NMR (400 MHz, CD₃OD, Figure C91) δ 3.28-3.24 (m, 2H), 3.02-2.94 (m, 1H), 2.90-2.80 (m, 2H), 2.69-2.65 (m, 2H), 2.54 (very br t, 4H), 2.40-2.33 (m, 1H), 2.10-2.02 (m, 1H), 1.78-1.68 (m, 1H), 1.64 (p, J = 5.6 Hz, 4H), 1.54-1.41 (m, 5H), 1.01 (t, J = 7.6 Hz, 3H); ¹³C NMR (100 MHz, CD₃OD, Figure C92) δ 166.8, 163.9, 157.9, 130.3, 126.4, 112.4, 67.8, 55.8, 38.2, 35.7, 30.9, 30.7, 29.0, 28.2, 28.1, 26.5, 25.2, 21.0, 10.4; LRMS m/z calcd for C₁₀H₂₈N₄S₂: 376.2; found 377.2 [M+H]+ (Figure C3E); Purity of the compound was further confirmed by RP-HPLC by using method 2: Rₜ = 22.53 min (96% pure, Figure C93).

**Preparation of compound 6d.** Compound 6d was prepared using general procedure E. Compound 38 (0.10 g, 0.42 mmol), Cs₂CO₃ (0.14 g, 0.42 mmol), TBAI (0.16 g, 0.42 mmol), and 2-(2-
chloroethyl)-1-methylpyrrolidine hydrochloride (0.11 g, 0.62 mmol) in anhydrous DMF (8 mL) were used to afford compound 6d (29 mg, 17%, Rf 0.41 in 9:1/CH2Cl2:MeOH with NH4OH (7 mL/L)) as a white solid after purification by recrystallization from Hexanes:EtOAc: 1H NMR (400 MHz, CDCl3, Figure C94) δ 5.22 (br s, 2H), 3.24 (ddd, J = 13.2, 8.6, 5.2 Hz, 1H), 3.12-3.06 (m, 1H), 3.06-3.02 (m, 1H), 3.00-2.94 (m, 1H), 2.84-2.74 (m, 2H), 2.56-2.46 (m, 1H), 2.33 (s, 3H), 2.28-2.20 (m, 1H), 2.20-2.13 (m, 1H), 2.12-2.05 (m, 2H), 2.04-1.98 (m, 1H), 1.86-1.66 (m, 3H), 1.69-1.50 (m, 2H), 1.43 (qd, J = 12.0, 5.6 Hz, 1H), 0.94 (s, 9H); 13C NMR (100 MHz, CDCl3, Figure C95) δ 168.1, 165.2, 157.3, 132.5, 125.6, 112.8, 65.8, 57.4, 44.9, 40.6, 33.5, 32.7, 30.6, 28.0, 27.5, 27.3, 27.1, 24.3, 22.1; LRMS m/z calcd for C21H32N4S2: 404.2; found 405.1 [M+H]+ (Figure C3F); Purity of the compound was further confirmed by RP-HPLC by using method 1: Rt = 15.56 min (97% pure, Figure C96).

Preparation of compound 7d. Compound 7d was prepared using procedure D. Compound 39 (0.20 g, 0.64 mmol), K2CO3 (0.20 g, 1.41 mmol), and 2-(2-chloroethyl)-1-methylpyrrolidine hydrochloride (0.10 g, 0.65 mmol) in anhydrous DMF (4 mL) were used to afford compound 7d (116 mg, 43%, Rf 0.39 in 9:1/CH2Cl2:MeOH with NH4OH (7 mL/L)) as a white solid after purification by column chromatography (SiO2, 9:1/CH2Cl2:MeOH): 1H NMR (400 MHz, CDCl3, Figure C97) δ 7.35-3.31 (m, 2H), 7.26-7.22 (m, 3H), 5.24 (br s, 2H), 3.25 (ddd, J = 13.8, 9.8, 5.3 Hz, 1H), 3.12-2.85 (m, 7H), 2.34 (s, 3H), 2.37-1.95 (m, 6H), 1.85-1.68 (m, 3H), 1.68-1.56 (m, 1H); 13C NMR (100 MHz, CDCl3, Figure C98) δ 168.1, 165.4, 157.4, 145.2, 131.2, 128.9, 127.0, 126.9, 125.4, 112.8, 66.0, 57.3, 40.5, 40.4, 33.3, 33.1, 30.5, 29.9, 28.0, 26.5, 22.1; LRMS m/z calcd for C23H32N4S2: 424.2; found 425.0 [M+H]+ (Figure C3G); Purity
of the compound was further confirmed by RP-HPLC by using method 1: $R_t = 14.85$ min (95% pure, Figure C99).

**Preparation of compound 8d.** Compound 8d was prepared using procedure D. Compound 40 (0.21 g, 0.82 mmol), K$_2$CO$_3$ (0.25 g, 1.82 mmol), and 2-(2-chloroethyl)-1-methylpyrrolidine hydrochloride (0.15 g, 1.01 mmol) in anhydrous DMF (4 mL) were used to afford compound 8d (153 mg, 52%, $R_f$ 0.43 in 9:1/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7 mL/L)) as a brown solid after purification by column chromatography (SiO$_2$, 19:1/CH$_2$Cl$_2$:MeOH): $^1$H NMR (400 MHz, CDCl$_3$, Figure C100) $\delta$ 5.45 (s, 2H), 3.20 (ddd, $J = 13.3, 9.4, 5.2$ Hz, 1H), 3.11-3.07 (m, 1H), 3.01 (ddd, $J = 13.3, 9.2, 6.7$ Hz, 1H), 2.92-2.89 (m, 2H), 2.80-2.77 (m, 2H), 2.32 (s, 3H), 2.25 (qd, $J = 8.4, 3.4$ Hz, 1H), 2.18 (q, $J = 9.3$ Hz, 1H), 2.12-1.96 (m, 2H), 1.87-1.80 (m, 2H), 1.75-1.70 (m, 6H), 1.69-1.63 (m, 1H), 1.62-1.54 (m, 1H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure C101) $\delta$ 166.3, 164.2, 157.3, 135.1, 130.4, 114.1, 65.5, 57.2, 40.4, 33.3, 30.6, 30.3, 29.9, 28.9, 27.7, 27.1, 26.4, 21.9; LRMS $m/z$ calcd for C$_{18}$H$_{26}$N$_4$S$_2$: 362.2; found 363.1 [M+H]$^+$ (Figure C3H); Purity of the compound was further confirmed by RP-HPLC by using method 2: $R_t = 22.80$ min (97% pure, Figure C102).

**Preparation of compound 1e.** Compound 1e was prepared using procedure D. Compound 33 (0.20 g, 0.92 mmol), K$_2$CO$_3$ (0.27 g, 1.97 mmol), and 3-chloromethyl-1-methylpiperidine hydrochloride (0.17 g, 0.91 mmol) in anhydrous DMF (4 mL) were used to afford compound 1e (50 mg, 16%, $R_f$ 0.12 in
9:1/CH₂Cl₂:MeOH with NH₄OH (7 mL/L)) as a brown solid after purification by column chromatography (SiO₂, 9:1/CH₂Cl₂:MeOH with NH₄OH (7 mL/L)): ¹H NMR (400 MHz, CD₃OD, Figure C103) δ 3.16-3.08 (m, 3H), 3.06-3.01 (m, 2H), 2.97-2.90 (m, 3H), 2.50 (p, J = 4.0 Hz, 2H), 2.35 (s, 3H), 2.12 (td, J = 12.0, 4.0 Hz, 1H), 2.06-1.91 (m, 3H), 1.81-1.71 (m, 1H), 1.60 (qt, J = 12.4, 4.0 Hz, 1H), 1.09 (qd, J = 13.6, 4.0 Hz, 1H); ¹³C NMR (100 MHz, (CD₃)₂SO, Figure C104) δ 171.5, 164.1, 157.1, 135.9, 134.1, 109.2, 60.1, 55.2, 45.7, 35.5, 33.6, 29.0, 28.8, 28.7, 27.2, 24.0; LRMS m/z calcd for C₁₆H₂₂N₄S₂: 334.1; found 335.1 [M+H]⁺ (Figure C3I); Purity of the compound was further confirmed by RP-HPLC by using method 2: Rᵣ = 20.80 min (97% pure, Figure C105).

**Preparation of compound 2e.** Compound 2e was prepared using procedure D. Compound 34 (0.20 g, 0.78 mmol), K₂CO₃ (0.24 g, 1.72 mmol), and 3-chloromethyl-1-methylpiperidine hydrochloride (0.15 g, 0.78 mmol) in anhydrous DMF (4 mL) were used to afford compound 2e (95 mg, 35%, Rᵣ 0.15 in 9:1/CH₂Cl₂:MeOH with NH₄OH (7 mL/L)) as a dark yellow solid after purification by column chromatography (SiO₂, 9:1/CH₂Cl₂:MeOH with NH₄OH (7 mL/L)): ¹H NMR (400 MHz, CDCl₃, Figure C106) δ 5.28 (br s, 2H), 3.14-3.03 (m, 3H), 2.85-2.80 (m, 2H), 2.76-2.73 (m, 2H), 2.33 (s, 3H), 2.19-2.15 (m, 2H), 2.04-2.02 (m, 3H), 1.94-1.84 (m, 6H), 1.72-1.66 (m, 2H); ¹³C NMR (100 MHz, (CD₃)₂SO, Figure C107) δ 166.6, 164.5, 158.0, 129.4, 127.2, 112.6, 60.5, 55.6, 46.1, 36.0, 34.0, 29.2, 25.8, 25.1, 24.4, 22.7, 22.4; LRMS m/z calcd for C₁₇H₂₄N₄S₂: 348.1; found 349.1 [M+H]⁺ (Figure C3J); Purity of the compound was further confirmed by RP-HPLC by using method 2: Rᵣ = 21.48 min (97% pure, Figure C108).
Preparation of compound 3e. Compound 3e was prepared using procedure E. Compound 35 (0.20 g, 0.80 mmol), Cs₂CO₃ (0.29 g, 0.90 mmol), TBAI (0.29 g, 0.78 mmol), and 3-chloromethyl-1-methylpiperidine hydrochloride (0.18 g, 1.21 mmol) in anhydrous DMF (4 mL) were used to afford compound 3e (180 mg, 62%, Rf 0.32 in 9:1/CH₂Cl₂:MeOH with NH₄OH (7 mL/L)) as a white solid: ¹H NMR (400 MHz, CD₃OD, Figure C109) δ 3.56-3.43 (m, 1H), 3.42-3.32 (m, 1H), 3.26-3.00 (m, 4H), 2.82-2.64 (m, 2H), 2.61 (s, 3H), 2.50 (m, 2H), 2.40 (t, J = 11.6 Hz, 1H), 2.20-1.60 (m, 8H), 1.27 (d, J = 7.2 Hz, 3H), 1.26-1.10 (m, 1H); ¹³C NMR (100 MHz, CDCl₃, Figure C110) δ 168.1, 164.9, 156.6, 131.2, 131.1, 112.4, 61.5, 56.3, 46.8, 36.4, 35.0, 29.9, 29.8, 29.6, 25.5, 25.3, 21.9, 17.9; LRMS m/z calcd for C₁₈H₂₆N₅S₂: 362.2; found 363.0 [M+H]⁺ (Figure C3K); Purity of the compound was further confirmed by RP-HPLC by using method 1: Rt = 13.03 min (95% pure, Figure C111).

Preparation of compound 4e. Compound 4e was prepared using procedure D. Compound 36 (0.10 g, 0.39 mmol), K₂CO₃ (0.06 g, 0.43 mmol), and 3-chloromethyl-1-methylpiperidine hydrochloride (0.08 g, 0.39 mmol) in anhydrous DMF (4 mL) were used to afford compound 4e (120 mg, 85%, Rf 0.21 in 9:1/CH₂Cl₂:MeOH with NH₄OH (7 mL/L)) as a white solid after purification by column chromatography (SiO₂, 19:1/CH₂Cl₂:MeOH): ¹H NMR (400 MHz, (CD₃)₂SO, Figure C112) δ 6.85 (br s, 2H), 3.06-2.65 (m, 7H), 2.34-2.26 (m, 1H), 2.19 (s, 3H), 1.96 (t, J = 10.8 Hz, 1H), 1.88-1.74 (m, 5H), 1.62 (dt, J = 13.2, 3.6 Hz, 2H), 1.49-1.34 (m, 2H), 1.03 (d, J = 6.8 Hz,
3H); $^{13}$C NMR (100 MHz, (CD$_3$)$_2$SO, Figure C113) δ 166.3, 164.1, 157.5, 128.5, 126.4, 112.0, 60.3, 55.3, 45.8, 35.6, 33.7, 32.7, 30.0, 28.9, 28.5, 25.1, 24.1, 21.1; LRMS m/z calcd for C$_{13}$H$_{26}$N$_4$S$_2$: 362.2; found 363.1 [M+H]$^+$ (Figure C3L); Purity of the compound was further confirmed by RP-HPLC by using method 1: $R_t = 11.84$ min (97% pure, Figure C114).

**Preparation of compound 5e.** Compound 5e was prepared using procedure D. Compound 37 (0.11 g, 0.37 mmol), K$_2$CO$_3$ (0.11 g, 0.81 mmol), and 3-chloromethyl-1-methylpiperidine hydrochloride (0.08 g, 0.37 mmol), and DMF (4 mL) were used to afford compound 5e (18 mg, 13%, $R_f$ 0.37 in 9:1/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7 mL/L)) as a white solid after purification by recrystallization from MeCN: $^1$H NMR (400 MHz, CDCl$_3$, Figure C115) δ 5.25 (s, 2H), 3.06 (d, $J = 6.4$ Hz, 2H), 2.95-2.87 (m, 2H), 2.83 (dd, $J = 16.0$, 5.2 Hz, 2H), 2.71 (d, $J = 11.2$ Hz, 1H), 2.36 (dd, $J = 16.4$, 10.0 Hz, 1H), 2.22 (s, 3H), 2.06-1.92 (m, 2H), 1.86 (td, $J = 10.4$, 2.0 Hz, 2H), 1.73-1.63 (m, 3H), 160-1.48 (m, 2H), 1.46-1.34 (m, 3H), 0.96 (t, $J = 7.2$ Hz, 3H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure C116) δ 167.9, 165.2, 157.2, 131.5, 125.5, 112.9, 61.6, 56.4, 46.9, 36.4, 35.9, 35.1, 31.4, 30.0, 28.8, 28.6, 26.1, 25.4, 11.7; LRMS m/z calcd for C$_{19}$H$_{28}$N$_4$S$_2$: 376.2; found 377.1 [M+H]$^+$ (Figure C4A); Purity of the compound was further confirmed by RP-HPLC by using method 1: $R_t = 14.22$ min (96% pure, Figure C117).

**Preparation of compound 6e.** Compound 6e was prepared using procedure D. Compound 38 (0.20 g, 0.70 mmol), K$_2$CO$_3$ (0.16 g, 1.08 mmol), and 3-chloromethyl-1-methylpiperidine hydrochloride
(0.10 g, 0.49 mmol) in anhydrous DMF (4 mL) were used to afford compound 6e (63 mg, 28%, \( R_f \) 0.42 in 9:1/CH\(_2\)Cl\(_2\):MeOH with NH\(_4\)OH (7 mL/L)) as a yellow solid after purification by recrystallization from MeCN: \(^1\)H NMR (400 MHz, CDCl\(_3\), Figure C118) \( \delta \) 5.41 (br s, 2H), 3.05-2.88 (m, 2H), 2.75 (dd, \( J = 16.4, 4.8 \) Hz, 2H), 2.70-2.68 (m, 1H), 2.52-2.42 (m, 1H), 2.21 (s, 3H), 2.05 (dd, \( J = 13.2, 3.6 \) Hz, 1H), 2.00-1.90 (m, 1H), 1.89-1.80 (m, 2H), 1.71-1.62 (m, 2H), 1.58-1.48 (m, 2H), 1.39 (qd, \( J = 12.0, 5.2 \) Hz, 1H), 0.98 (qd, \( J = 12.0, 4.0 \) Hz, 1H), 0.92 (s, 9H); \(^{13}\)C NMR (100 MHz, CDCl\(_3\), Figure C119) \( \delta \) 167.8, 165.1, 157.3, 132.2, 125.6, 112.7, 61.6, 56.3, 46.8, 44.7, 36.4, 35.1, 32.6, 30.0, 27.4, 27.2, 27.0, 25.4, 24.2; LRMS \( m/z \) calcd for C\(_{21}\)H\(_{32}\)N\(_4\)S\(_2\): 404.2; found 405.1 [M+H]\(^+\) (Figure C4B); Purity of the compound was further confirmed by RP-HPLC by using method 2: \( R_t \) = 26.67 min (97% pure, Figure C120).

**Preparation of compound 7e.** Compound 7e was prepared using procedure D. Compound 39 (0.20 g, 0.64 mmol), K\(_2\)CO\(_3\) (0.20 g, 1.42 mmol), and 3-chloromethyl-1-methylpiperidine hydrochloride (0.10 g, 0.65 mmol) in anhydrous DMF (4 mL) were used to afford compound 7e (149 mg, 55%, \( R_f \) 0.24 in 9:1/CH\(_2\)Cl\(_2\):MeOH with NH\(_4\)OH (7 mL/L)) as a tan solid after purification by column chromatography (SiO\(_2\), 19:1/CH\(_2\)Cl\(_2\):MeOH): \(^1\)H NMR (400 MHz, CD\(_3\)OD, Figure C121) \( \delta \) 7.31 (d, \( J = 4.4 \) Hz, 4H), 7.25-7.18 (m, 1H), 3.14-2.98 (m, 7H), 2.96-2.78 (m, 2H), 2.27 (s, 3H), 2.24-2.16 (m, 1H), 2.11-1.90 (m, 4H), 1.84-1.70 (m, 2H), 1.65-1.52 (m, 1H), 1.11-0.90 (m, 1H); \(^{13}\)C NMR (100 MHz, CDCl\(_3\), Figure C122) \( \delta \) 168.0, 165.4, 157.4, 145.2, 131.2, 128.9, 127.1, 126.9, 125.4, 112.8, 61.3, 56.2, 46.5, 40.4, 36.1, 35.0, 33.1, 29.9, 29.7, 26.5, 25.1; LRMS \( m/z \) calcd for C\(_{23}\)H\(_{28}\)N\(_4\)S\(_2\): 424.2; found 425.0 [M+H]\(^+\) (Figure S8).
Preparation of compound 8e. Compound 8e was prepared using procedure D. Compound 40 (0.20 g, 0.81 mmol), K$_2$CO$_3$ (0.25 g, 1.80 mmol), and 3-chloromethyl-1-methylpiperidine hydrochloride (1.50 g, 1.02 mmol) in anhydrous DMF (4 mL) were used to afford compound 8e (159 mg, 55%, $R_f$ 0.48 in 9:1/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7 mL/L)) as a yellow solid after purification by column chromatography (SiO$_2$, 19:1/CH$_2$Cl$_2$:MeOH): $^1$H NMR (400 MHz, CD$_3$OD, Figure C124) $\delta$ 3.07-3.00 (m, 5H), 2.87-2.84 (m, 2H), 2.82-2.78 (m, 1H), 2.28 (s, 3H), 2.02-1.89 (m, 5H), 1.85-1.71 (m, 6H), 1.57 (qt, $J =$ 12.4, 3.6 Hz, 1H), 1.06 (qd, $J =$ 12.4, 3.6 Hz, 1H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure C125) $\delta$ 166.7, 164.6, 157.4, 135.5, 130.5, 114.4, 61.3, 56.2, 46.6, 36.2, 35.1, 30.9, 30.2, 29.7, 29.2, 27.4, 26.7, 25.1; LRMS m/z calcd for C$_{18}$H$_{26}$N$_4$S$_2$: 362.2; found 363.1 [M+H]$^+$ (Figure C4D); Purity of the compound was further confirmed by RP-HPLC by using method 2: $R_t =$ 22.57 min (98% pure, Figure C126).

Preparation of compound 1f. Compound 1f was prepared using procedure E. Compound 33 (0.10 g, 0.45 mmol), Cs$_2$CO$_3$ (0.14 g, 0.42 mmol), TBAI (0.17 g, 0.45 mmol), and compound 41 (0.11 g, 0.62 mmol) in anhydrous DMF (8 mL) were used to afford compound 1f (33 mg, 21%, $R_f$ 0.33 in 9:1/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7 mL/L)) as light brown needles after purification by recrystallization from Hexanes:EtOAc and washed with Hexanes: $^1$H NMR (400 MHz,
CDCl$_3$, Figure C127) δ 8.07 (br s, 1H), 5.50 (s, 2H), 3.73 (s, 2H), 3.32 (q, $J$ = 5.6 Hz, 2H), 3.01-2.95 (m, 4H), 2.52 (p, $J$ = 7.2 Hz, 2H), 2.36 (t, $J$ = 5.6 Hz, 2H), 2.13 (s, 6H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure C128) δ 172.8, 170.2, 164.1, 157.2, 137.7, 134.6, 110.4, 58.1, 45.3, 37.3, 35.0, 29.7, 29.2, 28.0; LRMS $m/z$ calcd for C$_{15}$H$_{21}$N$_5$S$_2$: 351.1; found 352.0 [M+H]$^+$ (Figure C4E); Purity of the compound was further confirmed by RP-HPLC by using method 1: $R_t$ = 11.12 min (96% pure, Figure C129).

**Preparation of compound 2f.** Compound 2f was prepared using procedure D. Compound 34 (0.20 g, 0.78 mmol), K$_2$CO$_3$ (0.25 g, 1.72 mmol), and compound 41 (0.19 g, 0.78 mmol) in anhydrous DMF (4 mL) were used to afford compound 2f (159 mg, 59%, $R_f$ 0.35 in 9:1/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7 mL/L)) as a yellow solid after purification by flash column chromatography (9:1/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7mL/L)): $^1$H NMR (400 MHz, (CD$_3$)$_2$SO, Figure C130) δ 7.97 (t, $J$ = 4.8 Hz, 1H), 6.93 (br s, 2H), 3.75 (s, 2H), 3.15 (q, $J$ = 6.0 Hz, 2H), 2.85 (m, 2H), 2.70 (m, 2H), 2.67 (t, $J$ = 6.8 Hz, 2H), 2.099 (s, 3H), 2.096 (s, 3H), 1.79 (m, 4H); $^{13}$C NMR (100 MHz, (CD$_3$)$_2$SO, Figure C131) δ 167.9, 166.0, 163.5, 157.6, 129.3, 126.8, 112.3, 57.9, 45.0, 37.0, 34.1, 25.3, 24.7, 22.2, 21.9; LRMS $m/z$ calcd for C$_{16}$H$_{23}$N$_5$S$_2$: 365.1; found 366.0 [M+H]$^+$ (Figure C4F); Purity of the compound was further confirmed by RP-HPLC by using method 1: $R_t$ = 11.59 min (96% pure, Figure C132).

**Preparation of compound 3f.** Compound 3f was prepared using procedure E. Compound 35 (0.20 g, 0.80 mmol), Cs$_2$CO$_3$ (0.29 g,
0.90 mmol), TBAI (0.29 g, 0.78 mmol), and compound 41 (0.30 g, 1.21 mmol), and DMF (4 mL) were used to afford compound 3f (172 mg, 57%, Rf 0.40 in 9:1/CH2Cl2:MeOH with NH4OH (7 mL/L)) as a white solid after purification by flash column chromatography (9:1/CH2Cl2:MeOH with NH4OH (7mL/L)): 1H NMR (400 MHz, CD3OD, Figure C133) δ 3.88 (s, 2H), 3.59 (t, J = 6.0 Hz, 2H), 3.21 (t, J = 6.0 Hz, 2H), 3.22-3.14 (m, 1H), 2.85 (s, 6H), 2.78-2.62 (m, 2H), 1.94-1.74 (m, 4H), 1.24 (d, J = 6.8 Hz, 3H); 13C NMR (100 MHz, CD3OD, Figure C134) δ 173.4, 168.6, 164.5, 158.7, 133.3, 131.9, 113.6, 58.4, 44.2, 36.5, 35.6, 30.7, 30.3, 26.3, 22.4, 19.0; LRMS m/z calcd for C17H25N5OS2: 379.2; found 380.0 [M+H]+ (Figure C4G); Purity of the compound was further confirmed by RP-HPLC by using method 2: Rt = 22.49 min (95% pure, Figure C135).

Preparation of compound 4f. Compound 4f was prepared using procedure D. Compound 36 (0.28 g, 0.78 mmol), K2CO3 (0.25 g, 1.72 mmol), and compound 41 (0.19 g, 0.78 mmol) in anhydrous DMF (4 mL) were used to afford compound 4f (132 mg, 44%, Rf 0.35 in 9:1/CH2Cl2:MeOH with NH4OH (7 mL/L)) as a yellow solid after purification by flash column chromatography (9:1/CH2Cl2:MeOH with NH4OH (7mL/L)): 1H NMR (400 MHz, CD3OD, Figure C136) δ 3.76 (s, 2H), 3.32 (t, J = 4.0 Hz, 2H), 3.01-2.93 (m, 1H), 2.91-2.83 (m, 1H), 2.81 (dd, J = 16.8, 4.0 Hz, 1H) 2.39 (t, J = 6.4 Hz, 2H), 2.36-2.30 (m, 1H), 2.17 (s, 6H), 2.02-1.88 (m, 2H), 1.54-1.44 (m, 1H), 1.10 (d, J = 6.4 Hz, 3H); 13C NMR (100 MHz, (CD3)2SO, Figure C137) δ 167.9, 166.2, 163.5, 157.6, 128.8, 126.4, 112.1, 57.9, 45.0, 37.0, 34.1, 32.6, 30.0, 28.4, 25.0, 21.0; LRMS m/z calcd for C17H25N5OS2: 379.2; found 380.0 [M+H]+ (Figure C4H); Purity of the
compound was further confirmed by RP-HPLC by using method 2: $R_t = 17.70$ min (98% pure, Figure C138).

**Preparation of compound 5f.** Compound 5f was prepared using procedure D. Compound 37 (0.11 g, 0.37 mmol), K$_2$CO$_3$ (0.11 g, 0.81 mmol), and compound 41 (0.09 g, 0.37 mmol) in anhydrous DMF (4 mL) were used to afford compound 5f (5 mg, 4%, $R_f = 0.35$ in 9:1/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7 mL/L)) as a yellow solid after purification by recrystallization from MeCN: $^1$H NMR (400 MHz, CD$_3$OD, Figure C139) $\delta$ 3.78 (s, 2H), 3.32 (t, $J = 6.4$ Hz, 2H), 3.03-2.95 (m, 1H), 2.86 (dd, $J = 16.0$, 4.4 Hz, 1H), 2.91-2.79 (m, 1H), 2.42 (t, $J = 6.4$ Hz, 2H), 2.39-2.35 (m, 1H), 2.20 (s, 6H), 2.10-2.02 (m, 1H), 1.80-1.66 (m, 1H), 1.55-1.41 (m, 3H), 1.01 (t, $J = 7.6$ Hz, 3H); $^{13}$C NMR (100 MHz, CD$_3$OD, Figure C140) $\delta$ 172.6, 168.3, 164.9, 159.6, 132.1, 128.0, 114.1, 59.1, 45.5, 38.4, 37.3, 35.5, 32.3, 29.8, 29.7, 26.7, 11.9; LRMS $m/z$ calcd for C$_{18}$H$_{27}$N$_5$O$_2$: 393.2; found 394.1 [M+H]$^+$ (Figure C41); Purity of the compound was further confirmed by RP-HPLC by using method 1: $R_t = 13.45$ min (97% pure, Figure C141).

**Preparation of compound 6f.** Compound 6f was prepared using procedure D. Compound 38 (0.20 g, 0.70 mmol, K$_2$CO$_3$ (0.15 g, 1.08 mmol), and compound 41 (0.08 g, 0.39 mmol) in anhydrous DMF (4 mL) were used to afford compound 6f (20 mg, 10%, $R_f = 0.32$ in 9:1/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7 mL/L)) as a yellow solid after purification by recrystallization from MeCN: $^1$H NMR (400 MHz, CDCl$_3$, Figure C142) $\delta$ 8.04 (br t, 1H), 5.71 (s, 2H), 3.74 (d, $J = 14.8$ Hz,
1H), 3.69 (d, J = 14.8 Hz, 1H), 3.31 (q, J = 5.2 Hz, 2H), 2.97 (dd, J = 14.4, 4.8 Hz, 1H), 2.79 (dd, J = 16.4, 4.8 Hz, 2H), 2.56-2.46 (m, 1H), 2.36 (t, J = 6.0 Hz, 2H), 2.13 (s, 6H), 2.10-2.07 (m, 1H), 1.55 (td, J = 12.0, 4.0 Hz, 1H), 1.43 (qd, J = 12.0, 5.6 Hz, 1H), 0.94 (s, 9H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure C143) δ 170.2, 167.7, 164.0, 157.7, 133.2, 125.6, 113.1, 58.1, 45.3, 44.8, 37.2, 35.0, 32.7, 27.4, 27.3, 27.1, 24.3; LRMS m/z calcd for C$_{20}$H$_{31}$N$_{5}$OS$_{2}$: 421.2; found 422.2 [M+H]$^+$ (Figure C4J); Purity of the compound was further confirmed by RP-HPLC by using method 2: $R_t$ = 22.69 min (98% pure, Figure C144).

**Preparation of compound 7f.** Compound 7f was prepared using procedure D. Compound 39 (0.20 g, 0.64 mmol), K$_2$CO$_3$ (0.20 g, 1.41 mmol), compound 41 (0.16 g, 0.65 mmol) in anhydrous DMF (4 mL) were used to afford compound 7f (111 mg, 39%, $R_f$ 0.27 in 9:1/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7 mL/L)) as a tan solid after purification by column chromatography (SiO$_2$, 9:1/CH$_2$Cl$_2$:MeOH): $^1$H NMR (400 MHz, CD$_3$OD, Figure C145) δ 7.33-7.28 (m, 4H), 7.23-7.19 (m, 1H), 3.80 (s, 2H), 3.32 (t, J = 6.4 Hz, 2H), 3.07-2.92 (m, 4H), 2.92-2.81 (m, 1H), 2.42 (t, J = 6.4 Hz, 2H), 2.20 (s, 6H), 2.07-1.97 (m, 1H); $^{13}$C NMR (100 MHz, CD$_3$OD, Figure C146) δ 172.5, 168.4, 165.0, 159.6, 146.9, 131.9, 129.8, 128.1, 127.8, 127.7, 114.0, 59.1, 45.5, 41.7, 38.4, 35.5, 34.0, 31.1, 27.2; LRMS m/z calcd for C$_{22}$H$_{27}$N$_{5}$OS$_{2}$: 441.2; found 442.0 [M+H]$^+$ (Figure C4K); Purity of the compound was further confirmed by RP-HPLC by using method 1: $R_t$ = 12.14 min (98% pure, Figure C147).
Preparation of compound 8f. Compound 8f was prepared by using procedure D. Compound 39 (0.15 g, 0.59 mmol), K$_2$CO$_3$ (0.18 g, 1.29 mmol), and compound 41 (0.14 g, 0.59 mmol) in anhydrous DMF (4 mL) were used to afford compound 8f (122 mg, 55%, $R_f$ 0.28 in 9:1/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7 mL/L)) as a brown solid after purification by column chromatography (SiO$_2$, 9:1/CH$_2$Cl$_2$:MeOH): $^1$H NMR (400 MHz, CDCl$_3$, Figure C148) $\delta$ 8.05 (br t, 1H), 5.78 (s, 2H), 3.71 (s, 2H), 3.32 (q, $J$ = 6.0 Hz, 2H), 2.96-2.93 (m, 2H), 2.84-2.82 (m, 2H), 2.40 (t, $J$ = 6.0 Hz, 2H), 2.15 (s, 6H), 1.90-1.85 (m, 2H), 1.82-1.74 (m, 4H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure C149) $\delta$ 170.1, 166.1, 163.2, 157.6, 136.0, 130.3, 114.4, 57.9, 45.0, 36.9, 34.7, 30.5, 29.9, 28.9, 27.1, 26.4; LRMS $m/z$ calcd for C$_{17}$H$_{25}$N$_5$O$_5$S$_2$: 379.2; found 380.1 [M+H]$^+$ (Figure C4L); Purity of the compound was further confirmed by RP-HPLC by using method 2: $R_t$ = 22.53 min (95% pure, Figure C150).

Preparation of compound 1g. Compound 1g was prepared using procedure D. Compound 33 (0.20 g, 0.91 mmol), K$_2$CO$_3$ (0.27 g, 1.98 mmol), and compound 42 (0.25 g, 0.90 mmol) in anhydrous DMF (4 mL) were used to afford compound 1g (100 mg, 30%, $R_f$ 0.23 in 9:1/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7 mL/L)) as a brown solid after purification by flash column chromatography (9:1/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7mL/L)): $^1$H NMR (400 MHz, CD$_3$OD, Figure C151) $\delta$ 3.85 (s, 2H), 3.51 (t, $J$ = 6.4 Hz, 2H), 3.02 (t, $J$ = 6.4 Hz, 2H), 2.98 (q, $J$ = 7.6 Hz, 4H), 2.95 (t, $J$ = 7.6 Hz, 2H), 2.89 (t, $J$ = 7.2 Hz, 2H), 2.45 (p, $J$ = 3.2 Hz, 2H), 1.16 (t, $J$ = 7.2 Hz, 6H); $^{13}$C NMR (100 MHz, CD$_3$OD, Figure C152) $\delta$ 173.6 (2 carbons overlapping), 164.9, 159.3, 137.8, 137.2, 111.5, 52.8, 49.2, 37.2, 35.9, 30.6, 30.2, 29.2, 10.5; LRMS $m/z$ calcd for C$_{17}$H$_{25}$N$_5$O$_5$S$_2$:
Preparation of compound 2g. Compound 2g was prepared using procedure D. Compound 34 (0.20 g, 0.78 mmol), K$_2$CO$_3$ (0.21 g, 1.72 mmol), and compound 42 (0.21 g, 0.78 mmol) in anhydrous DMF (4 mL) were used to afford compound 2g (165 mg, 54%, R$_f$ 0.21 in 9:1/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7 mL/L)) as a white solid after purification by flash column chromatography (9:1/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7mL/L)): $^1$H NMR (400 MHz, CDCl$_3$, Figure C154) $\delta$ 7.65 (t, $J$ = 5.2 Hz, 1H), 5.92 (s, 2H), 3.68 (s, 2H), 3.21 (q, $J$ = 5.2 Hz, 2H), 2.72 (m, 2H), 2.62 (m, 2H), 2.43 (t, $J$ = 5.2 Hz, 2H), 2.38 (q, $J$ = 7.2 Hz, 4H), 1.76 (m, 4H), 0.82 (t, $J$ = 7.2 Hz, 6H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure C155) $\delta$ 169.8, 166.9, 163.1, 157.5, 131.6, 125.7, 113.0, 51.4, 46.7, 37.2, 34.5, 25.8, 25.2, 22.5, 22.3, 11.1; LRMS $m/z$ calcd for C$_{18}$H$_{27}$N$_5$OS$_2$: 393.2; found 394.1 [M+H]$^+$ (Figure C5B); Purity of the compound was further confirmed by RP-HPLC by using method 2: $R_t$ = 22.46 min (95% pure, Figure C156).

Preparation of compound 3g. Compound 3g was prepared using procedure E. Compound 35 (0.20 g, 0.80 mmol), Cs$_2$CO$_3$ (0.30 g, 0.91 mmol), TBAI (0.29 g, 0.77 mmol), and compound 42 (0.33 g, 1.20 mmol), and DMF (4 mL) were used to afford compound 3g (305 mg, 93%, R$_f$ 0.33 in 9:1/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7 mL/L)) as a white solid after purification by flash column chromatography (9:1/CH$_2$Cl$_2$:MeOH): $^1$H NMR (400 MHz, CD$_3$OD, Figure C157) $\delta$ 3.80 (s,
2H), 3.33-3.31 (m, 2H), 3.28-3.20 (m, 1H), 2.84-2.68 (m, 2H), 2.55 (m, 6H), 2.02-1.78 (m, 4H), 1.29 (d, J = 7.2 Hz, 3H), 0.96 (t, J = 7.2 Hz, 6H); \(^1\)C NMR (100 MHz, CDCl\(_3\), Figure C158) δ 169.2, 168.1, 163.5, 156.9, 131.9, 131.2, 112.8, 51.7, 47.1, 37.3, 34.8, 29.8, 29.6, 25.5, 22.0, 17.9, 11.4; LRMS m/z calcd for C\(_{19}\)H\(_{29}\)N\(_5\)OS\(_2\): 407.2; found 408.0 [M+H]+ (Figure C5C); Purity of the compound was further confirmed by RP-HPLC by using method 1: \(R_t = 10.85\) min (95% pure, Figure C159).

**Preparation of compound 4g.** Compound 4g was prepared using procedure D. Compound 36 (0.28 g, 0.78 mmol), K\(_2\)CO\(_3\) (0.25 g, 1.72 mmol), and compound 42 (0.21 g, 0.78 mmol) in anhydrous DMF (4 mL) were used to afford compound 4g (200 mg, 63%, \(R_f = 0.42\) in 9:1/CH\(_2\)Cl\(_2\):MeOH with NH\(_4\)OH (7 mL/L)) as a yellow solid after purification by flash column chromatography (9:1/CH\(_2\)Cl\(_2\):MeOH with NH\(_4\)OH (7mL/L)): \(^1\)H NMR (400 MHz, CDCl\(_3\), Figure C160) δ 7.89 (br t, 1H), 5.79 (s, 2H), 3.76 (s, 2H), 3.37 (q, \(J = 5.8\) Hz, 2H), 2.93-2.77 (m, 3H), 2.67 (t, \(J = 5.9\) Hz, 2H), 2.62 (q, \(J = 7.2\) Hz, 4H), 2.35 (ddt, \(J = 16.8, 9.3, 2.8\) Hz, 1H), 1.99-1.92 (m, 2H), 1.55-1.45 (m, 1H), 1.08 (d, \(J = 6.5\) Hz, 3H), 1.01 (t, \(J = 7.2\) Hz, 6H); \(^1\)C NMR (100 MHz, CDCl\(_3\), Figure C161) δ 170.4, 167.6, 163.6, 157.7, 131.9, 125.3, 113.1, 51.9, 47.4, 37.0, 34.7, 33.5, 30.7, 29.1, 25.9, 21.4, 10.8; LRMS m/z calcd for C\(_{19}\)H\(_{29}\)N\(_5\)OS\(_2\): 407.2; found 408.0 [M+H]+ (Figure C5D); Purity of the compound was further confirmed by RP-HPLC by using method 1: \(R_t = 11.42\) min (95% pure, Figure C162).
Preparation of compound 5g. Compound 5g was prepared using procedure E. Compound 37 (0.1 g, 0.38 mmol), Cs$_2$CO$_3$ (0.18 g, 0.57 mmol), TBAI (0.15 g, 0.38 mmol), and compound 42 (0.11 g, 0.41 mmol) in anhydrous DMF (2 mL) were used to afford compound 5g (15 mg, 10%, R$_f$ 0.34 in 9:1/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7 mL/L)) as a white solid after purification by recrystallization from Hexanes:EtOAc: $^1$H NMR (400 MHz, CD$_3$OD, Figure C163) $\delta$ 3.79 (s, 2H), 3.31 (t, $J = 7.2$ Hz, 2H), 3.01-2.92 (m, 1H), 2.84 (dd, $J = 16.8, 4.0$ Hz, 1H), 2.88-2.78 (m, 1H), 2.57 (t, $J = 7.2$ Hz, 2H), 2.54 (q, $J = 7.6$ Hz, 4H), 2.40-2.31 (m, 1H), 2.09-2.01 (m, 1H), 1.76-1.64 (m, 1H), 1.53-1.39 (m, 3H), 1.02 (t, $J = 7.6$ Hz, 6H), 0.97 (t, $J = 7.6$ Hz, 3H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure C164) $\delta$ 169.9, 167.7, 163.7, 157.6, 132.2, 125.6, 113.2, 51.7, 47.1, 37.4, 35.9, 34.8, 31.4, 28.7, 28.6, 26.0, 11.7, 11.5; LRMS m/z calcd for C$_{20}$H$_{31}$N$_5$OS$_2$: 421.2; found 422.0 [M+H]$^+$ (Figure C5E); Purity of the compound was further confirmed by RP-HPLC by using method 1: $R_t = 12.01$ min (98% pure, Figure C165).

Preparation of compound 6g. Compound 6g was prepared using procedure E. Compound 38 (0.21, 0.68 mmol), Cs$_2$CO$_3$ (0.34 g, 1.0 mmol), TBAI (0.26 g, 0.68 mmol), and compound 42 (0.21 g, 0.75 mmol) in anhydrous DMF (4 mL) were used to afford compound 6g (68 mg, 22%, $R_f$ 0.50 in 9:1/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7 mL/L)) as a white solid after purification by flash column chromatography (SiO$_2$, 9:1/CH$_2$Cl$_2$:MeOH with 7 mL/L NH$_4$OH) and then by recrystallization from Hexanes:EtOAc: $^1$H NMR (400 MHz, CD$_3$OD, Figure C166) $\delta$ 3.80 (s, 2H), 3.33 (t, $J = 6.4$ Hz, 2H), 3.08-3.02 (m, 1H), 2.84-2.76 (m, 2H), 2.60 (t, $J = 6.8$ Hz, 2H), 2.57 (t, $J = 7.6$ Hz, 4H), 2.20-2.12 (m, 1H), 1.57 (td, $J = 10.4, 3.6$ Hz, 1H), 1.43 (qd, $J = 11.6$, 7.6 Hz, 1H), 1.03 (t, $J = 7.6$ Hz, 3H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure C166) $\delta$ 169.9, 167.7, 163.7, 157.6, 132.2, 125.6, 113.2, 51.7, 47.1, 37.4, 35.9, 34.8, 31.4, 28.7, 28.6, 26.0, 11.7, 11.5; LRMS m/z calcd for C$_{20}$H$_{31}$N$_5$OS$_2$: 421.2; found 422.0 [M+H]$^+$ (Figure C5E); Purity of the compound was further confirmed by RP-HPLC by using method 1: $R_t = 12.01$ min (98% pure, Figure C165).
5.6 Hz, 1H), 0.99 (t, $J = 8.4$ Hz, 6H), 0.99 (s, 9H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure C167) δ 171.8, 167.6, 163.5, 158.0, 132.4, 125.9, 112.9, 52.3, 48.2, 44.7, 35.8, 34.7, 32.6, 27.4, 27.1 27.0, 24.2, 9.0; LRMS $m/z$ calcd for C$_{22}$H$_{35}$N$_3$OS$_2$: 449.2; found 450.2 [M+H]$^+$ (Figure C5F); Purity of the compound was further confirmed by RP-HPLC by using method 2: $R_t = 22.24$ min (96% pure, Figure C168).

**Preparation of compound 7g.** Compound 7g was prepared using procedure D. Compound 39 (0.20 g, 0.64 mmol), K$_2$CO$_3$ (0.20 g, 1.44 mmol), compound 42 (0.18 g, 0.67 mmol) in anhydrous DMF (4 mL) were used to afford compound 7g (77 mg, 26%, $R_f$ 0.26 in 9:1/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7 mL/L)) as a tan solid after purification by flash column chromatography (SiO$_2$, 9:1/CH$_2$Cl$_2$:MeOH): $^1$H NMR (400 MHz, CD$_3$OD, Figure C169) δ 7.31 (d, $J = 4.8$ Hz, 4H), 7.25-7.19 (m, 1H), 3.80 (s, 2H), 3.30 (t, $J = 5.2$ Hz, 2H), 3.14-2.98 (m, 4H), 2.96-2.86 (m, 1H), 2.54 (t, $J = 6.8$ Hz, 2H), 2.51 (q, $J = 6.8$ Hz, 4H), 2.24-2.18 (m, 1H), 2.12-2.01 (m, 1H), 0.96 (t, $J = 7.2$ Hz, 6H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure C170) δ 169.4, 167.5, 163.7, 157.4, 144.8, 131.5, 128.6, 126.8, 126.7, 125.3, 112.9, 51.4, 46.7, 40.0, 37.3, 34.6, 32.8, 29.6, 26.2, 11.5; LRMS $m/z$ calcd for C$_{24}$H$_{31}$N$_5$OS$_2$: 469.2; found 470.2 [M+H]$^+$ (Figure C5G); Purity of the compound was further confirmed by RP-HPLC by using method 2: $R_t = 23.49$ min (100% pure, Figure C171).

**Preparation of compound 8g.** Compound 8g was prepared by using procedure D. Compound 40 (0.11 g, 0.43 mmol), K$_2$CO$_3$
(0.14 g, 0.948 mmol), and compound 42 (0.12 g, 0.43 mmol) in anhydrous DMF (4 mL) were used to afford compound 8g (107 mg, 61%, Rf 0.28 in 9:1/CH₂Cl₂:MeOH with NH₄OH (7 mL/L)) as a tan solid after purification by column chromatography (SiO₂, 9:1/CH₂Cl₂:MeOH): ¹H NMR (400 MHz, CDCl₃, Figure C172) δ 7.72 (br t, J = 5.4 Hz, 1H), 5.78 (s, 2H), 3.73 (s, 2H), 3.29 (q, J = 5.9 Hz, 2H), 2.92-2.90 (m, 2H), 2.80-2.78 (m, 2H), 2.53 (t, J = 6.0 Hz, 2H), 2.47 (q, J = 7.2 Hz, 4H), 1.86-1.82 (m, 2H), 1.78-1.71 (m, 4H), 0.89 (t, J = 7.2 Hz, 6H); ¹³C NMR (100 MHz, CDCl₃, Figure C173) δ 169.6, 166.1, 162.8, 157.5, 135.8, 130.4, 114.3, 51.4, 46.7, 37.2, 34.5, 30.6, 29.9, 28.9, 27.1, 26.4, 11.3; LRMS m/z calcd for C₁₉H₂₉N₅O₂: 407.2; found 408.1 [M+H]⁺ (Figure C5H); Purity of the compound was further confirmed by RP-HPLC by using method 2: Rₜ = 24.35 min (96% pure, Figure C174).

**Preparation of compound 1h.** Compound 1h was prepared using procedure D. Compound 33 (0.20 g, 0.91 mmol), K₂CO₃ (0.28 g, 1.98 mmol), and compound 43 (0.27 g, 0.91 mmol) in anhydrous DMF (4.0 mL) were used to afford compound 1h (141 mg, 39%, Rf 0.35 in 9:1/CH₂Cl₂:MeOH with NH₄OH (7 mL/L)) as an off-white solid after purification by flash column chromatography (SiO₂, 9:1/CH₂Cl₂:MeOH with 7 mL/L NH₄OH): ¹H NMR (400 MHz, CDCl₃, Figure C175) δ 7.84 (br s, 1H), 5.63 (br s, 2H), 3.78 (s, 2H), 3.42 (q, J = 5.6 Hz, 2H), 2.95 (m, 4H), 2.59 (m, 4H), 2.51 (p, J = 7.6 Hz, 4H), 1.55 (m, 4H), 1.40 (m, 2H); ¹³C NMR (100 MHz, CDCl₃, Figure C176) δ 172.8, 169.7, 163.6, 157.0, 137.5, 134.6, 110.3, 57.4, 54.4, 36.7, 34.8, 29.7, 29.2, 28.0, 25.8, 24.4; LRMS m/z calcd for C₁₈H₂₅N₅O₂: 391.2; found 392.0 [M+H]⁺ (Figure C5I); Purity of the compound was further confirmed by RP-HPLC by using method 2: Rₜ = 21.78 min (95% pure, Figure C177).
**Preparation of compound 2h.** Compound 2h was prepared using procedure D. Compound 34 (0.20 g, 0.78 mmol), K$_2$CO$_3$ (0.25 g, 1.72 mmol), and compound 43 (0.23 g, 0.78 mmol) in anhydrous DMF (4 mL) were used to afford compound 2h (172 mg, 54%, R$_f$ 0.31 in 9:1/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7 mL/L)) as a brown solid after purification by flash column chromatography (SiO$_2$, 9:1/CH$_2$Cl$_2$:MeOH with 7 mL/L NH$_4$OH): $^1$H NMR (400 MHz, CD$_3$OD, Figure C178) δ 3.79 (s, 2H), 3.33 (t, $J$ = 6.4 Hz, 2H), 2.92-2.87 (m, 2H), 2.79-2.74 (m, 2H), 2.42 (t, $J$ = 6.4 Hz, 2H), 2.34 (m, 4H), 1.90 (m, 4H), 1.45 (p, $J$ = 6.0 Hz, 4H) 1.37-1.36 (m, 2H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure C179) δ 169.6, 167.3, 163.4, 157.3, 132.2, 125.4, 113.1, 57.2, 54.2, 36.4, 34.5, 26.0, 25.6, 25.2, 24.2, 22.6, 22.4; LRMS m/z calcd for C$_{19}$H$_{27}$N$_5$OS$_2$: 405.2; found 406.3 [M+H]$^+$ (Figure C5J); Purity of the compound was further confirmed by RP-HPLC by using method 2: $R_t$ = 18.53 min (97% pure, Figure C180).

**Preparation of compound 3h.** Compound 3h was prepared using procedure E. Compound 35 (0.20 g, 0.80 mmol), Cs$_2$CO$_3$ (0.29 g, 0.90 mmol), TBAI (0.29 g, 0.78 mmol), and compound 43 (0.35 g, 1.22 mmol) in anhydrous DMF (4 mL) were used to afford compound 3h (258 mg, 77%, R$_f$ 0.34 in 9:1/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7 mL/L)) as a white solid after purification by flash column chromatography (SiO$_2$, 9:1/CH$_2$Cl$_2$:MeOH with 7 mL/L NH$_4$OH)): $^1$H NMR (400 MHz, CD$_3$OD, Figure C181) δ 3.76 (s, 2H), 3.31-3.28 (m, 2H), 3.26-3.18 (m, 1H), 2.80-2.62 (m, 2H), 2.37 (t, $J$ = 6.8 Hz, 2H), 2.32-2.22 (m, 4H), 2.0-1.74 (m, 4H), 1.39 (p, $J$ = 5.2 Hz,
Preparation of compound 4h. Compound 4h was prepared using procedure D. Compound 36 (0.29 g, 0.78 mmol), K₂CO₃ (0.25 g, 1.72 mmol), compound 43 (0.24 g, 0.78 mmol) in anhydrous DMF (4 mL) were used to afford compound 4h (222 mg, 68%, R_f 0.31 in 9:1/CH₂Cl₂:MeOH with NH₄OH (7 mL/L) as a yellow solid after purification by flash column chromatography (SiO₂, 9:1/CH₂Cl₂:MeOH with 7 mL/L NH₄OH)): ¹H NMR (400 MHz, CDCl₃, Figure C184) δ 7.74 (br s, 1H), 5.60 (br s, 2H), 3.77 (s, 2H), 3.36 (q, J = 5.6 Hz, 2H), 2.93-2.78 (m, 3H), 2.49-2.34 (m, 7H), 1.99-1.95 (m, 2H), 1.56-1.46 (m, 5H), 1.36 (m, 2H), 1.09 (d, J = 6.4 Hz, 3H); ¹³C NMR (100 MHz, CDCl₃, Figure C185) δ 169.5, 167.4, 163.4, 157.3, 131.8, 125.1, 113.0, 57.2, 54.2, 36.5, 34.5, 33.2, 30.5, 28.9, 25.7, 25.6, 24.2, 21.1; LRMS m/z calcd for C₂₀H₂₉N₅O₅S²: 419.2; found 420.0 [M+H]⁺ (Figure C5L); Purity of the compound was further confirmed by RP-HPLC by using method 2: R_t = 20.03 min (100% pure, Figure C186).

Preparation of compound 5h. Compound 5h was prepared using procedure E. Compound 37 (0.05 g, 0.19 mmol), Cs₂CO₃
(0.06 g, 0.19 mmol), TBAI (0.07 g, 0.19 mmol), and compound 43 (0.08 g, 0.28 mmol) in anhydrous DMF (8 mL) were used to afford compound 5h (56 mg, 68%, $R_f$ 0.27 in 9:1/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7 mL/L)) as a white solid after purification by column chromatography (SiO$_2$, CH$_2$Cl$_2$, 49:1/CH$_2$Cl$_2$:MeOH, 19:1/CH$_2$Cl$_2$:MeOH, 9:1/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7 mL/L), then 7:3/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7 mL/L)) and then recrystallization from Hexanes:EtOAc: $^1$H NMR (400 MHz, CD$_3$OD, Figure C187) $\delta$ 3.78 (s, 2H), 3.33 (t, $J$ = 6.0 Hz, 2H), 3.03-2.95 (m, 1H), 2.87 (dd, $J$ = 16.8, 4.0 Hz, 1H), 2.91-2.80 (m, 1H), 2.40 (t, $J$ = 6.4 Hz, 2H), 2.38-2.34 (m, 1H), 2.32 (m, 4H), 2.12-2.04 (m, 1H), 1.80-1.68 (m, 1H), 1.56-1.42 (m, 3H), 1.44 (p, $J$ = 4.8 Hz, 4H), 1.40-1.32 (m, 2H), 1.02 (t, $J$ = 7.6 Hz, 3H); $^{13}$C NMR (100 MHz, CD$_3$OD, Figure C188) $\delta$ 172.5, 168.3, 164.7, 159.5, 132.1, 127.9, 114.1, 58.7, 55.4, 37.8, 37.3, 35.4, 32.2, 29.71, 29.69, 26.7, 26.6, 25.1, 11.9; LRMS m/z calcd for C$_{21}$H$_{31}$N$_5$OS$_2$: 433.2; found 434.0 [M+H]$^+$ (Figure C6A); Purity of the compound was further confirmed by RP-HPLC by using method 1: $R_t$ = 11.95 min (97% pure, Figure C189).

**Preparation of compound 6h.** Compound 6h was prepared using procedure E. Compound 38 (0.10 g, 0.34 mmol), Cs$_2$CO$_3$ (0.11 g, 0.34 mmol), TBAI (0.13 g, 0.34 mmol), and compound 43 (0.15 g, 0.51 mmol) in anhydrous DMF (8 mL) were used to afford compound 6h (88 mg, 56%, $R_f$ 0.61 in 9:1/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7 mL/L)) as a white solid after purification by column chromatography (SiO$_2$, CH$_2$Cl$_2$, 49:1/CH$_2$Cl$_2$:MeOH, 19:1/CH$_2$Cl$_2$:MeOH, 9:1/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7 mL/L), then 7:3/CH$_2$Cl$_2$:MeOH with NH$_4$OH(7 mL/L)): $^1$H NMR (400 MHz, CD$_3$OD, Figure C190) $\delta$ 3.84 (s, 2H), 3.54 (t, $J$ = 6.0 Hz, 2H), 3.10-2.94 (m, 3H), 3.03 (br t, 4H), 2.86-2.74 (m, 2H), 2.60-2.49 (m, 1H), 2.21-2.13
(m, 1H), 1.73 (p, J = 5.6 Hz, 4H), 1.62-1.52 (m, 3H), 1.43 (qd, J = 12.0, 5.2 Hz, 1H), 0.99 (s, 9H); \(^{13}\)C NMR (100 MHz, CDCl\(_3\), Figure C191) \(\delta\) 171.6, 167.8, 163.8, 158.0, 132.7, 125.8, 112.9, 57.5, 54.6, 44.8, 35.2, 34.8, 32.7, 27.4, 27.2, 27.1, 24.3, 23.1, 22.3; LRMS \(m/z\) calcd for \(\text{C}_{23}\text{H}_{35}\text{N}_{3}\text{OS}_{2}\): 461.2; found 462.1 [M+H]\(^+\) (Figure C6B); Purity of the compound was further confirmed by RP-HPLC by using method 1: \(R_t = 12.61\) min (95% pure, Figure C192).

Preparation of compound 7h. Compound 7h was prepared using procedure D. Compound 39 (0.20 g, 0.64 mmol), K\(_2\)CO\(_3\) (0.20 g, 1.43 mmol), and compound 43 (0.19 g, 0.65 mmol) in anhydrous DMF (4 mL) were used to afford compound 7h (151 mg, 49%, \(R_f\) 0.27 in 9:1/CH\(_2\)Cl\(_2\):MeOH with NH\(_4\)OH (7 mL/L)) as a tan solid after purification by column chromatography (SiO\(_2\), 9:1/CH\(_2\)Cl\(_2\):MeOH): \(^1\)H NMR (400 MHz, CD\(_3\)OD, Figure C194) \(\delta\) 7.31 (m, 4H), 7.25-7.19 (m, 1H), 3.80 (s, 2H), 3.34 (t, \(J = 6.4\) Hz, 2H), 3.21-2.99 (m, 4H), 2.94-2.87 (m, 1H), 2.43 (t, \(J = 6.4\) Hz, 2H), 2.35 (m, 4H), 2.22-2.16 (m, 1H), 2.11-2.00 (m, 1H), 1.46 (app. p, J = 5.6 Hz, 4H), 1.40-1.35 (m, 2H); \(^{13}\)C NMR (100 MHz, CDCl\(_3\), Figure C194) \(\delta\) 169.9, 167.7, 163.8, 157.7, 145.0, 131.7, 128.8, 127.0, 126.9, 125.5, 113.0, 57.4, 54.4, 40.2, 36.6, 34.7, 33.0, 29.8, 26.4, 25.7, 24.4; LRMS \(m/z\) calcd for \(\text{C}_{25}\text{H}_{31}\text{N}_{3}\text{OS}_{2}\): 481.2; found 482.0 [M+H]\(^+\) (Figure C6C); Purity of the compound was further confirmed by RP-HPLC by using method 1: \(R_t = 12.41\) min (96% pure, Figure C195).

Preparation of compound 8h. Compound 8h was prepared using procedure D. Compound 40 (0.08 g, 0.31 mmol), K\(_2\)CO\(_3\)
(0.10 g, 0.68 mmol), and compound 43 (0.09 g, 0.31 mmol) in anhydrous DMF (4 mL) were used to afford compound 8h (88 mg, 68%, R<sub>f</sub> 0.31 in 9:1/CH<sub>2</sub>Cl<sub>2</sub>:MeOH with NH<sub>4</sub>OH (7 mL/L)) as a brown solid after purification by column chromatography (SiO<sub>2</sub>, 9:1/CH<sub>2</sub>Cl<sub>2</sub>:MeOH): <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>, Figure C196) δ 7.67 (br t, J = 5.2 Hz, 1H), 5.71 (s, 2H), 3.75 (s, 2H), 3.33 (q, J = 5.6 Hz, 2H), 2.94-2.91 (m, 2H), 2.82-2.80 (m, 2H), 2.43 (t, J = 6.0 Hz, 2H), 2.33-2.30 (m, 4H), 1.87-1.83 (m, 2H), 1.79-1.72 (m, 4H), 1.43-1.37 (m, 4H), 1.34-1.30 (m, 2H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>, Figure C197) δ 169.7, 166.1, 162.8, 157.5, 135.8, 130.5, 114.3, 57.1, 54.1, 36.3, 34.4, 30.6, 29.9, 28.9, 27.1, 26.4, 25.4, 24.0; LRMS m/z calcd for C<sub>20</sub>H<sub>29</sub>N<sub>5</sub>O<sub>2</sub>: 419.2; found 420.1 [M+H]<sup>+</sup> (Figure C6D); Purity of the compound was further confirmed by RP-HPLC by using method 2: t<sub>R</sub> = 23.50 min (95% pure, Figure C198).

Preparation of compound 1i. Compound 1i was prepared using procedure D. Compound 33 (0.20 g, 0.91 mmol), K<sub>2</sub>CO<sub>3</sub> (0.27 g, 1.98 mmol), and compound 44 (0.26 g, 0.90 mmol) in anhydrous DMF (4.0 mL) were used to afford compound 1i (150 mg, 42%, R<sub>f</sub> 0.51 in 9:1/CH<sub>2</sub>Cl<sub>2</sub>:MeOH with NH<sub>4</sub>OH (7mL/L)) as a white solid after purification by flash column chromatography (SiO<sub>2</sub>, 9:1/CH<sub>2</sub>Cl<sub>2</sub>:MeOH with 7 mL/L NH<sub>4</sub>OH): <sup>1</sup>H NMR (400 MHz, CD<sub>3</sub>OD, Figure C199) δ 3.77 (s, 2H), 3.42 (t, J = 4.8 Hz, 4H), 3.30 (t, J = 6.0 Hz, 2H), 3.00 (tt, J = 7.6, 2.0 Hz, 2H), 2.93 (tt, J = 7.6, 1.6 Hz, 2H), 2.48 (p, J = 7.6 Hz, 2H), 2.39 (t, J = 6.0 Hz, 2H), 2.28 (t, J = 4.4 Hz, 4H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>, Figure C200) δ 172.7, 169.8, 163.7, 156.9, 137.6, 134.7, 110.3, 66.9, 57.1, 53.4, 36.3, 34.6, 29.7, 29.2, 28.0; LRMS m/z calcd for
C₁₇H₂₃N₅O₂S₂: 393.1; found 394.1 [M+H]⁺ (Figure C6E); Purity of the compound was further confirmed by RP-HPLC by using method 2: \( R_t = 22.73 \text{ min} \) (97% pure, Figure C201).

**Preparation of compound 2i.** Compound 2i was prepared using procedure D. Compound 34 (0.20 g, 0.78 mmol), K₂CO₃ (0.25 g, 1.72 mmol), and compound 44 (0.23 g, 0.78 mmol), and DMF (4.0 mL) were used to afford compound 2i (172 mg, 54%, \( R_f \) 0.40 in 9:1/CH₂Cl₂:MeOH with NH₄OH (7 mL/L)) as a white solid after purification by flash column chromatography (SiO₂, 9:1/CH₂Cl₂:MeOH with 7 mL/L NH₄OH): \(^1\)H NMR (400 MHz, (CD₃)₂SO, Figure C202) \( \delta \) 7.91 (t, \( J = 5.5 \text{ Hz}, 1\text{H} \)), 6.95 (br s, 2H), 3.74 (s, 2H), 3.45 (t, \( J = 4.7 \text{ Hz}, 4\text{H} \)), 3.17 (q, \( J = 6.4 \text{ Hz}, 2\text{H} \)), 2.85 (m, 2H), 2.71 (m, 2H), 2.32-2.26 (m, 6H), 1.79 (m, 4H); \(^{13}\)C NMR (100 MHz, (CD₃)₂SO, Figure C203) \( \delta \) 167.9, 166.0, 163.4, 157.6, 129.2, 126.8, 112.3, 66.0, 57.1, 53.1, 36.1, 34.0, 25.3, 24.7, 22.2, 21.9; LRMS \( m/z \) calcd for C₁₈H₂₅N₅O₂S₂: 407.1; found 408.2 [M+H]⁺ (Figure C6F); Purity of the compound was further confirmed by RP-HPLC by using method 1: \( R_t = 10.64 \text{ min} \) (99% pure, Figure C204).

**Preparation of compound 3i.** Compound 3i was prepared using procedure E. Compound 35 (0.20 g, 0.80 mmol), Cs₂CO₃ (0.30 g, 0.91 mmol), TBAI (0.29 g, 0.79 mmol), and compound 44 (0.34 g, 1.19 mmol) in anhydrous DMF (4 mL) were used to afford compound 3i (177 mg, 53%, \( R_f \) 0.30 in 9:1/CH₂Cl₂:MeOH with NH₄OH (7 mL/L)) as a white solid after purification by flash column chromatography (SiO₂, 9:1/CH₂Cl₂:MeOH): \(^1\)H NMR (400 MHz, CD₃OD, Figure
C205) δ 3.81 (d, J = 15.6 Hz, 1H), 3.77 (d, J = 15.6 Hz, 1H), 3.43 (t, J = 4.0 Hz, 4H), 3.33 (t, J = 6.0 Hz, 2H), 3.28-3.20 (m, 1H), 2.84-2.68 (m, 2H), 2.41 (t, J = 6.0 Hz, 2H), 2.31 (t, J = 4.4 Hz, 4H), 2.04-1.76 (m, 4H), 1.29 (d, J = 7.2 Hz, 3H); 13C NMR (100 MHz, CDCl3, Figure C206) δ 170.1, 168.0, 163.6, 156.9, 132.0, 112.8, 66.5, 57.2, 53.4, 36.1, 34.6, 29.9, 29.6, 25.5, 22.0, 17.9; LRMS m/z calcd for C19H27N5O2S2: 421.2; found 422.0 [M+H]+ (Figure C6G); Purity of the compound was further confirmed by RP-HPLC by using method 2: Rf = 24.81 min (100% pure, Figure C207).

Preparation of compound 4i. Compound 4i was prepared using procedure D. Compound 36 (0.28 g, 0.78 mmol), K2CO3 (0.25 g, 1.72 mmol), and compound 44 (0.23 g, 0.78 mmol) in anhydrous DMF (4 mL) were used to afford compound 4i (196 mg, 60%, Rf 0.35 in 9:1/CH2Cl2:MeOH with NH4OH (7 mL/L)) as a yellow solid after purification by flash column chromatography (SiO2, 9:1/CH2Cl2:MeOH with 7 mL/L NH4OH)). 1H NMR (400 MHz, CD3OD, Figure C208) δ 3.79 (s, 2H), 3.44 (t, J = 4.4 Hz, 4H), 3.32 (t, J = 6.0 Hz, 2H), 3.01-2.96 (m, 1H), 2.92-2.86 (m, 1H), 2.83 (dd, J = 16.8, 5.2 Hz, 1H), 2.41 (t, J = 6.0 Hz, 2H), 2.38-2.33 (m, 1H), 2.31 (t, J = 4.4 Hz, 4H), 2.04-1.90 (m, 2H), 1.57-1.46 (m, 1H), 1.12 (d, J = 6.4 Hz, 3H); 13C NMR (100 MHz, CDCl3, Figure C209) δ 169.6, 167.3, 163.4, 157.2, 131.9, 125.2, 112.9, 66.7, 56.9, 53.2, 36.1, 34.3, 33.2, 30.4, 28.9, 25.7, 21.1; LRMS m/z calcd for C19H27N5O2S2: 421.2; found 422.2 [M+H]+ (Figure C6H); Purity of the compound was further confirmed by RP-HPLC by using method 1: Rf = 11.16 min (96% pure, Figure C210).
Preparation of compound 5i. Compound 5i was prepared using procedure E. Compound 37 (0.05 g, 0.68 mmol), Cs₂CO₃ (0.06 g, 0.19 mmol), TBAI (0.07 g, 0.19 mmol), and compound 44 (0.08 g, 0.28 mmol) in anhydrous DMF (8 mL) were used to afford compound 5i (10 mg, 12%, Rₜ 0.45 in 9:1/CH₂Cl₂:MeOH with NH₄OH (7 mL/L)) as a white solid after purification by column chromatography (SiO₂, CH₂Cl₂, 49:1/CH₂Cl₂:MeOH, 19:1/CH₂Cl₂:MeOH, 9:1/CH₂Cl₂:MeOH with NH₄OH (7 mL/L), then 7:3/CH₂Cl₂:MeOH with NH₄OH (7 mL/L)): ¹H NMR (400 MHz, CD₃OD, Figure C211) δ 3.79 (s, 2H), 3.46 (t, J = 4.8 Hz, 4H), 3.31 (t, J = 6.0 Hz, 2H), 3.02-2.94 (m, 1H), 2.86 (dd, J = 16.8, 4.0 Hz, 1H), 2.90-2.79 (m, 1H), 2.44 (t, J = 6.0 Hz, 2H), 2.40-2.36 (m, 1H), 2.34 (t, J = 4.4 Hz, 4H), 2.10-1.92 (m, 1H), 1.78-1.68 (m, 1H), 1.55-1.39 (m, 3H), 1.01 (t, J = 7.2, 3H); ¹³C NMR (100 MHz, CDCl₃, Figure C212) δ 169.9, 167.6, 163.7, 157.4, 132.3, 125.6, 113.2, 66.8, 57.1, 53.5, 36.3, 35.9, 34.6, 31.4, 28.7, 28.6, 26.0, 11.7; LRMS m/z calcd for C₂₀H₂₉N₅O₂S₂: 435.2; found 436.0 [M+H]⁺ (Figure C61) Purity of the compound was further confirmed by RP-HPLC by using method 1: Rₜ = 11.77 min (96% pure, Figure C213).

Preparation of compound 6i. Compound 6i was prepared using procedure E. Compound 38 (0.21 g, 0.68 mmol), Cs₂CO₃ (0.33 g, 1.02 mmol), TBAI (0.25 g, 0.68 mmol), and compound 44 (0.22 g, 0.75 mmol) in anhydrous DMF (8 mL) were used to afford compound 6i (126 mg, 40%, Rₜ 0.49 in 9:1/CH₂Cl₂:MeOH with NH₄OH (7 mL/L)) as a white solid after purification by column chromatography (SiO₂, 49:1/CH₂Cl₂:MeOH, 19:1/CH₂Cl₂:MeOH, then 9:1/CH₂Cl₂:MeOH with NH₄OH (7 mL/L)): ¹H NMR (400 MHz, CD₃OD, Figure C214)
δ 3.76 (s, 2H), 3.43 (t, $J = 4.4$ Hz, 4H), 3.30 (t, $J = 6.8$ Hz, 2H), 3.06-2.99 (m, 1H), 2.83-2.72 (m, 2H), 2.38 (t, $J = 6.4$ Hz, 2H), 2.28 (t, $J = 4.4$ Hz, 4H), 2.17-2.10 (m, 1H), 1.59-1.50 (m, 1H), 1.41 (qd, $J = 12.4$, 5.2 Hz, 1H), 0.96 (s, 9H); $^{13}$C NMR (100 MHz, CD$_3$OD, Figure C215) δ 172.4, 168.3, 164.7, 159.4, 133.0, 127.9, 114.0, 67.7, 58.3, 54.6, 46.1, 37.5, 35.3, 33.4, 27.93, 27.90, 27.8, 25.4; LRMS m/z calcd for C$_{22}$H$_{33}$N$_5$O$_2$S$_2$: 463.2; found 464.1 [M+H]$^+$ (Figure C6J); Purity of the compound was further confirmed by RP-HPLC by using method 2: $R_t = 22.47$ min (97% pure, Figure C216).

Preparation of compound 7i. Compound 7i was prepared using procedure D. Compound 39 (0.20 g, 0.64 mmol), K$_2$CO$_3$ (0.20 g, 1.45 mmol), and compound 44 (0.19 g, 0.65 mmol) in anhydrous DMF (4 mL) were used to afford compound 7i (119 mg, 38%, $R_f$ 0.36 in 9:1/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7 mL/L)) as a tan solid after purification by column chromatography (SiO$_2$, 9:1/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7 mL/L) then SiO$_2$, 19:1/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7 mL/L)); $^1$H NMR (400 MHz, CD$_3$OD, Figure C217) δ 7.27 (d, $J = 4.4$ Hz, 4H), 7.24-7.16 (m, 1H), 3.78 (s, 2H), 3.45 (t, $J = 4.4$ Hz, 4H), 3.31 (t, $J = 6.4$ Hz, 2H), 3.52-2.98 (m, 4H), 2.96-2.86 (m, 1H), 2.42 (t, $J = 5.6$ Hz, 2H), 2.32 (m, 4H), 2.22-2.14 (m, 1H), 2.11-1.98 (m, 1H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure C218) δ 169.5, 167.4, 163.7, 157.4, 144.7, 131.6, 128.7, 126.8, 126.7, 125.4, 112.8, 66.7, 56.9, 53.2, 40.0, 36.1, 34.3, 32.8, 29.6, 26.2; LRMS m/z calcd for C$_{24}$H$_{29}$N$_5$O$_2$S$_2$: 483.2; found 484.0 [M+H]$^+$ (Figure C6K); Purity of the compound was further confirmed by RP-HPLC by using method 2: $R_t = 20.96$ min (95% pure, Figure C219).
Preparation of compound 8i. Compound 8i was prepared using procedure D. Compound 40 (0.20 g, 0.81 mmol), K₂CO₃ (0.25 g, 1.79 mmol), and compound 44 (0.23 g, 0.82 mmol) in anhydrous DMF (4 mL) were used to afford compound 8i (191 mg, 56%, Rᶠ 0.39 in 9:1/CH₂Cl₂:MeOH with NH₄OH (7 mL/L)) as a pink solid after purification by column chromatography (SiO₂, 9:1/CH₂Cl₂:MeOH): ¹H NMR (400 MHz, CD₃OD, Figure C220) δ 3.80 (s, 2H), 3.49 (very br t, 4H), 3.36 (t, J = 6.4 Hz, 2H), 3.04-3.01 (m, 2H), 2.88-2.86 (m, 2H), 2.60-2.38 (m, 6H), 1.93 (p, J = 6.4 Hz, 2H), 1.77 (p, J = 6.4 Hz, 4H); ¹³C NMR (100 MHz, CDCl₃, Figure C221) δ 169.7, 166.1, 162.9, 157.3, 136.0, 130.5, 114.3, 66.6, 56.9, 53.2, 36.0, 34.3, 30.6, 29.9, 28.9, 27.1, 26.4; LRMS m/z calcd for C₁₉H₂₇N₅O₂S₂: 421.2; found 422.1 [M+H]⁺ (Figure C6L); Purity of the compound was further confirmed by RP-HPLC by using method 2: Rₜ = 25.09 min (97% pure, Figure C222).

4.5.1.5. General procedure F for the preparation of compounds 41-44

Amine (1.1 eq) was dissolved in anhydrous CH₂Cl₂ (6 mL). After this solution was cooled at -10 °C for 20 min, bromoacetyl chloride (1 eq) was added. The reaction mixture was then removed from the ice bath and allowed to warm to rt. After reacting for about 19 h, the solvent was removed under reduced pressure to afford the desired products, which was used without any further purification.
Synthesis of compound 41. Compound 41 was synthesized using general procedure F. N,N-Diethylethylenediamine (0.42 mL, 2.96 mmol) and bromoacetyl chloride (0.47 g, 2.96 mmol) in anhydrous CH$_2$Cl$_2$ (6 mL) were used to afford compound 41 (805 mg, 99%) as a light pink solid, which was used without purification: LRMS m/z calcd for C$_6$H$_{14}$BrN$_2$O$: 209.0; found 209.0 [M]$^+$. 

Synthesis of compound 42. Compound 42 was synthesized using general procedure F. N,N-Dimethylethylenediamine (0.30 mL, 2.96 mmol) and bromoacetyl chloride (0.25 mL, 2.96 mmol) in anhydrous CH$_2$Cl$_2$ (6 mL) were used to afford compound 42 (641 mg, 91%) as a light pink solid, which was used without purification: LRMS m/z calcd for C$_8$H$_{18}$BrN$_2$O$: 237.1; found 237.1 [M]$^+$. 

Synthesis of compound 43. Compound 43 was synthesized using general procedure F. 1-(2-aminoethyl)piperidine (0.43 mL, 2.96 mmol) and bromoacetyl chloride (0.25 mL, 2.96 mmol) in anhydrous CH$_2$Cl$_2$ (6 mL) were used to afford compound 43 (845 mg, quant.) as a light pink solid, which was used without purification: LRMS m/z calcd for C$_9$H$_{18}$BrN$_2$O$: 249.1; found 249.1 [M]$^+$. 

Synthesis of compound 44. Compound 44 was synthesized using general procedure F. 4-(2-Aminoethyl)morpholine (0.42 g, 3.20 mmol) and
bromoacetyl chloride (0.25 mL, 3.00 mmol) in anhydrous CH₂Cl₂ (6 mL) were used to afford compound 44 (0.87 g, 99%) as a pale orange solid, which was used without any further purification: LRMS m/z caled for C₈H₁₆BrN₂O₂⁺: 251.0; found 251.0 [M]⁺.

4.5.2. Biochemical and biological methods

4.5.2.1. High-throughput screening

We expressed Eis protein from Mycobacterium tuberculosis (Eis_Mtb) using a previously described method. The following chemicals were required for biochemical/biological assays and acquired from Sigma-Aldrich (St. Louis, MO, USA): 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), Tween® 80, kanamycin A (KAN), acetyl-CoA (AcCoA), and chlorhexidine. Albumin-dextrose-catalase (ADC) was acquired from BD Biosciences (San Jose, CA, USA). The high-throughput screening (HTS) was performed at the Center for Chemical Genomics (CCG, University of Michigan) and screened for putative inhibitors of purified Eis enzymes from three different libraries: (i) a ChemDiv library (20,000 compounds), (ii) the BioFocus NCC library (1,000 compounds), and (iii) the MicroSource MS2000 library (2,000 compounds). The hit validation assay was done to confirm the inhibition of Eis_Mtb. We validated compound 2i by purchasing fresh powder from ChemDiv (San Diego, CA, USA). All small molecules synthesized (1a-i, 2a-i, 3a-i, 4a-i, 5a-i, 6a-i, 7a-i, 8a-i) in this study were dissolved in DMSO (10 mM stock) prior to testing. Please note that the concentrations listed below are the final concentrations in the assays. The pH of buffers was adjusted at rt.
4.5.2.2. Eis enzymatic assay

As previously described,\textsuperscript{108} the Ellman’s reagent (DTNB) was used to monitor acetylation at 412 nm. The reactions (40 µL) contained Tris-HCl (50 mM, pH 8.0), Eis (0.25 µM), NEO (100 µM), AcCoA (40 µM), DTNB (0.5 mM), and compounds 1a-i, 2a-i, 3a-i, 4a-i, 5a-i, 6a-i, 7a-i, 8a-i (20 µM). Chlorhexidine (5 µM) and DMSO (0.5%) served as controls. Plates were incubated at rt and read on a PHERAstar plate reader 5 min after initiation of the reaction. The average Z' score for the HTS assay was 0.65.

4.5.2.2. Antitubercular activity against Mtb cells

The Mtb K204 cell line is a cell line that has been engineered to overexpress the eis gene and maintained at a BSL 3 facility at the CDC. The Mtb Mc\textsuperscript{2}6020 cell line is BSL 2 and was a generous gift from Professor Bill Jacob’s laboratory. Sterile water (200 µL) was added to the outer wells of the plate. Middlebrook 7H9 (100 µL) supplemented with ADC (10%), Tween\textsuperscript{®} 80 (0.05%), and glycerol (0.4%) was added in columns 4-11. The same media (200 µL) was added to rows B-G. Compounds were added and serially diluted. The plate was then set aside while the bacteria culture was prepared. Media (5 mL) was added to a 15 mL screw-capped glass culture tubes. The culture tubes were placed in densitometer and the reference point was noted. The bacterial colonies were transferred to the culture tubes using a loop. The density of the culture tubes was aimed to be between 0.4 and 0.5 OD. The standard bacterial solution was diluted 100 fold using fresh media. The bacterial solution (100 µL) was then transferred to each well on the 96-well plate. The plate was incubated for 1-4 weeks and stained with 5 µL of 2.5 mg/mL resazurin solution to track for growth rate.
Chapter 5

Development of ebsulfur analogues as potent antibacterials against methicillin-resistant

*Staphylococcus aureus*

Note:

These molecules were initially developed as antitubercular compounds. We were excited when we came across literature indicating that they are potential inhibitors of Ag85 enzymes, a group of mycolyltransferases that are essential for the biosynthesis of the *Mtb* cell wall. However, in our hand, these ebsulfur analogues only displayed low antitubercular activity. With these molecules already developed, we decided to screen them for other antimicrobial applications. In this chapter, we reported the activity of ebsulfur/ebselen scaffold against methicillin-resistant *Staphylococcus aureus* (MRSA), which was not well investigated in the literature.

5.1. ABSTRACT

Antibiotic resistance is a worldwide problem that needs to be addressed. MRSA is one of the dangerous “ESKAPE” pathogens that rapidly evolve and evade many current FDA-approved antibiotics. Thus, there is an urgent need for new anti-MRSA compounds. Ebselen (also known as 2-phenyl-1,2-benzisoselenazol-3(2H)-one) has shown promising activity in clinical trials for cerebral ischemia, bipolar disorder, and noise-induced hearing loss. Recently, there
has been a renewed interest in exploring the antibacterial properties of ebselen. In this study, we synthesized an ebselen-inspired library of 33 compounds where the selenium atom has been replaced by sulfur (ebsulfur derivatives) and evaluated them against a panel of drug sensitive and drug resistant \textit{S. aureus} and non-\textit{S. aureus} strains. Within our library, we identified three outstanding analogues with potent activity against all \textit{S. aureus} strains tested (MIC values mostly \leq 2 mg/mL), and numerous additional ones with overall very good to good antibacterial activity (1-7.8 mg/mL). We also characterized the time-kill analysis, anti-biofilm ability, hemolytic activity, mammalian cytotoxicity, membrane-disruption ability, and reactive oxygen species (ROS) production of some of these analogues.

\textbf{5.2. INTRODUCTION}

Only two years after its introduction on the market in 1959, methicillin had experienced resistance by \textit{Staphylococcus aureus}.\textsuperscript{159} Ever since, methicillin-resistant \textit{S. aureus} (MRSA) strains have spread worldwide and have become resistant to many additional FDA approved antibiotics causing great harm to infected patients. In 2011, the Centers for Disease Control (CDC) estimated that the national incidence of invasive MRSA infections was 80,461 cases including 650 cases of death, which was the highest mortality rate among bacterial infections.\textsuperscript{160} \textit{S. aureus} is a Gram-positive bacterium residing mostly on the skin and nasal lining of up to one-third of healthy individuals and can be transferred from one host to another by skin contact. Typically, the bacterium causes no symptoms. However, once the skin layer is broken due to scratches or cuts, \textit{S. aureus} may lead to many problems varying from mild acne to life-threatening conditions such as bacteremia, pneumonia, endocarditis, and osteomyelitis.\textsuperscript{161} Furthermore, MRSA is one of the “ESKAPE” pathogens, which also include
Enterococcus faecium, Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa, and Enterobacter species. The “ESKAPE” pathogens are termed that way because of their ability to escape the therapeutic effects of many known antibiotics and are responsible for the majority of hospital infections. In fact, the Infectious Diseases Society of America (IDSA) has expressed concerns about the empty pipeline for novel antibacterials that can target these pathogens.\textsuperscript{162} Thus, there is a need for new drug candidates to combat MRSA and the rest of the “ESKAPE” pathogens.

In recent years, a number of novel scaffolds with promising activities and mechanism of actions against MRSA have been described. Among them, the compound 5-nitro-2-phenyl-(1H)-indole was discovered as a NorA, efflux pump, inhibitor at IC\textsubscript{50} values lower than 5.0 \textmu M.\textsuperscript{163} We also reported 6"-thioether tobramycin and kanamycin B analogues with long linear alkyl chains disrupting bacterial cell membranes and displaying good activity against \textit{S. aureus} strains.\textsuperscript{164-166} Another compound, AFN-125, was found to selectively inhibit \textit{S. aureus} enoyl-ACP reductase, and has even been tested in clinical trials.\textsuperscript{167} These recent advancements have certainly contributed towards our efforts of eradicating MRSA. However, the concerns about MRSA and “ESKAPE” pathogens have not been completely alleviated and novel scaffolds with potent antibacterial activities are still urgently needed.

Ebselen (also known as 2-phenyl-1,2-benzisoselenazol-3(2H)-one) was developed by Daiichi Sankyo Pharmaceuticals in 1997 for cerebral ischemia in Japan, but failed during phase 3 clinical trial due to insufficient efficacy.\textsuperscript{168-169} Since then, there has been a renewed interest in
this compound; in fact, ebselen is currently being evaluated in clinical trials for treatments of bipolar disorder and noise-induced hearing loss. Clinical applications of ebselen are hypothesized to be related to its ability to covalently bind to cysteine residues on targeted proteins or its antioxidant activity via mimicking glutathione peroxidase. In addition, ebselen was found to inhibit the growth of various Gram-positive and Gram-negative bacterial strains. In 2014, the crystal structure of antigen 85C, a putative drug target in *Mycobacterium tuberculosis* (*Mt*)b, was solved and revealed that ebselen covalently inhibit the antigen 85 complex and hence, explained its activity against *Mt*. From a high-throughput screen, ebselen was discovered to target the cysteine protease domain within the major virulence factors A and B of *Clostridium difficile*. Furthermore, ebselen and ebsulfur (2a) were found to inhibit bacterial thioredoxin reductase, suggesting that they may be useful agents against bacteria lacking the glutathione redox system. Finally, ebselen especially caught our attention because it demonstrated potent bactericidal activity against many clinical isolates of drug-resistant *S. aureus* and was effective in a murine model of MRSA skin infection. These findings prompted us to investigate the antibacterial activity of our own library of ebselen-inspired compounds.

Although no clear evidence has yet been established during clinical studies, there were initial concerns about selenium toxicity of ebselen as some systemic accumulation was observed. In lieu of this potential adverse effect, we decided to study ebsulfur, in which the selenium of ebselen is replaced by a sulfur atom. Additionally, in terms of synthesis, the ebsulfur scaffold or 2-phenyl-1,2-benzisothiazol-3(2H)-one is readily accessible via a convenient 2-step synthesis, which allows for simple scale-up and derivatization. Herein, we synthesized and
evaluated ebsulfur \((2a)\) and 32 of its analogues \((2b-4n)\) against a panel of methicillin-sensitive \textit{S. aureus} (MSSA), MRSA, and other bacterial strains. We also performed the time-kill analysis, established the anti-biofilm ability, hemolytic activity, membrane-disruption ability, and ROS production of some of these analogues.

5.3. RESULTS AND DISCUSSION

5.3.1. Chemistry

To synthesize the desired ebsulfur \((2a)\) and its derivatives \(2b-30\), we first prepared the common intermediate compound \(1\) in 80\% yield by refluxing 2,2'-dithio-dibenzoic acid with thionyl chloride (Figure 5.1). Compound \(1\) was then treated with a variety of aniline analogues and triethylamine in dichloromethane to afford compounds \(2a-o\) in 9-51\% yield, as shown in procedure \(A\). We initially tried to use procedure \(A\) for the preparation of ebsulfur analogues with aliphatic amines, but were unsuccessful as the undesired 2,2'-dithio-dibenzamide products were typically the major products observed. Thus, the undesired 2,2'-dithio-dibenzamide products were converted to the desired products \(3a-o\) in 14\% to quantitative yield by subsequent addition of \(N\)-chlorosuccinimide to the reaction mixture, as shown in procedure \(B\). During our synthesis, we also noticed that extra-long reaction time or poor-quality anhydrous dichloromethane led to the formation of the oxidized sulfoxide byproducts. On silica gel, the oxidized byproducts actually displayed retention times that were similar to the desired product and could be mistakenly isolated. Thus, we carefully monitored our reactions by TLC and verified their masses either by LRMS or HRMS. To test the effect of oxidation of the sulfur on the biological activity of our ebsulfur \((2a)\) derivatives, we
randomly selected three compounds (3e, f, and n) for which we let the reaction go longer to isolate the corresponding oxidized derivatives 4e, f, and n. All new molecules were characterized by $^1$H and $^{13}$C NMR as well as by mass spectrometry and were confirmed to be $\geq 95\%$ purity.
Figure 5.1. Synthetic scheme for the preparation of compounds 2a-o, 3a-o, and 4e, 4f, and 4n following two different experimental procedures (A and B).
5.3.2. Biochemistry and Biology

5.3.2.1. Evaluation of compounds 2a-4n as antibacterial agents

The antibacterial activity of 2a-4n was evaluated against a panel of S. aureus strains comprised of three MSSA strains (A-C) and 25 MRSA strains (D-AB) (Table 5.1). We also tested the activity of all these analogues against non-S. aureus strains such as S. epidermidis, E. faecalis, E. faecium, VRE, L. monocytogenes, and M. smegmatis (AC-AI) (Table 5.2). Furthermore, the controls amikacin (AMK), ebselen, and ebsulfur (2a), along with our best compound, 2h, were tested against a panel of additional bacterial strains, which included A. baumannii, E. cloacae, E. coli, K. pneumoniae, P. aeruginosa, S. enterica, and S. epidermidis (Table 5.3). The strains tested ranged from completely resistant to the control AMK (≥125 µg/mL, AD, AE, and AF) to very susceptible to AMK (≤0.50 µg/mL, B, T, and AI). Minimum inhibitory concentration (MIC) values were determined using the broth double dilution method.
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Table 5.1. MIC values (in µg/mL) determined for all compounds and for the control antibacterial agent (AMK) against various *S. aureus* strains.
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**Bacterial strains:** A = *S. aureus* ATCC 6538, B = *S. aureus* ATCC 25923, C = *S. aureus* ATCC 29213, D = *S. aureus* ATCC 33591, E = MRSA1, F = MRSA2, G = MRSA BR3, H = MRSA C1, I = MRSA C2, J = MRSA C7, K = MRSA C14, L = MRSA C16, M = MRSA C19, N = MRSA G1, O = MRSA G6, P = MRSA G12, Q = MRSA G14, R = MRSA MRSA NRS4, S = MRSA NRS51, T = MRSA NRS77, U = MRSA S14, V = MRSA S17, W = MRSA S22, X = MRSA S24, Y = MRSA USA100, Z = MRSA USA200, AA = MRSA USA300, AB = MRSA USA600.

**Table 5.2.** MIC values (in µg/mL) determined for all compounds and for the control antibacterial agent (AMK) against various non-*S. aureus* bacterial strains.
### Table 5.3

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**Bacterial strains:** AC = *S. epidermidis* ATCC 35984, AD = *E. faecalis* ATCC 29212, AE = *E. faecalis* ATCC 49533, AF = *E. faecium* BM4105-RF, AG = VRE, AH = *L. monocytogenes* ATCC 19115, AI = *M. smegmatis* MC2-155.
5.3.2.1a. Evaluation of compounds 2a-4n against various S. aureus strains

We commenced our study by evaluating ebselen against a panel of 28 S. aureus strains (Table 5.1). Ebselen displayed excellent (≤0.50 µg/mL), very good (1-2 µg/mL), and good (3.9-7.8 µg/mL) activity against 5 (D, E, J, U, and X), 16 (A, C, F, H, I, K, M-Q, S, V, W, Y, and Z), and 7 (B, G, L, R, T, AA, and AB) of these S. aureus strains, respectively. We then investigated the replacement of the selenium atom by sulfur by synthesizing and testing ebsulfur (2a). Compound 2a was found to have similar MIC values to those of ebselen against all S. aureus strains tested. As replacement of the selenium atom by sulfur could result in decreased toxicity, we decided to use compound 2a as our model compound for further derivatization and evaluation.

Previous literature on the antibacterial properties of ebselen suggested that the 1,2-benzisothiazol-3(2H)-one core could be required for antibacterial activity. We also thought of possibly removing the annulated benzene ring of the core to generate 1,2-isothiazolin-3-one analogues. However, we shied away from these analogues once we realized that there were reports suggesting that these compounds could be allergenic and neurotoxic against humans. Thus, we decided to keep the 1,2-benzisothiazol-3(2H)-one core intact and hypothesized that the phenyl group adjacent to the core scaffold would be a good site for our investigation. In search of chemical modifications to increase the biological activity of 2a, we first replaced the phenyl ring by the following moieties: substituted phenyl and other aromatic rings (2b-o), alkyl chains (3a-g), alkyl chains with a terminal phenyl group (3h-j), and aliphatic rings (3k-o). In general, all of our analogues displayed moderate to excellent activity against S. aureus (15.6 to ≤0.25 µg/mL), except for compounds 4e, 4f, and 4n, which
were found to be completely inactive (>125 µg/mL) against all S. aureus strains tested. In addition, compounds 2i, 2n, 2o, and 3o were not evaluated due to solubility issues in liquid Mueller-Hinton medium.

Most of the compounds with mono- (2b, 2c, 2d, 2f, and 2g) and disubstitutions (2i and 2j) at the 3- and 4-positions of the phenyl ring were found to display good to excellent activity (3.9 to ≤0.25 µg/mL), but the activity was very similar to that of 2a. The three overall best compounds of this series of analogues (2e, 2h, and 2k) were found to have very good to excellent activity (≤2.0 µg/mL) across all S. aureus strains tested (Table 5.1). Among these three, the relatively bulkier 4-isopropylphenyl (2e) and 3-isopropylphenyl (2h) analogues were our two best compounds and displayed up to 16-fold improvement in MIC values when compared to the parent compound 2a. We also noticed that the substitution pattern (p- vs m-isopropylphenyl (2e vs 2h) or p- vs m-bromophenyl (2d vs 2g)) did not have a substantial effect on the MIC values of these compounds (mostly within 2 fold dilutions). Intrigued by this result, we synthesized and tested more analogues with bulky substituents on the phenyl ring. Surprisingly, the 2,3-dimethoxyphenyl analogue (2k) also yielded very good to excellent MIC values (2 to ≤0.25 µg/mL). We decided to add even more bulkiness to the scaffold by synthesizing the naphthyl analogue (2l), which displayed very good to excellent MIC values (2 to ≤0.25 µg/mL). This result further suggested that adding different bulky groups to the phenyl ring is a favorable strategy to increase activity.
To further understand the structure-activity-relationship (SAR) of our ebsulfur scaffold, we decided to introduce a heteroatom into the benzene ring. Previously, the 3-chloropyridyl replacement of the phenyl ring in the ebselen scaffold was reported to greatly reduce toxicity in mammalian HEK293T cell line (IC$_{50}$ >160 µM). We applied this knowledge to our ebsulfur scaffold and synthesized the pyridyl (2m), 3-chloropyridyl (2n), as well as the bulky quinolinyl (2o) analogues. However, only 2m was soluble enough for biological testing, but yielded inferior MIC values (15.6 to 2 µg/mL) relative to the parent compound 2a, suggesting that introduction of a nitrogen atom into the phenyl ring may not be the way to pursue.

We next investigated the effect of replacing the phenyl ring by linear alkyl chains, which were previously shown to improve the antibacterial activity of another class of antibiotics, the aminoglycosides. Compounds 3a-d were synthesized to contain n-pentyl, n-hexyl, n-octyl, and n-dodecyl side chains instead of the typical phenyl ring found in 2a. Among these compounds, the n-octyl analogue (3c) displayed the best MIC values (3.9 to ≤0.25 µg/mL). Compounds 3a, 3b, and 3d displayed MIC values (62.5 to 0.25 µg/mL) that were similar to that of the parent compound 2a. Inspired by our best compounds in series 2 containing an isopropyl moiety, compounds 2e and 2h, we decided to explore the effect of branched alkyl chains by synthesizing and testing compounds 3e-g. These compounds displayed mostly good to moderate activity (3.9 to 15.6 µg/mL). However, they were definitely inferior when comparing to compounds 2e and 2h. Additionally, we were also able to isolate the sulfoxide analogues of these compounds (4e and 4f) and evaluated them. We were surprised to find that these oxidized compounds completely lost their antibacterial activity against S. aureus. This
finding was consistent with previous reports that the S-N bond is essential for biological activity by covalently binding to cysteine residues of targeted enzymes.\textsuperscript{175,177}

In an attempt to further understand the SAR of the phenyl ring, we explored whether having this ring directly attached to (compound \textit{2a}) or at a distance from the 1,2-benzisothiazol-3(2H)-one core made any difference. We synthesized compounds \textit{3h-j} with 1-3 carbon linkers separating the phenyl ring and the core. These compounds displayed good to excellent activity (3.9 to \leq 0.25 \mu g/mL). Lastly, to confirm that the aromaticity of the substituent is not required for antibacterial activity, we synthesized a series of compounds containing different-sized aliphatic rings (\textit{3k-o}). We found that compound \textit{3l} with a cyclohexyl ring displayed very similar MIC values (7.8 to 0.5 \mu g/mL) to its aromatic counterpart \textit{2a}. We noted that all of these compounds retained good to excellent antibacterial activity against \textit{S. aureus}, with the exception of the adamantyl derivative \textit{3o}, which could not be tested due to solubility issues.

\textbf{5.3.2.1b. Evaluation of compounds 2a-4n against various non-\textit{S. aureus} strains}

To further examine the antibacterial spectrum of compounds \textit{2a-4n}, we also tested them against a panel of non-\textit{S. aureus} strains. Overall, we found that many of our ebsulfur (\textit{2a}) analogues were more specific towards \textit{S. aureus} strains. We did observe that a few analogues actually displayed moderate to excellent activity (15.6 to \leq 0.25 \mu g/mL) against certain non-\textit{S. aureus} strains (Table 5.2). Interestingly, we found that ebselen still displayed good to excellent activity against many non-\textit{S. aureus} strains (7.8 to \leq 0.25 \mu g/mL), in contrary to what was previously observed when ebselen was tested against a different panel of bacterial
strains. We were especially enlightened to find that our best compounds, 2e and 2h, still retained their excellent activity (1 to ≤0.25 µg/mL) against selected strains such as *S. epidermidis* (AC), VRE (AG), *L. monocytogenes* (AH), and *M. smegmatis* (AI).

To gain a better understanding of the antibacterial spectrum of our ebsulfur (2a) analogues, we decided to test them against a biofilm-forming *S. epidermidis* (AC), another Gram-positive bacterium of the genus *Staphylococcus*. As expected, other than the previously inactive oxidized compounds 4e, 4f, and 4n, we found that our entire library of analogues still retained moderate to excellent activity that was similarly observed during our evaluation of these compounds against the panel of *S. aureus* strains presented in Table 5.1.

Next, we explored the activity of our analogues against examples of the genus *Enterococcus*. Against the *E. faecalis* strains (AD and AE) our ebsulfur analogues mostly showed moderate to poor activity (≥15.6 µg/mL), except in the case of compound 2k, which still displayed good to very good activity (7.8 to 2 µg/mL) (Table 5.2). When comparing the activity of our compounds against *E. faecalis* (strains AD and AE) and *E. faecium* (strain AF), we observed a slightly improved activity (lower MIC values) against *E. faecium*, with compounds 2a, 2d, 2f, 2h, 2k, 3b-f, 3g, 3h, and 3m-n displaying good to very good activity (7.8 to 2 µg/mL). Surprisingly, against the vancomycin-resistant *Enterococcus* (VRE) strain (AG), all of our analogues (except for 4e, 4f, and 4n) displayed good to excellent activity (7.8 to ≤0.25 µg/mL).
To continue our study, we explored the activity of our library against *L. monocytogenes* (AH) and *M. smegmatis* (AI). We found that most of our analogues (2a-h, 2k-m, 3a-f (except 3e,f against strain AH), and 3h-m) displayed good to excellent activity (7.8 to 0.5 µg/mL) against these strains.

Intrigued by our results against the non-*S. aureus* panel, we further investigated the activity of our best compound, 2h, along with ebselen and ebsulfur (2a) against some additional non-*S. aureus* strains (*A. baumannii, E. cloacaee, E. coli, K. pneumoniae, P. aeruginosa, S. enterica*, and an additional non-biofilm-forming *S. epidermidis*). However, we found that these compounds were mostly inactive against these strains (Table 5.3).

### 5.3.2.2. Evaluation of time-kill curve of compounds 2a, 3b, and 3c

To gain more insights into the antibacterial activity kinetics of our ebsulfur analogues, we performed time-kill assays\(^{185}\) of compounds 3b and 3c on MRSA S22 (strain W) over a period of 24 h and compared that to ebselen, ebsulfur (2a), and AMK (Figure 5.2). At sub-MIC concentration (0.5× MIC), as expected, we observed that strain W grew well in the presence of all these compounds. Unlike the control (untreated bacteria), we found that all of the compounds were bacteriostatic up until 3 to 6 h. At MIC concentration, we observed similar bacteriostatic profiles as described previously in our experiment at sub-MIC. We decided to conduct another study at 4× MIC, where we were surprised to find that ebselen was bactericidal and led to complete cell death at 24 h. Compounds 2a, 3b, and 3c reduced bacterial load by 1.5 orders of magnitude, but they still remained bacteriostatic. These results
are interesting and may suggest that the selenium atom has a role in promoting the bactericidal effect. However, it should be noted that the time-kill curve assays were conducted under different conditions than those used in the MIC broth double-dilution assays (inoculum size, air flow, and volumes of medium). These differences could lead to a change in the effective or therapeutic doses and thus, potentially explain why the 4× MIC dosages were required for strong efficacy. It is well established that increasing the inoculum size will require a different MIC to achieve therapeutic efficacy. To investigate whether the inoculum size affected our MIC values, we re-performed the MIC broth double-dilution assay on eight compounds (AMK, ebselen, 2a, 2g, 2h, and 3a-c) with the inoculum size used in time-kill assays (1×10⁶ CFU/mL) and found that the MIC values remained identical. Therefore, the selenium atom might indeed play a role in the bactericidal effect.
Figure 5.2. Representative time-kill studies of ebselen, AMK, 2a, 3c, and 3b against MRSA S22 (strain W). Bacterial cells were either treated with ebselen (white circle), AMK (white triangle), 2a (black inverted triangle), 3c (black square), and 3b (white square) at 0.5× MIC (top panel), 1× MIC (middle panel) and 4× MIC (bottom panel), respectively or no drug (black circle).
5.3.2.3. Activity against biofilms of *S. aureus* and *S. epidermidis*

Having established that our compounds displayed activity against biofilm-forming *S. aureus* (strain A) and *S. epidermidis* (strain AC), we wanted to explore whether these compounds could also reduce the biofilm mass of these strains. Bacterial biofilm is a matrix of bacterial cells that are more tolerant to most antibacterial compounds and are highly associated with chronic persistent infections.\textsuperscript{186} Recently, ebselen was reported to display potent biofilm reduction properties against established biofilms of *S. aureus* and *S. epidermidis*. When compared to the biofilm reduction activity of conventional antibiotics (linezolid, mupirocin, vancomycin, and rifampicin), the activity of ebselen was found to be significantly superior.\textsuperscript{178} We were interested in finding out whether our ebsulfur analogues also possess the same antibiofilm property, which would make them highly desirable in combating pathogenic bacteria associated with chronic infectious diseases. To evaluate the biofilm reduction activity,\textsuperscript{178} ebselen, ebsulfur (2a), as well as some of our best compounds (2e, 2h, 2k, and 3c) were tested against the established biofilm of two biofilm-forming strains of *S. aureus* ATCC 6538 (strain A) and *S. epidermidis* ATCC 35984 (strain AC) at 125x, 62.5x, 31.3x, 15.6x, 7.8x, 3.9x, and 1x MIC (Figure 5.3). Due to the observed ceiling effect (where the biofilm reduction plateaus), for each compound, we are reporting the lowest MIC folds at which the plateau begins.

Against the established biofilm of strain A, we found that ebselen (at 15.6 µg/mL or 7.8x MIC) reduced approximately 20% of the biofilm when comparing to the biofilm of the control (untreated biofilm) (Figure 5.3A). This is different from previous finding about the biofilm
reduction of this MRSA strain by ebselen, which was reported to be approximately 60% at 2 µg/mL.\textsuperscript{178} Ebsulfur (2a) (at 31.3 µg/mL or 3.9x MIC) displayed superior biofilm reduction, approximately 40%. Compound 2e also resulted in ~50% biofilm reduction at a much higher concentration of 125 µg/mL or 62.5x MIC. Meanwhile, compound 2h (at 62.5 µg/mL or 15.6x MIC) only reduced the biofilm by ~10%. Compound 3c was also found to result in low to no biofilm reduction. However, we found that compound 2k displayed 40% biofilm reduction at 3.9 µg/mL or 3.9x MIC, which is the lowest concentration tested in our assay.

Against the biofilm of strain AC, ebselen (at 31.3 µg/mL or 125x MIC) was found to result in ~20% reduction of the biofilm (Figure 5.3B). Ebsulfur (2a) was found to be slightly more effective, reducing the biofilm by ~50%, but at a much higher concentration (125 µg/mL or 125x MIC). Compounds 2e and 2h were found to have similar biofilm reduction activity (~60-50%) at 3.9 and 15.6 µg/mL (15.6x and 62.5x MIC), respectively. Compound 2k displayed about 50% biofilm reduction at 15.6 µg/mL or 62.5x MIC. Finally, we found that compound 3c displayed about 40% biofilm reduction at 31.3 µg/mL or 31.3x MIC.
Figure 5.3. Bar graphs showing the ability of selected compounds to reduce the amount of biofilm observed for A. *S. aureus* ATCC 6538 (strain A) and B. *S. epidermidis* ATCC 35984 (strain AC). The fold MIC for the optimal reduction for each compound is shown into parenthesis beside the compound name/number.

Overall, when compared to ebselen, we found that ebsulfur (2a) and compound 2e displayed more potent biofilm reduction, but at higher concentrations. Compounds 2h and 3c were found to be active against the biofilm of strain AC, but not against the biofilm of strain A. Finally, compound 2k displayed good biofilm disruption (40-50%) against both biofilm-forming strains at relatively low concentrations (3.9 and 15.6 µg/mL).
5.3.2.4. Evaluation of the hemolytic potential of compounds 2a, 2h, and 3c

As red blood cells are some of the more fragile mammalian cells, we were interested in assessing our analogues for their general mammalian cell cytotoxicity and establishing whether these compounds would be suitable for any applications in humans. Hemolytic assays\textsuperscript{187-188} were performed by testing ebselen, compounds 2a, 2h, and 3c, as well as AMK (as a negative control) at various concentrations against murine red blood cells (mRBCs) (Figure 5.4). We observed that our compounds tested did not show any significant hemolytic activity (≤20% hemolysis) up to 7.8 µg/mL. This result is relatively encouraging knowing that against \textit{S. aureus} strains, compound 2h was found mostly to display MIC values ranging from ≤0.25 to 1 µg/mL, while compound 3c mostly displayed MIC values ranging from ≤0.25 to 2 µg/mL. Another interesting observation was that 2a and its analogues 2h and 3c were less hemolytic when compared to ebselen (100% hemolytic at 15.6 µg/mL). Amongst the ebsulfur analogues tested, compound 2h was found to be the least hemolytic.
Figure 5.4. Hemolytic activity of ebselen (black circle), 2a (white circle), AMK (black inverted triangle), 3c (white triangle), and 2h (black square) on mouse red blood cells. The positive control (untreated) was found to display no hemolysis while the negative control (1% Triton X) was found to display 100% hemolysis (not shown on the graph).

5.3.2.5. Evaluation of mammalian toxicity potential of ebselen and compounds 2e, 2h, 2k, 2l, 3c, 3i, and 3k

Although the hemolysis data was encouraging, we realized that a more extensive in vitro cytotoxicity study would still be needed to further understand the mammalian toxicity potential of our compounds. Given that many current antibiotics are nephrotoxic, we selected the human embryonic kidney cell line HEK-293 as the model mammalian cell line for our cytotoxicity evaluation (Figure 5.5). Ebselen was used as the control because it was shown to be safe for human use. Compounds 2e, 2h, and 2l all did not display any significant cytotoxicity (>20% cell death) up until 10 µg/mL, which was very similar to what was found with ebselen. On the other hand, compounds 2k, 3c, 3i, and 3k were slightly more toxic against HEK-293 with >20% cell death observed at 1.25 µg/mL. Collectively, it was intriguing and encouraging that our best analogues 2e and 2h displayed stronger selectivity for
bacterial cells. Depending on the MRSA strains, there could be up to 40-fold difference when compared to their MIC values.

![Graph showing mammalian cytotoxicity](image)

**Figure 5.5.** Mammalian cytotoxicity of ebselen (orange bars), 2e (green bars), 2h (teal bars), 2k (blue bars), 2l (yellow bars), 3c (purple bars), 3i (light green bars), and 3k (pink bars). Untreated cells were used as positive control and cells treated with 1% Triton X were used as negative control (not shown on graph).

5.3.2.6. **Evaluation of ebselen and compounds 2a and 3b,c as bacterial membrane disruptors**

Based on previous literature on membrane-disrupting cationic amphiphilic aminoglycosides,\(^{164-166}\) we suspected that our linear alkyl ebsulfur analogues (3b,c) could potentially exert their antibacterial activity by their ability to disrupt bacterial cell membranes. Thus, we tested compounds 3b,c along with 2a and ebselen against *S. epidermidis* ATCC 35984 (strain AC) at their 1x and 4x MIC values and evaluated them for membrane disruption. We also used a tobramycin derivative with a linear alkyl chain of 14 carbons in
length attached in a thioether linkage to the 6"-position (C_{14}-TOB) and AMK as positive and negative controls, respectively. We used propidium iodide (PI) staining to visualize any damage to the bacterial cell membrane. If the bacterial cell membrane was compromised, the PI would penetrate and stain the cells red. Based on our results, we saw no sign of membrane disruption from any of our compounds (Figure 5.6). Thus, membrane disruption is likely not a viable antibacterial mechanism of action for these analogues. We then decided to switch our attention to study another possible mechanism of action for these compounds, the production of ROS.
Figure 5.6. Effect of ebsulfur (2a) and its analogues 3b and 3c on cell membrane integrity of S. epidermidis ATCC 35984 (strain AC). Bacterial cells were treated with no drug or AMK
(negative controls), C_{14}-TOB (positive control), or ebselen, \textbf{2a, 3b, and 3c}, at their 1× and 4× respective MIC values. Propidium iodine (PI) dye was used to monitor the uptake by bacterial cells.

\textbf{5.3.2.7. Detection of reactive oxygen species (ROS) production}

In bacteria lacking glutathione and glutaredoxin such as \textit{S. aureus} and others, the thioredoxin system is crucial for ROS regulation and thus, bacterial survival and proliferation.\textsuperscript{189-191} Ebselen was identified previously to inhibit bacterial thioredoxin reductase.\textsuperscript{177} To test the hypothesis that our analogues also inhibit thioredoxin reductase, we treated \textit{S. epidermidis} ATCC 35984 (strain \textbf{AC}) with ebselen, compounds \textbf{2a} and \textbf{3b,c} at various concentrations (1x and 4x their respective MIC values). 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA)\textsuperscript{192} was then used to detect and visualize ROS production (Figure 5.7). We found that ebselen and all of our analogues led to production of ROS and that inhibition of thioredoxin reductase is possibly the target responsible for antibacterial activity, which will be the subject of future studies.
**Figure 5.7.** Effect of ebsulfur (2a) and its analogues 3b and 3c on intracellular ROS production by *S. epidermidis* ATCC 35984 (strain AC). Bacterial cells were treated with no drug (negative control), 1 mM of H₂O₂ (positive control), or ebselen, 2a, 3b, and 3c, at their

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1× and 4× respective MIC values for 1 h at 37 °C. After staining with DCFH-DA (40 µg/mL), the samples were analyzed using a Zeiss Axovert 200M fluorescence microscope.

5.4. CONCLUSION
We synthesized an ebselen-inspired (ebsulfur) library comprised of 33 molecules based on previous reports of the antibacterial activity of ebselen against drug-resistant S. aureus clinical isolates. Our SAR analysis suggested that replacing the selenium atom with the sulfur atom in the 1,2-benzisoselenazol-3(2H)-one core did not significantly alter antibacterial activity. However, oxidizing the sulfur atom of the 1,2-benzisothiazol-3(2H)-one core, as in compounds 4e, 4f, and 4n, completely obliterated antibacterial activity. This finding demonstrated that the stereoelectronic nature of the S-N bond is indeed important for biological activity, which is consistent with previous reports.175, 177 During our search for analogues with improved antibacterial activity, we identified three compounds (2e, 2h, and 2k) with remarkably potent activity (MIC values mostly ≤2 µg/mL) against S. aureus clinical isolates. We then evaluated our compounds for their antibacterial spectrum by testing them against non-S. aureus strains. Our analogues were generally more selective towards Staphylococcus strains, but some of them also displayed good activities against VRE, L. monocytogenes, and M. smegmatis. Compounds with biofilm reduction are currently in high demand. Our evaluation showed that albeit at high concentrations, our analogues were able to reduce Staphylococcal established biofilms. We showed that the antibacterial activity of our compounds was highly correlated with ROS production. Lastly, we assessed our compounds for general mammalian toxicity by testing them against murine RBCs and HEK293. The mammalian toxicities of our most potent compounds were found to be acceptable. With
further optimizations, these ebsulfur analogues could potentially have clinical utilities in our
day against bacterial resistance.

5.5. MATERIALS AND INSTRUMENTATION

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and AK Scientific (Union
City, CA) and used without further purification. Deuterated solvents were purchased from
Cambridge Isotope Laboratories Inc. (Tewksbury, MA). Chemical reactions were monitored
by TLC (Merck, Silica gel 60 F254). Flash column chromatography was performed on silica
gel (Dynamic Adsorbents Inc., Flash silica gel 32-63u). Visualization was achieved using UV
light and a cerium-molybdate stain ((NH₄)₂Ce(NO₃)₆ (5g), (NH₄)₆Mo₇O₂₄•4H₂O (120 g),
H₂SO₄ (80 mL), and H₂O (720 mL)). NMR spectra were measured in δ (ppm) using the ¹H
NMR CDCl₃ (δ 7.24 ppm) and ¹³C NMR CDCl₃ (δ 77.23 ppm) as internal standards: where J
= coupling constants in Hz. Abbreviations used: s = singlet, d = doublet, t = triplet, q =
quartet, m = multiplet, dd = doublet of doublets, dt = doublet of triplets, td = triplet of
doublets, tt = triplet of triplets, qt = quartet of triplets, pd = pentet of doublets. NMR spectra
were recorded on a Varian Unity Inova 400 MHz instrument (Palo Alto, CA). High-resolution
electrospray mass spectra (HRMS) were recorded on a Q-TOF Tandem Mass Spectrometer
and low-resolution electrospray mass spectra (LRMS) were recorded on a liquid
chromatography-mass spectrometry using an Agilent 1200 series Quaternary LC system
equipped with a diode array detector, and Eclipse XDB-C₁₈ column (250 mm × 4.6 mm, 5
µm), and an Agilent 6120 Quadrupole MSD mass spectrometer. All compounds were found to
be ≥95% pure. Bacterial experiments utilized untreated 96-well plates (Corning), biofilm
measurements were performed using a SpectraMax M5 spectrometer (Molecular Devices, Sunnyvale, CA).

5.6. METHODS

5.6.1. Chemical methods

5.6.1.1. Preparation of compound 1

The known compound 1 was prepared using a published protocol.\textsuperscript{182} A mixture of 2,2'-dithio-dibenoic acid (3.0 g, 9.8 mmol) and SOCl\textsubscript{2} (25 mL) was heated under reflux for 48 h, and the solvent was removed by distillation followed by evaporation \textit{in vacuo}. The residue was triturated with hexanes, quickly filtered, and washed with hexanes and dried under vacuum to afford compound 1 (2.69 g, 80%) as a yellow solid: \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}, Figure C1, which matches the lit.\textsuperscript{182}) \(\delta 8.37 (d, J = 7.6 \text{ Hz}, 2H), 7.75 (d, J = 8.4 \text{ Hz}, 2H), 7.53 (t, J = 7.6 \text{ Hz}, 2H), 7.36 (t, J = 7.6 \text{ Hz}, 2H)\).

5.6.1.2. Preparation of compounds 2a-o following procedure A

Compounds 2a-o were synthesized using a one-step strategy that employed Et\textsubscript{3}N and various aniline derivatives in CH\textsubscript{2}Cl\textsubscript{2}.

\textbf{Preparation of compound 2a.} The known compound 2a was prepared using
a published protocol.\textsuperscript{182} To a stirred and ice-cooled mixture of aniline (0.13 mL, 1.0 mmol) and Et\textsubscript{3}N (0.45 mL, 3.2 mmol) in anhydrous CH\textsubscript{2}Cl\textsubscript{2} (40 mL), 2,2'-dithiobenzoyl chloride (I, 0.50 g, 1.5 mmol) in anhydrous CH\textsubscript{2}Cl\textsubscript{2} (20 mL) was added dropwise over 20 min. The reaction mixture was warmed-up to rt, stirred for 12 h, and washed with aq. sat. NaHCO\textsubscript{3} (30 mL) and H\textsubscript{2}O (30 mL). The organic layer was dried over MgSO\textsubscript{4}, concentrated \textit{in vacuo}, and the residue was purified by flash column chromatography (SiO\textsubscript{2}, 100% hexanes, 1:9/EtOAc:hexanes, 3:17/EtOAc:hexanes, 1:4/EtOAc:hexanes, 1:3/EtOAc:hexanes, 3:7/EtOAc:hexanes, then 100% EtOAc) and recrystallized in EtOH to afford compound 2a (30 mg, 9%, \textit{R}\textsubscript{f} 0.21 in 49:1/CH\textsubscript{2}Cl\textsubscript{2}:MeOH) as off-white needle crystals: \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}, Figure C2, which matches the lit.\textsuperscript{193}) \textdelta 8.09 (d, \textit{J} = 8.0 Hz, 1H), 7.69 (d, \textit{J} = 8.0 Hz, 2H), 7.65 (t, \textit{J} = 8.0 Hz, 1H), 7.57 (d, \textit{J} = 8.4 Hz, 1H), 7.48-7.42 (m, 3H), 7.31 (t, \textit{J} = 7.6 Hz, 1H).

\textbf{Preparation of compound 2b.} Compound 2b was synthesized following a procedure published for the preparation of similar compounds.\textsuperscript{182} To a mixture of 4-fluoroaniline (0.08 mL, 0.87 mmol) and Et\textsubscript{3}N (0.12 mL, 0.87 mmol) in anhydrous CH\textsubscript{2}Cl\textsubscript{2}(10 mL), 2,2'-dithiobenzoyl chloride (I, 0.30 g, 0.87 mmol) in anhydrous CH\textsubscript{2}Cl\textsubscript{2} (20 mL) was added dropwise over 15 min. The reaction mixture was stirred for 3 h at rt, and washed with 1 M HCl (50 mL), H\textsubscript{2}O (50 mL), and brine (50 mL). The organic layer was dried over anhydrous MgSO\textsubscript{4}, concentrated \textit{in vacuo}, and the residue was purified by flash column chromatography (SiO\textsubscript{2}, 1:4/EtOAc:hexanes) to afford compound 2b (102 mg, 48%, \textit{R}\textsubscript{f} 0.33 in 1:4/EtOAc:hexanes) as a white solid: \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}, Figure C3,
which matches the lit.\(^{194}\) δ 8.09 (d, \(J = 8.0\) Hz, 1H), 7.68-7.65 (m, 3H), 7.57 (d, \(J = 8.4\) Hz, 1H), 7.48-7.40 (m, 3H).

**Preparation of compound 2c.** Compound 2c was synthesized following a procedure published for the preparation of similar compounds.\(^{182}\) To a mixture of 4-chloroaniline (0.11 g, 0.87 mmol) and Et\(_3\)N (0.12 mL, 0.87 mmol) in anhydrous CH\(_2\)Cl\(_2\) (10 mL), 2,2'-dithiobenzoyl chloride (1, 0.30 g, 0.87 mmol) in anhydrous CH\(_2\)Cl\(_2\) (20 mL) was added dropwise over 15 min. The reaction mixture was stirred for 3 h at rt, and washed with 1 M HCl (50 mL), H\(_2\)O (50 mL), and brine (50 mL). The organic layer was dried over anhydrous MgSO\(_4\), concentrated *in vacuo*, and the residue was purified by flash column chromatography (SiO\(_2\), 1:4/EtOAc:hexanes) to afford compound 2c (89 mg, 39%, \(R_f\) 0.26 in 1:4/EtOAc:hexanes) as a white solid: \(^1\)H NMR (400 MHz, CDCl\(_3\), Figure C4, which matches the lit.\(^{195}\) δ 8.09 (d, \(J = 7.6\) Hz, 1H), 7.66 (t, \(J = 7.6\) Hz, 1H), 7.64 (dd, \(J = 8.8, 4.8\) Hz, 2H), 7.57 (d, \(J = 8.0\) Hz, 1H), 7.44 (t, \(J = 7.6\) Hz, 1H), 7.15 (t, \(J = 8.8\) Hz, 2H).

**Preparation of compound 2d.** The known compound 2d was synthesized following a procedure published for the preparation of similar compounds.\(^{182}\) To a mixture of 4-bromoaniline (0.34 g, 2.00 mmol) and Et\(_3\)N (0.28 mL, 1.00 mmol) in anhydrous CH\(_2\)Cl\(_2\) (10 mL), 2,2'-dithiobenzoyl chloride (1, 0.68 g, 2.00 mmol) in anhydrous CH\(_2\)Cl\(_2\) (20 mL) was added dropwise over 15 min. The reaction mixture was stirred for 3 h at rt, and washed with 1 M HCl (50 mL), H\(_2\)O (50 mL), and brine (50 mL). The organic layer was dried over anhydrous MgSO\(_4\), concentrated *in vacuo*, and the residue was
purified by flash column chromatography (SiO$_2$, 100% hexanes, 0.5:9.5/EtOAc:hexanes, 1:9/EtOAc:hexanes, 3:17/EtOAc:hexanes, 1:4/EtOAc:hexanes, 1:3/EtOAc:hexanes, then 100% EtOAc) and recrystallized in EtOH to afford compound 2d (199 mg, 33%, $R_f$ 0.25 in 1:3/EtOAc:hexanes) as a white solid: $^1$H NMR (400 MHz, CDCl$_3$, Figure C5, which matches the lit.$^{196}$) $\delta$ 8.08 (d, $J$ = 8.0 Hz, 1H), 7.68-7.64 (m, 1H), 7.62-7.56 (m, 5H), 7.46-7.42 (m, 1H).

**Preparation of compound 2e.** The known compound 2e was synthesized following a procedure published for the preparation of similar compounds.$^{182}$ To a mixture of 4-isopropylaniline (0.12 mL, 0.87 mmol) and Et$_3$N (0.12 mL, 0.87 mmol) in anhydrous CH$_2$Cl$_2$ (10 mL), 2,2'-dithiobenzoyl chloride (1, 0.30 g, 0.87 mmol) in anhydrous CH$_2$Cl$_2$ (20 mL) was added dropwise over 15 min at rt. The reaction mixture was stirred for an additional 3 h at rt, and then washed with 1 M HCl (50 mL), H$_2$O (50 mL), and brine (50 mL). The organic layer was dried over anhydrous MgSO$_4$, concentrated *in vacuo*, and the residue was purified by flash column chromatography (SiO$_2$, 1:4/EtOAc:hexanes) and recrystallized from MeOH to afford compound 2e (30 mg, 13%, $R_f$ 0.25 in 1:4/EtOAc:hexanes) as a white solid: $^1$H NMR (400 MHz, CDCl$_3$, Figure C6, which matches the lit.$^{194}$) $\delta$ 8.09 (d, $J$ = 7.6 Hz, 1H), 7.64 (td, $J$ = 7.6, 0.8 Hz, 1H), 7.58 (d, $J$ = 8.4 Hz, 2H), 7.56 (d, $J$ = 7.6 Hz, 1H), 7.43 (t, $J$ = 7.6, 0.8 Hz, 1H), 7.31 (d, $J$ = 8.4 Hz, 2H), 2.94 (septet, $J$ = 6.8 Hz, 1H), 1.26 (d, $J$ = 6.8 Hz, 6H).

**Preparation of compound 2f.** Compound 2f was synthesized following a procedure published for the preparation of similar compounds.$^{182}$ To a
mixture of 4-ethynylaniline (0.14 g, 1.00 mmol) and Et$_3$N (0.14 mL, 1.00 mmol) in anhydrous CH$_2$Cl$_2$ (10 mL), 2,2'-dithiobenzoyl chloride (1, 0.34 g, 1.00 mmol) in anhydrous CH$_2$Cl$_2$ (20 mL) was added dropwise over 15 min. The reaction mixture was stirred for 3 h at rt, and washed with 1 M HCl (50 mL), H$_2$O (50 mL), and brine (50 mL). The organic layer was dried over anhydrous MgSO$_4$, concentrated in vacuo, and the residue was purified by flash column chromatography (SiO$_2$, 1:5/acetone:hexanes) to afford compound 2f (56 mg, 22%, R$_f$ 0.18 in 1:5/acetone:hexanes) as a white solid: $^1$H NMR (400 MHz, CDCl$_3$, Figure C7) $\delta$ 8.08 (d, $J = 8.0$ Hz, 1H), 7.71 (d, $J = 8.8$ Hz, 2H), 7.65 (m, 1H), 7.56 (d, $J = 8.8$ Hz, 3H), 7.43 (m, 1H), 3.10 (s, 1H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure C8) $\delta$ 164.3, 139.8, 138.0, 133.3, 132.8, 127.5, 126.2, 125.0, 123.9, 120.6, 120.3, 83.0, 78.2; HRMS m/z calcd for C$_{15}$H$_9$NOS: 251.0405; found 252.0476 [M+H]$^+$.

Preparation of compound 2g. The known compound 2g$^{194}$ was synthesized following a procedure published for the preparation of similar compounds.$^{182}$ To a mixture of 3-bromoaniline (0.11 mL, 1.00 mmol) and Et$_3$N (0.14 mL, 1.00 mmol) in anhydrous CH$_2$Cl$_2$ (10 mL), 2,2'-dithiobenzoyl chloride (1, 0.34 g, 1.00 mmol) in anhydrous CH$_2$Cl$_2$ (20 mL) was added dropwise over 15 min at -15 °C. The reaction mixture was stirred for an additional 3 h at rt, and washed with 1 M HCl (50 mL), H$_2$O (50 mL), and brine (50 mL). The organic layer was dried over anhydrous MgSO$_4$, concentrated in vacuo, and the residue was purified by flash column chromatography (SiO$_2$, 1:3/EtOAc:hexanes) to afford compound 2g (107 mg, 35%, R$_f$ 0.39 in 1:3/EtOAc:hexanes) as a white solid: $^1$H NMR (400 MHz, CDCl$_3$, Figure C9) $\delta$ 8.08 (d, $J = 8.0$ Hz, 1H), 7.92 (s, 1H), 7.65 (d, $J = 7.6$ Hz, 2H), 7.57 (d, $J = 8.4$ Hz, 1H), 7.44 (t, $J = 8.0$ Hz, 2H), 7.31 (t, $J = 8.0$ Hz, 1H); $^{13}$C NMR (100
MHz, CDCl₃, Figure C10) δ 164.3, 139.8, 138.7, 132.8, 132.9, 130.8, 130.1, 127.5, 127.4, 126.2, 124.8, 123.0, 120.3; HRMS m/z calcd for C₁₃H₆BrNOS: 304.9510; found 305.9580 [M+H]+.

**Preparation of compound 2h.** Compound 2h was synthesized following a procedure published for the preparation of similar compounds.²⁻¹⁸² To a mixture of 3-isopropylaniline (0.04 mL, 0.29 mmol) and Et₃N (0.04 mL, 0.29 mmol) in anhydrous CH₂Cl₂ (5 mL), 2,2'-dithiobenzoyl chloride (1, 0.10 g, 0.29 mmol) in anhydrous CH₂Cl₂ (10 mL) was added dropwise over 15 min. The reaction mixture was stirred for 3 h at rt, and washed with 1 M HCl (30 mL), H₂O (30 mL), and brine (30 mL). The organic layer was dried over anhydrous MgSO₄, concentrated in vacuo, and the residue was purified by flash column chromatography (SiO₂, 1:4/EtOAc:hexanes) and recrystallized in EtOH to afford compound 2h (35 mg, 45%, Rf 0.39 in 1:4/EtOAc:hexanes) as a white solid: ¹H NMR (400 MHz, CDCl₃, Figure C11) δ 8.09 (d, J = 8.0 Hz, 1H), 7.64 (t, J = 8.0 Hz, 1H), 7.57-7.55 (m, 2H), 7.49-7.46 (m, 1H), 7.42 (t, J = 7.2 Hz, 1H), 7.37 (t, J = 8.0 Hz, 1H), 7.18 (d, J = 7.2 Hz, 1H), 2.95 (sep, J = 7.2 Hz, 1H), 1.27 (d, J = 7.2 Hz, 6H); ¹³C NMR (100 MHz, CDCl₃, Figure C12) δ 164.3, 150.6, 140.2, 137.3, 132.5, 129.4, 127.3, 126.0, 125.5, 125.1, 123.1, 122.4, 120.3, 34.3, 24.1; HRMS m/z calcd for C₁₆H₁₅NOS: 269.0874; found 270.0937 [M+H]+.

**Preparation of compound 2i.** Compound 2i was synthesized following a procedure published for the preparation of similar compounds.²⁻¹⁸² To a mixture of 3,5-dibromoaniline (0.22 g, 0.87 mmol) and Et₃N (0.12 mL, 0.87 mmol) in
anhydrous CH$_2$Cl$_2$ (10 mL), 2,2'-dithiobenzoyl chloride (1, 0.30 g, 0.87 mmol) in anhydrous CH$_2$Cl$_2$ (20 mL) was added dropwise over 15 min. The reaction mixture was stirred for 3 h at rt, and washed with 1 M HCl (50 mL), H$_2$O (50 mL), and brine (50 mL). The organic layer was dried over anhydrous MgSO$_4$, concentrated in vacuo, and the residue was purified by flash column chromatography (SiO$_2$, 1:4/EtOAc:hexanes) and recrystallized in EtOH to afford compound 2i (135 mg, 33%, R$_f$ 0.31 in 1:4/EtOAc:hexanes) as a white solid: $^1$H NMR (400 MHz, CDCl$_3$, Figure C13) δ 8.08 (d, $J = 8.0$ Hz, 1H), 7.89 (s, 2H), 7.68 (t, $J = 6.8$ Hz, 1H), 7.58 (s, 1H), 7.57 (d, $J = 8.0$ Hz, 1H), 7.45 (t, $J = 8.0$ Hz, 1H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure C14) δ 164.3, 139.7, 139.6, 133.2, 132.5, 127.6, 126.4, 125.8, 124.5, 123.5, 120.4; LRMS m/z calcd for C$_{13}$H$_7$Br$_2$NOS: 385.1; found 386.1 [M+H]$^+$.

Preparation of compound 2j. Compound 2j was synthesized following a procedure published for the preparation of similar compounds.$^{182}$ To a mixture of 3.5-dimethylaniline (0.12 g, 1.00 mmol) and Et$_3$N (0.14 mL, 1.00 mmol) in anhydrous CH$_2$Cl$_2$ (5 mL), 2,2'-dithiobenzoyl chloride (1, 0.34 g, 1.00 mmol) in anhydrous CH$_2$Cl$_2$ (10 mL) was added dropwise over 15 min. The reaction mixture was stirred for 3 h at rt, and washed with 1 M HCl (50 mL), H$_2$O (50 mL), and brine (50 mL). The organic layer was dried over anhydrous MgSO$_4$, concentrated in vacuo, and the residue was purified by flash column chromatography (SiO$_2$, 100% hexanes, 0.5:9.5/EtOAc:hexanes, 1:9/EtOAc:hexanes, 3:17/EtOAc:hexanes, 1:4/EtOAc:hexanes, 1:3/EtOAc:hexanes, then 100% EtOAc) and by preparative TLC (1:3/acetone:hexanes) to afford compound 2j (88 mg, 35%, R$_f$ 0.25 in 1:3/acetone:hexanes) as a white solid: $^1$H NMR (400 MHz, CDCl$_3$, Figure C15) δ 8.08 (d, $J = 8.0$ Hz, 1H), 7.63 (ddd, $J = 7.6, 7.2, 1.2$ Hz, 1H), 7.55 (d, $J = 8.0$ Hz, 1H),
7.41 (ddd, J = 8.0, 7.2, 1.2 Hz, 1H), 7.29 (s, 2H), 6.94 (s, 1H), 2.35 (s, 6H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure C16) δ 164.3, 140.2, 139.4, 137.1, 132.4, 129.2, 127.3, 125.9, 125.1, 122.7, 120.2, 21.5; LRMS m/z calcd for C$_{15}$H$_{13}$NOS: 255.1; found 256.1 [M+H]$^+$. 

**Preparation of compound 2k.** Compound 2k was synthesized following a procedure published for the preparation of similar compounds. To a mixture of 3.5-dimethoxylaniline (0.15 g, 1.00 mmol) and Et$_3$N (0.14 mL, 1.00 mmol) in anhydrous CH$_2$Cl$_2$ (10 mL), 2,2'-dithiobenzoyl chloride (1, 0.34 g, 1.00 mmol) in anhydrous CH$_2$Cl$_2$ (20 mL) was added dropwise over 15 min. The reaction mixture was stirred for 3 h at rt, and washed with 1 M HCl (50 mL), H$_2$O (50 mL), and brine (50 mL). The organic layer was dried over anhydrous MgSO$_4$, concentrated in vacuo, and the residue was purified by flash column chromatography (SiO$_2$, 1:4/acetone:hexanes) to afford compound 2k (96 mg, 33%, R$_f$ 0.18 in 1:4/acetone:hexanes) as a white solid: $^1$H NMR (400 MHz, CDCl$_3$, Figure C17) δ 8.07 (dd, J = 8.0 Hz, 1H), 7.63 (ddd, J = 8.4, 7.2, 1.2 Hz, 1H), 7.55 (d, J = 8.0 Hz, 1H), 7.42 (ddd, J = 8.0, 7.2, 1.2 Hz, 1H), 6.92 (d, J = 2.4 Hz, 2H), 6.40 (t, J = 2.0 Hz, 1H), 3.81 (s, 6H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure C18) δ 164.3, 161.3, 140.0, 139.1, 132.6, 127.3, 126.0, 125.2, 120.2, 102.9, 99.6, 55.8; HRMS m/z calcd for C$_{15}$H$_{13}$NO$_3$S: 287.0616; found 288.0682 [M+H]$^+$. 

**Preparation of compound 2l.** The known compound 2l was synthesized following a procedure published for the preparation of similar compounds. To a stirred and ice-cooled mixture of 1-naphthylamine (0.080 g, 0.50 mmol) and Et$_3$N (0.04
mL, 0.50 mmol) in anhydrous CH$_2$Cl$_2$ (5 mL), 2,2'-dithiobenzoyl chloride (1, 0.15 g, 0.50 mmol) in anhydrous CH$_2$Cl$_2$ (10 mL) was added dropwise over 20 min. The reaction mixture was warmed-up to rt, stirred for 12 h, and washed with aq. sat. NaHCO$_3$ (15 mL) and H$_2$O (15 mL). The organic layer was dried over MgSO$_4$, concentrated in vacuo, and the residue was purified by flash column chromatography (SiO$_2$, 1:4/EtOAc:hexanes) and recrystallized in MeOH to afford compound 2l (26 mg, 25%, $R_f$ 0.31 in 1:4/EtOAc:hexanes) as white needle crystals: $^1$H NMR (400 MHz, CDCl$_3$, Figure C19, which matches the lit., but our spectrum has better resolution$^{197}$) $\delta$ 8.14-8.12 (m, 2H), 7.92 (d, $J$ = 9.2 Hz, 1H), 7.88-7.84 (m, 3H), 7.67 (ddd, $J$ = 8.4, 7.2, 1.2 Hz, 1H), 7.60 (d, $J$ = 8.0 Hz, 1H), 7.51 (m, 2H), 7.46 (ddd, $J$ = 8.4, 7.2, 1.2 Hz, 1H).

Preparation of compound 2m. The known compound 2m was synthesized following a procedure published for the preparation of similar compounds.$^{182}$ To a mixture of 2-aminopyridine (0.10 g, 1.00 mmol) and Et$_3$N (0.14 mL, 1.00 mmol) in anhydrous CH$_2$Cl$_2$ (10 mL), 2,2'-dithiobenzoyl chloride (1, 0.34 g, 1.00 mmol) in anhydrous CH$_2$Cl$_2$ (20 mL) was added dropwise over 15 min at rt. The reaction mixture was stirred for an additional 3 h at rt, and washed with 1 M HCl (50 mL), H$_2$O (50 mL), and brine (50 mL). The organic layer was dried over anhydrous MgSO$_4$, concentrated in vacuo, and the residue was purified by flash column chromatography (SiO$_2$, 1:3/EtOAc:hexanes) to afford compound 2m (55 mg, 24%, $R_f$ 0.43 in 1:3/EtOAc:hexanes) as a white solid: $^1$H NMR (400 MHz, CDCl$_3$, Figure C20, which matches the lit.$^{182}$) $\delta$ 8.73 (d, $J$ = 8.4 Hz, 1H), 8.40 (d, $J$ = 4.4 Hz, 1H), 8.05 (d, $J$ = 7.6 Hz, 1H), 7.79 (t, $J$ = 8.0 Hz, 1H), 7.64 (t, $J$ = 7.6 Hz, 1H), 7.57 (d, $J$ = 8.4 Hz, 1H), 7.39 (t, $J$ = 7.6 Hz, 1H), 7.13 (t, $J$ = 6.4 Hz, 1H).
**Preparation of compound 2n.** The known compound 2n was synthesized following a procedure published for the preparation of similar compounds.\textsuperscript{182} To a mixture of 5-chloro-2-aminopyridine (0.06 g, 0.50 mmol) and Et\textsubscript{3}N (0.07 mL, 0.05 mmol) in anhydrous CH\textsubscript{2}Cl\textsubscript{2} (5 mL), 2,2'-dithiobenzoyl chloride (1, 0.15 g, 0.05 mmol) in anhydrous CH\textsubscript{2}Cl\textsubscript{2} (10 mL) was added dropwise over 15 min at rt. The reaction mixture was stirred for an additional 3 h at rt, and washed with 1 M HCl (50 mL), H\textsubscript{2}O (50 mL), and brine (50 mL). The organic layer was dried over anhydrous MgSO\textsubscript{4}, concentrated in vacuo, and the residue was purified by flash column chromatography (SiO\textsubscript{2}, 1:3/EtOAc:hexanes) to afford compound 2n (69 mg, 51%, \(R_f\) 0.56 in 1:3/EtOAc:hexanes) as a white solid: \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}, Figure C21, which matches the lit.\textsuperscript{182}) \(\delta\) 8.73 (d, \(J = 9.2\) Hz, 1H), 8.35 (d, \(J = 2.4\) Hz, 1H), 8.04 (d, \(J = 7.6\) Hz, 1H), 7.76 (dd, \(J = 9.2, 2.4\) Hz, 1H), 7.65 (t, \(J = 8.4\) Hz, 1H), 7.57 (d, \(J = 8.4\) Hz, 1H), 7.40 (t, \(J = 7.6\) Hz, 1H).

**Preparation of compound 2o.** Compound 2o was synthesized following a procedure published for the preparation of similar compounds.\textsuperscript{182} To a mixture of 2-aminooquinoline (0.14 g, 0.50 mmol) and Et\textsubscript{3}N (0.07 mL, 0.50 mmol) in anhydrous CH\textsubscript{2}Cl\textsubscript{2} (5 mL), 2,2'-dithiobenzoyl chloride (1, 0.15 g, 0.44 mmol) in anhydrous CH\textsubscript{2}Cl\textsubscript{2} (10 mL) was added dropwise over 15 min at rt. The reaction mixture was stirred for an additional 3 h at rt, and washed with 1 M HCl (50 mL), H\textsubscript{2}O (50 mL), and brine (50 mL). The organic layer was dried over anhydrous MgSO\textsubscript{4}, concentrated in vacuo, and the residue was purified by flash column chromatography (SiO\textsubscript{2}, 1:3/EtOAc:hexanes) to afford compound 2o (29 mg, 21%, \(R_f\) 0.50 in 1:3/EtOAc:hexanes) as a white solid: \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3},
5.6.1.3. Preparation of compounds 3a-4n following procedure B

Compounds 3a-4n were synthesized using a two-step strategy that employed Et$_3$N and various alkylamine derivatives in CH$_2$Cl$_2$ followed by use of N-chlorosuccinimide.

Preparation of compound 3a. Compound 3a was synthesized following a modified procedure published for the preparation of similar compounds.$^{198}$ To a mixture of amylamine (0.10 mL, 0.87 mmol) and Et$_3$N (0.12 mL, 0.86 mmol) in anhydrous CH$_2$Cl$_2$ (10 mL), compound 1 (0.27 g, 1.31 mmol) in anhydrous CH$_2$Cl$_2$ (20 mL) was added dropwise over 15 min at rt. A slurry solution was formed after another 5 min of stirring at rt. N-Chlorosuccinimide (0.13 g, 1.00 mmol) was added and the mixture was refluxed at 60 °C for an additional 1 h. The reaction mixture was stirred for an additional 3 h at rt, and then washed with 1 M HCl (50 mL), H$_2$O (50 mL), and brine (50 mL). The organic layer was dried over anhydrous MgSO$_4$, concentrated in vacuo, and the residue was purified by flash column chromatography (SiO$_2$, 1:4/EtOAc:hexanes) to afford compound 3a (65 mg, 34%, R$_f$ 0.36 in 1:4/EtOAc:hexanes) as a yellow oil: $^1$H NMR (400 MHz, CDCl$_3$, Figure C24) δ 8.00 (d, $J = 7.6$ Hz, 1H), 7.56 (td, $J = 8.0$, 0.8 Hz, 1H), 7.51 (d, $J = 8.0$ Hz, 1H), 7.36 (td, $J = 8.0$, 1.2 Hz, 1H), 7.81 (d, $J = 7.6$ Hz, 1H), 7.71 (t, $J = 7.6$ Hz, 1H), 7.66 (t, $J = 8.4$ Hz, 1H), 7.59 (d, $J = 7.6$ Hz, 1H), 7.49 (t, $J = 7.6$ Hz, 1H), 7.40 (t, $J = 8.0$ Hz, 1H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure C23) δ 164.6, 149.9, 146.5, 141.6, 138.8, 133.3, 130.5, 127.9, 127.8, 127.2, 127.00, 126.97, 126.0, 125.8, 120.5, 114.1; HRMS m/z calcd for C$_{16}$H$_{10}$N$_2$OS: 278.0514; found 279.0514 [M+H]$^+$. 

274
1H), 3.86 (t, J = 7.2 Hz, 2H), 1.73 (p, J = 7.2 Hz, 2H), 1.35-1.29 (m, 4H), 0.87 (t, J = 7.2 Hz, 3H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure C25) δ 165.5, 140.3, 131.8, 126.8, 125.6, 125.0, 120.5, 44.1, 29.4, 28.8, 22.4, 14.1; LRMS m/z calcd for C$_{12}$H$_{13}$NOS: 221.1; found 222.1 [M+H]$^+$. 

**Preparation of compound 3b.** Compound 3b was synthesized following a modified procedure published for the preparation of similar compounds.$^{198}$ To a mixture of hexylamine (0.11 mL, 0.87 mmol) and Et$_3$N (0.12 mL, 0.87 mmol) in anhydrous CH$_2$Cl$_2$ (10 mL), compound 1 (0.27 g, 1.50 mmol) in anhydrous CH$_2$Cl$_2$ (20 mL) was added dropwise over 15 min. A slurry solution was formed after another 5 min of stirring at rt. N-Chlorosuccinimide (0.13 g, 1.00 mmol) was added and the mixture was refluxed at 60 °C for an additional 1 h. The reaction mixture was stirred for 3 h at rt, and washed with 1 M HCl (50 mL), H$_2$O (50 mL), and brine (50 mL). The organic layer was dried over anhydrous MgSO$_4$, concentrated in vacuo, and the residue was purified by flash column chromatography (SiO$_2$, 1:4/EtOAc:hexanes) and recrystallized in EtOH to afford compound 3b (62 mg, 20%, R$_f$ 0.35 in 1:4/EtOAc:hexanes) as a yellow oil: $^1$H NMR (400 MHz, CDCl$_3$, Figure C26) δ 7.99 (d, J = 8.0 Hz, 1H), 7.54 (td, J = 8.0, 1.2 Hz, 1H), 7.50 (d, J = 7.6 Hz, 1H), 7.34 (td, J = 8.0, 1.2 Hz, 1H), 3.84 (t, J = 7.6 Hz, 2H), 1.71 (p, J = 7.2 Hz, 2H), 1.37-1.21 (m, 6H), 0.84 (t, J = 7.6 Hz, 3H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure C27) δ 165.4, 140.3, 131.7, 126.7, 125.5 125.0, 120.4, 44.1, 31.5, 29.6, 26.4, 22.6, 14.1; HRMS m/z calcd for C$_{13}$H$_{17}$NOS: 235.1031; found 236.1103 [M+H]$^+$. 

275
Preparation of compound 3c. Compound 3c was synthesized following a modified procedure published for the preparation of similar compounds. To a mixture of octylamine (0.17 mL, 1.50 mmol) and Et₃N (0.14 mL, 1.00 mmol) in anhydrous CH₂Cl₂ (10 mL), compound 1 (0.34 g, 1.00 mmol) in anhydrous CH₂Cl₂ (20 mL) was added dropwise over 15 min at rt. A slurry solution was formed after another 5 min of stirring at rt. N-Chlorosuccinimide (0.13 g, 1.00 mmol) was added and the mixture was refluxed at 60 °C for an additional 1 h. The reaction mixture was stirred for an additional 3 h at rt, and then washed with 1 M HCl (50 mL), H₂O (50 mL), and brine (50 mL). The organic layer was dried over anhydrous MgSO₄, concentrated in vacuo, and the residue was purified by flash column chromatography (SiO₂, CH₂Cl₂) to afford compound 3c (64 mg, 24%, Rf 0.43 in CH₂Cl₂) as a white solid: ¹H NMR (400 MHz, CDCl₃, Figure C28) δ 8.00 (d, J = 7.6 Hz, 1H), 7.56 (ddd, J = 8.4, 7.2, 1.2 Hz, 1H), 7.53 (t, J = 8.4 Hz, 1H), 7.36 (ddd, J = 8.4, 7.2, 1.2 Hz, 1H), 3.86 (t, J = 7.2 Hz, 2H), 1.73 (p, J = 7.2 Hz, 2H), 1.37-1.20 (m, 10H), 0.84 (t, J = 7.2 Hz, 3H); ¹³C NMR (100 MHz, CDCl₃, Figure C29) δ 165.5, 140.3, 131.8, 126.8, 125.6, 125.1, 120.5, 44.2, 31.9, 29.7, 29.35, 29.30, 26.8, 22.8, 14.3; HRMS m/z calcd for C₁₅H₂₁NOS: 263.1344; found 264.1415 [M+H⁺].

Preparation of compound 3d. The known compound 3d was synthesized following a modified procedure published for the synthesis of similar compounds. To a mixture of dodecylamine (0.46 mL, 2.00 mmol) and Et₃N (0.28 mL, 2.00 mmol) in anhydrous CH₂Cl₂ (10 mL), compound 1 (0.34 g, 1.00 mmol) in anhydrous CH₂Cl₂ (20 mL) was added dropwise over 15 min at rt. A slurry solution was formed after another 5 min of stirring at rt. N-Chlorosuccinimide (0.13 g, 1.00 mmol) was added and the mixture was
refluxed at 60 °C for an additional 1 h. The reaction mixture was stirred for an additional 3 h at rt, and then washed with 1 M HCl (50 mL), H2O (50 mL), and brine (50 mL). The organic layer was dried over anhydrous MgSO4, concentrated in vacuo, and the residue was purified by flash column chromatography (SiO2, CH2Cl2) to afford compound 3d (130 mg, 41%, Rf 0.30 in CH2Cl2) as a white solid: 1H NMR (400 MHz, CDCl3, Figure C30, which matches the lit.199) δ 8.01 (d, J = 7.6 Hz, 1H), 7.57 (t, J = 7.6 Hz, 1H), 7.53 (d, J = 8.0 Hz, 1H), 7.37 (t, J = 7.6 Hz, 1H), 3.86 (t, J = 7.2 Hz, 2H), 1.73 (p, J = 7.2 Hz, 2H), 1.35-1.22 (m, 18H), 0.85 (t, J = 6.8 Hz, 3H); 13C NMR (100 MHz, CDCl3, Figure C31) δ 165.5, 140.3, 131.8, 126.9, 125.6, 125.1, 120.5, 44.2, 32.1, 29.81, 29.76, 29.7, 29.5, 29.4, 26.8, 22.9, 14.3.

**Preparation of compound 3e.** Compound 3e was synthesized following a modified procedure published for the preparation of similar compounds.198 To a mixture of isobutylamine (0.20 mL, 2.00 mmol) and Et3N (0.14 mL, 1.00 mmol) in anhydrous CH2Cl2 (10 mL), compound 1 (0.34 g, 1.00 mmol) in anhydrous CH2Cl2 (20 mL) was added dropwise over 15 min at rt. The mixture was stirred for 1 h at rt to form a slurry solution. N-Chlorosuccinimide (0.13 g, 1.00 mmol) was added and the mixture was refluxed at 60 °C for an additional 1 h until the reaction mixture was clear. The reaction mixture was stirred for an additional 3 h at rt, and then washed with 1 M HCl (50 mL), H2O (50 mL), and brine (50 mL). The organic layer was dried over anhydrous MgSO4, concentrated in vacuo, and the residue was purified by flash column chromatography (SiO2, 1:4/EtOAc:hexanes) to afford compound 3e (180 mg, 87%, Rf 0.29 in 1:4/EtOAc:hexanes) as a yellow oil: 1H NMR (400 MHz, CDCl3, Figure C32) δ 8.02 (d, J = 8.4 Hz, 1H), 7.58 (td, J = 8.0, 0.8 Hz, 1H), 7.52 (d, J = 8.4 Hz, 1H), 7.38 (td, J = 8.0, 0.8 Hz, 1H), 3.69 (d, J = 7.2 Hz, 2H), 2.12 (nonet, J =
6.4 Hz, 1H), 0.97 (d, J = 6.4 Hz, 6H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure C33) δ 165.8, 140.4, 131.9, 127.0, 125.6, 124.9, 120.4, 51.4, 29.3, 20.1; LRMS m/z calcd for C$_{11}$H$_{13}$NOS: 207.1; found 208.1 [M+H]$^+$. 

Preparation of compound 3f. Compound 3f was synthesized following a modified procedure published for the preparation of similar compounds.$^{198}$ To a mixture of isoamylamine (0.23 mL, 2.00 mmol) and Et$_3$N (0.14 mL, 1.00 mmol) in anhydrous CH$_2$Cl$_2$ (10 mL), compound 1 (0.34 g, 1.00 mmol) in anhydrous CH$_2$Cl$_2$ (20 mL) was added dropwise over 15 min at rt. A slurry solution was formed after another 5 min of stirring at rt. N-Chlorosuccinimide (0.13 g, 1.00 mmol) was added and the mixture was stirred for an additional 4 h at rt, and then washed with 1 M HCl (50 mL), H$_2$O (50 mL), and brine (50 mL). The organic layer was dried over anhydrous MgSO$_4$, concentrated in vacuo, and the residue was purified by flash column chromatography (SiO$_2$, 1:4/EtOAc:hexanes) to afford compound 3f (221 mg, quantitative yield, R$_f$ 0.28 in 1:4/EtOAc:hexanes) as a yellow oil: $^1$H NMR (400 MHz, CDCl$_3$, Figure C34) δ 8.00 (d, J = 8.0 Hz, 1H), 7.57 (td, J = 8.0, 1.2 Hz, 1H), 7.52 (d, J = 8.4 Hz, 1H), 7.37 (td, J = 8.0, 0.8 Hz, 1H), 3.90 (t, J = 6.8 Hz, 2H), 1.67-1.60 (m, 3H), 0.96 (d, J = 6.4 Hz, 6H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure C35) δ 165.1, 140.0, 131.5, 126.5, 125.3, 124.8, 120.2, 42.2, 38.2, 25.5, 22.3; LRMS m/z calcd for C$_{12}$H$_{15}$NOS: 221.1; found 222.1 [M+H]$^+$. 

Preparation of compound 3g. The known compound 3g was synthesized following a modified procedure published for the preparation of similar
compounds. To a mixture of tert-butylamine (0.16 mL, 1.50 mmol) and Et₃N (0.14 mL, 1.00 mmol) in anhydrous CH₂Cl₂ (10 mL), compound 1 (0.34 g, 1.00 mmol) in anhydrous CH₂Cl₂ (20 mL) was added dropwise over 15 min at rt. A slurry solution was formed after another 5 min of stirring at rt. N-Chlorosuccinimide (0.13 g, 1.00 mmol) was added and the mixture was refluxed at 60 °C for an additional 1 h. The reaction mixture was stirred for an additional 3 h at rt, and then washed with 1 M HCl (50 mL), H₂O (50 mL), and brine (50 mL). The organic layer was dried over anhydrous MgSO₄, concentrated in vacuo, and the residue was purified by flash column chromatography (SiO₂, 1:4/EtOAc:hexanes) to afford compound 3g (87 mg, 56%, Rf 0.25 in 1:4/EtOAc:hexanes) as a white solid: ¹H NMR (400 MHz, CDCl₃, Figure C36, which matches the lit.¹⁹⁹) δ 7.90 (d, J = 7.2 Hz, 1H), 7.80 (d, J = 7.2 Hz, 1H), 7.74 (td, J = 7.6, 1.2 Hz, 1H), 7.69 (td, J = 7.2, 1.2 Hz, 1H), 1.73 (s, 9H).

Preparation of compound 3h. The known compound 3h was synthesized following a procedure published for the preparation of similar compounds.¹⁹⁸ To a mixture of benzylamine hydrochloride (0.14 g, 1.00 mmol) and Et₃N (0.42 mL, 3.00 mmol) in anhydrous CH₂Cl₂ (10 mL), 2,2′-dithiobenzoyl chloride (1, 0.34 g, 1.00 mmol) in anhydrous CH₂Cl₂ (20 mL) was added dropwise over 15 min. The reaction mixture was stirred for 3 h at rt, and washed with 1 M HCl (50 mL), H₂O (50 mL), and brine (50 mL). The organic layer was dried over anhydrous MgSO₄, concentrated in vacuo, and the residue was purified by flash column chromatography (SiO₂, CH₂Cl₂, 49:1/CH₂Cl₂:MeOH, then 19:1/CH₂Cl₂:MeOH) to afford compound 3h (120 mg, 50%, Rf 0.23 in CH₂Cl₂) as a white solid: ¹H NMR (400 MHz, CDCl₃, Figure C37, which matches the lit.¹⁹⁷) δ 8.05 (d, J = 7.6 Hz, 1H), 7.59-7.55 (m, 1H), 7.47 (d, J = 8.4 Hz, 1H), 7.40-7.36 (m, 1H), 7.35-7.30 (m, 5H), 5.04
Preparation of compound 3i. The known compound 3i\textsuperscript{200} was synthesized following a procedure published for the preparation of similar compounds.\textsuperscript{198} To a mixture of phenethylamine (0.11 mL, 0.87 mmol) and Et\textsubscript{3}N (0.12 mL, 0.87 mmol) in anhydrous CH\textsubscript{2}Cl\textsubscript{2} (30 mL), 2,2'-dithiobenzoyl chloride (1, 0.30 g, 0.87 mmol) was added in portions over 5 min at rt. A slurry solution is formed after another 5 min of stirring at rt. N-chlorosuccinimide (0.10 g, 0.87 mmol) was added and the mixture was refluxed at 60 °C for an additional 1 h. The reaction mixture was cooled to rt and washed with 1 M HCl (50 mL), H\textsubscript{2}O (50 mL), and brine (50 mL). The organic layer was dried over anhydrous MgSO\textsubscript{4}, concentrated \textit{in vacuo}, and the residue was purified by flash column chromatography (SiO\textsubscript{2}, 1:4/EtOAc:hexanes) and recrystallized from MeOH to afford compound 3i (61 mg, 27%, R\textsubscript{f} 0.25 in 1:4/EtOAc:hexanes) as a white solid: \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}, Figure C39, which matches the lit.\textsuperscript{200}) δ 8.02 (d, J = 8.0 Hz, 1H), 7.58 (td, J = 7.6, 1.2 Hz, 1H), 7.49 (d, J = 8.0 Hz, 1H), 7.37 (td, J = 8.0, 0.8 Hz, 1H), 7.32-7.20 (m, 5H), 4.11 (t, J = 7.6 Hz, 2H), 3.05 (t, J = 7.6 Hz, 2H).

Preparation of compound 3j. Compound 3j was synthesized following a modified procedure published for the preparation of similar compounds.\textsuperscript{198} To a mixture of phenylpropylamine (0.14 g, 1.00 mmol) and Et\textsubscript{3}N (0.14 mL, 1.00 mmol) in anhydrous CH\textsubscript{2}Cl\textsubscript{2} (10 mL), compound 1 (0.34 g, 1.00 mmol) in anhydrous CH\textsubscript{2}Cl\textsubscript{2} (20 mL)
was added dropwise over 15 min at rt. A slurry solution was formed after another 5 min of stirring at rt. N-Chlorosuccinimide (0.13 g, 1.00 mmol) was added and the mixture was refluxed at 60 °C for an additional 1 h. The reaction mixture was stirred for an additional 3 h at rt, and then washed with 1 M HCl (50 mL), H₂O (50 mL), and brine (50 mL). The organic layer was dried over anhydrous MgSO₄, concentrated in vacuo, and the residue was purified by flash column chromatography (SiO₂, 1:4/EtOAc:hexanes) to afford compound 3j (142 mg, 71%, Rᵣ 0.30 in 1:4/EtOAc:hexanes) as a white solid. ¹H NMR (400 MHz, CDCl₃, Figure C40) δ 7.98 (d, J = 7.2 Hz, 1H), 7.88 (d, J = 7.2 Hz, 1H), 7.85 (td, J = 7.6, 0.8 Hz, 1H), 7.76 (td, J = 7.2, 1.2 Hz, 1H), 7.28-7.24 (m, 2H), 7.20-7.14 (m, 3H), 4.00-3.92 (m, 1H), 3.86-3.79 (m, 1H), 2.73 (t, J = 7.6 Hz, 2H), 2.14 (pd, J = 7.6, 2.4 Hz, 2H); ¹³C NMR (100 MHz, CDCl₃, Figure C41) δ 165.6, 145.8, 141.0, 134.3, 133.4, 128.7, 128.6, 126.4, 126.3, 125.3, 41.3, 33.3, 31.0; LRMS m/z calcd for C₁₆H₁₅NOS: 269.1; found 270.1 [M+H]+.

Preparation of compound 3k. Compound 3k was synthesized following a modified procedure published for the preparation of similar compounds. To a mixture of cyclopentylamine (0.10 mL, 1.00 mmol) and Et₃N (0.14 mL, 1.00 mmol) in anhydrous CH₂Cl₂ (30 mL), 2,2'-dithiobenzoyl chloride (1, 0.34 g, 1.00 mmol) was added in portions over 5 min at rt. A slurry solution was formed after another 5 min of stirring at rt. N-chlorosuccinimide (0.13 g, 1.00 mmol) was added and the mixture was refluxed at 60 °C for an additional 1 h. The reaction mixture was cooled to rt and washed with 1 M HCl (50 mL), H₂O (50 mL), and brine (50 mL). The organic layer was dried over anhydrous MgSO₄, concentrated in vacuo, and the residue was purified by flash column chromatography (SiO₂, 1:4/EtOAc:hexanes) to afford compound 3k (30 mg, 14%, Rᵣ 0.30 in 1:4/EtOAc:hexanes) as a
white solid: $^1$H NMR (400 MHz, CDCl$_3$, Figure C42) $\delta$ 8.00 (d, $J = 7.6$ Hz, 1H), 7.53 (td, $J = 8.4$, 1.2 Hz, 1H), 7.53 (d, $J = 8.0$ Hz, 1H), 7.36 (td, $J = 8.0$, 1.2 Hz, 1H), 5.07 (p, $J = 7.2$ Hz, 1H), 2.20-2.08 (m, 2H), 1.90-1.81 (m, 2H), 1.80-1.64 (m, 4H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure C43) $\delta$ 165.6, 140.2, 131.7, 126.7, 125.63, 125.56, 120.5, 55.2, 32.4, 24.5; HRMS $m/z$ calcd for C$_{12}$H$_{13}$NOS: 219.0718; found 220.0792 [M+H]$^+$. 

Preparation of compound 3l. The known compound 3l was synthesized following a modified procedure published for the preparation of similar compounds.$^{198}$ To a mixture of cyclohexylamine (0.11 mL, 1.00 mmol) and Et$_3$N (0.14 mL, 1.00 mmol) in anhydrous CH$_2$Cl$_2$ (30 mL), 2,2'-dithiobenzoyl chloride (I, 0.34 g, 1.00 mmol) was added in portions over 5 min at rt. A slurry solution was formed after another 5 min of stirring at rt. N-chlorosuccinimide (0.13 g, 1.00 mmol) was added and the mixture was refluxed at 60 °C for an additional 1 h. The reaction mixture was cooled to rt and washed with 1 M HCl (50 mL), H$_2$O (50 mL), and brine (50 mL). The organic layer was dried over anhydrous MgSO$_4$, concentrated in vacuo, and the residue was purified by flash column chromatography (SiO$_2$, 1:4/EtOAc:hexanes) to afford compound 3l (156 mg, 66%, $R_f$ 0.30 in 1:4/EtOAc:hexanes) as a white solid: $^1$H NMR (400 MHz, CDCl$_3$, Figure C44, which matches the lit.$^{201}$) $\delta$ 8.03 (d, $J = 8.0$ Hz, 1H), 7.56 (td, $J = 8.0$, 1.2 Hz, 1H) 7.56-7.52 (m, 1H), 7.37 (td, $J = 8.4$, 1.6 Hz, 1H), 4.58 (tt, $J = 11.2$, 4.0 Hz, 1H), 2.08-2.00 (m, 2H), 1.89-1.80 (m, 3H), 1.74-1.68 (m, 1H), 1.55 (td, $J = 12.8$, 3.2 Hz, 2H), 1.45 (qt, $J = 12.8$, 3.6 Hz, 1H), 1.19 (qt, $J = 12.8$, 3.6 Hz, 1H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure C45) $\delta$ 165.0, 140.5, 131.6, 126.7, 125.7, 125.5, 120.5, 53.4, 33.1, 25.8, 25.5; HRMS $m/z$ calcd for C$_{13}$H$_{15}$NOS: 233.0874; found 234.0935 [M+H]$^+$. 

\[ \text{SN} \]
Preparation of compound 3m. Compound 3m was synthesized following a modified procedure published for the preparation of similar compounds.\textsuperscript{198} To a mixture of cycloheptylamine (0.25 mL, 2.00 mmol) and Et\textsubscript{3}N (0.14 mL, 1.00 mmol) in anhydrous CH\textsubscript{2}Cl\textsubscript{2} (10 mL), compound 1 (0.34 g, 1.00 mmol) in anhydrous CH\textsubscript{2}Cl\textsubscript{2} (20 mL) was added dropwise over 15 min at rt. A slurry solution was formed after another 1 h of stirring at rt. N-Chlorosuccinimide (0.13 g, 1.00 mmol) was added and the mixture was refluxed at 60 °C for an additional 1 h. The reaction mixture was stirred for an additional 3 h at rt, and then washed with 1 M HCl (50 mL), H\textsubscript{2}O (50 mL), and brine (50 mL). The organic layer was dried over anhydrous MgSO\textsubscript{4}, concentrated \textit{in vacuo}, and the residue was purified by flash column chromatography (SiO\textsubscript{2}, 1:4/EtOAc:hexanes) to afford compound 3m (255 mg, 51\%, R\textsubscript{f} 0.30 in 1:4/EtOAc:hexanes) as a white solid: \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}, Figure C46) \(\delta\) 8.01 (d, \(J = 8.0\) Hz, 1H), 7.56 (ddd, \(J = 8.4, 6.8, 1.6\) Hz, 1H), 7.55-7.50 (m, 1H), 7.36 (ddd, \(J = 8.4, 6.8, 1.6\) Hz, 1H), 4.79 (septet, \(J = 4.4\) Hz, 1H), 2.30-2.02 (m, 2H), 1.84-1.52 (m, 10H); \textsuperscript{13}C NMR (100 MHz, CDCl\textsubscript{3}, Figure C47) \(\delta\) 164.7, 140.4, 131.6, 126.7, 125.5 (2 carbons), 120.5, 55.4, 35.1, 28.1, 24.8; HRMS \textit{m/z} calcd for C\textsubscript{14}H\textsubscript{17}NOS: 247.1031; found 248.1104 [M+H]\textsuperscript{+}.

Preparation of compound 3n. Compound 3n was synthesized following a modified procedure published for the preparation of similar compounds.\textsuperscript{198} To a mixture of cyclooctylamine (0.27 mL, 2.00 mmol) and Et\textsubscript{3}N (0.14 mL, 1.00 mmol) in anhydrous CH\textsubscript{2}Cl\textsubscript{2} (10 mL), compound 1 (0.34 g, 1.00 mmol) in anhydrous CH\textsubscript{2}Cl\textsubscript{2} (20 mL).
was added dropwise over 15 min at rt. The mixture was allowed to stir for 1 h at rt. N-Chlorosuccinimide (0.13 g, 1.00 mmol) was added and the mixture was stirred for an additional 4 h at rt, and then washed with 1 M HCl (50 mL), H₂O (50 mL), and brine (50 mL). The organic layer was dried over anhydrous MgSO₄, concentrated *in vacuo*, and the residue was purified by flash column chromatography (SiO₂, 1:4/EtOAc:hexanes) to afford compound 3n (81 mg, 31%, Rᶠ 0.26 in 1:4/EtOAc:hexanes) as a yellow oil: ¹H NMR (400 MHz, CDCl₃, Figure C48) δ 8.00 (dt, J = 8.0, 1.2 Hz, 1H), 7.58-7.53 (m, 2H), 7.36 (ddd, J = 7.6, 6.4, 1.2 Hz, 1H) 4.86 (app. septet, J = 4.4 Hz, 1H), 1.99-1.58 (m, 14H); ¹³C NMR (100 MHz, CDCl₃, Figure C49) δ 164.7, 140.5, 131.6, 126.7, 125.51, 125.46, 120.5, 54.4, 33.1, 27.0, 26.0, 24.7; LRMS m/z calcd for C₁₅H₁₉NOS: 261.1; found 262.1 [M+H]⁺.

**Preparation of compound 3o.** Compound 3o was synthesized following a procedure published for the preparation of similar compounds.¹⁹⁸ To a mixture of amantadine hydrochloride (0.19 g, 1.00 mmol) and Et₃N (0.14 mL, 1.00 mmol) in anhydrous CH₂Cl₂ (10 mL), 2,2'-dithiobenzoyl chloride (1, 0.34 g, 1.00 mmol) in anhydrous CH₂Cl₂ (20 mL) was added dropwise over 15 min at -15 °C. N-Chlorosuccinimide (0.13 g, 1.00 mmol) was added and the reaction mixture was stirred for an addition 3 h at rt, and washed with 1 M HCl (50 mL), H₂O (50 mL), and brine (50 mL). The organic layer was dried over anhydrous MgSO₄, concentrated *in vacuo*, and the residue was purified by flash column chromatography (SiO₂, 1:3/EtOAc:hexanes) to afford compound 3o (39 mg, 14%, Rᶠ 0.60 in 1:3/EtOAc:hexanes) as a white solid: ¹H NMR (400 MHz, CDCl₃, Figure C50) δ 7.93 (d, J = 7.6, 1H), 7.53 (t, J = 7.2 Hz, 1H), 7.48 (d, J = 8.0 Hz, 1H), 7.33 (t, J = 6.8 Hz, 1H), 2.43 (s, 6H), 2.18 (s, 3H), 1.77 (q, J = 12.8 Hz, 6H); ¹³C NMR (100 MHz, CDCl₃, Figure C51) δ
Preparation of compound 4e. Compound 4e was synthesized following a modified procedure published for the preparation of similar compounds. To a mixture of isobutylamine (0.20 mL, 2.00 mmol) and Et₃N (0.14 mL, 1.00 mmol) in anhydrous CH₂Cl₂ (10 mL), compound 1 (0.34 g, 1.00 mmol) in anhydrous CH₂Cl₂ (20 mL) was added dropwise over 15 min at rt. The mixture was stirred for 1 h at rt. N-Chlorosuccinimide (0.13 g, 1.00 mmol) was added and the mixture was refluxed at 60 °C for an additional 1 h. The reaction mixture was stirred for an additional 36 h at rt, and then washed with 1 M HCl (50 mL), H₂O (50 mL), and brine (50 mL). The organic layer was dried over anhydrous MgSO₄, concentrated in vacuo, and the residue was purified by flash column chromatography (SiO₂, 1:4/EtOAc:hexanes) to afford compound 4e (239 mg, 58%, Rf 0.19 in 1:4/EtOAc:hexanes) as a yellow oil: ¹H NMR (400 MHz, CDCl₃, Figure C52) δ 8.00-7.97 (m, 1H), 7.90-7.88 (m, 1H), 7.78 (td, J = 7.6, 1.6 Hz, 1H), 7.74 (td, J = 7.6, 1.2 Hz, 1H), 3.75 (dd, J = 14.4, 7.6 Hz, 1H), 3.57 (dd, J = 14.4, 7.6 Hz, 1H), 2.19 (nonet, J = 6.8 Hz, 1H) 1.01 (d, J = 6.8 Hz, 3H), 0.96 (d, J = 6.8 Hz, 3H); ¹³C NMR (100 MHz, CDCl₃, Figure C53) δ 165.9, 145.9, 134.3, 133.4, 128.5, 126.4, 125.3, 48.6, 28.7, 20.4, 20.3; HRMS m/z calcd for C₁₁H₁₃NO₂S: 223.0667; found 224.0736 [M+H]⁺.

Preparation of compound 4f. Compound 4f was synthesized following a modified procedure published for the preparation of similar compounds. To
a mixture of isoamylamine (0.23 mL, 2.00 mmol) and Et$_3$N (0.14 mL, 1.00 mmol) in anhydrous CH$_2$Cl$_2$ (10 mL), compound 1 (0.34 g, 1.00 mmol) in anhydrous CH$_2$Cl$_2$ (20 mL) was added dropwise over 15 min at rt. A slurry solution was formed after another 5 min of stirring at rt. N-Chlorosuccinimide (0.13 g, 1.00 mmol) was added and the mixture was refluxed at 60 °C for an additional 1 h. The reaction mixture was stirred for an additional 36 h at rt, and then washed with 1 M HCl (50 mL), H$_2$O (50 mL), and brine (50 mL). The organic layer was dried over anhydrous MgSO$_4$, concentrated in vacuo, and the residue was purified by flash column chromatography (SiO$_2$, 1:4/EtOAc:hexanes) to afford compound 4f (193 mg, 87%, R$_f$ 0.25 in 1:4/EtOAc:hexanes) as a white solid: $^1$H NMR (400 MHz, CDCl$_3$, Figure C54) $\delta$ 7.97 (dd, $J = 6.8, 0.8$ Hz, 1H), 7.88 (d, $J = 7.6$ Hz, 1H), 7.76 (td, $J = 7.6, 1.6$ Hz, 1H), 7.73 (td, $J = 7.6, 0.8$ Hz, 1H), 3.98 (dt, $J = 14.4, 7.6$ Hz, 1H), 3.76 (dt, $J = 14.4, 7.6$ Hz, 1H), 1.74-1.60 (m, 3H), 0.97 (d, $J = 4.4$ Hz, 3H), 0.95 (d, $J = 4.4$ Hz, 3H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure C55) $\delta$ 165.5, 145.8, 134.3, 133.4, 128.7, 126.3, 125.2, 39.9, 38.2, 26.1, 22.6, 22.5; HRMS m/z calcd for C$_{12}$H$_{15}$NO$_2$S: 237.0823; found 238.0895 [M+H]$^+$. 

**Preparation of compound 4n.** Compound 4n was synthesized following a modified procedure published for the preparation of similar compounds. To a mixture of cyclooctylamine (0.27 mL, 2.00 mmol) and Et$_3$N (0.14 mL, 1.00 mmol) in anhydrous CH$_2$Cl$_2$ (10 mL), compound 1 (0.34 g, 1.00 mmol) in anhydrous CH$_2$Cl$_2$ (20 mL) was added dropwise over 15 min at rt. The mixture was allowed to stir for 1 h at rt. N-Chlorosuccinimide (0.13 g, 1.00 mmol) was added and the mixture was refluxed at 60 °C for an additional 1 h. The reaction mixture was stirred for an additional 3 h at rt, and then washed with 1 M HCl (50 mL), H$_2$O (50 mL), and brine (50 mL). The organic layer was dried over
anhydrous MgSO$_4$, concentrated in vacuo, and the residue was purified by flash column chromatography (SiO$_2$, 1:4/EtOAc:hexanes) to afford compound 4n (229 mg, 44%, R$_f$ 0.25 in 1:4/EtOAc:hexanes) as an off-white solid: $^1$H NMR (400 MHz, CDCl$_3$, Figure C56) $\delta$ 7.95 (dd, $J = 6.8$, 1.2 Hz, 1H), 7.85 (d, $J = 7.2$ Hz, 1H), 7.57 (td, $J = 7.2$, 1.6 Hz, 1H), 7.71 (td, $J = 7.2$, 1.2 Hz, 1H), 4.54 (tt, $J = 10.4$, 4.0 Hz, 1H), 2.22-2.02 (m, 3H), 2.00-1.92 (m, 1H), 1.90-1.76 (m, 2H), 1.72-1.58 (m, 8H); $^{13}$C NMR (100 MHz, CDCl$_3$, Figure C57) $\delta$ 165.3, 145.9, 134.1, 133.2, 129.0, 126.2, 125.0, 56.0, 33.0, 32.9, 26.9, 25.9, 25.1, 24.9; HRMS m/z calcd for C$_{15}$H$_{19}$NO$_2$S: 277.1136; found 278.1210 [M+H]$^+$. 

5.6.2. Biochemical and biological methods

5.6.2.1. Bacterial strains

*S. aureus* ATCC 6538 (strain A) and *S. epidermidis* ATCC 35984 (strain AC) were purchased from American Type Tissue Collection (ATCC, Manassas, VA). *S. aureus* ATCC 25923 (strain B), *S. aureus* ATCC 33591 (strain D), MRSA2 (strain F), *P. aeruginosa* ATCC 27853, *E. cloacae* ATCC 13407, and *S. epidermidis* ATCC 12228 were donated by Prof. Dev P. Arya (Clemson University). *S. aureus* ATCC 29213 (strain C), MRSA BRS3 (strain G), MRSA C1 (strain H), MRSA C2 (strain I), MRSA C7 (strain J), MRSA C14 (strain K), MRSA C16 (strain L), MRSA C19 (strain M), MRSA G1 (strain N), MRSA G6 (strain O), MRSA G12 (strain P), MRSA G14 (strain Q), MRSA NRS4 (strain R), MRSA NRS51 (strain S), MRSA NRS77 (strain T), MRSA S14 (strain U), MRSA S17 (strain V), MRSA S22 (strain W), MRSA S24 (strain X), MRSA USA100 (strain Y), MRSA USA200 (strain Z), MRSA USA300 (strain AA), MRSA USA600 (strain AB), *E. faecalis* ATCC 29212 (strain AD), *E.
faecalis ATCC 49533 (strain AE), *E. faecium* BM4105-RF (strain AF), *L. monocytogenes* ATCC 19115 (strain AH), *A. baumannii* ATCC 19606, *E. coli* MC1061, and *K. pneumoniae* ATCC 27736 were a generous gift from Prof. Paul J. Hergenrother (University of Illinois Urbana-Champaign). MRSA1 (strain E) and VRE (strain AG) were obtained from Prof. David H. Sherman (University of Michigan). Finally, *M. smegmatis* MC2-155 (strain AI) was provided by Prof. Sabine Erht (Cornell University).

5.6.2.2. Determination of MIC values against bacterial strains

The MIC values against bacterial strains were determined based on a previously published protocol. The double-dilution method was used to determine MIC values starting at 125 µg/mL. The tested compounds were dissolved in Mueller-Hinton (MH) broth and a 1:1000 dilution of bacterial cultures were added. Bacteria were allowed to grow at 37 °C overnight. MIC values were determined by sight or by staining with MTT (1 mg/mL, 50 µL) when needed. These data are presented in Tables 5.1-5.3.

5.6.2.3. Time-kill curves

The efficiency of the compounds to kill MRSA S22 (strain W) was monitored using a slightly modified previously published protocol. The initial inoculum used for this assay was 1-5×10^6 CFU/mL in MH medium at 37 °C in 50 mL Falcon tubes (10 mL volume) with continuous agitation (200 rpm). Ebselen, ebsulfur (2a), ebsulfur derivatives 3b and 3c, and AMK were used at concentrations of 0.5×, 1×, and 4× their respective MIC values. At 0, 3, 6, 9, and 24 h, 100 µL aliquots were removed from each solution and serially diluted in sterile
ddH₂O. Each dilution was spread onto MH-agar plates and incubated at 37 °C. Colony counts were determined after 24 h of incubation. The experiments were performed in duplicate (Figure 5.2). In order to confirm that the MIC values reported from the experiments described above, remained the same in the time-kill assays when 1×10⁶ CFU/mL was used as an inoculum, we determined the MIC values again for the compounds evaluated in time-kill assays (AMK, ebselen, 2a, 3b, and 3c) and additional ones (2g, 2h, and 3a) using the double-dilution method starting with 1×10⁶ CFU/mL. We found the MIC values determined by both methods (different starting inoculum) to be identical for the compounds tested.

5.6.2.4. Biofilm disruption

The ability of the compounds to disrupt biofilms was tested based on a previously published protocol. Briefly, S. aureus ATCC 6538 (strain A) and S. epidermidis ATCC 35984 (strain AC) were grown overnight in Trypticase soy broth (TSB) at 37 °C. The bacteria were then diluted 1:100 in TSB with 1% glucose and plated (100 µL) in 96-well plates. The cells were grown for 24 h at 37 °C before the medium and planktonic bacteria were removed with PBS washing (4×100 µL). At this point, antibacterial compounds, dissolved in TBS (100 µL), were added to the biofilms and incubated for an additional 24 h at 37 °C. The compounds and medium were removed by submerging the entire plate in tap H₂O. The biofilms were then stained with a 0.1% crystal violet solution by incubating 100 µL of the solution in the wells for 30 min. The excess crystal violet was removed by washing with ddH₂O (4×100 µL). The plates were allowed to dry for 1 h and the dye was solubilized with 95% EtOH. After
thorough mixing the absorbance at 595 nm was read on a SpectraMax M5 plate reader. All readings were normalized to the biofilm formation without compound (Figure 5.3).

5.6.2.5. Determination of hemolytic activity

Hemolytic activity was determined as previously described with minor modifications (Figure 5.4). To obtain red blood cells (RBCs), mouse RBCs (1 mL) were suspended in 9 mL of PBS (10 mM, pH 7.2) and centrifuged (1,200 rpm) for 10 min. RBCs were washed in PBS (4×) and resuspended in fresh PBS to achieve the final concentration (10⁷ RBCs/mL). Serial dilutions of compounds were prepared in Eppendorf tubes containing H₂O (100 µL) and RBCs suspension (100 µL) was added to achieve final concentrations of compounds ranging from (31.2 to 0.24 µg/mL) and of RBCs at 5×10⁶ RBCs/mL. After that, the tubes were incubated at 37 °C for 1 h. Tubes containing ddH₂O (200 µL) and Triton X-100® (1% v/v, 2 µL) served as negative (blank) and positive (100%) control, respectively. Percent hemolysis was calculated using the following equation: % hemolysis = [(absorbance of sample) – (absorbance of blank)]×100/(absorbance of positive control). Fifty percent hemolysis (HC₅₀) values were defined as the concentrations of compounds required to lyse 50% of the RBCs.

5.6.2.6. Determination of mammalian cytotoxicity

Mammalian cytotoxicity assays were performed as previously described with slight modifications. HEK-293 cells (ATCC CRL-1573) were cultured in DMEM medium (ATCC, Manasas, VA) with 10% FBS (ATCC, Manasas, VA) and 1% pen/strep (ATCC, Manasas, VA) at 37 °C with 5% CO₂. Cells were plated at 10,000 cells per well (100 µL well)
overnight in 96-well microtiter plates. The next day, ebselen as well as compounds 2e, 2h, 2k, 2l, 3c, 3i, and 3k were serially diluted and stored at 1000× stocks (10,000, 5,000, 2,500, 1,250, 630, 310, 160, 80, and 40 µg/mL) in DMSO. Then, 1 µL of 1000× compound stocks was added to 999 mL medium to achieve final compound concentrations of 10, 5, 2.5, 1.25, 0.63, 0.31, 0.16, 0.08, and 0.04 µg/mL with 0.1% DMSO, which was determined to not be toxic in HEK-293 cell line. The medium was removed from the 96-well microtiter plates and an additional 200 µL of medium with ebselen, 2e, 2h, 2k, 2l, 3c, 3i, and 3k were added to each well. The positive control consisted of cells treated with 0.1% DMSO, while the negative control consisted cells treated with Triton X-100® (1% v/v, 2 µL). The cells were incubated with the compounds for 24 h at 37 °C with 5% CO₂. After the incubation, each well was treated with 10 µL (25 mg/L) of resazurin sodium salt (Sigma-Aldrich) for 6 h. Live cells can convert the blue-fluorescent dye resazurin to the highly fluorescent pink dye resorufin, which can be detected at A₅₆₀ excitation and A₅₉₀ emission wavelengths using a SpectraMax M5 plate reader. Percent cell survival was calculated as: (test value/control value) x 100. Control value represents cells + resazurin without compounds and test value represents cells + resazurin + compounds. We thank Dr. Matthew S. Gentry (University of Kentucky) for kindly providing the HEK-293 cell line.

5.6.2.7. Cell membrane permeabilization assay using propidium iodide (PI) staining

A colony of *S. epidermidis* ATCC 35984 (strain AC) was used to inoculate 5 mL of MH broth in a Falcon tube and grown overnight at 37 °C at 200 rpm. The overnight culture was diluted by adding 200 µL of bacterial cells to 800 µL of MH broth. Cell suspension (50 µL) was then
added to the MH broth containing no drug (negative control) or ebselen, 2a, 3b, 3c, and AMK, at their 1× and 4× MIC values, respectively. C14-thioether tobramycin\textsuperscript{166} was used as the positive control. The cell suspensions were then treated for 1 h at 37 °C with continuous agitation (200 rpm). Afterwards, treated cells were centrifuged and resuspended in 500 μL of PBS buffer (pH 7.2). Propidium iodide (PI) (3 μM, final concentration) was added and the mixture was incubated for 10 min at rt in the dark (PI is light sensitive) and centrifuged to remove excess PI. Glass slides with 10-15 μL of each mixture were prepared and observed in bright field and fluorescence modes (Texas red filter set, excitation, and emission wavelengths of 535 and 617 nm, respectively) using a Zeiss Axovert 200M fluorescence microscope. Data were obtained from two independent experiments (Figure 5.6).

5.6.2.8. Assay for ROS production

We used 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) dye as a probe to measure the production of ROS in bacterial cells upon treatment with our compounds (ebselen, 2a, 3b, and 3c). This DCFH-DA was used as it can easily cross the cell membranes and be hydrolyzed by cellular esterases to the non-fluorescent 2',7'-dichlorodihydrofluorescein (DCFH), which can further be oxidized to the highly fluorescent 2',7'-dichlorofluorescein (DCF) by the intracellular ROS.\textsuperscript{192} A colony of S. epidermidis ATCC 35984 (strain AC) was used to inoculate 5 mL of MH broth in a Falcon tube and grown overnight at 37 °C at 200 rpm. The overnight culture was diluted by adding 200 μL of bacterial cells to 800 μL of MH broth. Cell suspension (50 μL) was then added to the MH broth containing no drug (negative control) or ebselen, 2a, 3b, and 3c, at their 1x and 4x MIC and treated for 1 h at 37 °C. Hydrogen
peroxide ($\text{H}_2\text{O}_2$) at 1 mM concentration was used as a positive control. Cells were centrifuged and washed twice with PBS buffer (pH 7.2). Cells were resuspended in the same buffer and incubated with DCFH-DA (40 $\mu$g/mL) for 30 min in the dark. Afterwards, cells were centrifuged, washed with PBS buffer to remove the excess DCFH-DA. Glass slides with 10-15 $\mu$L of each mixture were prepared and observed in bright field and fluorescence modes (FITC filter set, excitation, and emission wavelengths of 488 and 512 nm, respectively) using a Zeiss Axovert 200M fluorescence microscope (Figure 5.7).

5.7. ACKNOWLEDGMENTS

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5.8. AUTHORS’ CONTRIBUTIONS

HXN synthesized all compounds.

KDG, SKS, and HXN performed all biochemical and biological assays.

HXN and SGT analyzed data and wrote the manuscript.
Chapter 6

Introduction: fungal diseases, current antifungal agents, and resistance associated with fungal diseases

6.1. INTRODUCTION TO FUNGAL DISEASES

Fungal infections have become a major burden for society, especially with the current rise in the number of immunocompromised patients. *Candida albicans* is the major pathogen and is responsible for 50-70% cases of total fungal infections worldwide.\^202 Among the fungal infections, invasive candidiasis is one of the most common nosocomical fungal disease with an estimate of 250,000 cases and more than 50,000 deaths worldwide per year.\^202-203 Additionally, *Aspergillus* strains are also the culprits of numerous disease states. For instance, approximately 2.5% of adults with asthma also suffer with allergic bronchopulmonary aspergillosis (ABPA).\^204 According to the Centers for Disease Control, out of these roughly 4.8 million patients with ABPA, 400,000 end up with chronic pulmonary aspergillosis (CPA).\^204 As recently as 2012, in the USA, a multistate outbreak of fungal meningitis and associated diseases (749 reported cases) were discovered to be due to steroid injections contaminated with fungal pathogens prepared by a single compounding pharmacy.\^205 Based on some experts opinions, fungal diseases are expected to become even more prominent in the near future as a result of global warming and the rise of fungal strains that are capable of surviving and propagating at mammalian body temperature.\^206
The impact of fungi extends far beyond direct human infections. The main human food sources, comprised of livestock and plants, are also hosts for a variety of fungal pathogens. In fact, there are historical examples of food sources contaminated by fungi, which have presented and continue to present challenges to human civilization. A dark period in the history of the USA was the Salem witch trials. A well-known scientific explanation for the reported afflictions was that the alleged victims unknowingly consumed mouldy bread contaminated with a fungus called *Claviceps purpurea*, more commonly known as ergot. This fungus, infecting the head of rye and other grains, produces psychotropic natural products. In the nineteenth century, fungal diseases in crops were responsible for multiple famines. For example, the Irish Potato Famine resulted from a phytopathogenic fungus, *Phytophthora infestans*, which led to innumerable dead from starvation and triggered the emigration of an estimated half a million Irish citizens to the USA. Currently, the fungus *Puccina graminis tritici* Ug99, responsible for stem or black rust disease on wheat, is threatening to wipe out the world wheat production. Fungal diseases also affect livestock. Mastitis in dairy cattle is an inflammation of the udder often caused by infectious microorganisms. Recently, a group from Poland identified milk samples of mastitic cows to be contaminated with *Candida parapsilosis*, a pathogen capable of infecting humans.

It is therefore clear that fungal afflictions have arisen in various links of the food web throughout history (Figure 6.1A). Over the ages, fungi have come out of their hiding places and humans have sought new ways to overcome these pathogens. We are currently in an era where resistance to all the currently used antifungal agents is fast emerging. Thus, there is a need for new, safe, and effective antifungals to combat these pathogens. Unlike the case of
antibacterials, our repertoire of antifungals is limited, and the antifungal pharmaceutical pipeline is running dry. It is important to note that the most clinically useful antifungal classes (polyenes and azoles) were discovered at least 40 years ago and the azoles are used in livestock and plants as well. While also important, the literature on antifungal usage in livestock is limited and will not be directly addressed herein. On the contrary, since the introduction of the most commonly used antifungals, there has been an explosion in the number of manuscripts related to antifungals since the 1960s, as exemplified by the increase from ~443 and 39 to 4934 and 1088 manuscripts in PubMed and Scifinder, respectively, from 1960 to 2015 (Figure 6.1B).
Figure 6.1. **A.** Schematic showing that fungi can infect not only humans, but affect humans at multiple points of the food web. Lines indicate connections through food sources. Examples of pathogenic fungi are written adjacent to each point or connection. **B.** Line graph showing the increase in the publication of antifungal papers.

### 6.2. CURRENT ANTIFUNGAL AGENTS

The antifungal drug pipeline has received contributions from many chemical classes: natural products, synthetic organic molecules, and inorganic compounds (Figure 6.2). Collectively, synthetic organic molecules have been the predominant class in terms of applications in both humans and crops.\textsuperscript{215-216} There is currently an alarmingly high number of molecules used in crops that are either structurally or pharmacologically the same as the FDA-approved drugs.
utilized in medicine. This observed crossover could potentially contribute to widespread fungal resistance in the future. In the following sections we discuss the antifungal compounds currently used in both humans and agriculture. The most common antifungal targets include fungal ergosterol (e.g., the 14α-demethylases), β-1,3-β-d-glucan synthase, N-myristoyltransferase, aminoacyl tRNA synthase, chitin synthase, elongation factor, and the secreted aspartic protease. Detailed information about each of these targets can be found in a recent review article.217
**Figure 6.2. A.** Timeline showing the introduction to market (year of introduction into parentheses) of the antifungals for human use (top) and for use on crops (bottom) discussed in this chapter. AmB = amphotericin B, 5-FC = 5-fluorocytosine, NYS = nystatin, MCZ = miconazole, NAF = naftifine, FLC = fluconazole, KTC = ketoconazole, ITC = itraconazole, OH OH OH R

|-------------------------|------------|------------|------------|------------|------------|

|-------------------------|------------|------------|------------|------------|

**B.** Chemical structures of antifungals divided by class:

**Azoles:**

![Azoles diagram]

**Echinocandins:**

![Echinocandins diagram]

**Polenes:**

![Polenes diagram]

**Benzimidazoles:**

![Benzimidazoles diagram]

**Allylamines:**

![Allylamines diagram]

**Morpholines:**

![Morpholines diagram]

**Strobilurins:**

![Strobilurins diagram]
The first conjugated polyketide (PK) natural product, nystatin (NYS), discovered to display antifungal activity, was isolated from *Streptomyces noursei* (named for the dairy farmer William Nourse) in 1950. Although this discovery was a monumental achievement in the field of medical mycology, it was apparent that, due to its toxicity, NYS would not be an ideal drug for systemic fungal infections, a complication for healthcare providers at the time. Hence, new antifungal compounds capable of treating systemic fungal diseases were necessary. However, this discovery put the spotlight on antifungal natural product isolation, specifically the polyenes. Eventually, amphotericin B (AmB), a heptaene PK, was isolated in 1953 from a soil sample from Venezuela. The discovery of AmB was essential considering that it was the first drug that could be used to treat deep-seated systemic fungal infections. Despite its potent antifungal activity, AmB has severe off-target effects and is often reserved for life-threatening infections. Patients treated with AmB frequently experience infusion-related adverse effects and/or dose-limiting nephrotoxicity. The liposomal formulations of AmB have alleviated some of its
toxicity concerns, but not to a satisfactory extent. And in addition, AmB formulations are required to be administered parenterally.

In fact, it took 30 years after the isolation of AmB for the first oral antifungal agent, fluconazole (FLC), to be introduced for clinical use. FLC is part of theazole class of antifungals that inhibit 14α-demethylase (ERG11 for Candida spp. or CYP51 for Aspergillus spp.), an enzyme essential in the biosynthesis of a major component of the fungal cell membrane, ergosterol via lanosterol. Inhibition of 14α-demethylase causes the accumulation of toxic methylated sterols, eventually leading to fungal cell death. Unlike AmB, FLC exhibits excellent oral bioavailability, predictable linear pharmacokinetics, broader scope of affected tissues, and fewer adverse effects. As a result, FLC quickly became one of the most prescribed antifungal agents in clinics worldwide. However, some fungal strains, such as Candida glabrata, Candida krusei, Aspergillus spp., Fusarium spp., and the Mucorales were found to exhibit intrinsic resistance to FLC. Consequently, to expand the clinical utilities of azoles, new FLC analogues with broader spectrum of activity, such as itraconazole (ITC), voriconazole (VOR), and posaconazole (POS), were developed and introduced in 1992, 2002, and 2006, respectively (Figure 6.2B). ITC and POS contain modified and extended hydrophobic side chains, which allow for additional interactions with the enzyme targets, leading to an enhanced spectrum of activity. Theseazole compounds have been essential in our fight against many types of fungal diseases. However, azoles have their own shortcomings. Despite their tremendous clinical utilities, they also inhibit the human cytochrome P450 (CYP) enzymes interfering with the metabolism of many other concomitant drugs. Indeed, to correct the problem ofazole-related drug
interactions, many research groups have been designing novel azole analogues that selectively target the fungal 14α-demethylase enzyme (See the modification of current antifungals and other FDA-approved drugs section).

An alternative strategy that has been used to overcome the problem of selectivity towards the fungal enzymes, is the development of another class of compounds, the allylamines, which inhibit a different target in the ergosterol biosynthetic pathway. The first FDA-approved allylamines included terbinafine (TER) and naftifine (NAF), which were put on the market in 1996 and 1988, respectively (Figure 6.2).\(^{233}\) These compounds inhibit squalene epoxidase, the enzyme responsible for generating a sterol upstream of ergosterol.\(^{234}\) TER has been shown to accumulate more in the skin and nail beds relative to blood plasma, possibly due to its lipophilicity.\(^{235}\) Thus, TER and the other allylamines are highly effective against dermatophytes and employed for the treatment of onychomycosis and cutaneous fungal infections.\(^{236}\)

Despite the improvements brought by the allylamines, in order to further differentiate between mammalian and fungal cells, their distinct cell walls have been targeted by the echinocandins. This latest milestone in medical mycology provided us with caspofungin (CFG), micafungin (MFG), and anidulafungin (AFG), which were introduced in 2001, 2005, and 2006, respectively.\(^{237-238}\) More specifically, these compounds target glucan synthesis, which is the most notable aberration between fungal and mammalian cells, by inhibiting the β-1,3-\(d\)-glucan synthase. The polymer β-glucan is an essential component of many fungal cell walls.\(^{239}\) As a result of this unique mechanism of action, the echinocandins addressed an
important scientific challenge in medical mycology, which is selective toxicity against the eukaryotic fungal cell. Indeed, when comparing the rate of treatment discontinuation due to adverse effects, the aforementioned echinocandins were found to be more tolerable than AmB in all formulations, ITC, and VOR. The echinocandins are fungicidal against most *Candida* spp. and fungistatic against *Aspergillus* spp. Unfortunately, they are generally not active against Zygomycetes, *Cryptococcus neoformans*, or *Fusarium* spp. Recent work by Nett and Andes provides a full account of the antifungals currently approved and used in the medical field, their spectrum of activity, resistance, pharmacology, clinical indications of use, toxicities, and any drug-drug interactions. Although good antifungal agents exist, based on the number of affected individuals and the number of deaths from candidiasis, aspergillosis, and cryptococcal meningitis, among other diseases (Figure 6.3), it is clear that new and improved antifungals are urgently needed.

**Figure 6.3.** Bar graphical representation of the number of individuals living with and deaths caused by fungal infections. Data for this graph were obtained from the Global Action Fund for Fungal Infections website ([www.gaffi.org](http://www.gaffi.org)).
6.3. RESISTANCE ASSOCIATED WITH FUNGAL DISEASES

Much like their bacterial counterparts, the number of fungi resistant to the current antifungals and fungicides is rapidly increasing. Below, we briefly summarize the main mechanisms of resistance for human pathogens and some phytopathogens. Although phytopathogens are abundant, the literature examining their mechanisms of resistance to fungicides is sparse, at best. For full coverage of the resistance mechanisms found in human fungal pathogens, please refer to the recent review by Sanglard on resistant fungal pathogens.

There are two types of resistance found in fungi: (i) acquired resistance through exposure to antifungal agents and (ii) intrinsic resistance, a genetic predisposition that precludes the activity of a particular antimycotic agent. The former has been reported for nearly every major fungal pathogen. Molecularly, resistance occurs in one of three ways: (i) decrease in effective drug concentration, (ii) drug target alterations, and (iii) metabolic bypasses. A decrease in intracellular concentration can be achieved in several ways including active efflux, target overexpression, and compartmentalization through the formation of biofilms. One recent case reported in phytopathogens is the development of resistance in *P. capsici* due to a mutation in the *CesA3* gene, involved in cellulose synthesis. Drug targets are altered primarily through mutation of the given enzyme (e.g., 14α-lanosterol demethylase and β-1,3-glucan synthase) so that the antifungal agent no longer efficiently prevents the enzyme from performing its dedicated task. Metabolic bypasses can include alternative metabolic or shunt pathways that convert the toxic metabolites, created by antifungal treatment, into a secondary metabolic or excretion route. In the following sections, we discuss the new
antifungal compounds that either overcome these methods of resistance, or avoid the currently targeted metabolic pathways.

6.4. ACKNOWLEDGMENTS

We thank all of those working in the field of antifungal drug discovery and apologize if their work is not cited due to the scope of the review presenting examples from 2010-2016. Work in S.G.-T. laboratory on resistance is supported by a grant from the National Institute of Health NIH AI90048 and by startup funds from the University of Kentucky.


6.5. AUTHORS’ CONTRIBUTIONS

HXN, SGT, and KDG performed literature search and wrote the manuscript.
Chapter 7

Identification of ebsulfur analogues with broad-spectrum antifungal activity

7.1. ABSTRACT

Invasive fungal infections are on the rise due to an increased population of critically ill patients as a result of HIV infections, chemotherapies, and organ transplantations. Current antifungal drugs are helpful, but insufficient in addressing the problem of drug-resistant fungal infections. Thus, there is a growing need for novel antimycotics that are safe and effective. The ebselen scaffold has been evaluated in clinical trials and has been shown to be safe in humans. This makes ebselen an attractive scaffold for facile translation from bench to bedside. We have recently reported a library of ebselen-inspired ebsulfur analogues with antibacterial properties, but their antifungal activity has not been characterized. Herein, we repurposed ebselen, ebsulfur, and 32 additional ebsulfur analogues as antifungal agents by evaluating their antifungal activity against a panel of 13 clinically relevant fungal strains. The effect of induction of reactive oxygen species (ROS) by three of these compounds was evaluated. Their hemolytic and cytotoxicity activities were also determined using mouse erythrocytes and mammalian cells. The MIC values of these compounds were in the ranges of 0.02-12.5 μg/mL against the fungal strains tested. Notably, yeast cells treated with our compounds showed the accumulation of ROS, which may further contribute to the growth
inhibitory effect against fungi. This study provides new lead compounds for the development of antimycotic agents.

7.2. INTRODUCTION

Fungal infections have become an emerging public health threat mainly due to the increasing size of the immunocompromised patient population. This population includes patients with AIDS, primary immune deficiency, and those who are immunocompromised due to chemotherapy or organ and bone marrow transplantation. Globally, *Candida* species are the predominant causes of invasive systemic fungal infections with the prevalence reported at 6.9 cases per 1000 patients. In the United States, *Candida* infections rank fourth among all hospital-acquired systemic infections in intensive care units. In most population-based studies, *Candida* infections represent the seventh to tenth most common bloodstream infections. Additionally, many patients are now infected with other fungal species including *Aspergillus fumigatus*, *Aspergillus nidulans*, and *Cryptococcus neoformans*.

Common therapeutic classes used to treat systemic fungal infections include azoles (e.g., fluconazole (FLC), itraconazole (ITC), posaconazole (POS), and voriconazole (VOR)), polyenes (e.g., amphotericin B (AmB), nystatin (NYS), and candicidin (CAN)), and echinocandins (e.g., micafungin, caspofungin, and anidulafungin). These drugs function by different mechanisms of action: (i) inhibition of the cytochrome P450 enzyme 14α-demethylase (azoles), (ii) introduction of transmembrane channel leading to monovalent ion leakage (polyenes), and (iii) inhibition of synthesis of glucan in the fungal cell wall via the enzyme 1,3-β-glucan synthase (echinocandins).
Due to improper usage of these antifungal agents, more drug-resistant fungal strains have evolved. Specifically, these improper usages include insufficient dosages and durations of treatment. Additionally, new evidence suggests that antibacterials also contribute to this development of fungal resistance. Overall, fungal resistance is still relatively uncommon, but this problem is on the rise and expected to become a major healthcare problem. Thus, we have a critical need for the development of novel antifungal compounds.

Currently, three strategies to overcome antifungal drug resistance have been employed. The first strategy is the development of compounds with novel mechanisms of action distinct from previous antifungal agents. For instance, compound E1210 was discovered as a novel first-in-class antifungal compound by the Tsukuba Research Laboratories of Eisai Co., Ltd. This compound was discovered to inhibit fungal glycosylphosphatidylinositol (GPI) biosynthesis and validated in murine models of candidiasis, aspergillosis, and fusariosis. The second strategy is the combination of two antifungal agents. In the literature, there have been plenty of examples using two compounds in conjunction to produce synergistic antifungal activity and reduce resistance as well as toxicity. Specifically, in patients diagnosed with cryptococcal meningitis, the combination therapy of flucytosine and AmB was shown to be essential for successful clinical outcomes. Recently, it was also found that a combination of azoles and analogues of the aminoglycoside antibiotics tobramycin and kanamycin B resulted in favorable synergistic effects against drug-resistant Candida albicans strains. Lastly, the third strategy is the repurposing of existing compounds for new applications. For example,
the decongestant drug octodrine was identified as a broad-spectrum antifungal compound.\textsuperscript{264} In this study, we employed a combination of the first and third strategy to address the problem of antifungal drug resistance. We originally attempted to utilize all three strategies, but found that our novel compounds did not display synergy with currently used antifungal agents.

Ebselen (1, Figure 7.1) is an organoseleno compound, which has completed phase I clinical trial for general safety in human use. Ebselen (1) has very diverse therapeutic applications and has been studied in several clinical trials.\textsuperscript{265} During the phase I study, up to 1,600 mg of ebselen (1) was dosed orally and found to be very well tolerated compared to placebo in 32 healthy male and female subjects.\textsuperscript{171} Ebselen (1) is currently in phase II clinical trials for the treatment of chemotherapy-induced hearing loss and Meniere’s disease (\url{http://clinicaltrials.gov}). Furthermore, ebselen (1) completed a 300-patient phase III clinical trial for cerebral ischemia in Japan.\textsuperscript{169} The ebsulfur or 1,2-benzisothiazol-3(2H)-one scaffold has been demonstrated to have a very narrow spectrum of antibacterial activity (only really being active against methicillin-resistant \textit{Staphylococcus aureus} (MRSA)), in our previous work.\textsuperscript{266} We hypothesized that ebsulfur (2a, Figure 7.1) would have a similar safety profile compared to that of ebselen (1). This scaffold is interesting because it is structurally very similar to ebselen (1, Figure 7.1). Therefore, we hypothesized that ebsulfur (2a, Figure 7.1) and its analogues would have a safety profile comparable to that of ebselen (1). In the literature, the antifungal activity of the ebsulfur scaffold had not been well characterized. Herein, we explored the antifungal properties of ebselen (1) as well as ebsulfur (2a) and 32 of its analogues (2b-4n, Figure 7.1) by using a combination of minimum inhibitory concentration (MIC) study, time-kill study, and reactive oxygen species (ROS) assays. The
safety of these compounds was also assessed and compared to ebselen (1) via mammalian cytotoxicity and hemolytic assays. Our study provides us with a better understanding of the structure-activity-relationship (SAR) of ebselen (1) and the 1,2-benzisothiazol-3(2H)-one scaffold as well as their potential as a new class of antifungal agents.
**Figure 7.1.** Chemical structures of our library featuring ebselen (1), ebsulfur (2a), and 32 ebsulfur analogues.
7.3. RESULTS AND DISCUSSION

7.3.1. Antifungal activity

Compounds 1-3a-o and 4e-n were evaluated for whole-cell activity against a panel of clinically relevant fungal strains (Table 7.1). Our library of compounds featured ebselen (1) and ebsulfur (2a) as the main scaffolds. From the ebsulfur scaffold, the library was further organized into three sub-series: analogues with aromatic substituents (2 series, 2a-o), analogues with aliphatic substituents (3 series, 3a-o), and oxidized sulfoxide analogues (4 series, 4e, 4f, and 4n). Series 2 contained aromatic substituents such as mono- and disubstituted phenyl rings (2a-k), naphthyl (2l), and nitrogen-containing aromatic heterocycles (2m-o). Series 3 contained analogues with substituents such as linear alkyl chains (3a-d), branched alkyl chains (3e-g), alkyl with terminal phenyl ring (3h-j), aliphatic rings (3k-n), and adamantyl (3o). In our previous work, we have verified that all the compounds tested were at least 95% pure by NMR and HRMS. We used the commercially available AmB, FLC, ITC, POS, and VOR as positive controls. The MIC values listed for the controls were either tested herein or acquired from some of our previously published manuscripts on unrelated antifungal agents. For the controls, AmB, as expected, was the most active against both Candida and Aspergillus strains with MIC values ranging from 0.98-15.6 mg/mL. Despite its potent antifungal activity, it should be noted that AmB, even with the liposomal formulations, has been well known for its severe and potentially lethal side effects such as nephrotoxicity and hypokalemia. FLC, the most popular and well-tolerated FDA approved antifungal agent, was fairly inactive against our panel of fungal strains with MIC values mostly from >31.2->125 µg/mL (except against Candida parapsilosis ATCC 22019 (strain J), MIC = 1.95 µg/mL). ITC, POS, and VOR displayed similar activity against our
strains with MIC values mostly ranging from <0.03-31.2 μg/mL. The azole compounds, however, are potent inhibitors of human cytochrome P450 enzymes, which somewhat limit their applications due to drug-drug interactions with co-administered drugs.\textsuperscript{268} ITC and VOR are also generally not as well tolerated as FLC.\textsuperscript{241} To effectively evaluate the activities of our compounds, we define poor, good, very good, and excellent activity as $\geq 12.5$ μg/mL, 1.56-6.25 μg/mL, 0.39-0.78 μg/mL, and ≤0.10 μg/mL, respectively. In our broth dilution assays, the concentration of DMSO used is less than 1%, which was previously experimentally determined to not cause any DMSO-related cytotoxicity effect.
Table 7.1. MIC values\textsuperscript{a,b} (in \(\mu\)g/mL) (and \(\mu\)M in parentheses)\textsuperscript{c} determined for compounds 1-4n and for five control antifungal agents (AmB, FLC, ITC, POS, and VOR) against various yeast strains and filamentous fungi.

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<th>Yeast strains</th>
<th>Filamentous fungi</th>
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<tr>
<td>3k</td>
<td>0.39 (1.7)</td>
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<td>Yeast strains:</td>
<td>A = <em>Candida albicans</em> ATCC 10231, B = <em>C. albicans</em> ATCC 64124, C = <em>C. albicans</em> ATCC MYA-2876(S), D = <em>C. albicans</em> ATCC 90819(R), E = <em>C. albicans</em> ATCC MYA-2310(S), F = <em>C. albicans</em> ATCC MYA-1237(R), G = <em>C. albicans</em> ATCC MYA-1003(R), H = <em>Candida glabrata</em> ATCC 2001, I = <em>Candida krusei</em> ATCC 6258, J = <em>Candida parapsilosis</em> ATCC 22019. NOTE: Here, the (S) and (R) indicate that ATCC reports these strains to be susceptible (S)</td>
</tr>
</tbody>
</table>
and resistant (R) to ITC and FLC.
Filamentous fungi: K = Aspergillus flavus ATCC MYA-3631, L = Aspergillus nidulans ATCC 38163, M = Aspergillus terreus ATCC MYA-3633.
Known antifungal agents: AmB = amphotericin B, FLC = fluconazole, ITC = itraconazole, POS = posaconazole, and VOR = voriconazole.
* These values were previously reported in ref #166.
* For yeast strains: MIC-0 values are reported for compounds 1-4n and AmB, whereas MIC-2 values are reported for azoles. For filamentous fungi, MIC-0 values are reported for all compounds.
* Values in µM are presented so that the reader can visualize the potential impact of the varied molecular weights of the compounds.
ND indicates that MIC values were not determined due to solubility issues with the compound.

We first tested ebselen (1) against our panel of Candida strains (A-J). Ebselen (1) displayed good activity against C. albicans ATCC 90819(R) (strain D), C. albicans ATCC MYA-2310(S) (strain E), C. glabrata ATCC 2001 (strain H), C. krusei (strain I), and C. parapsilosis ATCC 22019 (strain J) (1.56-6.25 mg/mL) and poor activity against strains C. albicans ATCC 10231 (strain A), C. albicans ATCC 64124 (strain B), C. albicans ATCC MYA-2876(S) (strain C), C. albicans ATCC MYA-1237(R) (strain F), and C. albicans ATCC MYA-1003(R) (strain G) (≥12.5 µg/mL). When compared to the controls, these MIC values were generally better than the MIC values of the azoles (except against strains A and H-J), but were worse than those of AmB. Next, we evaluated ebsulfur (2a) to gain insight into the importance of the sulfur atom for antifungal activity. Ebsulfur (2a) displayed a very similar anti-Candida profile to that of ebselen (1). In fact, we noticed that in general, they displayed good and poor activity against the same Candida strains. With the exception of strain J, 2a was active against strains D, E, H, and I (1.56-6.25 µg/mL) and poorly active against strains A, B, C, F, G, and J (≥12.5 µg/mL). This finding demonstrates that replacing the Se atom with the S atom does not compromise antifungal activity.
In search for a chemical modification that would increase the activity of the parent scaffolds, we first decided to assess our analogues with substituents at the para- and meta-positions of the phenyl ring adjacent to the 1,2-benzisothiazol-3(2H)-one (ebsulfur) core. Compounds 2b-d were systematically prepared to contain p-substituted halogen atoms that increased in bulkiness with F < Cl < Br. The SAR comparison for these compounds, however, was flat with all three compounds generally displaying MIC values from 1.56-6.25 µg/mL. We then tested the p-substituted isopropyl analogue (2e), which was previously found to be among the best ebsulfur antibacterial analogues. Compound 2e displayed good MIC values (3.13-6.25 µg/mL) similarly to those of 2b-d. Lastly, we tested the p-ethinyl analogue (2f) and found that 2f displayed mostly poor activity against Candida strains (≥12.5 µg/mL). We speculated that substitution at the para-position was not well tolerated due to steric hindrance with the putative target(s) and that a meta-substitution might show a different pattern of activities.

Based on this assumption, we next examined the m-monosubstituted analogues (2g,h) and the 3,5-disubstituted analogues (2i-k). While the m-Br substitution in 2g was not beneficial at all (≥12.5 µg/mL) against Candida strains, the m-iPr (2h), m,m-di-Br (2i), m,m-di-Me (2j), and m,m-di-OMe (2k) analogues were overall better tolerated with good to moderate MIC values (3.13->12.5 µg/mL). By comparing the p-substituted analogues 2d,e and their m-substituted counterparts 2g,h, we noticed that switching from p-Br (2d) to m-Br (2g) led to loss of activity, whereas switching from p-iPr (2e) and m-iPr (2h) led to compounds which displayed very similar MIC values. Overall, the activity of these compounds appeared to weakly correlate with the number or the positions of the substituents on the phenyl ring.
Moving away from the substituted phenyl strategy, we next explored the analogues with more complex aromatic rings such as the naphthyl (2l), pyridyl (2m,n), and quinolinyl (2o). We found that these relatively bulkier rings were not as well tolerated. Compounds 2l,m displayed good to poor activity (6.25-≥12.5 µg/mL), while compound 2n was poorly active (≥12.5 µg/mL) and 2o could not be evaluated due to solubility issues in our RPMI 1640 medium. In conclusion, the chemical strategy of installing flat aromatic moieties to the core scaffold of the 2 series was able to generate many analogues with mostly good MIC values that are comparable to the parent ebsulfur (2a). We identified compounds 2d, 2e, 2h, and 2i that displayed incrementally improved MIC values when compared to those of ebsulfur (2a), but these improvements were still insufficient. Next, we pondered whether modifications with substituents possessing more geometric freedom and flexibility would be able to generate more substantially potent analogues.

Inspired by the observation that coupling linear alkyl chains to aminoglycoside antibiotics resulted in a significant improvement of their antifungal activity, we systematically generated and examined the antifungal activities of ebsulfur analogues with linear alkyl chains of 5-12 carbons (C₅, C₆, C₈, and C₁₂, 3a-d). Based on our previous work with aminoglycosides where tobramycin and kanamycin analogues with C₁₂ and C₁₄ alkyl chains displayed the best antifungal activity, we hypothesized that our longer C₁₂ ebsulfur analogue (3d) would be the most active. Surprisingly, we observed an opposite trend than that displayed by the aminoglycosides; we found that our shorter C₅ (3a) and C₆ (3b) analogues
were remarkably effective with very good to good MIC values against all *Candida* strains (0.39-1.56 µg/mL). The C₈ analogue (3c) was slightly worse when compared to the C₅ (3a) and C₆ (3b) analogues (specifically against strains A and C), and our C₁₂ analogue (3d) displayed poor MIC values (>12.5 µg/mL), which was the worst amongst the analogues.

We were intrigued to identify the C₅ (3a) and C₆ (3b) linear alkyl analogues as the best anti-*Candida* agents in our library, thus far. Compared to FLC, 3a was 20- to 320-fold more potent in MIC values (except against strain J). When compared to AmB, 3a was 1.25- to 10-fold more active. Since the C₅ and C₆ alkyl chains were extremely well tolerated, we speculated whether our putative target(s) could also tolerate branched alkyl chains with similar chain lengths. We went ahead and evaluated the *iso*-butyl (3e) and *iso*-amyl (3f) analogues. Interestingly, both 3e and 3f were equally as effective as 3a and 3b (0.39-1.56 µg/mL). We then examined the final branched analogue in our library, the *tert*-butyl compound 3g. Compound 3g was also as effective as 3a-f (within 2-fold dilution, 0.78-3.13 µg/mL). Against *Candida* strains, analogues with aliphatic alkyl chains (linear or branched) were found to be very beneficial, which could possibly be attributed to the added rotational flexibility.

As we found that additional methylene groups were highly favorable, we hypothesized that adding methylene linkers (C₁ (3h), C₂ (3i), and C₃ (3j)) in between the 1,2-benzisothiazol-3(2H)-one core and the phenyl ring could provide the added rotational flexibility needed to generate analogues with improved MIC values when compared to the parent compound (2a), which was flat and rigid. The C₁ and C₂-linker analogues (3h,i) indeed had better MIC values
comparing to 2a (0.39-1.56 µg/mL). However, the C₃-linker analogue (3j) was not as potent (1.56-62.5 µg/mL). From these observations, we noticed that addition of flexible methylene linkers were well tolerated up to two carbons.

To further understand the correlation between the flexibility of the R group and activity, we tested our non-aromatic analogues (3k-o) as non-aromatic rings are considerably more flexible than their aromatic counterparts. We first tested the cyclopentyl analogue (3k) and found it to be just as active (0.39-1.56 µg/mL) as some of our best analogues 3a-c described above. On the other hand, the cyclohexyl analogue (3l) still displayed very good to good activity (0.78-3.13 µg/mL), but overall was slightly worse relative to 3k. The cycloheptyl (3m) and cyclooctyl (3n) analogues were also not as good as 3k,l. We also attempted to test whether an ultra-bulky ring such as the adamantane could still be accommodated, but our adamantyl analogue (3o) was not soluble in the RPMI 1640 medium that we used for determination of MIC values. Collectively, the SAR showed a modest preference for smaller size ring, as systematically expanding the ring size resulted in a gradual loss in activity.

Lastly, we tested the oxidized analogues 4e, 4f, and 4n. Oxidizing the sulfur atom to sulfoxide completely abolish antifungal activity. This finding was in accord with our previous report of these compounds as antibacterials and with other reports in the literature that the biological activity of ebselen (1) and ebsulfur (2a) was highly dependent on the Se-N or S-N bonds. Ebselen (1) has been reported to utilize the electrophilic Se-N bond to covalently bind to cysteine residues of multiple enzyme targets.
Invasive aspergillosis is highly correlated with fulminant development and poor prognosis. Compounds with potent anti-Aspergillus activity are considered to be of great valuable. To evaluate the antifungal spectrum of our compounds, we tested them against freshly harvested spores of three Aspergillus strains: A. flavus ATCC MYA-3631 (strain K), A. nidulans ATCC 38163 (strain L), and A. terreus ATCC MYA-3633 (strain M). Overall, our compounds were mostly active against Aspergillus strains and the SAR trends observed from our study with Candida strains were highly translatable to Aspergillus strains. Aromatic analogues (2a-o) remained to have either good to poor activity against strains K-M (1.56-12.5 µg/mL). We were especially intrigued to observe that our linear-chain C₅, C₆, and C₈ analogues (3a-c) displayed excellent activity at ng/mL concentrations (≤0.02-0.20 µg/mL). These results were equivalent or slightly better when comparing them to VOR (0.03-0.24 µg/mL), the gold standard for the treatment of invasive aspergillosis. Other analogues (3e-m) displayed very good activity (0.10-0.78 µg/mL), but they were not as effective as 3a-c. We were also surprised to find that our cyclooctyl analogue (3n) displayed excellent activity against Aspergillus strains (≤0.02-0.05 µg/mL). These values were equivalent to our best anti-Aspergillus analogues 3a-c. We realized that many of our potent analogues were lipophilic. Thus, we investigated the log P values of all the compounds by two log P calculators (ChemDraw and molinspiration) (Table 7.2). In general, we observed that compounds with extremely high or very low lipophilicity did not display good antifungal activity. However, we found that increasing lipophilicity, to a certain extent, generally correlated with increase in antifungal activity.
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<th>Cpd #</th>
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Table 7.2. log P values for all compounds studied.

*a* Calculated from ChemDraw and from Molinspiration (in parenthesis). *b* Indicates that ChemDraw did not provide a log P value.
7.3.2. Time-kill curves

To gain insight for the rate of fungicidal activity of our compounds, we performed time-kill assays with ebsulfur (2a) and our most potent analogue 3a (Figure 7.2). We then compared the results of our compounds to ebselen (1) and the clinically potent and widely used antifungal agent AmB, which also served as our positive control. We selected ebsulfur (2a) because we previously observed that this compound was bacteriostatic and we pondered whether a similar fungistatic effect would be observed.266 We commenced our study by dosing all of our tested compounds (ebselen (1), ebsulfur (2a), 3a, and AmB) at 1× their respective MIC values (Figure 7.2A). For ebsulfur (2a), although the MIC value for ebsulfur (2a) against strain B was greater than 12.5 µg/mL, we decided to test this compound at 12.5 µg/mL because we were concerned that higher concentration may lead to precipitation of the compounds. We observed that ebselen (1) (at 12.5 µg/mL) displayed potent fungicidal activity leading to complete fungal cell death at the 6-h mark, which was even quicker than AmB (at 3.9 µg/mL). Our ebsulfur (2a) (at 12.5 µg/mL) and 3a (at 0.39 µg/mL) displayed fungistatic effects. However, at their 1× MIC, ebsulfur (2a) and 3a were not able to completely inhibit fungal re-growth even after 24 h incubation. Hence, we decided to double the doses of our compounds for our additional time-kill analysis experiments.
Figure 7.2. Time-kill analysis of ebselen (1) (black inverted triangles), ebsulfur (2a) (white triangle), compound 3a (black squares) at 0, 3, 6, 9, 12, and 24 h. A. Cultures were exposed to compounds at 1× their respective MIC values. B. Cultures were exposed to compounds at 2× their respective MIC values. C. Cultures were exposed to compounds at 4× their respective MIC values. Untreated culture (black circles) was used as the negative control and AmB (white circles) was used the positive control. Data was combined from two independent
experiments. The first experiment was conducted with the standard 1× and 2× respective MIC values. The second experiment was needed to supplement the analysis and was performed at 4× their respective MIC values. Each data points were collected in duplicates. The error bars were reported as ± standard deviations.

At 2× MIC (Figure 2B), Ebselen (1) (at 25 µg/mL) and AmB (at 7.8 µg/mL) were completely fungicidal from 3 and 6 h, respectively. At the higher concentration, ebsulfur (2a) (25 µg/mL) interestingly became fungicidal. Conversely, compound 3a (at 0.78 µg/mL) remained fungistatic with a 4-log reduction of fungal cells at approximately the 24-h mark. We were very intrigued by this result and pondered whether fungicidal and fungistatic effects could be concentration dependent. Thus, we further increase the concentration of compound 3a to 4× its MIC value with the hope that 3a would also switch to the fungicidal mode. We performed the assay and actually found that 3a at 4× MIC (at 1.56 µg/mL) still remained fungistatic (Figure 2C). These findings suggested that in order for ebsulfur (2a) and compound 3a to be effective antifungal agents, they would have to be dosed at ≥2× their respective MIC values while albeit at high concentration, ebselen (1) could still be effective at 1× MIC.

Based on the time-kill curves, we observed that ebselen (1) and ebsulfur (2a) were fungicidal at high concentrations (12.5 and 25 µg/mL or 45.6 and 110 µM, respectively) and our best compound from the MIC determination assays, 3a was fungistatic at 0.78 and 1.56 µg/mL (3.5 and 7.0 µM), which were much lower than the fungicidal concentrations of the other
compounds. Fungistatic property does not necessarily mean that compound 3a is not as good as ebselen (1) and ebsulfur (2a) as an antifungal agent because the most popular antifungal compound (FLC) is also fungistatic. This data, however, gave us hints that compound 3a may potentially not be suitable for some specific fungal infections that absolutely require fungicidal effect for clinical efficacy such as cryptococcal meningitis. Additionally, the fact that these benzisothiazolinone compounds exhibit fungicidal effect at high concentrations may mean that they target a different fungal enzymatic pathway when the concentrations are high.

7.3.3. Hemolytic assay

Although we were excited to discover new analogues with improved antifungal activity relatively to ebselen (1), we pondered whether this cytotoxic property could be more selective towards fungal cells than mammalian cells. We were cautiously optimistic that our analogues would still retain some of the good tolerability properties that were highly desirable in the original ebselen (1) scaffold. Previously, our lab studied aminoglycoside analogues with linear alkyl chains and reported that aminoglycoside analogues with linear alkyl chains could potentially be toxic to red blood cells (RBCs) as these RBCs have ultra thin cell membrane and thus, are prone to hemolysis.\textsuperscript{165} Therefore, we decided to evaluate some of our compounds with linear alkyl chains, the C\textsubscript{5} analogue 3a and the C\textsubscript{8} analogue 3c against murine red blood cells (mRBCs) and compare their results to ebselen (1) (Figure 7.3). Although the C\textsubscript{6} analogue 3b was also one of our top analogues, we did not test this analogue because its chain length (C\textsubscript{6}) was extremely similar structurally to the C\textsubscript{5} compound 3a. We also excluded the C\textsubscript{12} analogue 3d because it was completely inactive against fungal strains. Ebsulfur (2a) was chosen because we were interested to verify that the linear alkyl chain would be a required
feature for hemolysis. Most of the compounds (ebselen (1), ebsulfur (2a), and compound 3c) tested did not show any significant hemolytic activity until 15.6 µg/mL (56.9 µM for ebselen (1), 68.6 µM for ebsulfur (2a), and 59.2 µM for 3c). At first glance, compound 3a appeared to be very hemolytic at approximately 3.9 µg/mL (17.6 µM). However, it should be noted that this compound showed remarkable potency against fungal cells. The MIC values of compound 3a were at least 5- to 195-fold lower than the hemolytic concentrations for Candida and Aspergillus strains, respectively. Thus, we could observe that there was still some cytocidal selectivity towards fungal cells. Initially, we expected the C₈ analogue 3c to be more hemolytic because analogues with longer linear alkyl chains tend to perforate cell membranes easily. Thus, it was unexpected to find the C₅ analogue 3a to be hemolytic. This observation led us to speculate that the hemolytic activity was not due to disruption of the cell membrane of the mRBCs, but was simply an artifact of general mammalian cytotoxicity. This prompted us to evaluate our three best compounds 3a, 3b, and 3g (in terms of their overall antifungal activity against both Candida and Aspergillus strains) against two different mammalian cell lines, which have normal cell membranes and are not susceptible to membrane-lytic compounds.
Figure 7.3. Hemolytic assays of ebselen (1), ebsulfur (2a), compound 3a, and compound 3c against murine red blood cells (mRBCs). Ebselen (1), ebsulfur (2a), compound 3a, and compound 3c are represented as black circles, white circles, inverted orange triangle, and upright white triangle, respectively. The data points of ebselen (1), ebsulfur (2a), and compound 3c were previously present in one of our publications and are used here for comparison. Each data point was collected in duplicates. The error bars were reported as ± standard deviations.

7.3.4 Mammalian cytotoxicity assay

Compounds 3a, 3b, and 3g were evaluated for their cytotoxicity against HEK293 and J774 cell lines using a resazurin assay. The concentration of DMSO used in the cytotoxicity assay is 0.1%, which was ensured to not demonstrate any DMSO-related toxicity to the cells. We compared the percentage of surviving cells treated with our analogues versus the percentage of surviving cells treated with ebselen (1) (Figure 7.4). Against the HEK293 cell line (Figure 7.4A), we noticed that all of our analogues (3a, 3b, and 3g) were slightly more toxic but overall, quite comparable to ebselen (1) at all concentrations tested. Overall, for all the tested compounds (ebselen (1), 3a, 3b, and 3g) the IC\textsubscript{50} values were greater than 10 µg/mL.
This corresponds to IC$_{50}$ values of 36.5 µM for ebselen (1), 45.2 µM for 3a, 42.5 µM for 3b, and 48.2 µM for 3g. We verified that the cytotoxicity data of ebselen (1) found in our study was fairly consistent with other reported in vitro mammalian cytotoxicity studies of ebselen (1). Given the good tolerability of ebselen (1) during clinical trials, it was perplexing to us that ebselen (1) displayed some in vitro cytotoxicity. The HEK293 cell line was chosen because we were interested to determine whether our compounds could potentially cause kidney injury. The kidney is a highly perfused organ and comes in contact with many compounds due to renal excretion. Thus, many compounds such as AmB are highly nephrotoxic and cause great burden to patients with compromised renal function.

Next, we evaluated our compounds against J774 (Figure 7.4B), a murine macrophage cell line. This cell line was selected because macrophages are the first-line of defense against fungal infection and we were hopeful that our compounds would not interfere with the survival of host macrophages. Against the J774 cell line, we observed a trend similar to the HEK293 cell line and found that our analogues (3a and 3b) were slightly more toxic but still comparable to ebselen (1) with IC$_{50}$ values approximately at 10 µg/mL. It was interesting that compounds 3g actually did not show any toxicity at all up to 5 µg/mL (24.1 µM). Typically, the difference between the toxic dose in mammalian cells and the fungal MIC value should be at least 10-fold. The fact that this was the case for our most potent antifungal compounds is highly encouraging.
We acknowledge that there are concerns in the literature regarding the highly reactive benzisothiazolinone moiety of the ebsulfur (2a) scaffold, which possibly explains the toxicity effects observed against mammalian cell lines.\textsuperscript{276} This concern is valid considering that the parent compound ebselen (1) has also been found to target different proteins.\textsuperscript{265} However, we argue that this scaffold still merits further consideration as a potential antifungal candidate based on two particular reasons. First, since many potent antifungal compounds are only available intravenously, there is currently a dire clinical need for orally active antifungals to assist the azoles as an alternative option for step-down therapy.\textsuperscript{221} These azoles often complicate drug dosing due to interactions with the metabolism of many drugs and have experienced an increased rate of resistance.\textsuperscript{277-278, 279} The ebsulfur analogues would most likely be orally active due to its similarity to ebselen (1), which was successfully administered orally.\textsuperscript{168, 171} Secondly, while we are also concerned about the high reactivity of the benzisothiazolinone moiety towards non-specific cysteine residues, ebselen (1) with the benzisoselenazolinone moiety has been shown to be well-tolerated during clinical trials. There are also examples of other clinically successful small-molecule drugs with highly reactive pharmacophores within the FDA-approved chemical space. Some of these compounds are penicillin, fosfomycin, or bendamustine.\textsuperscript{280} Compounds 3a and 3b displayed MIC values against \textit{Candida} strains at 780 ng/mL and \textit{Aspergillus} strains at $\leq$20 ng/mL, which are much lower than their IC\textsubscript{50} values against mammalian cells. This could potentially be due to the fact that 3a and 3b may have a fungal-specific mechanism of action at lower concentrations. To gain insights on the mechanism of action, we decided to first look at ROS induction of these compounds.
**Figure 7.4.** Mammalian cell cytotoxicity of ebselen (1) (yellow bars), and compounds 3a (orange bars), 3b (turquoise bars), and 3g (purple bars) against A. HEK 293 cell line and B. J774 cell line. Triton-X 100® (1%, v/v) was used as the positive control (data not shown). Each data point was collected in quadruplicates. The error bars were reported as ± standard deviations.

### 7.3.5. ROS production

Recently, we showed that ebselen (1) and our ebsulfur analogues with antibacterial activity were highly correlated with ROS production in MRSA bacterial cells. Another group independently reported that ebselen (1) induced ROS-mediated cytotoxicity in *Saccharomyces cerevisiae* via inhibition of glutamate dehydrogenase. Thus, we sought to determine
whether our analogues would also induce ROS against *C. albicans* via inhibition of the *C. albicans* glutamate dehydrogenase. Ebselen (1), ebsulfur (2a), and compound 3a were tested against *C. albicans* ATCC 10231 cells (strain A) at 1× and 2× their respective MIC values. 2′,7′-dichlorodihydrofluorescin diacetate (DCFH-DA) was then used to detect and visualize ROS production (Figure 7.5). As a positive control, we treated cells with H$_2$O$_2$, which is an inducer of hydroxyl radical formation. After 1-h treatment, we found that all the compounds tested and the positive control were highly fluorescent, which indicated ROS induction. We also compared the samples that were treated with different doses of compounds (1× and 2× their respective MIC values) and observed that the amount ROS induction could potentially be concentration dependent. It is certainly possible that this ROS induction in *C. albicans* spp. is due to inhibition of *C. albicans* glutamate dehydrogenase as we hypothesized. However, this finding still does not rule out other potential drug targets such as fungal enzymes responsible for ROS regulation or it is simply a downstream secondary effect as the ebselen (1) and ebsulfur (2a) scaffolds inhibit enzymes that are unrelated to ROS generation. Assays to determine the specific molecular target(s) of our best compound, 3a, are ongoing and will be the subject of future reports.
Figure 7.5. ROS induction assay of ebselen (1), ebsulfur (2a), and compound 3a against C. albicans ATCC 10231 (strain A). Candida cells were treated with no drug (negative control), 1 mM of H$_2$O$_2$ (positive control), or ebselen, 2a, and 3a at their 1x and 2x respective MIC values for 1 h at 37 °C. DCFH-DA (40 µg/mL) was added to detect ROS and the samples were analyzed using a Zeiss Axovert 200M fluorescence microscope.
7.4. CONCLUSION

In summary, we expanded our knowledge of this scaffold in terms of the antifungal activity against a panel of clinically relevant Candida and Aspergillus strains. In light of our SAR analysis, we identified that the addition of flexible chemical moieties to the 1,2-benzisothiazol-3(2H)-one scaffold is a viable strategy to generate analogues with potent antifungal activities. Interestingly, the majority of our compounds displayed comparable or, in most cases, enhanced antifungal activities against all fungal strains when compared to ebselen (1) and the reference drugs used in this study. Although, our best compound (3a) exhibited some hemolytic activity, its effect on nucleated mammalian cells was found to be in the acceptable range considering their antifungal efficacies. Finally, our preliminary study on mechanism of action indicated that the growth inhibitory effect of fungi by these compounds might be due to the elevating concentration of ROS in the yeast cells. However, the biological activities of the ebselen (1) and ebsulfur (2a) scaffolds are complex due to their general electrophilicity. An extensive study is required to identify the specific mode of action against fungal strains. Once the mechanism of action is established, we feel that this scaffold would have merit for further evaluation in a fungal infected murine model.

7.5. MATERIALS AND INSTRUMENTATION

7.5.1. Antifungals and equipment

The antifungal agents amphotericin B (AmB), fluconazole (FLC), itraconazole (ITC), posaconazole (POS), and voriconazole (VOR) were obtained from AK Scientific (Union City, CA, USA) and used without further purification. AmB, FLC, ITC, POS, and VOR were
dissolved in DMSO at a final concentration of 5 mg/mL. All these antifungal agent stocks were stored at -20 °C. Fungal MIC determination experiments were performed using untreated 96-well plates (Corning). Cells were counted either by using a hemocytometer (Hausser Scientific, Horsham, PA, USA) or by measuring optical density at attenuation of 600 nm (OD\(_{600}\)) by using a Genesys 20 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Spectrophotometric and colorimetric measurements in 96-well plates were performed using a SpectraMax M5 spectrometer (Molecular Devices, Sunnyvale, CA, USA).

### 7.5.2. Fungal strains

The yeast strains *Candida albicans* ATCC 10231 (strain A), *C. albicans* ATCC 64124 (strain B), and *C. albicans* ATCC MYA-2876(S) (strain C) were kindly provided by Dr. Jon Y. Takemoto (Utah State University, Logan, UT, USA). *C. albicans* ATCC MYA-90819(R) (strain D), *C. albicans* ATCC MYA-2310(S) (strain E), *C. albicans* ATCC 1237(R) (strain F), *C. albicans* ATCC MYA-1003(R) (strain G), *Candida glabrata* ATCC 2001 (strain H), *Candida krusei* ATCC 6258 (strain I), and *Candida parapsilosis* ATCC 22019 (strain J) were obtained from the American Type Culture Collection (ATCC, Manasas, VA, USA). The (S) and (R) indicate that ATCC reports these strains to be susceptible (S) and resistant (R) to ITC and FLC. The filamentous fungal strains *Aspergillus flavus* ATCC MYA-3631 (strain K), and *Aspergillus terreus* ATCC MYA-3633 (strain M) were also obtained from the American Type Culture Collection (ATCC, Manasas, VA, USA). *Aspergillus nidulans* ATCC 38163 (strain L) was kindly provided by Dr. Jon S. Thorson (University of Kentucky, Lexington, KY, USA), respectively. Yeast strains were cultured at 35 °C. Filamentous fungal strains were cultured at 25 °C and the spores were harvested. All fungal strains were cultured in RPMI 1640 medium.
(catalog # R6504, Sigma-Aldrich Chemical Co., St. Louis, Mo.) buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) buffer (Sigma-Aldrich Chemical Co.).

7.5.3. Mammalian cells
The human embryonic kidney cell line HEK-293 (ATCC CRL-1573) and the murine macrophage cell line J774A.1 (ATCC TIB-67) were kindly provided by Dr. Matthew S. Gentry and Dr. David J. Feola (University of Kentucky, Lexington, KY, USA), respectively. The HEK-293 cell line was grown in Dulbecco’s Modified Eagle’s Medium (DMEM) (ATCC, Manasas, VA, USA) with 10% fetal bovine serum (FBS) (ATCC, Manasas, VA, USA) and 1% Pen/Strep (ATCC, Manasas, VA, USA). The J774A.1 cell line was grown under the same conditions, except that the medium used was a different type of DMEM (catalog # 30-2002, ATCC, Manasas, VA, USA). The HEK-293 cell line was passaged by trypsinization with 0.05%-trypsin-0.53 mM EDTA (ATCC, Manasas, VA, USA). The J774A.1 cell line was passaged mechanically by cell scrapers (ATCC, Manasas, VA, USA). Cell confluency was observed by using a Nikon Eclipse TS100 microscope (Minato, Tokyo, Japan).

7.6. METHODS

7.6.1. Determination of MIC values against fungal strains
The MIC values against fungal strains were determined based on a previously published protocol. MIC values for ebsulfur (2a) analogues against fungal cells were evaluated in 96-well plates as described in the CLSI document M27-A3 with minor modifications. Some of the fungal strains, such as C. albicans ATCC 64124 (strain B) tend to produce pseudohyphae
(filaments) in RPMI 1640 medium, which was found to hinder cell counting when using a hemocytometer. Therefore, potato dextrose broth (PDB) was used to grow the yeast inocula of all strains tested, which were later diluted in RPMI 1640 medium to perform determination of MIC values. Minor modifications included growing yeast cells in PDB for 24-48 h at 35 °C at 200 rpm, diluting in RPMI 1640 medium to a concentration of $1 \times 10^6$ cells/mL (as determined by using a hemocytometer or an OD$_{600}$ of 0.12) and using a final inoculum size of $5 \times 10^3$ CFU/mL for all the assays. The tested compounds (10 mg/mL) were diluted to the working stocks (500 µg/mL) by addition of DMSO. Two-fold serial dilution of the working stocks was prepared by addition of RPMI 1640 medium (100 µL) and cell suspension (100 µL) to 96-well microtiter plate to achieve final drug and inoculum concentrations ranging from 12.5-0.02 µg/mL and $5 \times 10^3$ CFU/mL, respectively. Plates were incubated for 48 h at 35 °C. The MIC values for all tested compounds studied were defined as the lowest drug concentration that inhibits the visible growth of fungal strains after a 48-h incubation period. MIC assays for the spore-forming filamentous fungi, such as strain *A. flavus* ATCC MYA-3631 (strain K), were performed in a similar fashion. The filamentous fungal strains were first cultured at 25 °C on potato dextrose agar (PDA) plates for 3-5 days or until confluent. We collected the spores by washing the surface of the agar plates with ddH$_2$O (5 mL) and then isolated the spores by gravity filtration (the spores are H$_2$O soluble). The spores were then counted by using a hemocytometer and added to the MIC assays to achieve a final concentration of $5 \times 10^3$ cells/mL. Researchers working with spores should wear a facemask to prevent spore inhalation. These MIC data are presented in Table 7.1.
7.6.2. Mammalian cytotoxicity assays

Mammalian cytotoxicity assays were performed as previously described with minor modifications (Figure 7.4). The HEK-293 and J774A.1 cell lines were grown in various Dulbecco’s Modified Eagle’s Medium (DMEM) (see mammalian cell lines section above) with 10% fetal bovine serum (FBS) and 1% Pen/Strep at 37 °C with 5% CO₂. The confluent cells were either trypsinized with 0.05%-trypsin-0.53 mM EDTA (HEK-293 cell line) or mechanically removed by cell scrapers (J774A.1 cell line). The cells were transferred into 96-well microtiter plates at a density of 1 × 10⁴ cells/mL (HEK-293 cell line) or 2 × 10⁴ cells/mL (J774A.1 cell line) and were grown for 16 h overnight. The following day, the media were replaced by fresh media (100 µL) containing no compound (negative control), triton-X 100® (positive control) (1%, v/v), and serially diluted ebselen (1), 3a, 3b, and 3g at final concentrations of 10-0.02 µg/mL. Every well contained 0.1% DMSO, which is not toxic against these mammalian cell lines. The cells were incubated with tested compounds for another 24 h at 37 °C with 5% CO₂. Cell survival was assessed by resazurin assay. Each well was treated with resazurin (10 µL of a 25 mg/L solution) for 6 h. Live cells produced the highly fluorescent pink dye resorufin, which was detected at λ₅₆₀ absorption and λ₅₉₀ emission by a SpectraMax M5 plate reader. Dead cells remain purple/blue. The percentage of survival rate was calculated by using the following formula: \[
\frac{\text{test value}}{\text{control value}} \times 100
\]. The control value is obtained from the wells, which have cells and resazurin, but no tested compounds.
7.6.3. Assay for reactive oxygen species (ROS) production

ROS production assay was performed as previously described with minor modifications.\textsuperscript{192, 266} The 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) probe was used to measure the production of ROS in fungal cells after treatment of cells with ebselen, ebsulfur (\textit{2a}), and \textit{3a}. Once entering the cells, the DCFH-DA probe is first hydrolyzed to the non-fluorescent 2',7'-dichlorodihydrofluorescein (DCFH) by cellular esterases. After that, DCFH is oxidized to the highly fluorescent 2',7'-dichlorofluorescein (DCF) by intracellular ROS. A colony of \textit{C. albicans} ATCC 10231 (strain \textit{A}) was used to inoculate 5 mL of PDB in a Falcon tube and grown overnight at 35 °C at 200 rpm. In the morning, we diluted the culture by addition of fungal cells (200 µL) to RPMI 1640 medium (800 µL). After that, we added the newly diluted cell suspension (100 µL) to the RPMI 1640 medium (900 µL) containing no drug (negative control) or ebselen, \textit{2a}, and \textit{3a}, at their 1x and 2x MIC values and incubated for 1 h at 37 °C. Glass slides (with 10-15 of each mixture) were prepared and observed in bright field and fluorescence modes (FITC filter set, λ\textsubscript{ex} = 488 nm and λ\textsubscript{em} = 512 nm excitation) using a Zeiss Axovert 200M fluorescence microscope (Figure 7.5).

7.7. ACKNOWLEDGMENTS

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7.8. AUTHORS’ CONTRIBUTIONS

HXN synthesized all compounds.

HXN and SKS performed all biochemical and biological assays.

HXN and SGT analyzed data and wrote the manuscript.
Chapter 8

Ongoing research

8.1. EXPANSION OF THE THIENO[2,3-\textit{D}]PYRIMIDINE LIBRARY

Chapter 4 introduced the thieno[2,3-\textit{D}]pyrimidine library. We were able to further expand this library of compounds and generated a series of analogues. The 4-aminopyrimidine adjacent to the thiophene ring was replaced by a pyrimidone. The following molecules were prepared and added to our library using the protocol that we previously established in Chapter 4 (Figure 8.1). These compounds will help us further understand the SAR of this scaffold against \textit{Eis} as well as their unique toxicity activity against \textit{Mtb} cells.
Figure 8.1. Synthetic scheme to expand the thieno[2,3-\textit{d}]pyrimidine library.

8.1.1. Preparation of compounds 3-4

Synthesis of compounds 3-4 was done following a modified published protocol.\textsuperscript{155} Ethyl cyanoacetate (1 eq) and sulfur (1 eq) were added to a solution of a cycloalkyl ketone (1 eq) in EtOH (30 mL). Morpholine (5 mL) was added to the mixture. After stirring at rt for 1-15 h, the reaction was quenched by the addition of ddH\textsubscript{2}O. The product was filtered, washed with ddH\textsubscript{2}O, and dried to afford the desired product.
Preparation of compound 3. The known compound 3 was prepared as described above. Ethyl cyanoacetate (4.74 mL, 44.6 mmol), sulfur (1.43 g, 59.5 mmol), 4-methyl cyclohexanone (compound 1) (5.47 mL, 44.6 mmol), and morpholine (5 mL) in EtOH (30 mL) were used to afford compound 3 (10.6 g, quant.) as a yellow solid after reacting for 15 h. This product was without any further purification: $^1$H NMR (400 MHz, (CDCl$_3$) δ 5.90 (br s, 2H), 4.25 (q, $J$ = 7.2 Hz, 2H), 2.88-2.81 (m, 1H), 2.62-2.49 (m, 2H), 2.15-2.07 (m, 1H), 1.88-1.77 (m, 2H), 1.31 (t, $J$ = 7.2 Hz, 3H), 1.02 (d, $J$ = 4.0 Hz, 3H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 166.3, 162.0, 132.3, 117.4, 105.9, 59.6, 32.9, 31.3, 29.7, 26.9, 21.7, 14.7.

Preparation of compound 4. The known compound 4 was prepared as described above. Ethyl cyanoacetate (4.20 mL, 39.6 mmol), sulfur (1.27 g, 39.6 mmol), 4-ethyl cyclohexanone (compound 2) (5.60 mL, 39.6 mmol), and morpholine (5 mL) in EtOH (30 mL) were used to afford compound 4 (8.0 g, 80%) as a yellow solid after reacting for 15 h. This product was purified via recrystallization in i-OH: $^1$H NMR (400 MHz, CDCl$_3$) δ 5.89 (br s, 2H), 4.24 (q, $J$ = 7.2 Hz, 2H), 2.89-2.82 (m, 1H), 2.60-2.50 (m, 2H), 2.16-2.08 (m, 1H), 1.90-1.83 (m, 1H), 1.65-1.58 (m, 1H), 1.42-1.26 (m, 3H), 1.31 (t, $J$ = 7.2 Hz, 3H), 0.93 (t, $J$ = 7.6 Hz, 3H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 166.1, 161.7, 132.4, 117.3, 105.6, 59.3, 36.3, 30.5, 28.9, 28.7, 26.8, 14.4, 11.5.
8.1.2. Preparation of compounds 7-8

Synthesis of compounds 5-6 was done following a published protocol\(^{155}\). Benzoyl isothiocyanate (1 eq) was added to a solution of a thiophene derivative (1 eq) in anhydrous 1,4-dioxane (20 mL). After stirring at rt for 1-3 h, the product was filtered and washed with a 4:1/Hexanes:EtOAc mixture to afford the desired product, which was used in the next step without any further purification.

Synthesis of compounds 7-8 was done following a published protocol\(^{155}\). 2 N NaOH (50 mL) was added to a mixture of benzamides (38.3 mmol) in EtOH (70 mL). After refluxing at 100 °C for 30 min to 2 h, the solution was cooled to rt and then neutralized in an ice bath by adding 10% AcOH until the pH reached 7. The resultant precipitate was filtered and washed with a 4:1/H\(_2\)O:EtOH mixture to afford the desired product.

**Preparation of compound 7.** Compound 7 was prepared as described above. 2 N NaOH (50 mL) and compound 5 (5.14 g, 12.8 mmol) in EtOH (50 mL) were used to afford compound 7 (2.64 g, 82%) as a yellow solid after refluxing for 2 h: \(^1\)H NMR (400 MHz, (CD\(_3\))\(_2\)SO) \(\delta\) 13.29 (s, 1H), 12.27 (s, 1H), 2.97-2.90 (m, 1H), 2.78-2.70 (m, 1H), 2.67-2.55 (m, 1H), 2.28-2.20 (m, 1H), 1.91-1.77 (m, 2H), 1.40-1.25 (m, 1H), 1.02 (d, \(J = 6.8\) Hz, 3H).

**Preparation of compound 8.** Compound 8 was prepared as described above. 2 N NaOH (50 mL) and compound 5 (5.00 g, 12.0 mmol) in EtOH (50 mL) were used to afford compound 7 (1.61 g, 50%) as a yellow solid after refluxing for 2
h: $^1$H NMR (400 MHz, (CD$_3$)$_2$SO) $\delta$ 3.07-2.97 (m, 1H), 2.83-2.75 (m, 1H), 2.73-2.60 (m, 1H), 2.35-2.23 (m, 1H), 2.00-1.90 (m, 1H), 1.75-1.63 (m, 1H), 1.50-1.30 (m, 3H), 0.98 (t, $J = 7.2$ Hz, 3H).

8.1.3. Preparation of compounds 9-13

The synthesis of these compounds was done following a published thiol alkylation protocol.$^{158}$ Cs$_2$CO$_3$ (1.1 eq) and TBAI (1 eq) were added to a solution of thieno[2,3-\textit{d}]pyrimidin-4amines (1 eq) in anhydrous DMF and stirred at 80 °C for 30 min. Alkyl halide (1.5 eq) was added, and the mixture was heated at 80 °C for an additional 1-4 h. The reaction was cooled to rt and quenched by addition of aq. NaHCO$_3$, extracted with EtOAc three times, and washed with brine three times. The organic layer was then dried over MgSO$_4$ and evaporated to dryness under reduced pressure to give a residue, which was purified by column chromatography to afford the desire product.

Preparation of compound 9. Compound 9 was prepared as described above. Compound 7 (0.10 g, 0.40 mmol), Cs$_2$CO$_3$ (0.13 g, 0.40 mmol), TBAI (0.15 g, 0.40 mmol), and 1-(3-chloropropyl)piperidine monohydrochloride (0.12 g, 0.59 mmol) in anhydrous DMF (8 mL) were used to afford compound 9 (103 mg, 69%, $R_f$ 0.48 in 9:1/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7 mL/L)) as a white solid after purification by column chromatography (SiO$_2$, 9:1/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7 mL/L)): $^1$H NMR (400 MHz, CD$_3$OD) $\delta$ 3.70-3.45 (m, 2H), 3.43-3.35 (m, 1H), 3.31 (t, $J = 6.8$ Hz, 2H), 3.20-3.10 (m, 2H), 2.87-2.75 (m, 3H), 2.40-
2.25 (m, 4H), 2.15-1.85 (m, 6H), 1.70-1.50 (m, 2H), 1.47-1.35 (m, 2H), 1.07 (d, $J = 6.4$ Hz, 3H); $^{13}$C NMR (100 MHz, (CD$_3$)$_2$SO) δ 162.7, 158.2, 155.5, 130.2, 129.8, 118.9, 55.1, 52.3, 32.3, 30.0, 28.8, 27.2, 24.9, 23.9, 23.0, 22.0, 21.2.

**Preparation of compound 10.** Compound 10 was prepared as described above. Compound 7 (0.10 g, 0.40 mmol), Cs$_2$CO$_3$ (0.13 g, 0.40 mmol), TBAI (0.15 g, 0.40 mmol), and 2-bromo-$N$-(2-dimethylaminoethyl)acetamide (0.13 g, 0.53 mmol) in anhydrous DMF (8 mL) were used to afford compound 10 (57 mg, 38%, $R_f$ 0.26 in 9:1/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7 mL/L)) as a white solid after purification by column chromatography (SiO$_2$, 9:1/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7 mL/L)): $^1$H NMR (400 MHz, CD$_3$OD) δ 4.01 (s, 2H), 3.64 (t, $J = 6.0$ Hz, 2H), 3.30 (t, $J = 6.0$ Hz, 2H), 3.10-3.00 (m, 1H), 2.92 (s, 6H), 2.82-2.66 (m, 2H), 2.34-2.24 (m, 1H), 1.94-1.84 (m, 2H), 1.44-1.32 (m, 1H), 1.08 (d, $J = 6.4$ Hz, 3H); $^{13}$C NMR (100 MHz, CD$_3$OD) δ 171.6, 165.0, 160.4, 155.7, 132.6, 131.7, 120.5, 58.2, 44.0, 36.3, 34.9, 33.8, 31.6, 30.6, 26.3, 21.8.

**Preparation of compound 11.** Compound 11 was prepared as described above. Compound 7 (0.10 g, 0.40 mmol), Cs$_2$CO$_3$ (0.13 g, 0.40 mmol), TBAI (0.15 g, 0.40 mmol), and 2-bromo-$N$-(2-diethylaminoethyl)acetamide (0.16 g, 0.58 mmol) in anhydrous DMF (8 mL) were used to afford compound 11 (42 mg, 26%, $R_f$ 0.51 in 9:1/CH$_2$Cl$_2$:MeOH with NH$_4$OH (7 mL/L)) as a white solid after purification by column chromatography (SiO$_2$, 9:1/CH$_2$Cl$_2$:MeOH with
NH₄OH (7 mL/L): ¹H NMR (400 MHz, (CD₃)₂SO) δ 8.40 (br t, 1H), 3.91 (s, 2H), 3.32 (q, J = 6.4 Hz, 2H), 3.06-2.96 (m, 1H), 2.90-2.80 (m, 6H), 2.75-2.60 (m, 2H), 2.40-2.20 (m, 1H), 2.00-1.80 (m, 2H), 1.50-1.30 (m, 1H), 1.07 (t, J = 7.2 Hz, 6H), 1.04 (t, J = 6.4 Hz, 3H); ¹³C NMR (100 MHz, (CD₃)₂SO) δ 167.7, 163.0, 158.6, 155.7, 130.6, 130.3, 119.4, 50.6, 46.9, 34.4, 32.7, 30.4, 29.2, 25.3, 21.6, 10.1.

**Preparation of compound 12.** Compound 12 was prepared as described above. Compound 7 (0.10 g, 0.40 mmol), Cs₂CO₃ (0.13 g, 0.40 mmol), TBAI (0.15 g, 0.40 mmol), and 2-bromo-N-[2-(piperidin-1-yl)ethyl]acetamide (0.17 g, 0.59 mmol) in anhydrous DMF (8 mL) were used to afford compound 12 (55 mg, 36%, Rₚ 0.30 in 9:1/CH₂Cl₂:MeOH with NH₄OH (7 mL/L)) as a white solid after purification by column chromatography (SiO₂, 9:1/CH₂Cl₂:MeOH with NH₄OH (7 mL/L)): ¹H NMR (400 MHz, CD₃OD) δ 3.90 (s, 2H), 3.49 (t, J = 6.4 Hz, 2H), 3.12-3.00 (m, 1H), 2.82 (t, J = 6.4 Hz, 2H), 2.80-2.67 (m, 6H), 2.34-2.23 (m, 1H), 1.96-1.82 (m, 2H), 1.66 (p, J = 5.6 Hz, 4H), 1.50 (p, J = 5.6 Hz, 2H), 1.45-1.30 (m, 1H), 1.07 (d, J = 6.8 Hz, 3H); ¹³C NMR (100 MHz, CD₃OD) δ 170.0, 163.9, 160.8, 155.8, 130.5, 130.1, 119.0, 56.7, 53.6, 35.5, 33.5, 32.5, 30.3, 29.2, 25.0, 24.0, 22.5, 20.4.

**Preparation of compound 13.** Compound 13 was prepared as described above. Compound 8 (0.10 g, 0.38 mmol), Cs₂CO₃ (0.12 g, 0.38 mmol), TBAI (0.14 g, 0.38 mmol), and 1-(3-chloropropyl)piperidine monohydrochloride (0.11 g, 0.56 mmol) in anhydrous DMF (8 mL)
were used to afford compound 13 (24 mg, 16%, Rf 0.30 in 9:1/CH₂Cl₂:MeOH with NH₄OH (7 mL/L)) as a white solid after purification by column chromatography (SiO₂, 9:1/CH₂Cl₂:MeOH with NH₄OH (7 mL/L)): ¹H NMR (400 MHz, CD₃OD) δ 3.87 (s, 2H), 3.41 (t, J = 6.4 Hz, 2H), 3.20-3.05 (m, 1H), 2.90-2.80 (m, 1H), 2.80-2.70 (m, 1H), 2.62 (t, J = 6.4 Hz, 2H), 2.60-2.50 (m, 4H), 2.40-2.30 (m, 1H), 2.05-1.95 (m, 1H), 1.80-1.65 (m, 1H), 1.57 (p, J = 5.6 Hz, 4H), 1.53-1.35 (m, 5H), 1.00 (t, J = 7.6 Hz, 3H); ¹³C NMR (100 MHz, CD₃OD) δ 171.8, 166.1, 163.3, 158.1, 132.4, 132.3, 121.0, 58.9, 55.7, 38.0, 37.8, 35.4, 32.4, 30.3, 30.0, 27.0, 26.4, 25.0.

8.2. EBSELEN ANALOGUES

Previously, an ebsulfur analogue library was generated in Chapter 5. However, we were also interested in comparing those to their selenium counterparts. To complement our previous ebsulfur studies, a series of ebselen analogues were generated and biological evaluations of these molecules are ongoing (Figure 8.2).

![Figure 8.2. Synthetic scheme to generate ebselen analogues.](image-url)
8.2.1. Preparation of 2-Iodobenzamides (compounds 15-19)

**Preparation of compound 15.** To a solution of aniline (0.40 mL, 4.50 mmol) and Et₃N (0.90 mL, 9 mmol) in anhydrous CH₂Cl₂ (20 mL), 2-iodobenzoyl chloride (0.5 mL, 3.75 mmol) was added dropwise. The product was washed with 1 N HCl (50 mL) and a saturated solution of sodium bicarbonate (50 mL) and dried with MgSO₄. The organic layer was removed *in vacuo* and the crude product was purified via recrystallization in *i*-PrOH to afford the known compound 15²⁸³ (299 mg, 25%) as a pink solid: ᵃ¹H NMR (400 MHz, CDCl₃) δ 7.89 (d, J = 8.4 Hz, 1H), 7.62 (d, J = 8.0 Hz, 2H), 7.51 (d, J = 6.8 Hz, 1H), 7.48-7.30 (m, 4H), 7.20-7.10 (m, 2H); ᵃ¹³C NMR (100 MHz, CDCl₃) δ 167.4, 142.3, 140.2, 137.7, 131.7, 129.3, 128.7, 128.5, 125.1, 120.3, 92.6.

**Preparation of compound 16.** To a solution of benzylamine hydrochloride (0.65 g, 4.50 mmol) and Et₃N (0.90 mL, 9 mmol) in anhydrous CH₂Cl₂ (20 mL), 2-iodobenzoyl chloride (0.5 mL, 3.75 mmol) was added dropwise. The product was washed with 1 N HCl (50 mL) and a saturated solution of sodium bicarbonate (50 mL) and dried with MgSO₄. The organic layer was removed *in vacuo* and the crude product was purified via recrystallization in *i*-PrOH to afford the known compound 16²⁸³ (639 mg, 51%) as a white solid: ᵃ¹H NMR (400 MHz, CD₃OD) δ 7.88 (d, J = 8.4 Hz, 1H), 7.62 (d, J = 8.0 Hz, 2H), 7.51 (d, J = 6.8 Hz, 1H), 7.48-7.37 (m, 3H), 7.24-7.28 (m, 1H), 7.14 (app. tt, J = 7.6, 2.0 Hz, 1H), 4.53 (s, 2H); ᵃ¹³C NMR (100 MHz, CDCl₃) δ 169.4, 142.3, 140.1, 137.7, 131.4, 129.0, 128.5, 128.4 (2 carbons), 127.9, 92.6, 44.4.
**Preparation of compound 17.** To a solution of phenethylamine (1.1 mL, 9.0 mmol) and Et₃N (2.0 mL, 15.0 mmol) in anhydrous CH₂Cl₂ (20 mL), 2-iodobenzoyl chloride (1.0 mL mL, 3.75 mmol) was added dropwise. The product was washed with 1 N HCl (50 mL) and a saturated solution of sodium bicarbonate (50 mL) and dried with MgSO₄. The organic layer was removed *in vacuo* to afford the known compound 17 (2.6 g, quantitative yield) as a yellow solid: $^1$H NMR (400 MHz, CD₃OD) $\delta$ 7.86 (d, $J$ = 8.0 Hz, 1H), 7.38 (app. tt, $J$ = 8.0, 1.2 Hz, 1H), 7.32-7.24 (m, 4H), 7.24-7.16 (m, 2H), 7.20-6.80 (m, 1H), 3.56 (t, $J$ = 8.0 Hz, 2H), 2.92 (t, $J$ = 7.6 Hz, 2H); $^{13}$C NMR (100 MHz, CDCl₃) $\delta$ 169.6, 142.4, 140.0, 138.8, 131.2, 129.1, 129.0, 128.9, 128.3, 126.8, 92.6, 41.3, 35.6.

**Preparation of compound 18.** To a solution of isoamylamine (1.0 mL, 9.0 mmol) and Et₃N (2.0 mL, 15.0 mmol) in anhydrous CH₂Cl₂ (20 mL), 2-iodobenzoyl chloride (1.0 mL mL, 3.75 mmol) was added dropwise. The product was washed with 1 N HCl (50 mL) and a saturated solution of sodium bicarbonate (50 mL) and dried with MgSO₄. The organic layer was removed *in vacuo* to afford the known compound 18 (2.4 g, quantitative yield) as a yellow solid: $^1$H NMR (400 MHz, CDCl₃) $\delta$ 7.81 (d, $J$ = 8.0 Hz, 1H), 7.33 (d, $J$ = 3.6 Hz, 2H), 7.10-7.00 (m, 1H), 5.78 (s, 1H), 3.43 (q, $J$ = 7.6 Hz, 2H), 1.69 (nonet, $J$ = 6.4 Hz, 1H), 1.49 (q, $J$ = 7.6 Hz, 2H), 0.93 (d, $J$ = 6.4 Hz, 6H); $^{13}$C NMR (100 MHz, CDCl₃) $\delta$ 169.5, 142.7, 140.0, 131.1, 128.4, 128.3, 92.6, 38.6, 38.4, 26.1, 22.6.

**Preparation of compound 19.** To a solution of cyclohexylamine (1.0 mL, 9.0 mmol) and Et₃N (2.0 mL, 15.0 mmol) in anhydrous CH₂Cl₂ (20 mL), 2-
iodobenzoyl chloride (1.0 mL mL, 3.75 mmol) was added dropwise. The product was washed with 1 N HCl (50 mL) and a saturated solution of sodium bicarbonate (50 mL) and dried with MgSO₄. The organic layer was removed in vacuo to afford the known compound 19 (836 mg, 34%) as a yellow solid: \( ^1\text{H} \text{NMR} (400 \text{ MHz}, \text{CDCl}_3) \delta 7.81 (d, J = 8.0 \text{ Hz}, 1\text{H}), 7.40-7.28 (m, 2\text{H}), 7.28-7.20 (m, 1\text{H}), 5.65 (br s, 1\text{H}), 4.02-3.90 (m, 1\text{H}), 2.10-2.00 (m, 2\text{H}), 1.73 (dp, J = 13.2, 3.6 \text{ Hz}, 2\text{H}), 1.62 (dp, J = 12.8, 3.6 \text{ Hz}, 1\text{H}), 1.40 (m, 2\text{H}), 1.30-1.10 (m, 3\text{H}); ^{13}\text{C} \text{NMR} (100 \text{ MHz}, \text{CDCl}_3) \delta 168.7, 142.8, 139.9, 131.1, 128.4, 128.3, 92.7, 49.1, 33.1, 25.7, 25.0.

8.2.2. Preparation of isoselenazolone compounds (compounds 20-23)

**Preparation of compound 20.** The known compound 15 was prepared using a previously published protocol.\(^{284}\) Compound 1 (0.299 g, 1 mmol), copper(I) iodide (0.18 g, 1 mmol), 1,10-phenanthroline (0.17 g, 1 mmol), cesium carbonate (0.81 g, 2.5 mmol), and potassium selenocyanate (0.16 g, 1.2 mmol) were suspended in DMF (5 mL). The mixture turned red and was heated to 100 °C for 12 h. The reaction mixture was cooled to room temperature, diluted with EtOAc (40 mL), and filtered through Celite®. The filtrate was washed with cold H₂O (2×20 mL) and brine (2×20 mL), and dried with MgSO₄. The organic layer was removed in vacuo and the crude product was purified via recrystallization from EtOH and flash column chromatography (SiO₂, 49:1/CH₂Cl₂:MeOH) to afford the known compound 20\(^{284}\) (14 mg, 6%) as colorless needles: \( ^1\text{H} \text{NMR} (400 \text{ MHz}, \text{CDCl}_3) \delta 8.10 (d, J = 7.6 \text{ Hz}, 1\text{H}), 7.68-7.56 (m, 4\text{H}), 7.48-7.43 (m, 1\text{H}), 7.41 (t, J = 8.0 \text{ Hz}, 2\text{H}), 7.26 (t, J = 7.6 \text{ Hz}, 1\text{H}). \)
Preparation of compound 21. The known compound 16 was prepared using a previously published protocol.\textsuperscript{284} Compound 2 (0.34 g, 1 mmol), copper (I) iodide (0.19 g, 1 mmol), 1,10-phenanthroline (0.18 g, 1 mmol), cesium carbonate (0.70 g, 2 mmol), and potassium selenocyanate (0.16 g, 1.2 mmol) were suspended in DMF (5 mL). The mixture turned red and was heated to 100 °C for 12 h. The reaction mixture was cooled to room temperature, diluted with EtOAc (40 mL), and filtered through Celite\textregistered. The filtrate was washed with cold H\textsubscript{2}O (2×20 mL) and brine (2×20 mL), and dried with MgSO\textsubscript{4}. The organic layer was removed \textit{in vacuo} and the crude product was purified \textit{via} recrystallization from i-PrOH and flash column chromatography (SiO\textsubscript{2}, 49:1/CH\textsubscript{2}Cl\textsubscript{2}:MeOH then 19:1/CH\textsubscript{2}Cl\textsubscript{2}:MeOH) to afford the known compound 21\textsuperscript{284} (38 mg, 13%) as white solid: \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}) \(\delta\) 8.04 (d, \(J = 7.6\) Hz, 1H), 7.91 (d, \(J = 6.4\) Hz, 1H), 7.76-7.64 (m, 2H), 7.39 (d, \(J = 7.2\) Hz, 2H), 7.36-7.24 (m, 3H), 5.36 (d, \(J = 14.8\) Hz, 1H), 4.60 (d, \(J = 14.8\) Hz, 1H); \textsuperscript{13}C NMR (100 MHz, CDCl\textsubscript{3}, which matches the literature\textsuperscript{284}) \(\delta\) 168.2, 145.1, 136.7, 134.5, 133.3, 130.8, 129.3, 129.0, 128.7, 128.6, 126.6, 45.5.

Preparation of compound 22. Compound 17 (0.35 g, 1 mmol), copper (I) iodide (0.19 g, 1 mmol), 1,10-phenanthroline (0.18 g, 1 mmol), cesium carbonate (0.70 g, 2 mmol), and potassium selenocyanate (0.16 g, 1.2 mmol) were suspended in DMF (5 mL). The mixture turned red and was heated to 100 °C for 3 h. The reaction mixture was cooled to room temperature, diluted with EtOAc (40 mL), and filtered through Celite\textregistered. The filtrate was washed with cold H\textsubscript{2}O (2×20 mL) and brine (2×20 mL), and dried with MgSO\textsubscript{4}. The organic layer was removed \textit{in vacuo} and the crude product was purified \textit{via} recrystallization from i-PrOH and twice \textit{via} flash column chromatography (SiO\textsubscript{2},
49:1/CH₂Cl₂:MeOH then 19:1/CH₂Cl₂:MeOH) to afford the known compound 22 (12 mg, 4%) as white solid: ¹H NMR (400 MHz, CDCl₃) δ 8.03 (d, J = 8.0 Hz, 1H), 7.56 (d, J = 3.6 Hz, 2H), 7.42-7.36 (m, 1H), 7.32-7.20 (m, 6H), 4.09 (t, J = 6.8 Hz, 2H), 3.02 (t, J = 7.2 Hz, 2H); ¹³C NMR (100 MHz, CDCl₃) δ 167.1, 148.3, 138.0, 137.9, 131.9, 129.02, 128.96, 128.8, 128.6, 127.1, 126.7, 126.1, 123.9, 46.2, 36.4.

**Preparation of compound 23.** Compound 18 (0.32 g, 1 mmol), copper (I) iodide (0.19 g, 1 mmol), 1,10-phenanthroline (0.18 g, 1 mmol), cesium carbonate (0.70 g, 2 mmol), and potassium selenocyanate (0.16 g, 1.2 mmol) were suspended in DMF (5 mL). The mixture turned red and was heated to 100 °C for 3 h. The reaction mixture was cooled to room temperature, diluted with EtOAc (40 mL), and filtered through Celite®. The filtrate was washed with cold H₂O (2×20 mL) and brine (2×20 mL), and dried with MgSO₄. The organic layer was removed *in vacuo* and the crude product was purified *via* recrystallization from EtOAc and twice *via* flash column chromatography (SiO₂, 49:1/CH₂Cl₂:MeOH then 19:1/CH₂Cl₂:MeOH) to afford the known compound 23 (26 mg, 10%) as white solid: ¹H NMR (400 MHz, CDCl₃, which matches the literature²⁸⁵) δ 8.01 (d, J = 7.6 Hz, 1H), 7.61 (d, J = 8.0 Hz, 1H), 7.55 (t, J = 7.6 Hz, 1H), 7.39 (t, J = 7.6 Hz, 1H), 3.86 (t, J = 6.8 Hz, 2H), 1.67 (nonet, J = 6.4 Hz, 1H), 1.59 (q, J = 6.4 Hz, 2H), 0.95 (d, J = 6.8 Hz, 6H); ¹³C NMR (100 MHz, CDCl₃, which matches the literature²⁸⁵) δ 167.3, 137.8, 132.0, 129.0, 127.9, 126.4, 124.1, 43.4, 39.6, 25.9, 22.7.
Chapter 9

Conclusion and future directions

9.1. EIS INHIBITORS

It has been shown that Eis from *Mtb* can be successfully inhibited by a variety of small-molecule scaffolds. During my time working on Eis inhibitors in the Garneau-Tsodikova laboratory, we were able to optimize the potency of the following classes of Eis inhibitors (Chapters 2, 3, and 4): (1) pyrrolo[1,5-*a*]pyrazine, (2) 1,2,4-triazino[5,6-*b*]indole-3-thioether, and (3) thieno[2,3-*d*]pyrimidine. Additionally, we found that these inhibitors occupies part of the Eis active site and compete for the AG-binding pocket. Uniquely, the 1,2,4-triazino[5,6-*b*]indole-3-thioether-based inhibitors were found to interact with an additional region of the Eis active site that was not previously seen in other Eis inhibitors. In all the classes of inhibitors, we found that Eis inhibitors were able to fully or partially recover the activity of KAN against an Eis-mediated-KAN-resistant cell line, *Mtb* K204.

However, it is still unclear why, in many instances, *in vitro* Eis inhibition does not perfectly correlate with recovery of KAN activity in *Mtb* K204 cells. Further studies are needed to clarify this observation. Additionally, the biology of Eis has not fully been elucidated. First, since Eis is expressed and purified as highly stable hexamers, it is unclear whether inhibiting one monomer of Eis would be sufficient to shut down the activity of the hexameric enzyme.
Further biochemical optimization to separate the hexameric and monomeric forms of Eis would be exciting future research topics. Second, various research groups have hypothesized that Eis can play an important role in enabling *Mtb* cells to survive within human macrophages. It would be interesting to investigate whether the Eis inhibitors in this dissertation can disrupt the ability of *Mtb* cells to survive in macrophages.

In order to further investigate the clinical relevance of these inhibitors, we need to understand more about the absorption, disposition, metabolism, and excretion (ADME) properties of these compounds and their *in vivo* efficacies. Ongoing collaboration with the laboratory of Professor Tanya Parish at the Infectious Diseases Research Institute at University of Washington is aimed to explore the PK properties of these molecules. To develop clinically relevant AG adjuvants, we must ensure that the PK properties of the adjuvants and KAN are completely compatible. Patients must be able to concurrently experience exposures of both agents. Thus, future results from this collaboration would be crucial for ADME optimization of these inhibitors to compatibly co-administer with KAN or other AGs. Additionally, once we successfully identify inhibitors with compatible PK properties, these inhibitors and KAN should be evaluated for *in vivo* efficacies in a MDR-TB mouse model. Since mice exhibit a different physiological form of TB granuloma from human granuloma, further *in vivo* efficacy studies in larger animals such as pigs or monkeys will also be necessary before a first-in-human Phase I trial.
9.2. EBSULFUR/EBSELEN ANALOGUES

In Chapter 5 and 7, we successfully generated and repurposed the ebsulfur scaffold as potent MRSA and antifungal agents. The ebsulfur/ebselen scaffold is valuable because ebselen has successfully overcome many safety hurdles during clinical trials. However, the mechanism of action responsible for their antibacterial and antifungal activities remains to be elucidated. Our preliminary results showed that the mechanism might be associated with ROS generation. Ongoing research efforts are to generate biotin tagged ebsulfur and ebselen analogues to perform pull down experiments with streptavidin to identify the protein targets. Additionally, we are hoping to start evaluating these compounds against bacterial and fungal mouse models.

We are also actively looking for other applications of this scaffold. Recent collaborations with Professor Vincent Lee at the University of Maryland revealed that these compounds are active against *Pseudomonas aeruginosa*, an important pathogen in patients with cystic fibrosis, through inhibition of alginate production. We are working closely with the Lee laboratory to further understand the action of ebsulfur/ebselen analogues on the specific protein target within the alginate biosynthesis.
Figure A1: $^1$H NMR spectrum for compound 4 in CDCl$_3$ (400 MHz).
Figure A2: $^1$H NMR spectrum for compound 5 in CDCl$_3$ (400 MHz).

Figure A3: $^1$H NMR spectrum for compound 6 in CDCl$_3$ (400 MHz).
Figure A4: $^{13}$C NMR spectrum for compound 6 in CDCl$_3$ (100 MHz).

Figure A5: $^1$H NMR spectrum for compound 7 in CDCl$_3$ (400 MHz).
Figure A6: $^{13}$C NMR spectrum for compound 7 in CDCl$_3$ (100 MHz).

Figure A7: $^1$H NMR spectrum for compound 8 in CDCl$_3$ (400 MHz).
Figure A8: $^1$H NMR spectrum for compound 9 in CDCl$_3$ (400 MHz).

Figure A9: $^1$H NMR spectrum for compound 10 in CDCl$_3$ (400 MHz).
Figure A10: $^{13}$C NMR spectrum for compound 10 in CDCl$_3$ (100 MHz).

Figure A11: $^1$H NMR spectrum for compound 1a in CDCl$_3$ (400 MHz).
**Figure A12:** $^{13}$C NMR spectrum for compound 1a in CDCl$_3$ (100 MHz).

**Figure A13:** HPLC trace for compound 1a. $R_t = 9.94$ min.
Figure A14: $^1$H NMR spectrum for compound 1b in (CD$_3$)$_2$SO (500 MHz).

Figure A15: $^{13}$C NMR spectrum for compound 1b in (CD$_3$)$_2$SO (125 MHz).
Figure A16: HPLC trace for compound 1b. $R_t = 8.75$ min.

Figure A17: $^1$H NMR spectrum for compound 1c in CDCl$_3$ (400 MHz).
Figure A18: $^{13}$C NMR spectrum for compound 1c in CDCl$_3$ (100 MHz).

Figure A19: HPLC trace for compound 1c. $R_t = 8.49$ min.
Figure A20: $^1$H NMR spectrum for compound 1d in CDCl$_3$ (400 MHz).

Figure A21: $^{13}$C NMR spectrum for compound 1d in CDCl$_3$ (100 MHz).
Figure A22: HPLC trace for compound 1d. $R_t = 8.23$ min.

Figure A23: $^1$H NMR spectrum for compound 1e in (CD$_3$)$_2$SO (500 MHz).

Figure A23: $^{13}$C NMR spectrum for compound 1e in (CD$_3$)$_2$SO (125 MHz).
Figure A25: HPLC trace for compound 1e. \( R_t = 9.66 \) min.

Figure A26: \(^1\)H NMR spectrum for compound 1f in (CD\(_3\)\(_2\)SO (500 MHz).
Figure A27: $^{13}$C NMR spectrum for compound 1f in (CD$_3$)$_2$SO (125 MHz).

Figure A28: HPLC trace for compound 1f. $R_t = 9.25$ min.
Figure A29: $^1$H NMR spectrum for compound 1g in (CD$_3$)$_2$SO (500 MHz).

Figure A30: $^{13}$C NMR spectrum for compound 1g in (CD$_3$)$_2$SO (125 MHz).
**Figure A31:** HPLC trace for compound 1g. $R_t = 8.87$ min.

**Figure A32:** $^1$H NMR spectrum for compound 1h in (CD$_3$)$_2$SO (500 MHz).
Figure A33: $^{13}$C NMR spectrum for compound 1h in (CD$_3$)$_2$SO (125 MHz).

Figure A34: HPLC trace for compound 1h. $R_t = 8.66$ min.
Figure A35: $^1$H NMR spectrum for compound 1i in (CD$_3$)$_2$SO (500 MHz).

Figure A36: $^{13}$C NMR spectrum for compound 1i in (CD$_3$)$_2$SO (125 MHz).
Figure A37: HPLC trace for compound 1i. $R_t = 8.99$ min.
Figure A38: $^1$H NMR spectrum for compound 1j in (CD$_3$)$_2$SO (500 MHz).
**Figure A39:** $^{13}$C NMR spectrum for compound 1j in CDCl$_3$ (100 MHz).

**Figure A40:** HPLC trace for compound 1j. $R_t = 8.96$ min.
**Figure A41:** $^1$H NMR spectrum for compound **1k** in CDCl$_3$ (400 MHz).
Figure A42: $^{13}$C NMR spectrum for compound 1k in CDCl$_3$ (100 MHz).
Figure A43: HPLC trace for compound 1k. $R_t = 10.52$ min.

Figure A44: $^1$H NMR spectrum for compound 2a in CDCl$_3$ (400 MHz).
Figure A45: $^{13}$C NMR spectrum for compound 2a in CDCl$_3$ (100 MHz).

Figure A46: HPLC trace for compound 2a. $R_t = 9.85$ min.
**Figure A47:** $^1$H NMR spectrum for compound 2b in CDCl$_3$ (400 MHz).

**Figure A48:** $^{13}$C NMR spectrum for compound 2b in CDCl$_3$ (100 MHz).
Figure A49: HPLC trace for compound 2b. $R_t = 9.22$ min.

Figure A50: $^1$H NMR spectrum for compound 2c in (CD$_3$)$_2$SO (400 MHz).
Figure A51: $^{13}$C NMR spectrum for compound 2c in (CD$_3$)$_2$SO (100 MHz).
Figure A52: HPLC trace for compound 2c. $R_t = 9.07$ min.
**Figure A53:** $^1$H NMR spectrum for compound 2d in (CD$_3$)$_2$SO (400 MHz).

**Figure A54:** $^{13}$C NMR spectrum for compound 2d in (CD$_3$)$_2$SO (100 MHz).

**Figure A55:** HPLC trace for compound 2d. $R_t = 8.39$ min.
Figure A56: $^1$H NMR spectrum for compound 2e in (CD$_3$)$_2$SO (400 MHz).
**Figure A57:** $^{13}$C NMR spectrum for compound 2e in CDCl$_3$ (100 MHz).

**Figure A58:** HPLC trace for compound 2e. $R_t = 9.44$ min.
Figure A59: $^1$H NMR spectrum for compound 2f in (CD$_3$)$_2$SO (400 MHz).
**Figure A60.** $^{13}$C NMR spectrum for compound 2f in (CD$_3$)$_2$SO (100 MHz).
Figure A61: HPLC trace for compound 2f. \( R_t = 8.25 \) min.

Figure A62: \(^1\)H NMR spectrum for compound 2g in (CD\(_3\))\(_2\)SO (400 MHz).
Figure A63: $^{13}$C NMR spectrum for compound 2g in CDCl$_3$ (100 MHz).

Figure A64: HPLC trace for compound 2g. $R_t = 8.89$ min.
Figure A65: $^1$H NMR spectrum for compound 2h in CDCl$_3$ (400 MHz).

Figure A66: $^{13}$C NMR spectrum for compound 2h in CDCl$_3$ (100 MHz).
Figure A67: HPLC trace for compound 2h. $R_t = 8.97$ min.

Figure A68: $^1$H NMR spectrum for compound 2i in (CD$_3$)$_2$SO (400 MHz).
**Figure A69:** $^{13}$C NMR spectrum for compound 2i in CDCl$_3$ (100 MHz).

**Figure A70:** HPLC trace for compound 2i. $R_t = 9.52$ min.
Figure A71: $^1$H NMR spectrum for compound 2j in (CD$_3$)$_2$SO (400 MHz).
**Figure A72:** $^{13}$C NMR spectrum for compound 2j in CDCl$_3$ (100 MHz).

**Figure A73:** HPLC trace for compound 2j. $R_t$ = 9.53 min.
Figure A74: $^1$H NMR spectrum for compound 2k in CDCl$_3$ (400 MHz).
Figure A75: $^{13}$C NMR spectrum for compound 2k in CDCl$_3$ (100 MHz).
Figure A76: HPLC trace for compound 2k. $R_t = 9.98$ min.

Figure A77: $^1$H NMR spectrum for compound 3a in CDCl$_3$ (400 MHz).

Figure A78: $^{13}$C NMR spectrum for compound 3a in CDCl$_3$ (100 MHz).
Figure A79: HPLC trace for compound 3a. $R_t = 8.79$ min.

Figure A80: $^1$H NMR spectrum for compound 3d in CDCl$_3$ (400 MHz).
Figure A81: $^{13}$C NMR spectrum for compound 3d in CDCl$_3$ (100 MHz).

Figure A82: HPLC trace for compound 3d. $R_t = 9.94$ min.
Figure A83: $^1$H NMR spectrum for compound 3h in CDCl$_3$ (400 MHz).

Figure A84: $^{13}$C NMR spectrum for compound 3h in CDCl$_3$ (100 MHz).
Figure A85: HPLC trace for compound 3h. $R_t = 9.02$ min.

Figure A86: $^1$H NMR spectrum for compound 3k in CDCl$_3$ (400 MHz).
Figure A87: $^{13}$C NMR spectrum for compound 3k in CDCl$_3$ (100 MHz).

Figure A88: HPLC trace for compound 3k. $R_t = 9.58$ min.
Figure A89: $^1$H NMR spectrum for compound 11 in CDCl$_3$ (400 MHz).
Figure A90: $^1$H NMR spectrum for compound 12 in CDCl$_3$ (400 MHz).

Figure A91: $^1$H NMR spectrum for compound 1a* in CDCl$_3$ (400 MHz).
Figure A92: $^{13}$C NMR spectrum for compound 1a* in CDCl$_3$ (100 MHz).

Figure A93: HPLC trace for compound 1a*. $R_t = 8.99$ min.
Figure A94: $^1$H NMR spectrum for compound 1b* in CDCl$_3$ (400 MHz).

Figure A95: $^{13}$C NMR spectrum for compound 1b* in CDCl$_3$ (100 MHz).
**Figure A96**: HPLC trace for compound 1b*. $R_t = 8.69$ min.

**Figure A97**: $^1$H NMR spectrum for compound 1c* in CDCl$_3$ (400 MHz).
Figure A98: $^{13}$C NMR spectrum for compound $1c^*$ in CDCl$_3$ (100 MHz).
Figure A99: HPLC trace for compound 1c*. $R_t = 9.14$ min.

Figure A100: $^1$H NMR spectrum for compound 1d* in CDCl$_3$ (400 MHz).
Figure A101: $^{13}\text{C}$ NMR spectrum for compound 1d* in CDCl$_3$ (100 MHz).

Figure A102: HPLC trace for compound 1d*. $R_t = 8.95$ min.
Figure A103: $^1$H NMR spectrum for compound 1e* in CDCl$_3$ (400 MHz).
Figure A104: $^{13}$C NMR spectrum for compound 1e* in CDCl$_3$ (100 MHz).

Figure A105: HPLC trace for compound 1e*. $R_t = 9.24$ min.

Figure A106: $^1$H NMR spectrum for compound 1f* in CDCl$_3$ (400 MHz).
Figure A107: $^{13}$C NMR spectrum for compound 1f* in CDCl$_3$ (100 MHz).
Figure A108: HPLC trace for compound 1f*. \( R_t = 8.00 \text{ min.} \)

Figure A109: \(^1\text{H} \) NMR spectrum for compound 1g* in CDCl\(_3\) (400 MHz).
Figure A110: $^{13}$C NMR spectrum for compound 1g* in CDCl$_3$ (100 MHz).

Figure A111: HPLC trace for compound 1g*. $R_t = 8.86$ min.
Figure A112: $^1$H NMR spectrum for compound 1h* in CDCl$_3$ (400 MHz).
Figure A113: $^{13}$C NMR spectrum for compound 1h* in CDCl$_3$ (100 MHz).

Figure A114: HPLC trace for compound 1h*. $R_t = 8.67$ min.
Figure A115: $^1$H NMR spectrum for compound 1i* in CDCl$_3$ (400 MHz).
Figure A116: $^{13}$C NMR spectrum for compound 1i* in CDCl$_3$ (100 MHz).

Figure A117: HPLC trace for compound 1i*. $R_t = 9.25$ min.

Figure A118: $^1$H NMR spectrum for compound 1j* in CDCl$_3$ (400 MHz).
**Figure A119**: $^{13}$C NMR spectrum for compound 1j* in CDCl$_3$ (100 MHz).

**Figure A120**: HPLC trace for compound 1j*. $R_t = 9.25$ min.
Figure A121: $^1$H NMR spectrum for compound $1k^*$ in CDCl$_3$ (400 MHz).
**Figure A122:** $^{13}$C NMR spectrum for compound 1k* in CDCl$_3$ (100 MHz).

**Figure A123:** HPLC trace for compound 1k*. $R_t = 9.25$ min.
Figure A124: $^1$H NMR spectrum for compound 2a* in CDCl$_3$ (400 MHz).

Figure A125: $^{13}$C NMR spectrum for compound 2a* in CDCl$_3$ (100 MHz).
Figure A126: HPLC trace for compound 2a*. $R_t = 9.43$ min.

Figure A127: $^1$H NMR spectrum for compound 2b* in CDCl$_3$ (400 MHz).
Figure A128: $^{13}$C NMR spectrum for compound 2b* in CDCl$_3$ (100 MHz).
Figure A129: HPLC trace for compound 2b*. $R_t = 8.48$ min.

Figure A130: $^1$H NMR spectrum for compound 2c* in CDCl$_3$ (400 MHz).
Figure A131: $^{13}$C NMR spectrum for compound 2e* in (CD$_3$)$_2$SO (100 MHz).
**Figure A132**: HPLC trace for compound 2c*. $R_t = 8.59$ min.

**Figure A133**: $^1$H NMR spectrum for compound 2d* in CDCl$_3$ (400 MHz).
Figure A134: $^{13}$C NMR spectrum for compound 2d* in (CD$_3$)$_2$SO (100 MHz).
Figure A135: HPLC trace for compound 2d*. $R_t = 9.26$ min.
Figure A136: $^1$H NMR spectrum for compound 2e* in CDCl$_3$ (400 MHz).
Figure A137: $^{13}$C NMR spectrum for compound 2e* in (CD$_3$)$_2$SO (100 MHz).
Figure A138: HPLC trace for compound 2e*. $R_t = 8.89$ min.

Figure A139: $^1$H NMR spectrum for compound 2f* in CDCl$_3$ (400 MHz).
Figure A140: $^{13}$C NMR spectrum for compound 2f* in (CD$_3$)$_2$SO (100 MHz).
Figure A141: HPLC trace for compound 2f*. $R_t = 7.66$ min.

Figure A142: $^1$H NMR spectrum for compound 2g* in CDCl$_3$ (400 MHz).
Figure A143: $^{13}$C NMR spectrum for compound 2g* in CDCl$_3$ (100 MHz).
**Figure A144:** HPLC trace for compound 2g*. $R_t = 9.45$ min.

**Figure A145:** $^1$H NMR spectrum for compound 2h* in CDCl$_3$ (400 MHz).
Figure A146: $^{13}$C NMR spectrum for compound 2h* in CDCl₃ (100 MHz).
Figure A147: HPLC trace for compound 2h*. $R_t = 8.31$ min.

Figure A148: $^1$H NMR spectrum for compound 2i* in CDCl$_3$ (400 MHz).
Figure A149: $^{13}$C NMR spectrum for compound 2i* in CDCl$_3$ (100 MHz).
Figure A150: HPLC trace for compound 2i*. $R_t = 9.13$ min.

Figure A151: $^1$H NMR spectrum for compound 2j* in CDCl$_3$ (400 MHz).
**Figure A152:** $^{13}$C NMR spectrum for compound 2j* in CDCl$_3$ (100 MHz).

**Figure A153:** HPLC trace for compound 2j*. $R_t = 9.30$ min.
Figure A154: $^1$H NMR spectrum for compound 2k* in CDCl$_3$ (400 MHz).

Figure A155: $^{13}$C NMR spectrum for compound 2k* in CDCl$_3$ (100 MHz).
**Figure A156:** HPLC trace for compound 2k*. $R_t = 9.62$ min.
APPENDIX B

Spectroscopic spectra B1-B78 (\(^1\)H, \(^{13}\)C NMR, and HPLC) for Chapter 3

**Figure B1:** \(^1\)H NMR spectrum for compound 32b in (CD\(_3\))\(_2\)SO.

**Figure B2:** \(^{13}\)C NMR spectrum for compound 32b in (CD\(_3\))\(_2\)SO.
**Figure B3:** HPLC trace for compound 32b. $R_t = 5.02$ min.

**Figure B4:** $^1$H NMR spectrum for compound 34b in CDCl$_3$. 
Figure B5: $^{13}$C NMR spectrum for compound 34b in CDCl$_3$.

Figure B6: HPLC trace for compound 34b. $R_t = 7.90$ min.
Figure B7: $^1$H NMR spectrum for compound 35b in CDCl$_3$.

Figure B8: $^{13}$C NMR spectrum for compound 35b in CDCl$_3$.
Figure B9: HPLC trace for compound 35b. $R_t = 7.38$ min.
Figure B10: $^1$H NMR spectrum for compound 36b in CDCl$_3$. 
Figure B11: $^{13}$C NMR spectrum for compound 36b in CDCl$_3$. 
Figure B12: HPLC trace for compound 36b. $R_t = 7.02$ min.
Figure B13: $^1$H NMR spectrum for compound 37b in CDCl$_3$. 
Figure B14: $^{13}$C NMR spectrum for compound 37b in CDCl$_3$. 
**Figure B15:** HPLC trace for compound 37b. $R_t = 7.71$ min.

**Figure B16:** $^1$H NMR spectrum for compound 37h in CDCl$_3$. 
Figure B17: $^{13}$C NMR spectrum for compound 37h in CDCl$_3$. 
Figure B18: HPLC trace for compound 37h. $R_t = 9.49$ min.
Figure B19: $^1$H NMR spectrum for compound 38b in CDCl$_3$. 
**Figure B20:** $^{13}$C NMR spectrum for compound 38b in CDCl$_3$. 
Figure B21: HPLC trace for compound 38b. $R_t = 7.73$ min.
Figure B22: $^1$H NMR spectrum for compound 39b in CDCl$_3$. 
Figure B23: $^{13}$C NMR spectrum for compound 39b in CDCl$_3$.

Figure B24: HPLC trace for compound 39b. $R_t = 8.18$ min.
Figure B25: $^1$H NMR spectrum for compound 42b in CDCl$_3$. 
Figure B26: $^{13}$C NMR spectrum for compound 42b in CDCl$_3$. 
Figure B27: HPLC trace for compound 42b. $R_t = 10.36$ min.
Figure B28: $^1$H NMR spectrum for compound 43b in CDCl$_3$. 
Figure B29: $^{13}$C NMR spectrum for compound 43b in CDCl$_3$. 
Figure B30: HPLC trace for compound 43b. $R_t = 7.41$ min.

Figure B31: $^1$H NMR spectrum for compound 43d in CDCl$_3$. 
Figure B32: $^{13}$C NMR spectrum for compound 43d in CDCl$_3$.

Figure B33: HPLC trace for compound 43d. $R_t = 6.29$ min.
Figure B34: $^1$H NMR spectrum for compound 44b in CDCl$_3$. 
Figure B35: $^{13}$C NMR spectrum for compound 44b in CDCl$_3$. 
**Figure B36:** HPLC trace for compound 44b. \( R_t = 8.58 \text{ min} \).

**Figure B37:** \(^1\)H NMR spectrum for compound 45b in CDCl₃.
Figure B38: $^{13}$C NMR spectrum for compound 45b in CDCl$_3$.

Figure B39: HPLC trace for compound 45b. $R_t = 8.11$ min.

Figure B40: $^1$H NMR spectrum for compound 46b in CDCl$_3$. 
**Figure B41:** $^{13}$C NMR spectrum for compound 46b in CDCl₃.

**Figure B42:** HPLC trace for compound 46b. $R_t = 8.84$ min.
Figure B43: $^1$H NMR spectrum for compound 48b in CDCl$_3$. 
Figure B44: $^{13}$C NMR spectrum for compound 48b in CDCl$_3$. 
Figure B45: HPLC trace for compound 48b. $R_t = 8.26$ min.

Figure B46: $^1$H NMR spectrum for compound 48c in CDCl$_3$. 
**Figure B47:** $^{13}$C NMR spectrum for compound 48c in CDCl$_3$.

**Figure B48:** HPLC trace for compound 48c. $R_t = 8.55$ min.
Figure B49: $^1$H NMR spectrum for compound 48d in CDCl$_3$. 
Figure B50: $^{13}$C NMR spectrum for compound 48d in CDCl$_3$. 
Figure B51: HPLC trace for compound 48d. $R_t = 8.96 \text{ min}$.

Figure B52: $^1$H NMR spectrum for compound 48f in CDCl$_3$. 
Figure B53: $^{13}$C NMR spectrum for compound 48f in CDCl$_3$. 
Figure B54: HPLC trace for compound 48f. $R_t = 9.13$ min.

Figure B55: $^1$H NMR spectrum for compound 48h in CDCl$_3$. 
Figure B56: $^{13}$C NMR spectrum for compound 48h in CDCl$_3$.

Figure B57: HPLC trace for compound 48h. $R_t = 9.35$ min.
Figure B58: $^1$H NMR spectrum for compound 49b in CDCl$_3$. 

Figure B59: $^{13}$C NMR spectrum for compound 49b in CDCl$_3$. 

489
**Figure B60:** HPLC trace for compound 49b. $R_t = 8.13$ min.

**Figure B61:** $^1$H NMR spectrum for compound 33b in CDCl$_3$. 
Figure B62: $^{13}$C NMR spectrum for compound 33b in CDCl$_3$.

Figure B63: HPLC trace for compound 33b. $R_t = 8.05$ min.
Figure B64: $^1$H NMR spectrum for compound 47b in CDCl$_3$.

Figure B65: $^{13}$C NMR spectrum for compound 47b in CDCl$_3$. 
Figure B66: HPLC trace for compound 47b. \( R_t = 7.92 \) min.
Figure B67: $^1$H NMR spectrum for compound 60b in CDCl$_3$. 
Figure B68: $^{13}$C NMR spectrum for compound 60b in CDCl$_3$. 
Figure B69: HPLC trace for compound 60b. \( R_t = 7.92 \) min.
Figure B70: $^1$H NMR spectrum for compound 61b in CDCl$_3$. 
Figure B71: $^{13}$C NMR spectrum for compound 61b in CDCl$_3$. 
Figure B72: HPLC trace for compound 61b. $R_t = 5.99$ min.

Figure B73: $^1$H NMR spectrum for compound 52 in CDCl$_3$. 

499
Figure B74: $^{13}$C NMR spectrum for compound 52 in CDCl$_3$. 
Figure B75: HPLC trace for compound 52. $R_t = 3.30$ min.
Figure B76: $^1$H NMR spectrum for compound 53 in CDCl$_3$. 
Figure B77: $^{13}$C NMR spectrum for compound 53 in CDCl$_3$. 
Figure B78: HPLC trace for compound 53. $R_t = 4.97$ min.
APPENDIX C

Spectroscopic spectra C1-C222 (\(^1\)H, \(^{13}\)C NMR, and HPLC) for Chapter 4

**Figure C1:** Mass spectra for compounds 1a-4b.
Figure C2: Mass spectra for compounds 5b-8c.
Figure C3: Mass spectra for compounds 1d-4e.
Figure C4: Mass spectra for compounds 5e-8f.
Figure C5: Mass spectra for compounds 1g-4h.
Figure C6: Mass spectra for compounds 5h-8i.
Figure C7: $^1$H NMR spectrum for compound 1a in CD$_3$OD.
Figure C8: $^{13}$C NMR spectrum for compound 1a in (CD$_3$)$_2$SO.

Figure C9: HPLC trace for compound 1a. $R_t = 19.29$ min.
Figure C10: $^1$H NMR spectrum for compound 2a in (CD$_3$)$_2$SO.

Figure C11: $^{13}$C NMR spectrum for compound 2a in (CD$_3$)$_2$SO.
Figure C12: HPLC trace for compound 2a. $R_t = 19.96$ min.

Figure C13: $^1$H NMR spectrum for compound 3a in CD$_3$OD.
Figure C14: $^{13}$C NMR spectrum for compound 3a in CDCl$_3$.

Figure C15: HPLC trace for compound 3a. $R_t = 20.82$ min.
Figure C16: $^1$H NMR spectrum for compound 4a in CD$_3$OD.
**Figure C17**: $^{13}$C NMR spectrum for compound 4a in CD$_3$OD.
Figure C18: HPLC trace for compound 4a. $R_t = 11.72$ min.

Figure C19: $^1$H NMR spectrum for compound 5a in CD$_3$OD.
Figure C20: $^{13}$C NMR spectrum for compound 5a in (CD$_3$)$_2$SO.
**Figure C21**: HPLC trace for compound 5a. $R_t = 22.41$ min.

**Figure C22**: $^1$H NMR spectrum for compound 6a in CDCl$_3$. 
**Figure C23:** $^{13}$C NMR spectrum for compound 6a in CDCl$_3$.

**Figure C24:** HPLC trace for compound 6a. $R_t = 15.69$ min.
**Figure C25:** $^1$H NMR spectrum for compound 7a in CDCl$_3$.

![H NMR spectrum](image)

**Figure C26:** $^{13}$C NMR spectrum for compound 7a in CDCl$_3$.

![C NMR spectrum](image)
**Figure C27:** HPLC trace for compound 7a. $R_t = 14.85$ min.

**Figure C28:** $^1$H NMR spectrum for compound 8a in (CD$_3$)$_2$SO.
Figure C29: $^{13}$C NMR spectrum for compound 8a in CDCl$_3$.

Figure C30: HPLC trace for compound 8a. $R_t = 13.24$ min.
**Figure C31**: $^1$H NMR spectrum for compound 1b in CDCl$_3$.

**Figure C32**: $^{13}$C NMR spectrum for compound 1b in (CD$_3$)$_2$SO.
Figure C33: HPLC trace for compound 1b. $R_t = 20.59$ min.
Figure C34: $^1$H NMR spectrum for compound 2b in CDCl$_3$. 
Figure C35: $^{13}$C NMR spectrum for compound 2b in (CD$_3$)$_2$SO.

Figure C36: HPLC trace for compound 2b. $R_t = 20.21$ min.
**Figure C37:** $^1$H NMR spectrum for compound 3b in CD$_3$OD.

**Figure C38:** $^{13}$C NMR spectrum for compound 3b in CDCl$_3$. 

529
**Figure C39:** HPLC trace for compound 3b. $R_t = 13.68$ min.

**Figure C40:** $^1$H NMR spectrum for compound 4b in (CD$_3$)$_2$SO.
**Figure C41:** $^{13}$C NMR spectrum for compound 4b in (CD$_3$)$_2$SO.

**Figure C42:** HPLC trace for compound 4b. $R_t = 20.74$ min.
Figure C43: $^1$H NMR spectrum for compound 5b in CD$_3$OD.

Figure C44: $^{13}$C NMR spectrum for compound 5b in CDCl$_3$. 
**Figure C45:** HPLC trace for compound 5b. $R_t = 14.82$ min.

**Figure C46:** $^1$H NMR spectrum for compound 6b in CDCl$_3$. 

**Figure C47:** $^{13}$C NMR spectrum for compound 6b in CDCl$_3$.

**Figure C48:** HPLC trace for compound 6b. $R_t = 16.03$ min.
**Figure C49:** $^1$H NMR spectrum for compound 7b in CDCl$_3$.

**Figure C50:** $^{13}$C NMR spectrum for compound 7b in CDCl$_3$.

**Figure C51:** HPLC trace for compound 7b. $R_t = 22.54$ min.
**Figure C52:** $^1$H NMR spectrum for compound 8b in CDCl$_3$.

**Figure C53:** $^{13}$C NMR spectrum for compound 8b in CDCl$_3$. 
Figure C54: HPLC trace for compound 8b. $R_t = 23.15$ min.

Figure C55: $^1$H NMR spectrum for compound 1c in (CD$_3$)$_2$SO.
Figure C56: $^{13}$C NMR spectrum for compound 1c in (CD$_3$)$_2$SO.

Figure C57: HPLC trace for compound 1c. $R_1 = 24.10$ min.
Figure C58: $^1$H NMR spectrum for compound 2c in CD$_3$OD.
Figure C59: $^{13}$C NMR spectrum for compound 2c in (CD$_3$)$_2$SO.
**Figure C60:** HPLC trace for compound 2c. \( R_t = 18.58 \) min.

**Figure C61:** \(^1\)H NMR spectrum for compound 3c in CD\(_3\)OD.
**Figure C62:** $^{13}$C NMR spectrum for compound 3c in CDCl$_3$.

**Figure C63:** HPLC trace for compound 3c. $R_t = 11.81$ min.
Figure C64: $^1$H NMR spectrum for compound 4c in CD$_3$OD.
Figure C65: $^{13}$C NMR spectrum for compound 4c in CDCl$_3$.

Figure C66: HPLC trace for compound 4c. $R_t = 13.58$ min.
**Figure C67:** $^1$H NMR spectrum for compound 5c in CD$_3$OD.
**Figure C68**: $^{13}$C NMR spectrum for compound 5c in CD$_3$OD.

**Figure C69**: HPLC trace for compound 5c. $R_t = 14.47$ min.
Figure C70: $^1$H NMR spectrum for compound 6c in CD$_3$OD.

Figure C71: $^{13}$C NMR spectrum for compound 6c in CDCl$_3$. 
Figure C72: HPLC trace for compound 6c. $R_t = 15.83$ min.
Figure C73: $^1$H NMR spectrum for compound 7c in CD$_3$OD.

Figure C74: $^{13}$C NMR spectrum for compound 7c in CDCl$_3$. 
**Figure C75:** HPLC trace for compound 7c. $R_t = 15.61$ min.

**Figure C76:** $^1$H NMR spectrum for compound 8c in CDCl$_3$. 
**Figure C77:** $^{13}$C NMR spectrum for compound 8c in CDCl$_3$.

**Figure C78:** HPLC trace for compound 8c. $R_t = 20.59$ min.
Figure C79: $^1$H NMR spectrum for compound 1d in CD$_3$OD.

Figure C80: $^{13}$C NMR spectrum for compound 1d in (CD$_3$)$_2$SO.
**Figure C81:** HPLC trace for compound 1d. $R_t = 23.38$ min.

**Figure C82:** $^1$H NMR spectrum for compound 2d in CD$_3$OD.
**Figure C83:** $^{13}$C NMR spectrum for compound 2d in CD$_3$OD.

**Figure C84:** HPLC trace for compound 2d. $R_t = 12.45$ min.
Figure C85: $^1$H NMR spectrum for compound 3d in CD$_3$OD.

Figure C86: $^{13}$C NMR spectrum for compound 3d in CDCl$_3$. 
**Figure C87:** HPLC trace for compound 3d. $R_t = 20.18$ min.

**Figure C88:** $^1$H NMR spectrum for compound 4d in CD$_3$OD.
Figure C89: $^{13}$C NMR spectrum for compound 4d in CDCl$_3$.

Figure C90: HPLC trace for compound 4d. $R_t = 24.85$ min.
Figure C91: $^1$H NMR spectrum for compound 5d in CD$_3$OD.
**Figure C92**: $^{13}$C NMR spectrum for compound 5d in CD$_3$OD.
Figure C93: HPLC trace for compound 5d. $R_t = 22.53$ min.

Figure C94: $^1$H NMR spectrum for compound 6d in CDCl$_3$. 
Figure C95: $^{13}$C NMR spectrum for compound 6d in CDCl$_3$.

Figure C96: HPLC trace for compound 6d. $R_t = 15.56$ min.
Figure C97: $^1$H NMR spectrum for compound 7d in CDCl$_3$. 
**Figure C98:** $^{13}$C NMR spectrum for compound 7d in CDCl$_3$.

**Figure C99:** HPLC trace for compound 7d. $R_t = 14.85$ min.
Figure C100: $^1$H NMR spectrum for compound 8d in CDCl$_3$.

Figure C101: $^{13}$C NMR spectrum for compound 8d in CDCl$_3$. 
Figure C102: HPLC trace for compound 8d. $R_t = 22.80$ min.
Figure C103: $^1$H NMR spectrum for compound 1e in CD$_3$OD.
Figure C104: $^{13}$C NMR spectrum for compound 1e in (CD$_3$)$_2$SO.

Figure C105: HPLC trace for compound 1e. $R_t = 20.80$ min.

Figure C106: $^1$H NMR spectrum for compound 2e in CDCl$_3$. 
Figure C107: $^{13}$C NMR spectrum for compound 2e in (CD$_3$)$_2$SO.
Figure C108: HPLC trace for compound 2e. $R_t = 21.48$ min.

Figure C109: $^1$H NMR spectrum for compound 3e in CD$_3$OD.

Figure C110: $^{13}$C NMR spectrum for compound 3e in CDCl$_3$. 
Figure C111: HPLC trace for compound 3e. $R_I = 13.03$ min.

Figure C112: $^1$H NMR spectrum for compound 4e in (CD$_3$)$_2$SO.
**Figure C113:** $^{13}$C NMR spectrum for compound 4e in (CD$_3$)$_2$SO.

**Figure C114:** HPLC trace for compound 4e. $R_t = 11.84$ min.
Figure C115: $^1$H NMR spectrum for compound 5e in CDCl$_3$.

Figure C116: $^{13}$C NMR spectrum for compound 5e in CDCl$_3$. 
Figure C117: HPLC trace for compound 5e. $R_t = 14.22$ min.

Figure C118: $^1$H NMR spectrum for compound 6e in CDCl$_3$. 
**Figure C119:** $^{13}$C NMR spectrum for compound 6e in CDCl$_3$.

**Figure C120:** HPLC trace for compound 6e. $R_t = 26.67$ min.
Figure C121: $^1$H NMR spectrum for compound 7e in CD$_3$OD.

Figure C122: $^{13}$C NMR spectrum for compound 7e in CDCl$_3$. 
**Figure C123:** HPLC trace for compound 7e. $R_t = 25.33$ min.

**Figure C124:** $^1$H NMR spectrum for compound 8e in CD$_3$OD.
Figure C125: $^{13}$C NMR spectrum for compound 8e in CDCl$_3$.

Figure C126: HPLC trace for compound 8e. $R_t = 22.57$ min.
**Figure C127:** $^1$H NMR spectrum for compound 1f in CDCl₃.

**Figure C128:** $^{13}$C NMR spectrum for compound 1f in CDCl₃.
Figure C128: HPLC trace for compound 1f. $R_t = 11.12$ min.
Figure C130: $^1$H NMR spectrum for compound 2f in (CD$_3$)$_2$SO.
**Figure C131:** $^{13}$C NMR spectrum for compound 2f in (CD$_3$)$_2$SO.

**Figure C132:** HPLC trace for compound 2f. $R_t = 11.59$ min.
Figure C133: $^1$H NMR spectrum for compound 3f in CD$_3$OD.

Figure C134: $^{13}$C NMR spectrum for compound 3f in CD$_3$OD.

Figure C135: HPLC trace for compound 3f. $R_t = 22.49$ min.
**Figure C136:** $^1$H NMR spectrum for compound 4f in CD$_3$OD.

**Figure C137:** $^{13}$C NMR spectrum for compound 4f in (CD$_3$)$_2$SO.
Figure C138: HPLC trace for compound 4f. $R_t = 17.70$ min.
Figure C139: $^1$H NMR spectrum for compound 5f in CD$_3$OD.

Figure C140: $^{13}$C NMR spectrum for compound 5f in CD$_3$OD.
Figure C141: HPLC trace for compound 5f. $R_t = 13.45$ min.

Figure C142: $^1$H NMR spectrum for compound 6f in CDCl$_3$. 
**Figure C143:** $^{13}$C NMR spectrum for compound 6f in CDCl$_3$.

**Figure C144:** HPLC trace for compound 6f. $R_t = 22.69$ min.
Figure C145: $^1$H NMR spectrum for compound 7f in CD$_3$OD.
**Figure C146**: $^{13}$C NMR spectrum for compound 7f in CD$_3$OD.

**Figure C147**: HPLC trace for compound 7f. $R_t = 12.14$ min.
Figure C148: $^1$H NMR spectrum for compound 8f in CDCl$_3$.

Figure C149: $^{13}$C NMR spectrum for compound 8f in CDCl$_3$. 
Figure C150: HPLC trace for compound 8f. $R_t = 22.53$ min.

Figure C151: $^1$H NMR spectrum for compound 1g in CD$_3$OD.
Figure C152: $^{13}\text{C}$ NMR spectrum for compound 1g in CD$_3$OD.

Figure C153: HPLC trace for compound 1g. $R_t = 17.54$ min.
Figure C154: $^1$H NMR spectrum for compound 2g in CDCl$_3$. 
Figure C155: $^{13}$C NMR spectrum for compound 2g in CDCl$_3$.

Figure C156: HPLC trace for compound 2g. $R_t = 22.46$ min.
Figure C157: $^1$H NMR spectrum for compound 3g in CD$_3$OD.

Figure C158: $^{13}$C NMR spectrum for compound 3g in CDCl$_3$.
**Figure C159**: HPLC trace for compound 3g. $R_t = 10.85$ min.

**Figure C160**: $^1$H NMR spectrum for compound 4g in CDCl$_3$. 
**Figure C161:** $^{13}$C NMR spectrum for compound 4g in CDCl$_3$.

**Figure C162:** HPLC trace for compound 4g. $R_t = 11.42$ min.
**Figure C163:** $^1$H NMR spectrum for compound 5g in CD$_3$OD.
Figure C164: $^{13}$C NMR spectrum for compound 5g in CDCl$_3$.

Figure C165: HPLC trace for compound 5g. $R_t = 12.01$ min.
Figure C166: $^1$H NMR spectrum for compound 6g in CD$_3$OD.

Figure C167: $^{13}$C NMR spectrum for compound 6g in CDCl$_3$. 
Figure C168: HPLC trace for compound 6g. $R_t = 22.24$ min.
Figure C169: $^1$H NMR spectrum for compound 7g in CD$_3$OD.

Figure C170: $^{13}$C NMR spectrum for compound 7g in CDCl$_3$. 
Figure C171: HPLC trace for compound 7g. $R_t = 23.49$ min.
**Figure C172:** $^1$H NMR spectrum for compound 8g in CDCl$_3$.

**Figure C173:** $^{13}$C NMR spectrum for compound 8g in CDCl$_3$.

**Figure C174:** HPLC trace for compound 8g. $R_t = 24.35$ min.
Figure C175: $^1$H NMR spectrum for compound 1h in CDCl$_3$.

Figure C176: $^{13}$C NMR spectrum for compound 1h in CDCl$_3$. 
Figure C177: HPLC trace for compound 1h. $R_t = 21.78$ min.
Figure C178: $^1$H NMR spectrum for compound 2h in CD$_3$OD.

Figure C179: $^{13}$C NMR spectrum for compound 2h in CDCl$_3$. 
**Figure C180:** HPLC trace for compound 2h. $R_t = 18.53$ min.

**Figure C181:** $^1$H NMR spectrum for compound 3h in CD$_3$OD.
Figure C182: $^{13}$C NMR spectrum for compound 3h in CD$_3$OD.

Figure C183: HPLC trace for compound 3h. $R_t = 10.97$ min.
**Figure C184:** $^1$H NMR spectrum for compound 4h in CDCl$_3$.

**Figure C185:** $^{13}$C NMR spectrum for compound 4h in CDCl$_3$. 
Figure C186: HPLC trace for compound 4h. $R_t = 20.03$ min.

Figure C187: $^1$H NMR spectrum for compound 5h in CD$_3$OD.
Figure C188: $^{13}$C NMR spectrum for compound 5h in CD$_3$OD.
**Figure C189:** HPLC trace for compound 5h. $R_t = 11.95$ min.

**Figure C190:** $^1$H NMR spectrum for compound 6h in CD$_3$OD.
Figure C191: $^{13}$C NMR spectrum for compounds 6h in CDCl$_3$.

Figure C192: HPLC trace for compound 6h. $R_t = 12.61$ min.
Figure C193: $^1$H NMR spectrum for compound 7h in CD$_3$OD.

Figure C194: $^{13}$C NMR spectrum for compound 7h in CDCl$_3$. 
**Figure C195:** HPLC trace for compound 7h. $R_t = 12.41$ min.
Figure C196: $^1$H NMR spectrum for compound 8h in CDCl$_3$.

![Figure C196: $^1$H NMR spectrum for compound 8h in CDCl$_3$.](image)

Figure C197: $^{13}$C NMR spectrum for compound 8h in CDCl$_3$.

![Figure C197: $^{13}$C NMR spectrum for compound 8h in CDCl$_3$.](image)

Figure C198: HPLC trace for compound 8h. $R_t = 23.50$ min.

![Figure C198: HPLC trace for compound 8h. $R_t = 23.50$ min.](image)
Figure C199: $^1$H NMR spectrum for compound 1i in CD$_3$OD.

Figure C200: $^{13}$C NMR spectrum for compound 1i in CDCl$_3$. 
**Figure C201:** HPLC trace for compound 1i. $R_t = 22.73$ min.

**Figure C202:** $^1$H NMR spectrum for compound 2i in $(CD_3)_2$SO.
Figure C203: $^{13}$C NMR spectrum for compound 2i in (CD$_3$)$_2$SO.

Figure C204: HPLC trace for compound 2i. $R_t = 10.64$ min.
**Figure C205:** $^1$H NMR spectrum for compound 3i in CD$_3$OD.

**Figure C206:** $^{13}$C NMR spectrum for compound 3i in CDCl$_3$. 
Figure C207: HPLC trace for compound 3i. $R_t = 24.81$ min.
**Figure C208:** $^1$H NMR spectrum for compound 4i in CD$_3$OD.

![H NMR spectrum](image)

**Figure C209:** $^{13}$C NMR spectrum for compound 4i in CDCl$_3$.

![C NMR spectrum](image)

**Figure C210:** HPLC trace for compound 4i. $R_t = 11.16$ min.
Figure C211: $^1$H NMR spectrum for compound 5i in CD$_3$OD.
Figure C212: $^{13}$C NMR spectrum for compound 5i in CDCl$_3$.

Figure C213: HPLC trace for compound 5i. $R_t = 11.77$ min.
Figure C214: $^1$H NMR spectrum for compound 6i in CD$_3$OD.

Figure C215: $^{13}$C NMR spectrum for compound 6i in CD$_3$OD.
Figure C216: HPLC trace for compound 6i. $R_t = 22.47$ min.
**Figure C217:** $^1$H NMR spectrum for compound 7i in CD$_3$OD.

**Figure C218:** $^{13}$C NMR spectrum for compound 7i in CDCl$_3$. 
Figure C219: HPLC trace for compound 7i. $R_t = 20.96$ min.
Figure C220: $^1$H NMR spectrum for compound 8i in CD$_3$OD.
Figure C221: $^{13}$C NMR spectrum for compound 8i in CDCl$_3$.

Figure C222: HPLC trace for compound 8i. $R_t = 25.09$ min.
APPENDIX D

Spectroscopic spectra D1-D57 ($^1$H, $^{13}$C NMR, and HPLC) for Chapter 5

Figure D1: $^1$H NMR spectrum for compound 1 in CDCl$_3$ (400 MHz).

Figure D2: $^1$H NMR spectrum for compound 2a in CDCl$_3$ (400 MHz).
Figure D3: $^1$H NMR spectrum for compound 2b in CDCl$_3$ (400 MHz).

Figure D4: $^1$H NMR spectrum for compound 2c in CDCl$_3$ (400 MHz).

Figure D5: $^1$H NMR spectrum for compound 2d in CDCl$_3$ (400 MHz).
Figure D6: $^1$H NMR spectrum for compound 2e in CDCl$_3$ (400 MHz).
**Figure D7:** $^1$H NMR spectrum for compound 2f in CDCl$_3$ (400 MHz).

![H NMR spectrum for compound 2f](image)

**Figure D8:** $^{13}$C NMR spectrum for compound 2f in CDCl$_3$ (100 MHz).

![C NMR spectrum for compound 2f](image)

**Figure D9:** $^1$H NMR spectrum for compound 2g in CDCl$_3$ (400 MHz).

![H NMR spectrum for compound 2g](image)
Figure D10: $^{13}$C NMR spectrum for compound 2g in CDCl$_3$ (100 MHz).
Figure D11: $^1$H NMR spectrum for compound 2h in CDCl$_3$ (400 MHz).
Figure D12: $^{13}$C NMR spectrum for compound 2h in CDCl$_3$ (100 MHz).

Figure D13: $^1$H NMR spectrum for compound 2i in CDCl$_3$ (400 MHz).

Figure D14: $^{13}$C NMR spectrum for compound 2i in CDCl$_3$ (100 MHz).
**Figure D15:** $^1$H NMR spectrum for compound 2j in CDCl$_3$ (400 MHz).

**Figure D16:** $^{13}$C NMR spectrum for compound 2j in CDCl$_3$ (100 MHz).
Figure D17: $^1$H NMR spectrum for compound 2k in CDCl$_3$ (400 MHz).

Figure D18: $^{13}$C NMR spectrum for compound 2k in CDCl$_3$ (100 MHz).
Figure D19: $^1$H NMR spectrum for compound 2l in CDCl$_3$ (400 MHz).

Figure D20: $^1$H NMR spectrum for compound 2m in CDCl$_3$ (400 MHz).
Figure D21: $^1$H NMR spectrum for compound 2n in CDCl$_3$ (400 MHz).
**Figure D22:** $^1$H NMR spectrum for compound 2o in CDCl$_3$ (400 MHz).
Figure D23: $^{13}$C NMR spectrum for compound 20 in CDCl$_3$ (100 MHz).
Figure D24: $^1$H NMR spectrum for compound 3a in CDCl$_3$ (400 MHz).

Figure D25: $^{13}$C NMR spectrum for compound 3a in CDCl$_3$ (100 MHz).
**Figure D26:** $^1$H NMR spectrum for compound 3b in CDCl$_3$ (400 MHz).

**Figure D27:** $^{13}$C NMR spectrum for compound 3b in CDCl$_3$ (100 MHz).
Figure D28: $^1$H NMR spectrum for compound 3c in CDCl$_3$ (400 MHz).

Figure D29: $^{13}$C NMR spectrum for compound 3c in CDCl$_3$ (100 MHz).
Figure D30: $^1$H NMR spectrum for compound 3d in CDCl$_3$ (400 MHz).

Figure D31: $^{13}$C NMR spectrum for compound 3d in CDCl$_3$ (100 MHz).

Figure D32: $^1$H NMR spectrum for compound 3e in CDCl$_3$ (400 MHz).
Figure D33: $^{13}$C NMR spectrum for compound 3e in CDCl$_3$ (100 MHz).
Figure D34: $^1$H NMR spectrum for compound 3f in CDCl$_3$ (400 MHz).

Figure D35: $^{13}$C NMR spectrum for compound 3f in CDCl$_3$ (100 MHz).
Figure D36: $^1$H NMR spectrum for compound 3g in CDCl$_3$ (400 MHz).
Figure D37: $^1$H NMR spectrum for compound 3h in CDCl$_3$ (400 MHz).

Figure D38: $^{13}$C NMR spectrum for compound 3h in CDCl$_3$ (100 MHz).

Figure D39: $^1$H NMR spectrum for compound 3i in CDCl$_3$ (400 MHz).
Figure D40: $^1$H NMR spectrum for compound 3j in CDCl$_3$ (400 MHz).

Figure D41: $^{13}$C NMR spectrum for compound 3j in CDCl$_3$ (100 MHz).
**Figure D42:** $^1$H NMR spectrum for compound 3k in CDCl$_3$ (400 MHz).

**Figure D43:** $^{13}$C NMR spectrum for compound 3k in CDCl$_3$ (100 MHz).
Figure D44: $^1$H NMR spectrum for compound 3l in CDCl$_3$ (400 MHz).

Figure D45: $^{13}$C NMR spectrum for compound 3l in CDCl$_3$ (100 MHz).
Figure D46: $^1$H NMR spectrum for compound 3m in CDCl$_3$ (400 MHz).

Figure D47: $^{13}$C NMR spectrum for compound 3m in CDCl$_3$ (100 MHz).
Figure D48: $^1$H NMR spectrum for compound 3n in CDCl$_3$ (400 MHz).
Figure D49: $^{13}$C NMR spectrum for compound 3n in CDCl$_3$ (100 MHz).

Figure D50: $^1$H NMR spectrum for compound 3o in CDCl$_3$ (400 MHz).
Figure D51: $^{13}$C NMR spectrum for compound 3o in CDCl$_3$ (100 MHz).
**Figure D52:** $^1$H NMR spectrum for compound 4e in CDCl$_3$ (400 MHz).

**Figure D53:** $^{13}$C NMR spectrum for compound 4e in CDCl$_3$ (100 MHz).
Figure D54: $^1$H NMR spectrum for compound 4f in CDCl$_3$ (400 MHz).

Figure D55: $^{13}$C NMR spectrum for compound 4f in CDCl$_3$ (100 MHz).
Figure D56: $^1$H NMR spectrum for compound 4n in CDCl$_3$ (400 MHz).

Figure D57: $^{13}$C NMR spectrum for compound 4n in CDCl$_3$ (100 MHz).
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