SYNTHESIS AND EVALUATION OF PYRIDINIUM DERIVATIVES AS CENTRAL NERVOUS SYSTEM NICOTINIC ACETYLCHOLINE RECEPTOR LIGANDS

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ABSTRACT OF DISSERTATION

Joshua Thomas Longen Ayers

The Graduate School
University of Kentucky
2006
SYNTHESIS AND EVALUATION OF PYRIDINIUM DERIVATIVES AS CENTRAL NERVOUS SYSTEM NICOTINIC ACETYLCHOLINE RECEPTOR LIGANDS

ABSTRACT OF 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

Director: Dr. Peter A. Crooks,
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Lexington, Kentucky
2006

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ABSTRACT OF DISSERTATION

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This project utilized synthesis and in vitro assays to generate antagonist SARs at various nAChR subtypes. Alkylation of the pyridino nitrogen of the nicotine molecule afforded subtype specific antagonists at a4ß2* nAChR subtypes and nAChR subtypes that mediate nicotine-evoked dopamine release. Using this data, a series of mono-azaaromatic quaternary salts were produced and evaluated in binding and functional assays for a4ß2* and a7* nAChR subtypes and nAChR subtypes that mediate nicotine-evoked dopamine release. Additionally, bis-azaaromatic quaternary salts were synthesized and evaluated in the same assays. Two potent lead compounds were identified. N-n-dodecylnicotinium iodide (NDDNI) was found to be very potent at both a4ß2* nAChR subtypes and nAChR subtypes that mediate nicotine-evoked dopamine release. And the most promising candidate was N-N’-bis-dodecylpicolinium dibromide (bDDPiB), which was selective for the nAChR subtypes that mediate nicotine-evoked dopamine release (IC_{50} = 9 nM).

Additionally, using the data from the SARs, predictive computer models were generated to assist in future compound assessment without in vitro assays. Three self-organizing map (SOMs) models were generated from three different sets of compounds. The groups consisted of the mono-substituted compounds, the bis-substituted compounds, and both sets combined. The models were able to successfully "bin" the test set of compounds after developing a model from a similar set of training compounds. Additionally, using genetic functional activity (GFA) algorithms an evolutionary approach to generating predictive model equations was
applied to the compounds. Three separate equations were generated in order to form a predictive method for evaluating affinities at the $\alpha 4\beta 2*$ receptor subtype.

In addition to the modeling and SAR work of the quaternary ammonium compounds, novel synthetic methods were also employed to develop enantiomerically pure nicotine analogs. Efficient enantioselective syntheses of (S)- and R-(+)-nornicotine, (S)- and R-(+)-anabasine, and (S)- and R-(+)-anatabine have been developed, affording isomers in high enantiomeric excess.

KEYWORDS: Nicotinic receptors, Antagonists, Nicotine, Enantiomeric synthesis, Self-organizing maps

Joshua T. L. Ayers

07 July 2006
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DISSERTATION

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By
Joshua Thomas Longen Ayers

Lexington, Kentucky

Director: Dr. Peter A. Crooks,
Professor of Pharmaceutical Sciences, College of Pharmacy

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2006

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In Memory of my Grandfather
Lawrence N. Longen
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Chapter 1: Overview and Research Plan

1.1. **Overview**

In order to bring a drug to market, it first must go through two major areas of research: drug discovery and drug development. Drug discovery incorporates both chemical and biological methodologies in order to identify a compound or a group of compounds, which exhibit favorable *in vitro* activity in specific pharmacological assays. After the initial identification of a lead compound, the development process then incorporates formulation studies and human clinical trials to assess the bioavailability, stability, and safety of the drug candidate (Figure 1.1).

Within the discovery process, there are several important issues to assess. The first is the determination of an active compound. Once a structure is determined to be bioactive in *in vitro* screens, a series of structurally similar compounds are synthesized and evaluated and structure-activity relationships (SAR) are defined in an attempt to optimize biological activity, since the initial compound “hit” is rarely the most efficacious. The synthetic compounds are prioritized according to their bioactivity, and a lead candidate is then identified. The data obtained can be compiled to form a library of information or an SAR database relating the structure of the compounds to their bioactivities. From this information, computer-assisted models of the compound’s interaction with the receptor may be developed.

Depending on the system being evaluated, a variety of techniques may be used to generate computer models for assessing biological activity. Those compounds that
interact with a biological target that previously has been crystallized, e.g. an enzyme or protein molecule, can be modeled in the active site using *in silico* methods involving molecular modeling and docking to the crystallized target. For those biological targets that have not been identified crystallographically, e.g. receptors, several methodologies can be utilized in order to achieve a three-dimensional model of the molecular recognition site from SAR data. Pharmacophore models (three-dimensional approximation of the active binding site) can be generated from large numbers of compounds and their activities predicted using comparative molecular field analysis (CoMFA). Additionally, mathematical models, genetic functional approximation algorithms (GFAs), and self-organizing maps (SOMs) can be used as methodologies to predict activities or trends at a given receptor or enzyme active site.

Further drug development includes the pre-clinical processes associated with toxicity screening, formulation, and characterization of the absorption, distribution, metabolism, and elimination (ADME) properties of the potential drug molecule. After full characterization of the compound, as well as determination of dosing parameters, drug candidates enter human clinical studies. In order to obtain approval for clinical studies, an Investigational New Drug application (IND) must be filed. This document contains all pertinent data to the drug’s safety and efficacy as determined in the studies mentioned above. If accepted, trials in healthy human volunteers (Phase I) may begin followed by patient volunteers (Phase II-IV). After all information on safety, efficacy, and dosage regimes is collected, drug approval is sought from the Food and Drug Administration (FDA) through a New Drug Application (NDA).
In addition to their therapeutic uses, the development of compounds may also be useful as diagnostic and pharmacological tools in the analysis of perspective drug candidates. In \textit{in vitro} binding assays, a test ligand that interacts with the receptor binding site is used as a measurement tool in order to assess the relative affinities of the drug candidates for the target binding site. These ligands must be selective and potent at their site of action in order to be used as diagnostic tools.

An objective of this research is to involve aspects of the drug discovery process to aid in identifying potential drug candidates for therapeutic use or as analytical tools to help determine biological functions.
Figure 1.1 Components of the Drug Discovery and Drug Development Process

**Drug Discovery**

- Lead Identification
- Toxicity
- Pre-Clinical Studies

**Drug Development**

- Clinical Trials Phase I-IV
- Non-Clinical Formulation
- File with FDA

**In vitro** Assessment

Structure-Activity Relationships

Computer-Assisted Drug Design

Synthesis
1.2. Molecular Target and Lead Identification

Nicotinic acetylcholine receptors (nAChRs) belong to a ligand-gated ion channel superfamily of receptors located in both the peripheral and central nervous systems. Neuronal nAChRs are composed of five subunits that assemble in both heterologous (α- and β-subunits) and homologous forms (α-subunits). Several different nAChR combinations, or subtypes, have been identified, with the α4β2* being the most studied heterologous subtype and the α7* being the most studied homologous subtype. Since definitive subunit combinations have yet to be determined, an asterisk (*) is used following subtype nomenclature to indicate the reported subunit make-up. In addition to the above receptor subtypes, which are thought to produce a pharmacological effect without other nAChR subtypes, there are other receptor populations where multiple subtypes contribute to one pharmacological response. While these heterologous subunit combinations have not yet been fully assigned, they are referred to by their biological function that they initiate (e.g. the nicotinic receptor that facilitates dopamine (DA) release. These subtypes, which have recently begun to be identified as the α4β2*, α4α5β2*, α6β2β3*, and α6α4β2β3*, all contributing to nicotine-evoked DA release.

Research in the field of nAChRs has focused mainly on the development of receptor agonists, and to a lesser extent antagonists, that act at specific nicotinic receptor subtypes in order to elucidate function, as well as to serve as potential drug therapies in disease states such as Alzheimer's and Parkinson's disease, schizophrenia, pain management, and nicotine addiction.
Previous research in the Crooks and Dwoskin laboratories has focused on the development of ligands, specifically antagonists that may be more efficacious and specific than the more established “classical” antagonists (e.g. DHßE). In this respect, alkylation of the pyridyl nitrogen of the nicotine (NIC) molecule afforded two lead compounds, N-octynicotinium iodide (NONI), an antagonist at the nAChR subtypes that facilitate DA release, and N-decynicotinium iodide (NDNI), an antagonist at the α4β2* receptor subtype (Crooks et al. 1995; Dwoskin et al. 1999). Thus, quaternary ammonium compounds of this type were considered as useful lead candidates for exploring the complex biological activities at the molecular level through SAR studies at individual nAChR receptor subtypes.

1.3. **Hypothesis and Specific Aims of Research**

The nicotinic receptor has been implicated in many disease states, including Alzheimer’s and Parkinson’s disease and schizophrenia. With the diverse subtype architecture of the nicotinic receptor, novel selective ligands are needed for both furthering research and use as treatments for diseases. Current research has focused in the development of agonists for these purposes. However, the development of novel antagonists at various nAChR subtypes should also give insight into the pharmacology of these receptors. This research will utilize synthesis and in vitro assays to generate antagonist SARs at various nAChR subtypes. These SARs will be used to develop potent and selective antagonists at individual nicotinic receptor subtypes. Additionally, using the data from the SARs, predictive computer models will be generated to assist in future compound assessment without in vitro assays.
Specific Aim 1
SARs directed at alteration of the nicotine molecule will result in optimization of the potency, activity, and nicotinic receptor subtype selectivity of these novel classes of nicotinic receptor ligands. Alterations will include changing the pyridino head group, e.g. pyridinium and picolinium groups, increasing the carbon chain length, and creating both mono- and bis-substituted molecules.

Specific Aim 2
Utilizing the SARs developed in specific aim 1, computer generated models can be developed for screening of compounds at α4β2* receptor subtypes. Modeling techniques will include GFA and SOM model generation.

Specific Aim 3
The synthesis of quaternary ammonium salts outlined in specific aim 1, which utilize commercially available pyridine moieties, e.g. pyridine and S-(−)-nicotine, are useful for discovering new nAChR antagonists. In addition to those commercially available compounds, new potential nicotinic receptor ligands head groups will be developed. Novel enantioselective synthetic procedures of nornicotine, anabasine, and anatabine will be developed and optimized.
Chapter 2: Background

2.1. **Molecular Pharmacology**

Nicotinic acetylcholine receptors (nAChRs) are members of the ligand-gated ion channel superfamily of receptors, activated by endogenous acetylcholine (ACh), and are widely distributed throughout the body. Other members of this superfamily include, gamma-aminobutyric acid (GABA\textsubscript{A}), serotonin (5-HT), glycine, and several glutamate receptors (e.g. \textit{N}-methyl-D-aspartate (NMDA), \textit{\alpha}-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), and kinate). Members of this family of receptors all share a pair of disulphide-bonded cystines in their N-terminus extracellular halves that are separated by 13 residues (Kao and Karlin 1986; Karlin and Akabas 1995; Ortells and Lunt 1995). Nicotinic receptors modulate a wide range of physiological areas, including the central and peripheral nervous systems and the function of the cardiovascular, immune, and neuromuscular systems and are located at the neuromuscular junction in autonomic ganglia, and in the central nervous system (CNS) (Sargent 1993; Lloyd and Williams 2000). nAChRs are preferentially located presynaptically, where they play a role in neurotransmitter release (McGehee and Role 1995; Wonnacott 1997). Postsynaptic nAChRs, however, have also been identified in brain regions, although their function and importance is still being investigated (Colquhoun and Patrick 1997).

2.2. **Structure of the nAChR**

It is generally accepted that nAChRs are membrane-bound pentameric complexes composed of individual protein subunits. When activated by interaction with acetylcholine or other appropriate agonists, the receptor allows passage of cations, e.g.
Na\(^+\), Ca\(^{2+}\), and K\(^+\), through the central pore. These complexes have an approximate molecular weight of 300 kDa. The protein subunits assemble with a 5-fold axis of symmetry and form the ion conduction pathway of the receptor (Karlin and Akabas 1995; Schmitt 2000) (Figure 2.1A). Each subunit is composed of a large N-terminus loop followed by four transmembrane spanning hydrophobic regions, M1-M4 (Claudio et al. 1983; Leonard et al. 1988). The portions of the M1 and the complete segments of the M3 and M4 transmembrane regions interact with the lipid bilayer, separating the pore from the hydrophobic membrane (Karlin 2002; Miyazawa et al. 2003). M2 has a high degree of segregated hydrophilic and hydrophobic portions of the amino acid helical cylinder and forms the inner portion of the ion channel with some sections of the M1 (Leonard et al. 1988; Changeux et al. 1998; Grutter and Changeux 2001; Karlin 2002; Miyazawa et al. 2003). Additionally, alterations to the amino acid sequence of the M2 portion affect the ion selectivity of the channel (Imoto et al. 1988; Adcock et al. 1998) (Figure 2.1B).

Smit et al. (2001) provided the high-resolution structure of the Acetylcholine Binding Protein (AChBP), which provided a number of insights into the structure and function of nAChRs. AChBP is a soluble protein synthesized in snail neuronal glial cells and modulates synaptic transmissions (Brejc et al. 2001; Smit et al. 2001). This protein binds many of the same ligands as the peripheral and neuronal nAChRs, including nicotine, epibatidine, (+)-tubocurarine, and \(\alpha\)-bungarotoxin. The affinities for these ligands closely resemble those found in the \(\alpha7^*\) subtype, though with higher overall affinities. This similarity can be attributed in part to the AChBP’s similar amino acid residues and a
significant sequence alignment (20 - 24%, α7 ~ 24%) with the extracellular portions of the α subunits (Grutter and Changeux 2001; Karlin 2002; Smit et al. 2003).

As stated above, several of the nAChR subtypes have a conserved 13 residue Cys-loop at the N-terminus. This loop is present in the AChBP; it is only 12 amino acid residues and is not conserved. The nAChR subunits are typically hydrophobic residues that make up the loop, while the AChBP’s subunits are mostly hydrophilic (Karlin 2002; Smit et al. 2003). It has been proposed that differences in the 13 amino acid sequence of the Cys-loop are because nAChR subtypes are membrane bound and interacting with the transmembrane domain of the receptor. The AChBP is not membrane bound, therefore, it would not need to have the conserved residues in order to function (Karlin 2002; Smit et al. 2003). The AChBP also provided insight into the binding site of the nAChR. Examination of the peripheral nAChR ACh-binding site by mutagenesis and photoaffinity labeling studies identified the residues involved in ACh-binding (Corringer et al. 2000). When compared to the peripheral nAChR subtype, the AChBP has high conservation of residues that line the ACh-binding site (Smit et al. 2001; Karlin 2002). It was originally thought that the position of the binding site was contained within the α-subunit within the ion channel (Miyazawa et al. 1999). The binding site of the AChBP, however, was found to be at the outside of the complex approximately midway in between the top and bottom of the receptor. Additionally, access to receptor binding site was found to be from the outside not through the ion channel (Changeux and Edelstein 2001; Karlin 2002). Studies of the AChBP binding site also demonstrated that the site was located at the interface of two subunits. The same binding phenomenon was
determined for peripheral and neuronal nAChR subtypes (Corringer et al. 2000). Luetje and Patrick (1991) demonstrated that both the \( \alpha \)- and \( \beta \)-subunits are important in agonist, as well as antagonist, sensitivity to nAChRs. The heteropentamer’s interfacial binding site was split into two components, the principal (\( \alpha \)-subunit) and the complementary (the neighboring subunit, e.g. \( \beta \)-subunits in the CNS and \( \gamma/\delta \) in the periphery). Homopentamer’s binding sites are also split into the principle and complementary components but both are contributed by the \( \alpha \)-subunits. The principal component is composed of three loops, A (Tyr93, \( \alpha 1 \)-numbering), B (Trp149), and C (Tyr190, Cys 192, Cys 193, and Tyr 198). The complementary component is also composed of three loops, D (Trp55 and Glu57, \( \gamma \)-numbering), E (Leu109, Tyr111, Tyr117, and Leu119), and F (Asp 174 and Glu176) (Smit et al. 2003; Grutter et al. 2004). Tyr93 (methyl groups), Trp149 (ammonium ion), and Loop C (ester moiety) all interact with the binding of ACh (Cohen et al. 1991; Zhong et al. 1998; Grutter et al. 2000). Additionally, Sine et al. (2002) have reported that the AChBP binding of HEPES has been crystallized and the charged ammonium group was found to interact with the Trp143 (Trp149 in the peripheral nAChR) residue, confirming the calculations of Zhong et al. (1998). Docking experiments using models of the extracellular domains of nAChR subtypes based on the AChBP structure have been performed with success (Zhong et al. 1998; Fruchart-Gaillard et al. 2002; Le Novere et al. 2002; Schapira et al. 2002). The use of such experiments may lead to increased understanding of the nAChR; however, without crystallized structures of each individual subtype, data collected will not be definitive.
2.3. **NACHR Subunit Nomenclature and Composition**

Nicotinic receptors have been characterized into two different classes, peripheral and neuronal. The peripheral muscle-type nicotinic receptor has been extensively characterized due to the abundance and reproducibility of the receptor. When various toxins were found to bind to the acetylcholine binding site of the muscle nicotinic receptor, radiolabeled forms of these toxins were utilized to identify, quantitate, and purify these receptors. From these studies, the major binding subunit of the nicotinic receptor has become known as the $\alpha$ subunit. This subunit is identified by two adjacent cysteine residues (residues 192 and 193) (Figure 2.1A), as well as a highly conserved binding domain possessing aromatic amino acids involved in $\pi$-interactions (Grutter and Changeux 2001; Sharpless and Wonnacott 2001). From the information gathered using these criteria, ten such subunits have been identified, $\alpha1-\alpha10$, in various peripheral and central nervous systems (Sargent 1993; Elgoyhen *et al.* 2001).
Figure 2.1 Subunit designation and composition

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The $\alpha$ subunit has been found in combination with other subunits, $\beta$, $\gamma$, $\epsilon$, and $\delta$, in the periphery. These other subunits were initially identified as structural subunits. Similarly, $\alpha$ subunits in the central nervous system were found with subunits that lacked the disulfide bridge. These non-alpha or $\beta$ subunits have been identified as $\beta1$-$\beta4$ for heterologously expressed neuronal receptors (Sargent 1993). Additionally, homologously expressed neuronal receptors that only contain the $\alpha$ subunit have also been discovered. The various subunits found have been further organized into four subfamilies, I-IV. Subfamilies I and II contain the homologously expressed subunits, $\alpha9$ and $\alpha10$ (subfamily I) and $\alpha7$ and $\alpha8$ (subfamily II). The peripheral muscle-type receptor subunits ($\alpha1$, $\beta1$, $\gamma$, $\epsilon$, and $\delta$) compose subfamily IV. Finally the largest of the subfamilies, subfamily III ($\alpha2$-$\alpha6$ and $\beta2$-$\beta4$), has been further subdivided into three tribes. Tribe 1 contains the binding subunits, $\alpha2$, $\alpha3$, $\alpha4$, and $\alpha6$. The $\beta$ subunits, $\beta2$ and $\beta4$, make up tribe 2. Tribe 3 is composed of $\alpha5$ and $\beta3$ (Corringer et al. 2000).

Skeletal muscle nicotinic receptors were found to have a subunit composition of $(\alpha1)_2:\beta1:\gamma:\delta$ or $(\alpha1)_2:\beta1:\epsilon:\delta$ depending on the age of the receptor subtype (Karlin and Akabas 1995). The $\epsilon$ subunit replaces the $\gamma$ subunit in adult mammalian muscle receptors. The pentameric structure of this subtype is $\alpha1:\gamma:\alpha1:\delta:\beta1$ or $\alpha1:\epsilon:\alpha1:\delta:\beta1$. Additionally, contrary to the initial findings of the ACh binding site, the $\alpha1$, $\gamma$ or $\epsilon$, and $\delta$ all contributing to the binding of acetylcholine, with the majority of the binding sites located in the $\alpha1$ subunit (Adcock et al. 1998) (Figure 2.1C).
As stated above, CNS nicotinic receptor subunits tend to associate in two modes, e.g. heterologously and homologously. However, in contrast to the significant amount of information on the muscle-type receptors, the subunit make-up and stoichiometries of the neuronal nAChRs are not as well characterized. Members of subfamilies I and IV and the α8 subunit (subfamily II) are not expressed in the mammalian brain (Corringer et al. 2000; Elgoyhen et al. 2001). A number of researchers have isolated cDNA or gene clones of several α subunits, α2, α3, α4, α5, α6, and α7, as well as three β subunits, β2, β3, and β4 (Boulter et al. 1987; Goldman et al. 1987; Deneris et al. 1988; Wada et al. 1988; Deneris et al. 1989; Boulter et al. 1990; Seguela et al. 1993). Subunit mRNA for different α and β subunits have been identified in various overlapping brain regions, suggesting subunit complexity (Wilkens 2001). The binding site of the heterologously expressed nAChR subtypes is formed from both tribe 1 (α2, α3, α4, and α6) and tribe 2 (β2 and β4). Additionally, the function of the receptors requires that at least one subunit of α and one of β be present. Tribe 3 subunits (α5 and β3) have also been proposed to exist in such multi α or β complexes as modulators of the other paired subunits (Lin and Meyer 1998; Girod et al. 1999) (Figure 2.1C). Therefore, the subunit composition of the pentameric system is two binding site pairs and a fifth subunit from tribes 2 or 3 (Luetje 2004).

Homologously expressed receptor subtypes demonstrated a five α subunit binding site structure (suggesting a pentameric structure), each of which may exhibit different affinities for nicotinic ligands (Palma et al. 1996; Orr-Urtreger et al. 1997; Rakhilin et al. 1999). Only the α7 subunit from the homomeric subfamily II exists in the CNS.
Therefore, the only homologously expressed subtype is often referred to as the $\alpha 7^*$ nAChR subtype.

### 2.4. nAChR Subtype Formation

It should be noted that before there is any discussion about subunit assembly, there is still much to be learned about the composition of naturally expressed nAChR subtypes. Although many researchers have presented compelling evidence towards a particular subtype combination, other more complex assemblies may exist *in vivo*, and therefore an asterisk (*) follows native subtypes that are putatively assigned, after the convention adopted by Lukas *et al.* (Lukas *et al.* 1999).

**Heterologously expressed nAChR Subtypes**

Affinity labeling, immunoprecipitation, [$^3$H]-nicotine binding, *in situ* hybridization, and constitutive gene knockout studies all demonstrate that the $\alpha 4\beta 2^*$ receptor subtype is the predominant neuronal nicotinic receptor for which nicotine has high affinity (Clarke *et al.* 1985; Marks *et al.* 1986; Whiting *et al.* 1987; Wada *et al.* 1989; Flores *et al.* 1992; Picciotto *et al.* 1998; Zoli *et al.* 1998). *Xenopus* oocytes express $\alpha 4\beta 2$ receptors with a stoichiometry of $(\alpha 4)_2(\beta 2)_3$, with the presumed order being $\alpha 4\beta 2;\alpha 4\beta 2;\beta 2$ (Anand *et al.* 1991; Cooper *et al.* 1991). Two identical agonist binding sites exist at the $\alpha 4$ and $\beta 2$ subunit interfaces. It has also been proposed that an $\alpha 5$ subunit may be associated with some populations of $\alpha 4\beta 2^*$ receptors (Conroy *et al.* 1992; Sharpless and Wonnacott 2001). $\alpha 4\beta 2^*$ nAChR have been associated with anxiety, depression, pain, cognition, and neurodegeneration (Lloyd and Williams 2000).
Another form of heterologously expressed nAChR subtype is the nicotinic receptors that mediate NIC-evoked DA release. Although not as prevalent in brain tissue as $\alpha_4\beta_2^*$ subtypes (Schulz et al. 1991), these subunit assemblies are very important in regulation of dopamine release, as determined from antagonist studies involving several known compounds that interact at the nAChR subtype (Schulz and Zigmond 1989; Grady et al. 1992; Crooks et al. 1995; Cartier et al. 1996). The existence of DA release facilitating nAChR subtypes is further supported by autoradiography studies, where a potent, non-specific agonist, $[^3H]$(+)-epibatidine, is proposed to bind to areas of the brain containing $\alpha_4\beta_2^*$ nAChR subtypes as well as a second receptor class that were also marked by $[^{125}\text{I}]$neuronal bungarotoxin, a sensitive ligand to the nAChR subtype facilitating DA release (Holladay et al. 1997). Although, initially defined as one subtype, $\alpha_3\beta_2^*$, the exact assignment of the subunit composition of these DA releasing nAChR subtypes was uncertain for many years. mRNA of several nAChR subunits were shown to be present in areas of the brain associated with dopamine release, including $\alpha_4$, $\alpha_5$, $\alpha_6$, $\alpha_7$, $\beta_3$, and $\beta_4$ subunits located in the substantia nigra, which projects to the striatum, a known area involved in dopamine release (Deneris et al. 1989; Wada et al. 1989; Wonnacott 1997; Charpantier et al. 1998). Additionally, mRNA of the modulatory subunits $\alpha_6$ and $\beta_3$ had high expression levels in the substantia nigra (Deneris et al. 1989; Goldner et al. 1997; Charpantier et al. 1998). Studies involving $\alpha_5$ subunits (closely related structurally to $\beta$ subunits) revealed that the replacement of the non-binding $\beta_4$ subunit with an $\alpha_5$ subunit leads to a change in the pore $\text{Ca}^{2+}$ permeabilities and desensitization rates (Wang et al. 1996; Gerzanich et al. 1998) (Figure 2.1C). Moreover, increased agonist potencies were
observed when incorporating α5 into the oocyte α3β2 subunit assembly to yield a subunit composition of α3β2:α5β2:α3 (Gerzanich et al. 1998). These findings generated considerable debate as to the exact subunit composition of nAChR subtypes mediating NIC-evoked DA release (Klink et al. 2001; Azam et al. 2002; Champtiaux et al. 2002; Whiteaker et al. 2002; Zoli et al. 2002), and there remained a large focus on elucidating the specific nAChR subtypes responsible for DA release. The discovery and use of the α-conotoxins (α-ctx), particularly α-ctx MII, has greatly increased understanding of this receptor class. Two receptor classes, α-ctx MII sensitive and α-ctx MII resistant, have been determined using α-ctx MII. The α-ctx MII sensitive receptors were identified as an α3β2* subtype due to α-ctx MII sensitivity for oocytes expressing the α3β2 subtype. However, α3 knockout mice studies did not eliminate [125I]α-ctx MII binding in the brain (Whiteaker et al. 2002). Further studies found that deletion of α6 and β3 subunits led to a loss of [125I]α-ctx MII binding (Champtiaux et al. 2003; Cui et al. 2003). α6 containing receptors were found to be sensitive to α-ctx MII, neuronal bungarotoxin, and α-ctx PnIa, all reported α3β2* subtype antagonists (Kuryatov et al. 2000; Dowell et al. 2003; Everhart et al. 2003). In addition to the α6 subunit investigations, knockout mice have been used to determine which individual subunits are responsible for functional receptor subtypes. Loss of function was demonstrated for α4-, α5-, α6-, β2-, and β3-null mutant mice, implicating their role modulating NIC-evoked DA release (Grady et al. 2001; Champtiaux et al. 2003; Cui et al. 2003; Salminen et al. 2004). Salminen et al. (2004) also determined that β4- and α7-null mutant mice have no loss of receptor function. By coupling the knockout mice data with the α-ctx MII data, Luetje (2004) has
identified four distinct DA releasing subtypes: \( \alpha \)-ctx MII sensitive subtypes, \( \alpha 6\beta 2\beta 3 \) and \( \alpha 6\alpha 4\beta 2\beta 3 \), where \( \alpha 6 \) and \( \alpha 4 \) form with the \( \beta 2 \) subunit to form the binding sites and \( \alpha \)-ctx MII resistant subtypes consisting of \( \alpha 4\beta 2 \) and \( \alpha 4\alpha 5\beta 2 \) with the \( \alpha 4 \) and \( \beta 2 \) subunits forming the agonist binding sites (Luetje 2004). For purposes of this publication, this family of receptors will be referred to by its functional designation, e.g. the receptors that mediate NIC-evoked DA release.

**Homologously expressed nAChr Subtypes**

In addition to heterologously expressed nAChR subtypes, there exists a second class of homologously expressed receptor subtypes. Originally, these homomers were identified using the same methods utilized in identifying muscle-type nAChRs. Using radiolabeled \( \alpha \)-bungarotoxin, a known \( \alpha 1\beta 1\gamma \delta \) ligand (Lee 1972), researchers found a receptor population which demonstrated a high affinity for the compound (Marks et al. 1986). Using \( \alpha 7 \) mRNA brain distributions as well as the autoradiographic binding data, this receptor subtype was identified as an \( \alpha 7 \) homomer (Clarke et al. 1985; Wada et al. 1989; Seguela et al. 1993; Chen and Patrick 1997). This class of homomeric receptor subtype is almost as prevalent as the \( \alpha 4\beta 2^* \) population of nAChRs in the brain. However, these receptors are expressed differently in different brain regions (Holladay et al. 1997). The \( \alpha 7^* \) receptor subtypes are highly permeable to \( \text{Ca}^{2+} \) ions (Seguela et al. 1993; de Fiebre et al. 1995), and rapidly desensitize in the presence of agonists (Castro and Albuquerque 1993). Synapse formation, mediation of nicotine-induced seizures, and modulation of neurotransmitter release are some functions potentially regulated by the \( \alpha 7^* \) nAChR (Orr-Urtreger et al. 1997).
2.5. **Pharmacology of Nicotine at the nAChR**

As stated above, nicotinic receptors mediate a wide variety of effects on many systems in the body. Nicotine, the principal alkaloid and active compound in tobacco, has been found to interact with the acetylcholine binding site on nicotinic receptor subtypes. Nicotine exposure has been shown to enhance memory and attention, increase arousal, decrease anxiety, and cause weight loss (Arneric and Brioni 1999). Additionally, nicotine has been shown to be highly addictive due to its ability to affect the modulation of dopamine release. Like acetylcholine and other agonists, nicotine has been shown to first activate the opening of the receptor ion channel. After activation, nicotine exposure causes a second conformational change in which the receptor becomes refractory or desensitized (Corringer et al. 1998). In its desensitized state, the ion channel is closed, and the receptor is in a resting state. Before activation by an agonist, the receptor has a high affinity for binding nicotine or other agonist molecules. Interestingly, after binding of nicotine, the subsequent desensitized state also has an increased affinity for nicotine, even though it is unable to function (Sharpless and Wonnacott 2001). Additionally, at the binding site, the β-subunit may control the extent of desensitization or inactivation of the receptor after long-term agonist exposure (Fenster et al. 1997; Kuryatov et al. 2000; Kuo et al. 2005). Concentrations needed to desensitize the receptor are also much less then those required to activate the receptor (Rowell and Hillebrand 1994; Fenster et al. 1997; Grady et al. 1997). An increase in receptor number, or up-regulation, also occurs when a4β2* receptors are exposed to chronic nicotine treatments (Wonnacott et al. 1990). However, this upregulation is not due to an increase in receptor mRNA production, and
may be due to a recruitment of nicotinic receptors from a reserve pool (Marks et al. 1992; Bencherif et al. 1995). It should also be noted that different nAChR subtypes respond to agonists differently, and their desensitization rates and function can vary when exposed long term to NIC (Nguyen et al. 2004; Grilli et al. 2005). As stated above, α7 receptors are characterized by their very rapid desensitization, whereas receptors modulating DA release are more resistant to desensitization (Castro and Albuquerque 1993; Gray et al. 1996; Yu et al. 1996). Interestingly, the opposite is true for regulation of these receptor subtypes using chronic NIC infusion. a7* receptor subtypes are more resistant to regulation by NIC, whereas a6* containing receptors (DA mediating nAChR subtypes) are down-regulated when exposed to chronic NIC (Collins et al. 1994; Lai et al. 2005). Desensitization, as well as up-regulation, has allowed researchers not only to search for agonists that are selective for various subtypes, but also to explore subtype-selective antagonists as therapeutic agents (Arneric and Brioni 1999).

2.6. Existing Assays for Evaluating Affinity and Functional Responses at nAChRs

Radioligand Binding Studies

Only a limited number of pharmacological tools are available to evaluate affinity and function at the various nicotinic receptors. Radioligand binding assays are often used as the first step in determining ligand affinity for individual nAChRs, due to methodological ease and the capacity for rapid screening. These assays are carried out using native tissue, or utilizing receptors expressed in Xenopus oocytes or other stably-transfected cell lines.
Ligand binding assays developed for the \( \alpha_4\beta_2^* \) receptor subtype are performed using several different ligands, including \([^3\text{H}]\text{S}-(\text{-})\text{-nicotine (NIC)}, \[^3\text{H}]\text{cytisine}, \[^3\text{H}]\text{(+)-epibatidine, and }[^3\text{H}]\text{ACh. Several different brain regions may be used to obtain native tissue nAChRs, including the thalamus, cortex, and striatum. Cell line K-177, a line of human embryonic kidney (HEK) 293 cells that stably express the human } \alpha_4\beta_2 \text{nAChR, has also been utilized in binding assays (Gopalakrishnan et al. 1996). } \alpha_7^* \text{nAChR subtypes are also assayed via radioligand binding methods; the most widely used ligands for these studies have been }[^3\text{H}]\text{-}\alpha\text{-bungarotoxin and }[^3\text{H}]\text{-methyllycaconitine. Native tissue receptors, as well as stable cell lines expressing } a7 \text{ receptors have been used in these binding assays (Gopalakrishnan et al. 1995; Quik et al. 1996; Whiteaker et al. 1999). A third class of neuronal nAChRs, the subtypes that mediate NIC-evoked DA release, has primarily utilized }[^3\text{H}]\text{(+)-epibatidine as the radioligand in the binding assay. However, due to its non-specificity (} \alpha_4\beta_2 \text{ activity), this radioligand must be used in conjunction with unlabeled cytisine to block }[^3\text{H}]\text{(+)-epibatidine binding to the } \alpha_4\beta_2^* \text{ subtype (Marks et al. 1998). Cloned cell lines have revealed several possible candidates for these studies. } \alpha_3\alpha_5\beta_2, \alpha_3\beta_2, \text{ and } \alpha_3\beta_4 \text{nAChR clones have all shown an affinity for } n\text{-bungarotoxin and epibatidine (Lin and Meyer 1998). }[^{125}\text{I}]-\alpha\text{-ctx MII has also been used as a ligand for the nAChR subtype mediating NIC-evoked DA release (Holladay et al. 1997).}
**Functional Assays**

In order to differentiate between agonists and antagonist at nAChR subtypes, functional studies must be utilized in addition to the binding studies. These functional assays must also be used to assess what is occurring at the actual receptor site. Binding studies only represent the affinity of ligands for the desensitized form of the receptor and may not give an accurate indication of the ligand’s interaction with a functional receptor (e.g. the non-desensitized form). As in the binding studies, functional assays must be subtype specific. Current approaches incorporate the use of slices or synaptosomes from various brain regions, as well as recombinant receptors expressed in *Xenopus* oocytes, or in stably-transfected cell lines. $\alpha 4\beta 2^*$ subtypes have traditionally been assayed using ion flux assays involving the radioactive ion $[^{86}\text{Rb}]^+$. $^{86}\text{Rb}^+$ is an ion considered to be equivalent to the endogenous cation $\text{K}^+$. Rodent brain synaptosomes or stably transfected cell lines (K177 cells or IMR-32 cells) are preloaded with $^{86}\text{Rb}^+$ and superfusion systems in the presence of agonist are used to efflux the radiolabel through the ion channel. Functional response is designated once the compound either elicits efflux (agonist), or inhibits efflux when NIC is introduced into the system as an agonist (antagonist). Data from $^{86}\text{Rb}^+$ efflux and $[^3\text{H}]$-nicotine binding assays have shown correlative $\text{EC}_{50}$ and $\text{IC}_{50}$ values for agonist-induced efflux, respectively, as well as correlation of similar sites of action within brain regions. It is important to note that $\alpha 7$ $[^{125}\text{I}]$-bungarotoxin binding data does not correlate with $^{86}\text{Rb}^+$ efflux brain regions, offering further proof that $^{86}\text{Rb}^+$ efflux is a functional assay for $\alpha 4\beta 2^*$ nAChRs (Marks *et al.* 1998).
Generally, ion flux functional assays for α7 subunits have proven to be unsuccessful, due to rapid α7* receptor desensitization, which has been reported to occur over several milliseconds (Seguela et al. 1993). Consequently, electrophysiology studies have been used to characterize these homomeric receptor subtypes. Whole-cell voltage clamp experiments using either hippocampal interneurons or Xenopus oocytes expressing α7 subtypes have been shown to be useful in determining functional activity before the receptor is desensitized (Frazier et al. 1998; Papke and Thinschmidt 1998; Papke et al. 2000).

As mentioned above, the nAChR has been implicated in the mediation of NIC-evoked dopamine release. Therefore, nAChR subtypes that mediate NIC-evoked DA release have been assayed utilizing neurotransmitter (NT) release methodologies. These assays, usually utilize brain slices or synaptosomes, oocytes, or stably-transfected cell lines, which are preloaded with [3H]-dopamine and superfused to monitor the efflux of the radiolabel in the presence of agonist. Drug candidates can be added to the system to measure their intrinsic (agonist) activity, and compounds can also be tested against a known agonist (e.g. NIC) to determine the ability of the compound to inhibit agonist-evoked dopamine response (antagonist response). Native tissues, such as striatum, as well as cell lines, including IMR-32 human neuroblastoma cells and rat PC12 cells, both of which are ganglionic in nature and express an α3-containing subtype (possibly α3β4), have been employed using this assay method (Lukas 1993; Donnelly-Roberts et al. 1998). Although this assay does not differentiate between the four possible subtypes that
mediate DA release, it has been invaluable in identifying non-\(\alpha 4\beta 2\) and non-\(\alpha 7\) selective ligands.

Researchers may choose to use several types of tissues when conducting any of the assays outlined above. However, both native tissue and expressed cells have benefits and limitations. Using neuronal tissue affords a more accurate representation of the native receptor but limits the effective determination of nAChR subtype compositions, due to the complexity of the neuronal tissue, as well as the limitations of the pharmacological assays. Additionally, the rodent brain (e.g. rat or mouse) is small, and large amounts of tissue may be needed in order to fully assess a series of compounds, adding a high cost to the process. Expressed cells, in contrast, can be grown in large quantities, are quite easy to manipulate, and provide an accurate determination of subunit composition. These cells are also cost-effective, when screening large numbers of compounds. However, perceived strengths of homogeneity and basic cell function are also weaknesses for these tissue preparations. In addition, while the subunit composition of nAChRs is known in both *Xenopus* oocytes and transfected cells, they are not necessarily identical to the corresponding native receptor subtypes, with regard to subunit combination, stoichiometry, post-translational modification, and placement within the brain regions.
2.7. **Nicotinic Ligands of the nAChR**

2.7.1. **Agonists**

**Alkaloids**

The tobacco plant, *Nicotinia tobacum*, contains many different naturally occurring alkaloids. The most abundant, NIC (1), has a varied pharmacological profile, as discussed above, throughout the body, both peripherally and centrally. R-(+)-Nicotine (R-NIC, 2), the enantiomer of NIC, which is formed during pyrolysis of tobacco products and is present in tobacco smoke, also has activity at nAChRs. In addition to NIC, *N. tobacum* contains several other alkaloids, including, S-(-)-nornicotine (3), S-(−)-cotinine (4), S-(−)-anabasine (5), and S-(−)-anatabine (6). R-NIC has lower affinity for the α4β2* receptor subtype than that reported for (1), as determined by inhibition of [3H]-NIC binding (Ki = 46 and 2 nM, respectively) (Crooks et al. 1995). Stimulation of DA release in striatal tissue with (2) afforded an equivalent efficacy but a lower potency than (1) (Garvey et al. 1994). Compounds 3 and 4 have been evaluated in the NIC-evoked [3H]-DA release assay and both evoked [3H]-DA release but neither was as potent as NIC. S-(−)-Nornicotine (EC\textsubscript{50} = 1.0 μM) was more potent than S-(−)-cotinine (EC\textsubscript{50} = 30.0 μM) (Teng et al. 1997; Dwoskin et al. 1999). [3H]-NIC binding affinities for (3) and (4) were also determined in comparison to that of NIC and were 259-fold and 3900-fold lower in affinity, respectively (Garvey et al. 1994; Lin and Meyer 1998). Compound 5 exhibits a 30-fold decrease in affinity when compared to NIC in the [3H]-NIC binding assay, a 40% efficacy in the $^{86}$Rb$^{+}$ efflux assay compared to NIC, and an EC\textsubscript{50} value of 15.4 μM in the NIC-evoked [3H]-DA release assay (Marks et al. 1993; Dwoskin et al. 1995).
Anabaseine and Anabaseine Analogs

Anabaseine (7) is a 1’, 2’-dehydroanabasine analog. It is found in cured tobacco and in the marine worm *Hoponemetines hydrobiologia*. Compound 7 affords about a 20-fold decrease in rat brain [³H]-NIC binding affinity when compared to NIC, and has a similar selectivity for α4β2* nAChR subtypes (Ki = 32 nM) over α7* nAChR subtypes (Ki = 58 nM) (de Fiebre et al. 1995; Kem et al. 1997). However, intrinsic α7 activity is 200% higher for (7) over NIC in expressed oocytes. A 3’-substituted anabaseine analog, GTS-21 (8), exhibits partial agonist activity at rat α7 receptors expressed in *Xenopus* oocytes (ca. 28% of the ACh response) and low activities at α4β2 oocyte-expressed receptors. In contrast, human α7 receptor activity elicited by (8), when compared to that elicited at rat α7 nAChRs, was less efficacious (Briggs et al. 1997; Meyer et al. 1998). 4-OH-GTS-21 (9), a GTS-21 metabolite, showed a similar increase in affinity for the α7 receptor subtype when compared to (8) in rat oocyte PC-12 cells utilizing the [¹²⁵I]α-Btx binding assay [e.g. Ki = 45 nM (4-OH-GTS-21) and 31 nM (GTS-21)]. Additionally, the 4-OH metabolite had more affinity for the binding sites of human SK-N-SH cells when assayed
in the $[^{125}I]$$\alpha$-Btx binding assay (Ki = 170 nM (9) and 23 $\mu$M (8)) (Meyer et al. 1998; Papke et al. 2000).

Figure 2.3 Structures of Anabaseine and Modified Anabaseine Molecules

Two series of restricted nicotine analogs (10-12) have affinities for nAChRs. A syn-rotamers of NIC (10a) elicited antinociceptive effects in the tail flick assay (a purported $\alpha$4$\beta$2$^*$ mediated effect), with the (+)-enantiomer having a 12-fold greater effect over the (-)-enantiomer (Sabey et al. 1999; Sarkar et al. 2004). Interestingly, affinity values of these enantiomers in the $[^3]$H-NIC binding assay were reversed; the (+)- and (-)-enantiomers of 10) having Ki values of >10,000 and 605 nM, respectively. The racemic desmethyl analog (10b) had a higher affinity for $\alpha$4$\beta$2$^*$ nAChRs (Ki = 167 nM) when compared to the N-methyl enantiomers (Chavadarian et al. 1983; Glassco et al. 1993). Xu et al. (2001) obtained similar values for the syn-rotamer compounds 10a and 10b (Ki = 400 and 220 nM, respectively) in the $[^3]$H-NIC binding assay, and these compounds demonstrated different affinities in the $[^3]$H-MLA binding assay, Ki = 590 nM (10a) and 20.2 $\mu$M (9b). Binding affinities for the anti-rotameric analogs (11a and 11b) of NIC and
nornicotine indicated no appreciable affinity of these compounds at the \( \alpha 7 \) receptor subtype (Ki >100 \( \mu M \) for both compounds) and only weak micromolar affinity in the \[^3H\]-NIC binding assay (Ki = 12.2 and 10.5 \( \mu M \), respectively) (Xu et al. 2001). Compound 12, a racemic 6-methoxy substituted analog of (10b), had a much lower affinity (Ki = 1.9 \( \mu M \)) than that of NIC in the \[^3H\]-NIC binding assay using rat brain homogenate, but exhibited strong antinociceptive effects in the rat tail flick assay (McDonald et al. 1996).

Figure 2.4 Structures of Conformationally Restricted NIC Analogs

![Figure 2.4 Structures of Conformationally Restricted NIC Analogs](image)

**Cytisine**

Cytisine (Cyt, 13), an alkaloid found in laburnum of the *Leguminosae* family, has exhibited selectivity for neuronal nAChR subtypes over those in the periphery (Astles et al. 2002). Cyt has demonstrated high affinity and activity at \( \alpha 4 \beta 2^* \) nAChRs. In \[^3H\]-NIC binding assays, Ki values ranging from 0.14-2.7 nM for Cyt have been reported (Marks et al. 1993; Court et al. 1994; Whiteaker et al. 1998). Cyt has been shown to favor \( \alpha 4 \)-containg subtypes over the a7* nAChR subtype (Astles et al. 2002). Functional studies have revealed that Cyt elicits ion flux in both brain thalamic synaptosomes, as well as in cells expressing \( \alpha 4 \beta 2^* \) nicotinic receptors (EC\(_{50} = 0.019 – 71.4 \) nM) (Marks et
al. 1993; Peng et al. 1994; Marks et al. 1996). Cyt has low μM affinity (Ki = 1.6 μM) in the \[^{125}\text{I}]-\alpha\text{-Btx binding assay using rat brain preparations (}\alpha7\text{ nAChRs)}\) (Marks et al. 1993). In the \[^{3}\text{H}]-\text{DA release assay from rat striatal slices, Cyt was a partial agonist (Sacaan et al. 1995). However, Grady et al. (1992) reported that Cyt was a full agonist when evaluating DA release from mouse striatal synaptosomes (Grady et al. 1992). Cyt is also able to distinguish between the \(\beta4\) and \(\beta2\) subunits using oocyte-expressed cells systems, with the \(\alpha4\beta4\) nAChR subtype eliciting a much larger agonist response than the \(\alpha4\beta2\) subtype (Luetje and Patrick 1991).

**Epibatidine**

Epibatidine (Epi, 14), an alkaloid originally isolated from *Epipedobates tricolor* (an Ecuadorian poisonous frog), was first structurally determined by Daly et al. (1991) to be an azabicycloheptane (Daly et al. 1991; Spande et al. 1992; Badio and Daly 1994). To date, Epi is the most potent natural agonist at nAChRs. Epi has demonstrated very little enantioselectivity at various nAChRs (Badio and Daly 1994; Gerzanich et al. 1995). In rat homogenate binding assays using various specific radiolabeled ligands, Epi had picomolar affinities at \(\alpha4\beta2\) receptor subtypes (Ki = 70 pM), low nanomolar affinities at \(\alpha7\) (Ki = 21 nM), and low micromolar affinities at \(\alpha1\gamma\delta\) receptor subtypes (2.7 μM, respectively) (Gopalakrishnan et al. 1996; Sullivan and Bannon 1996). Cell expressed nAChRs demonstrated similar \(\alpha4\beta2\) affinities (Ki = 19 pM) and slightly weaker \(\alpha7\) affinities (Ki = 200 nM). Affinities for \(\alpha3\beta2\) and \(\alpha3\beta4\) nAChRs were determined to be over 10-fold lower than for \(\alpha4\beta2\) nAChRs (Ki = 230 and 380 pM, respectively) (Gerzanich et al. 1995; Xiao et al. 1998). In functional assays, Epi has been evaluated in
the NIC-evoked $[^{86}\text{Rb}^+]$ efflux assay using K177 cells expressing the $\alpha4\beta2^*$ nAChR ($EC_{50} = 17$ nM), as well as oocyte-expressed $\alpha7$ nAChRs ($EC_{50} = 1.3$ $\mu$M) (Gopalakrishnan et al. 1996; Sullivan and Bannon 1996). Epi was also 150-fold more potent ($EC_{50} = 0.4$ nM) than nicotine ($EC_{50} = 60$ nM) in evoking DA release from rat striatal slices, and 40% more efficacious (Sullivan et al. 1994).

Figure 2.5 Structures of Cytisine, Epibatidine, Anatoxin-a

![Structures of Cytisine, Epibatidine, Anatoxin-a](image)

**Anatoxin-a**

(+)-Anatoxin-a (Atx, 15), isolated from the freshwater blue-green algae *Anabaena flos-aquae*, has an azabicyclononene skeleton and exhibits a wide variety of nAChR activities. Atx has a similar affinity to NIC (Atx, $Ki = 3.5$ nM; NIC, $Ki = 1-3$ nM) in the $[^3\text{H}]-\text{NIC}$ binding assay using rat brain homogenate (Wonnacott et al. 1991). Binding studies using rat $\alpha7^*$ native receptors and $[^{125}\text{I}]-\alpha$-Btx revealed that (+)-Atx had a 60-fold higher binding affinity than NIC at this receptor subtype. Using mouse thalamic synaptosomes and M10 cells, 15 has been shown to be more efficacious than NIC in the NIC-evoked $[^{86}\text{Rb}^+]$ efflux assay ($EC_{50} = 48 - 93$ nM and 180 - 2500 nM, respectively) (Thomas et al. 1993; Marks et al. 1996). Using $\alpha7$ receptors expressed in *Xenopus* oocyte cells, ion flux assays demonstrated a 50-fold increase in potency for Atx over NIC (Thomas et al. 1993; Marks et al. 1996).
Additionally, (+)-anatoxin is 10-fold more potent than NIC in PC-12 cells expressing a ganglionic-type receptor (Daly et al. 1991). The (−)-Atx enantiomer has 1000-fold less affinity for α4β2* receptors and 50-fold less affinity for α7* receptors, compared to (+)-Atx, when assaying rat brain tissue (Macallan et al. 1988). Reduction of the olefinic bond in the octene ring of (+)-Atx causes a 20-fold decrease in affinity at both [3H]-NIC (α4β2*) and α-Btx (α7*) binding sites in rat brain tissue.

### 2.7.2. Antagonists

**Competitive Antagonists**

d-Tubocurarine (d-TC, 16) is a classical nAChR antagonist isolated from the South American bush Chondodendron tomentosum. Originally used as a poison on the tips of arrows, d-TC was found to be a potent antagonist at muscle type nAChR [(α1)2βγδ or (α1)2βεδ] (Sharpless and Wonnacott 2001). d-TC binds to many different peripheral and neuronal nicotinic receptor subtypes with a low micromolar affinity, and is able to antagonize several different functional responses of nicotinic receptors at micromolar concentrations in *Xenopus* oocytes (Chiappinelli et al. 1993; Cachelin and Rust 1995; Chavez-Noriega et al. 1997). Similar activities were seen in native neuronal receptor assays for α4β2* nAChR subtypes and receptor subtypes mediating DA release (Grady et al. 1992), further supporting the observation that d-TC is not a selective ligand at nAChR subtypes. It should also be noted that, while d-TC does exhibit competitive antagonistic properties in the systems mentioned above, it also may have a non-competitive component to its receptor inhibition profile (Cachelin and Rust 1994; Chavez-Noriega et al. 1997).
Dihydro-β-erythroidine (DHβE, 17), an alkaloid isolated from *Erythrina* seeds, competitively binds to nAChRs, with affinities in the nanomolar range, at both α4β2* nAChR subtypes and receptor subtypes mediating DA release (Crooks *et al.* 1995; Khiroug *et al.* 2004). Characterization of DHβE in a number of oocyte expression systems demonstrated selectivity between nAChR subtypes. A 3-fold selectivity α4β2 receptor subtype over the α2β2 receptor subtype and a 10-fold selectivity for the α4β4 receptor subtype over the α4β2 receptor subtype. Additionally, an approximate 100-fold increase in selectivity for α4β4 receptors over α3β4 receptors was observed (Harvey and Luetje 1996; Chavez-Noriega *et al.* 1997; Khiroug *et al.* 2004). DHβE binds similarly to NIC, and the receptor blockade can be overcome with high concentrations of NIC or another agonist. DHβE does not act as an antagonist at α7* receptor subtypes (McQuinston and Madison 1999). Erysodine (18), a structurally related DHβE analog, also isolated from *Erythrina* seeds, has also been investigated. Erysodine is more active at α4β2* receptor subtypes than DHβE, but like DHβE has relatively weak affinity for α7* nAChRs (Decker *et al.* 1995). Erysodine also exhibits antagonistic properties similar to those of DHβE at nAChR subtypes mediating DA release.

Methyllycaconitine (MLA, 19) isolated from *Delphinium brownii*, is a tertiary diterpenoid alkaloid. It is a potent and competitive antagonist at the homologously expressed and native α7* nAChR subtypes with a low nanomolar affinity (Ki =1 nM). MLA also has the ability to block α7 mediated currents recorded from *Xenopus* oocytes or hippocampal neurons (Alkondon *et al.* 1992; Palma *et al.* 1996; Davies *et al.* 1999).
addition to the homomeric α7* nAChR subtypes, MLA has been shown to inhibit a population of α7-like nAChR subtypes which are not labeled by other α7 antagonists (Yum et al. 1995; Davies et al. 1999). When compared to other receptor subtypes, both muscle and α4β2* subtypes require micromolar concentrations of MLA to achieve inhibition of nAChR function (Wonnacott et al. 1993). Early studies have shown MLA has little antagonistic activity in the nicotine-evoked dopamine release assay (Drasdo et al. 1992). However, more recent studies have demonstrated that MLA may antagonize α6-containing nAChR subtypes, one of the purported nAChR subtypes involved in mediating DA release (Mogg et al. 2002; Karadsheh et al. 2004). SAR on the MLA molecule reveals the ring E moiety is an important feature for activity, specifically the succinimide moiety of the multi-ring structure. Removal of various portions of the succinimide moiety significantly decreases binding affinity 20- to 2000-fold (Hardick et al. 1995). In addition, Ring E racemic analogs of MLA exhibited moderate to equivalent activity compared to that of MLA, and demonstrates the importance of the methyl group in the succinimide moiety (Jacyno et al. 1996; Bergmeier et al. 1999).
Neurotoxins

Several small peptide antagonists have been identified from the venom of *Bungarus multicinctus*, the Formosan banded krait. α-Bungarotoxin (α-Btx), the most studied of these peptides, is an 8kDa protein that was found to have high affinity at neuronal α7* homomeric nAChR subtypes (Ki = 1 nM) (Marks *et al.* 1986; Davies *et al.* 1999). Homomeric avian α8* nAChRs and α9* nAChRs of cochlear hair cells are also inhibited with low nanomolar concentrations of α-Btx (Gotti *et al.* 1994; Elgoyhen *et al.* 2001). α-
Btx does not seem to antagonize any neuronal heteromeric nAChR assemblies (McGehee and Role 1995).

A minor component of the venom of *Bungarus multicinctus*, is neuronal bungarotoxin (n-Btx or κ-Btx). n-Btx has been shown to inhibit some α3*- and α6*- containing neuronal nicotinic receptor subtypes, as well as α7* subtypes (Chiappinelli 1983; Luetje *et al.* 1998; Luetje 2004). n-Btx inhibits nicotine-evoked dopamine release, as well as inhibiting $[^3]H$-(+)-epibatidine non-α4β2* binding sites (Grady *et al.* 1992; Houghtling *et al.* 1995). Due to its limited availability and non-selective pharmacological profile, n-Btx is not as widely utilized in research as other nAChR antagonists.

Other neurotoxins exist in nature that act at various nAChRs, including neosurugatoxin (NSTX, 20) from the Japanese mollusk *Babylonia japonica*, which exhibits non-competitive antagonism at several different nAChR subtypes and lophotoxin (LTX, 21) and its analog LTX-1 are cyclic diterpenes isolated from coral, which block neurotransmission of muscle type and ganglionic nAChRs (Abramson *et al.* 1988; Groebe and Abramson 1995). Additionally, other toxins identified, include α-cobratoxin (α-CBT), erabutoxin-a (ETX-a) and erabutoxin-b (ETX-b), and histriionicotoxin, all of which have been isolated from various species of snake (Luetje *et al.* 1990; Adams and Olivera 1994). ETX-a has been identified as an inhibitor of NIC-evoked DA release in rat striatal tissue, while α-CBT had no inhibitory effect in the same system (Dajas-Bailador *et al.* 1998).
**a-Conotoxins**

Venoms from the *Conus* marine snails have been isolated and found to target various nAChR subtypes. The α-conotoxin compounds are peptides of 12-19 amino acids in length and contain key disulphide bonds integral to maintaining their structure (Janes 2005). α-Conotoxin MII (22a) has been shown to potently and selectively antagonize α3β2 nAChRs expressed in *Xenopus* oocytes at concentrations of 100 nM, which was 2 to 4 orders of magnitude higher than any other subtype tested (Harvey et al. 1997; Kaiser et al. 1998). Partial inhibition of nicotine-evoked dopamine release was observed when rat striatal synaptosomes were exposed to α-conotoxin MII (Kaiser et al. 1998). This partial inhibition was due to the presence of two classes of nAChR subtypes involved in mediating DA release. The α6-containing α-ctx MII sensitive receptor subtypes are blocked by α-ctx MII (Salminen et al. 2004). Related α-conotoxins, α-conotoxin ImI (22b) and α-conotoxin AuBI (22c), were shown to be selective for α7 and α3β4 receptors, respectively (Johnson et al. 1995; Grady et al. 2001). However, recent evaluation of α-ImI and a close analog, α-ImII (75% homology), both demonstrate activity at α3β2 (IC50 = 40.8 nM and 9.61 μM, respectively) and α7 (IC50 = 595 nM and 571 nM, respectively) receptor subtypes expressed in *Xenopus* oocytes. Therefore, the evaluation of the selectivity of α-ImI is undecided at this time. α-ImII did act at a different α7 binding region than traditional α7 antagonists, including α-ImI. This α-conotoxin could act as a potential probe for a novel α7 binding site (Ellison et al. 2003; Ellison et al. 2004). α-Conotoxin BuIA (22d), isolated from *Conus bullatus*, demonstrated a broad spectrum of activity at several nAChR subtypes expressed in *Xenopus* oocytes. BuIA was most active in the inhibiting α6/α3β2 nAChRs (IC50 = 0.258
nM) followed by a6/a3ß4 nAChRs (IC$_{50}$ = 1.54 nM). a7 and a4ß2 nAChR subtypes were not as active with IC$_{50}$ values of 272 nM and 10.4 µM, respectively. Additionally, BuIA was able to differentiate between ß2- and ß4-containing subtypes due to different dissociation rates when a common α-subunit was used (Azam et al. 2005).

**Nicotinium Salts**

$N$-$n$-alkylnicotinium iodide salts have been prepared by regioselective $N$-alkylation of the $S$-(-)-nicotine pyridino-$N$ moiety (Crooks et al. 1995; Dwoskin et al. 1999). These compounds exhibited a range of inhibitory potency at various nAChR subtypes. Compounds 23a-d were the most potent inhibitors. In [$^3$H]-NIC binding experiments utilizing rat striatal slices, NDNI (Ki = 93 nM) and NDDNI (140 nM) had similar affinities for the $\alpha$4$\beta$2* receptor subtype, with NNNI (840 nM) having moderate affinity, and NONI (20 µM) demonstrating weaker affinity for this receptor subtype. Functional responses (IC$_{50}$) were also determined in NIC-evoked $^{86}$Rb$^+$ efflux assays, IC$_{50}$ values obtained were: NDNI (14 nM) = NNNI (9 nM) > NDDNI (40 nM) > NONI (10 µM). Nicotine-evoked [$^3$H]-DA release assays, these $N$-alkylnicotinium salts exhibited a different inhibitory profile than that observed in the $^{86}$Rb$^+$ efflux assays, with inhibitory potencies (IC$_{50}$) ranging from the low nanomolar to high micromolar range; IC$_{50}$ values were as follows: NDDNI (9 nM) > NNNI (140 nM) > NONI (620 nM) > NDNI (>100 µM). From the functional and binding data, it can be concluded that NNNI and NDDNI are nonselective antagonists at $\alpha$4$\beta$2* nAChR subtypes and at nAChR subtypes mediating NIC-evoked DA release, while NDNI and NONI, due to their functional profiles, are selective and competitive receptor antagonists at $\alpha$4$\beta$2* nAChR subtypes.
and at nAChR subtypes mediating NIC-evoked DA release, respectively. In addition, none of the N-alkylated compounds 23a-d had any affinity for α7 [3H]-MLA binding sites (Wilkins et al. 2003). When unsaturation was introduced to the alkyl chains of the N-n-alkylnicotinium salts, the NONI analogs gained affinity for α4β2* nAChR subtypes (0.08 – 4.49 µM), while maintaining or increasing their activity at nAChR subtypes mediating NIC-evoked DA release (80 – 250 nM). Additionally, the NDNI analogs maintained or lost affinity for α4β2* nAChR subtypes (0.05 – 7.47 µM), but demonstrated antagonist activity at nAChR subtypes mediating NIC-evoked DA release (20 – 140 nM) (Sumarithan et al. 2005).

Figure 2.7 Structures of Nicotinium and Structurally Modified Nicotinium Salts

A second series of conformationally restricted nicotinium analogs (24 and 25) were alkylated with various long carbon chain lengths (Crooks et al. 2000). Restriction of the pyrrolidine ring rotation about the pyridine moiety caused complete loss of affinity for the α4β2* nAChR subtype, and none of these compounds had any activity in the [3H]-MLA binding assay. However, analysis of these compounds in the [3H]-nicotine-evoked dopamine release assay demonstrated that a general increase in carbon chain length from
C₈ to C₁₂ produced a nearly 100-fold increase in antagonism at the nAChR mediating DA release (IC₅₀ = 30 – 660 nM). The sole exception was the dodecyl analog BCDD, which was devoid of inhibitory activity at this receptor, although interestingly the isomeric analog ACDD exhibited an IC₅₀ of 220 nm.

**Other Antagonists**

Lobeline (LOB, 26), an alkaloid found in *Lobelia inflata* plants is the active ingredient in what is referred to as “Indian tobacco”. LOB has many pharmacological effects related to those of nicotine, and has historically been regarded as an agonist at nicotinic receptors; however, it may bind to the nicotinic receptor in a novel manner (Decker *et al.* 1995; Damaj *et al.* 1997). Both LOB and nicotine bind to the α4β2* nAChR with low nanomolar affinity (Ki = 4 - 30 nM), while exhibiting little or no α7 affinity (>10,000 nM) (Abood *et al.* 1988; Reavill *et al.* 1990; Bhat *et al.* 1991; Court *et al.* 1994; Brioni *et al.* 1997). LOB has recently been shown to competitively inhibit NIC-evoked [³H]dopamine overflow (IC₅₀ = 300 nM), as well as inhibiting NIC-evoked ^⁸⁶Rb⁺ efflux (IC₅₀ = 700 nM) in rat striatal slices and in rat thalamus synaptosomes, respectively (Miller *et al.* 2000). It should be noted that at concentrations greater than 1 µM, LOB potently releases dopamine from rat striatal slices in a manner that is not nAChR mediated, since the release cannot be inhibited by mecamylamine (Teng *et al.* 1997). These data lead to the classification of LOB as a competitive, nonselective antagonist at α4β2* nAChR subtypes and at nAChR subtypes mediating NIC-evoked DA release.
Strychnine (27) has been shown to competitively inhibit the α7* nAChR subtype, in addition to its original use as an antagonist at the glycine-gated chloride channel (Matsubayashi et al. 1998). In Xenopus oocytes expressing nAChRs individually composed of homomeric α7, α8, and α9 nAChR subtypes, strychnine acts as an inhibitor at toxicologically relevant concentrations (Gerzanich et al. 1994; Peng et al. 1994). Additionally, strychnine has also been found to block α4β2* nAChRs non-competitively (Matsubayashi et al. 1998).

bis-Isoquinolines, tetrandrine, hernandezine and E6-berbamine were evaluated as ligands to block ACh-evoked currents at α3* and α7 nAChR subtypes endogenously expressed in PC12 cells. Tetrandrine, hernandezine and E6-berbamine exhibited antagonistic activity at the α3* nAChR subtypes (IC50 = 8.1, 5.8, and 5.1 µM, respectively). [3H]MLA binding studies revealed that all three compounds displaced the radiolabeled ligand (IC50 = 8.1 µM (tetrandrine), 5.8 µM (hernandezine), and 5.1 µM (E6-berbamine)). Additionally, the functional assay demonstrated that tetrandrine and hernandezine inhibited ACh-evoked currents at α7 nAChR subtypes (IC50 = 407 and 372 nM, respectively) (Virginio et al. 2005).
Prevailing drug discovery endeavors have lead researchers to explore structural modifications of tobacco alkaloids in an attempt to find novel agonists that are potent and subtype-selective. More recently, there has been a growing interest in the development of novel receptor antagonists that are selective and potent at various nAChR subtypes. Abbot laboratories have disclosed several antagonist candidates that have stemmed from
their development of a series of pyridyl ether agonists. Compounds 29-36 have been evaluated in the IMR-32 expressed cell assay utilizing the nicotine-evoked $^{86}$Rb$^+$ efflux assay to determine the extent of $\alpha_3\beta_4^*$ nAChR antagonist activity. Compound 29a and 29b inhibited 50% of the $^{86}$Rb$^+$ efflux at concentrations of 1 $\mu$M, although 29b had 15% intrinsic activity at the same concentration implying that it is a partial agonist. Under the same conditions, compound 30a produced 50% inhibition (1 $\mu$M) and 60% inhibition (10 $\mu$M), while compound 30b exhibited 20-90% inhibition over the concentration range 1-10 $\mu$M. Two compounds in the 3-(2-pyrrolidinylethoxy)pyridine series (31a and 31b) produced moderate antagonist activity (IC$_{50}$ = 75 and 6 $\mu$M, respectively), with no observed intrinsic activity and compound 32 (1 $\mu$M) also exhibited 55% inhibition of NIC-evoked $^{86}$Rb$^+$ efflux and produced no intrinsic activity. Therefore, a pyridyl ether linkage can demonstrate antagonist activities at the $\alpha_3\beta_4^*$ nAChR subtype. The substituted pyridyl ethers (33-36) were also assessed using IMR-32 expressed cells in nicotine (100 $\mu$M)-evoked $^{86}$Rb$^+$ efflux assays. Compound 33a at a 1 $\mu$M exhibited 66% inhibition of NIC-evoked $^{86}$Rb$^+$ efflux, while compound 33b at 10 $\mu$M exhibited 100% inhibition, indicating potent $\alpha_3\beta_4^*$ antagonism. The 5-substituted pyridyl ethers 34a and 34b were found to inhibit 90% of NIC-evoked $^{86}$Rb$^+$ efflux at 10 $\mu$M; while 34b exhibited an IC$_{50}$ = 2 $\mu$M. Compounds 35a and 35b, exhibited ~50% inhibition, while the R- and S- enantiomers of 35c (only S-(-) shown) had comparable IC$_{50}$ values of 9 $\mu$M. Compound 36 (1 $\mu$M) produced 50% inhibition of NIC-evoked $^{86}$Rb$^+$ efflux. Taken together the substituted pyridyl ethers demonstrate an ability to inhibit $\alpha_3\beta_4^*$ nAChR subtypes at the $\mu$M level. The inclusion of a chlorine molecule on either aromatic ring in compounds 33a or 33b resulted in the most potent compounds in this series.
Additionally, this series of compounds demonstrates that the 5-position of the pyridine ring can be altered with large substituents and still fit into the binding site.

Figure 2.9 Conformationally Restricted Pyridyl Ether Analogs

Compounds 37-40 were assayed in the IMR-32 cell assay described above and/or in K177 cells expressing the α4β2* nAChR subtype. A rigid aromatic analog of compound 30b, compound 37, exhibits nonselective antagonist activity in both the K177 cell assay (55% inhibition and 6% intrinsic activity) and the IMR cell assay (38% inhibition and 7% intrinsic activity). This indicates a significant difference in inhibitory capability, since 30b was assayed in the IMR cell assay at 10 µM (90% inhibition). The difference in IMR cell assay activities between 37 and 30b may be attributed to increased aromatic characteristics of 37 as well as the additional flexibility of 30b. Compounds 38-40 incorporate an extra degree of conformational rigidity, due to the substitution of a hexahydro-1H-pyrrolizine moiety for the N-methyl pyrrolidine ring. The 7a-substituted hexahydro-1H-pyrrolizine analog, 38, was a low-potency antagonist (43% inhibition,
with minimal intrinsic activity). Compound 39 (30 µM), a dehalogenated, rigid analog of 37, exhibited no intrinsic activity, while exhibiting 50% inhibition in the IMR cell assay. The 55% inhibition produced by compound 40 is not significantly different from that afforded by its stereoisomer 39 in the IMR cell assay.

Figure 2.10 Structural Analogs of Anabaseine and NIC

In addition to the structural modifications of the agonist molecules listed above, other researchers have attempted to structurally modify the minor tobacco alkaloids, anabaseine and anabasine, in order to identify possible new nAChR antagonists. The partial agonist GTS-21 (7; also known as DMXB) is a structural analog of anabaseine and has been discussed previously in this chapter (de Fiebre et al. 1995; Meyer et al. 1997; Meyer et al. 1998). 3-Trans-cinnamylidene analogs have been developed to alter the agonist/antagonist profile of GTS-21 in an attempt to create antagonists that are more potent. Using an Xenopus oocyte α7 expression system, compounds 41a, 41b, and 41c were shown to exhibit nanomolar affinities for [125I]-α-Btx binding sites (IC50 = 8-80
nM), and cause activation of α7 receptor channels (EC\textsubscript{50} = 3.2 – 15.9 µM). These compounds also inhibited acetylcholine-elicited electrical responses after incubation for five minutes with various concentrations of the analog. The following order of potencies (IC\textsubscript{50}) was obtained: 2-MeOCA (41b) (2.3 µM) > 4-MeOCA (41c) (5.0 µM) > 3-CA (41a) (15 µM). Due to the similar concentration ranges for both IC\textsubscript{50} and EC\textsubscript{50} values, these compounds have antagonist characteristics but are viewed as partial agonists, rather than antagonists (Meyer \textit{et al.} 1998).

Conformationally restricted piperidine ring analogs of anabasine have been shown to be potent antagonists at α4β2* nAChRs. Compound 42 has high affinity for the [³H]-nicotine binding site (Ki = 1 nM) in rat brain membranes, and blocks nicotine (10µM)-evoked \(^{86}\)Rb\(^+\) efflux in rat thalamic synaptosomes (IC\textsubscript{50} = 154 nM) (Crooks \textit{et al.} 1998). However, in the nicotine-evoked [³H]-dopamine release assay using rat striatal synaptosomes, compound 42 had an EC\textsubscript{50} of 2 nM, E\textsubscript{max} = 40%, and this compound activated α3β4* (EC\textsubscript{50} = 1100 nM) and muscle-type (EC\textsubscript{50} = 59 nM) nAChRs (Bencherif \textit{et al.} 1998). Therefore, 42 is potent antagonist at α4β2* nAChRs and an agonist at other nAChR subtypes. The R-isomer appears to be the more active of the two enantiomers, with a Ki value of 0.5 µM at α4β2* nAChRs, and an EC\textsubscript{50} value of 9 nM, E\textsubscript{max} = 55% at nAChR subtypes mediating NIC-evoked DA release (Dwoskin and Crooks 2001). Compounds 43a and 43b are adamantane analogs of anabasine, developed by R. J. Reynolds (Co. 1999, 1999, 1999, 2000). Compound 43b was found to antagonize NIC (100 µM)-evoked \(^{86}\)Rb\(^+\) efflux in rat thalamic synaptosomes with an IC\textsubscript{50} value of 630 nM. Additionally, both 43a and 43b were found to inhibit NIC-evoked [³H]-DA release
using rat striatal synaptosomes with values in the high nanomolar range (IC$_{50} =$659 and 846 μM respectively) and were devoid of intrinsic activity. Thus, these 1-aza-2-(5-ethoxy-(3-pyridyl))tricyclo-[3.3.1.1$^{3,7}$]decanes appear to be nonselective and potent antagonist at α4β2* nAChR subtypes and nAChR subtypes mediating NIC-evoked DA release. Compounds 44 and 45 are methylene bridged analogs of 2-(3-pyridyl)-1-azabicyclo[2.2.2]octane (42) and have high affinity for the [3H]-NIC binding site in rat cortical membranes (Ki = 37 and 73 nM respectively). Although no functional antagonism has been reported in the $^{86}$Rb$^+$ efflux assay, no agonist activity was observed in the $^{86}$Rb$^+$ efflux assay, using rat thalamic synaptosomes. Also, no agonist activity was observed in either muscle-type (α1β1γδ) or α3β4* nAChRs (Dwoskin et al. 2000; Dwoskin and Crooks 2001).

2.8. **Channel Blockers**

One of the first nAChR open channel blockers (OCB) was identified as hexamethonium (HEX, 46), a six-carbon bis-trimethylammonium alkyl compound, which acts within the pores of nAChR subtypes on autonomic ganglia (Paton and Zaimis 1951; Blackman et al. 1963; Ascher et al. 1979). HEX is considered a prototypical OCB, since it meets the following criteria: (1) it causes a voltage dependent blocking effect, (2) it displays a use-dependent mode of action, and (3) it produces a more pronounced inhibitory effect when there is an increase in agonist concentrations (Ascher et al. 1979; Buisson and Bertrand 1998). Compound 46 has also been shown to exhibit antagonist effects on reconstituted chick and rat α4β2* nAChRs (Bertrand et al. 1990; Charnet et al. 1992).
Mecamylamine (MEC, 47) has become one of the most utilized nAChR blockers due to its ability to block the physiological, behavioral, and reinforcing effects of nicotine (Papke et al. 2001). MEC, a neuronal and peripheral OCB, has been shown to block several neuronal nAChR subtypes at low micromolar affinities, but has lower activities at muscle-like nAChRs (Cachelin and Rust 1995; Briggs and McKenna 1996). Moderate nanomolar inhibition (IC$_{50}$ = 150 – 190 nM) of rat and *Xenopus* oocyte-expressed $\alpha$3$\beta$4 nAChRs has been observed (Cachelin and Rust 1995; Webster et al. 1999). Separation of the R- and S-enantiomers of MEC demonstrated very little pharmacological benefit. However, S-(+)-MEC enantiomer appears to dissociate more slowly than R-(−)-MEC at $\alpha$4$\beta$3* and $\alpha$4$\beta$2* nAChRs (Papke et al. 2001). Suchocki et al. (1991) prepared a series of C1, C2, C3, and C7 analogs of MEC and showed that compounds with methyl substituents at the C3 position were important for channel blockage activity (Suchocki et al. 1991). Creasy et al. (1996) determined that substitutions on the C2 amino functionality with large groupings were detrimental to antinociception and hypomotility.
activities (Creasy et al. 1996). Webster et al. (1999) found that point mutations of the M2 region of the β4 subunit in Xenopus oocytes expressing ganglionic (α4β3) neuronal nAChRs, caused a decrease in sensitivity to MEC, indicating a possible binding location of MEC. Another well studied OCB is the bis-quaternary antagonist, chlorisondamine (48). This compound has no effect on [3H]-ACh or [3H]-NIC binding to rat brain (Schwartz et al. 1982). However, chlorisondamine does block NIC-evoked DA release from rat striatal synaptosomes both in vitro and when administered over a period of weeks in vivo (10 mg/kg/day) (el-Bizri and Clarke 1994, 1994). This suggests that the quaternary ammonium molecule is able to pass the blood brain barrier, although the exact mechanism is not known. In addition, it is not known if metabolic changes could account for its CNS activity. Central nervous system accumulation of chlorisondamine has been suggested to explain the blockade effects; however, this cannot provide a complete explanation of the long-term blockade effects of the drug (el-Bizri and Clarke 1994; Reuben et al. 1998). Buisson and Bertrand have demonstrated that several OCBs have activity at human α4β2* receptors. Classical NMDA OCBs, amantadine (49), memantine (50), and dizocilpine ((+)-MK-801, 51), as well as, the Ca\(^{2+}\) channel antagonist 8-(N,N-diethylamino)-octyl 3,4,5,-trimethoxybenzoate (TMB-8, 52) were evaluated using stably expressed α4β2* receptors from embryonic kidney 293 cells. ACh-elicited electrophysiological current blockade was measured in the presence of various concentrations of each of a number of antagonist molecules. Potency values (IC\(_{50}\)) were as follows: amantadine (3 µM) > memantine (7 µM) > TMB-8 (15 µM) ~ MK-801 (15 µM). These studies demonstrated that OCBs tested non-competitively antagonized α4β2* nAChRs and were supported by a mechanism of action mathematical
model detailing the interaction between the nAChR and OCBs (Buisson and Bertrand 1998). TMB-8 has also been shown to potently inhibit agonist-induced flux through muscle-type and ganglionic type nAChRs (IC$_{50}$ ~400 nM), as well as, exhibiting potent inhibition (IC$_{50}$ ~500 nM) in NIC-evoked $[^{3}]$H]DA release assays using rat brain synaptosomes (Bench erif et al. 1995). However, all nAChR binding affinities for TMB-8 were in the moderate to high micromolar range (IC$_{50}$ = 30 - 200 µM). Additionally, in the muscle-type nAChR ion flux assay, in the presence of TMB-8, increased agonist (ACh) concentration caused a more potent blockade of the receptor. MK-801 was also examined in human α7 nAChRs expressed in Xenopus oocytes, and exhibited a non-competitive inhibition of ACh elicited currents (IC$_{50}$ = 14 - 15 µM). Stereoselectivity was not a criteria for blockade since both the (+)- and (-)-MK-801 isomers had similar activities and the blockade observed was dependent on cell potential, a hallmark of channel blockade (Briggs and McKenna 1996).

2.9. The Nicotinic Pharmacophore

The nicotinic pharmacophore has been studied for over fifty years. Theories in the early 1950s and 60s suggested a positive center located near an onium site (e.g. N$^+$) (Hey 1952; Sekul and Holland 1961). In the late sixties, the theory was revised to include a partially negative site near (4.85 Å) an onium site (Kier 1968). Beers and Reich proposed a model that is considered to be the first for the nicotinic pharmacophore. They proposed a two element system using both agonist and antagonist molecules: element 1- a protonated nitrogen, and element 2- an electronegative atom that can act as a hydrogen bond acceptor. They also proposed a specific distance of 5.9 Å as the optimum length between
the center of the charged moiety and a van der Waals interaction area in the receptor binding site (Beers and Reich 1970). Sheridan et al. (1986) suggested that a third element, an aryl centroid moiety, was needed for binding site recognition, a point or atom that was close to the H-bond acceptor site and that was in-line with the acceptor atom, in addition to altering the Beers-Reich distance to 4.8 Å (See Figure 2.12A) (Sheridan et al. 1986). Over the last decade, several nAChR ligands have been identified and modifications to the Sheridan model have been necessary. One major issue was some compounds with affinity for the nAChR were not in accordence the Sheridan distance. Therefore, a model had to be developed for “long” and “short” ligands. It is also important to note that stereochemistry plays an important part in receptor recognition, and since several enantiomerically pure ligands have dramatically different affinities compared to their optical isomers (Hacksell and Mellin 1989). Tonder et al. (2001) proposed the following nAChR pharmacophore: 1- interaction between the protonated atom of the ligand (N\(^+\)) with an anionic site (a) located on the receptor, 2- a hydrogen bond acceptor moiety (X) on the ligand that is paired with a H-bond donator (b) on the receptor surface, 3- \(\pi\)-system interaction of the ligand with \(\pi\)-systems (or positively charged residues) of the receptor (aryl centroid, c), 4- interactions between a protonated N-atom in the ligand and \(\pi\)-systems of the receptor (possibly Trp-149), and steric interactions around the aliphatic and heteroaromatic portions of the molecule (Figure 2.12b) (Tonder and Olesen 2001; Tonder et al. 2001; Glennon and Dukat 2004). Another model proposed involved the use of a water molecule to change “short” ligands into “long” ligands. This water molecule may interact with the protonated atom or the hydrogen bonding acceptor (Glennon et al. 2004). Many ligands, however, have shown
different affinities and activities at various nAChRs. Therefore, different pharmacophore models may need to be developed for each individual receptor subtype, and maybe even for the assays as well, due to the varied characteristics of the pharmacological tools used.

Figure 2.12 Pharmacophore models proposed for the α4β2* receptor subtype

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The pharmacological tools themselves pose a challenge as it is unclear which molecular species is interacting with the binding site. Huang et al. (2005) modeled multiple free base and protonation states of NIC and (R)-descholoroepibatidine (DCEH) using a variety of molecular modeling and docking techniques to explore a general strategy of “from-microscopic-to-phenomenological” approach. This procedure involved prediction of binding energies to each of the different states of NIC and DCEH. Furthermore, hydrogen-bonding and cation-π interactions were found to be the dominant forces that differentiated the binding strengths of the molecular states of NIC and DCEH. These types of studies increase modeling ability beyond a static structure to a more dynamic
approach that can correlate binding energies with multiple states of molecule (Huang et al. 2005).

2.10. Disease States

Alzheimer’s Disease

Alzheimer’s disease (AD) is diagnosed as the loss of ability to learn by memory and attention deficits and usually affect the population over the age of 65 years and older (Lindstrom 1997; Tuppo and Arias 2005). As mentioned above, NIC improves cognitive function in healthy humans. Additionally, treatment of Alzheimer’s patients with nicotine has attenuated the decline of cognitive deficits (Everitt and Robbins 1997; Lawrence and Sahakian 1998). Diagnosed Alzheimer’s patients also had a decreased number of high affinity $[^3\text{H}]-$NIC binding sites in various brain regions (Flynn and Mash 1986; Whitehouse et al. 1988; Sugaya et al. 1990; Nordberg et al. 1995; Rusted et al. 2000). On a genetic level, β2 knockout mice (e.g. mice unable to express β2 subunits) lack high affinity nicotine binding sites. In addition, these knockout mice demonstrated learning and memory abnormalities, implicating the $\alpha_4\beta_2^*$ receptor subtype as playing a key role in cognitive function (Picciotto et al. 1995; Zoli et al. 1999). Neuroinflammation has also been linked to the onset of Alzheimer’s disease, although the role it plays is not fully understood (Tuppo and Arias 2005). $\alpha_7^*$ binding sites are unaffected in Alzheimer’s patients; however, a high concentration of Ca$^{2+}$ ions may implicate this receptor subtype in neurodegeneration, due to the high Ca$^{2+}$ permeability of the $\alpha_7^*$ nAChR (Sugaya et al. 1990; Perry et al. 2000). Additionally, strong correlations have been found between expression of $\alpha_7^*$ nAChR subtypes and the presence and rate
of accumulation of amyloid beta (1-42), the predominant Aβ species in amyloid plaques. The two proteins strongly interact and interference in this interaction may lead to the neurodegeneration. With this information, α7* nAChR subtypes may be targets for the treatment of Alzheimer’s disease (D’Andrea et al. 2002; D’Andrea and Nagele 2006). To date, several subtype selective nAChR ligands are being investigated for their potential therapeutic value. Among them are ABT-418 (Abbott Laboratories), RJR-2403 (R. J. Reynolds/Targacept), and GTS-21 (Decker et al. 1994; Lippiello et al. 1996; Kem 1997; Gotti et al. 2000). Administration of the nAChR channel blocker, mecamylamine, decreased cognitive function in all populations tested. This blockade of the channel is overcome by administration of NIC. Interestingly, younger healthy volunteers had a decreased sensitivity to the mecamylamine induced cognitive deficits, while AD patients had the highest sensitivity (Rusted et al. 2000)

**Parkinson’s Disease**

Parkinson’s disease (PD) has been closely associated with Alzheimer’s disease in that patients in both disease states are found to have deficiencies in neurotransmitter systems, as well as decreased nAChR expression within the CNS. Parkinsonian patients suffer from an inability to control hyper-motor function. This is manifested as difficulties in initiating and sustaining motions (Lloyd et al. 1975; Olanow 2004; Samii et al. 2004). These motor control problems occur due to loss of dopaminergic neurons in the substantia nigra (German et al. 1989). L-Dihydroxyphenylanine (L-DOPA), a dopamine prodrug, treatments have been used as a form of dopamine substitute therapy, after conversion of L-DOPA to dopamine in the CNS. Although not a cure for the disease, L-
DOPA treatments can retard the degenerative effects for three to five years before peripheral side effects prevent its use. Nicotine has also been linked to the treatment of Parkinson’s disease. Monkeys given NIC with L-DOPA experienced a synergistic effect to reverse parkinsonian symptoms (Schneider et al. 1998; Domino et al. 1999). An inverse relationship exists between smoking cigarettes and staving off the effects of PD. The occurrence of PD was decreased in smokers and non-smokers were two times as likely to contract PD (Fratiglioni and Wang 2000). Studies suggest that targeting heterologously expressed receptors, non-\(\alpha 7\), may be a valuable treatment for PD (Quik et al. 2005). \(\alpha 6^*\) nAChR subtypes involved in mediating DA release have recently been suggested as a therapeutic target for PD. Stimulation of these nAChR subtypes may result in relief from the PD symptoms without the side effects (O’Neill et al. 2002; Quik et al. 2004; Quik and McIntosh 2006). Nicotine is thought to be acting as a protective agent in the CNS (Morens et al. 1995; Baron 1996). SIB-1508Y (Sibia) has entered Phase 2 trials as a possible drug treatment for PD, due to its lower toxicity and increased potency for various nicotinic receptors (Sacaan et al. 1997; Arneric and Brioni 1999).

**Schizophrenia**

An excess of dopamine in the CNS has been hypothesized to cause Schizophrenia. Symptoms of this disease include delusions, hallucinations, blunt affect, and chaotic thinking and speech (Arnold and Trojanowski 1996). Schizophrenics have sensory imbalances, thereby making it difficult to regulate auditory and visual stimuli. Smoking is pervasive among the schizophrenic community (Lohr and Flynn 1992). It is postulated that those suffering from this disease smoke cigarettes in order to self-medicate
themselves with nicotine (Freedman et al. 1997; Sacco et al. 2005). Nicotine normalizes the gating deficit seen in patients participating in the P50 auditory-evoked response test (Adler et al. 1993; Bencherif and Schmitt 2002). Due to this finding, it has been suggested that nAChRs may be involved in schizophrenia. α7* receptors are decreased in schizophrenics (Freedman et al. 1995). Additionally, α7* antagonists impair auditory gating in rodents, indicating a possible loss, or reduced function of α7* nAChRs in schizophrenics’ gating response (Luntz-Leybman et al. 1992; Lena and Changeux 1997). A genetic polymorphism in the a7* nicotinic receptor D15S1360 has been linked to a deficiency in the normal inhibition of the P50 auditory-evoked response (Freedman et al. 1997). De Luca et al. (2004) demonstrated a genetic difference in the D15S1360 marker between Parkinson’s patients who smoke and those that do not, though larger studies are still needed to determine the relevance of these findings (De Luca et al. 2004).

**Pain Management**

Pain therapies have traditionally centered around two classes of compounds, nonsteroidal anti-inflammatory drugs (NSAIDs) and opioids. Nicotine in higher doses, however, affords a moderate and short-lived antinociceptive effect (Sahley and Berntson 1979; Tripathi et al. 1982). The discovery of the non-selective nAChR agonist, epibatidine, stimulated interest in the pain management aspect of nicotinic receptors. Epibatidine has demonstrated a potent mecamylamine-sensitive antinociceptive effect in animals (Badio and Daly 1994; Sullivan et al. 1994). However, due to its non-selectivity, epibatidine is not considered a drug candidate for the treatment of pain (Decker and Meyer 1999). Specific nAChR subtypes involved in analgesia have yet to be identified, although α4β2*...
and $\alpha_3\beta_4^{*}$ ganglionic receptor subtypes have been implicated (Badio et al. 1997; Badio et al. 1997; Marubio et al. 1999). Additionally, nicotinic agonist-induced antinociceptive behavior is most likely resulting from involvement of the $\alpha_4\beta_2^{*}$ subtype causing or mediating neurotransmitter release in the spinal cord (Hogg and Bertrand 2004). Affinity for the $\alpha_4\beta_2^{*}$ subtype, however, does not necessarily denote antinociceptive activity in a compound (Meyer et al. 2000). Studies assessing the possible role of $\alpha_7^{*}$ nAChRs in pain therapy have had mixed results. MLA and $\alpha$-Btx were unable to block the antinociceptive effects of nicotine, cytosine, and other nAChR agonists in the tail-flick latency test (Rao et al. 1996; Damaj et al. 1998). When choline, an $\alpha_7$-specific agonist, was used to elicit the effect of analgesia, MLA and $\alpha$-Btx were able to block the antinociceptive effect (Damaj et al. 2000).

**Tourette’s Syndrome**

Tourette’s syndrome is a hyperkinetic motor disorder characterized by involuntary movements and vocalizations. Although the exact mechanism of action is unknown, antipsychotics (e.g. haloperidol) are used to attenuate the symptoms of the condition. However, this treatment is not effective in ~30% of the cases and the drug causes some side effects (Erenberg et al. 1987; Shapiro et al. 1989). Nicotine reduces the symptoms of Tourette’s syndrome in animal models, as well as in human subjects (Moss et al. 1989; Sanberg et al. 1989; Sanberg et al. 1997). Presently, the nicotinic mechanism of these therapies is thought to be the inactivation of $\alpha_4\beta_2^{*}$ or $\alpha_7^{*}$ receptor subtypes, as well as a prolonged desensitization period (Lindstrom 1997; Sanberg et al. 1997). Mecamylamine
has also been used as a potential therapy for the attenuation of the tics associated with this disease (Silver et al. 2000; Young et al. 2001).

**Smoking Cessation**

Due to the addictive properties of NIC, the active alkaloid in tobacco products, smoking cessation has long been associated with the nicotinic receptors (Benowitz 1996; Rose 1996). Nicotine replacement therapy has been employed *via* nicotine gums, patches, and nasal sprays in order to achieve abatement of smoking (Hajek et al. 1999). Further research has also shown that the combination of the channel blocker mecamylamine with nicotine patches decreases cravings for cigarettes (Rose et al. 1994; Rose et al. 1998; Rose et al. 2001). Researchers uses the Rose *et al.* studies as the basis for using a partial agonist, which combines agonist and antagonist properties, to provide relief to smoking cessation patients. Cystine analogs, including varenicline, a $\alpha 4\beta 2*$ nicotinic receptor subtype partial agonist, have been evaluated as agents for smoking cessation by Pfizer Laboratories (Coe *et al.* 2005; Coe *et al.* 2005). The alkaloid (-)-lobeline was in clinical trials as treatment for smoking cessation (Schneider and Olsson 1996). However, further study revealed that lobeline use in patient groups was not statistically different than those of the placebo group, though one of three sites did demonstrate significant improvements (Glover *et al.* 1998). Therefore, the use of lobeline as a smoking cessation agent is not fully understood and not a viable option currently (Dwoskin and Crooks 2002).

Bupropion, a phenylaminoketone atypical antidepressant, has been utilized as a sustained release therapy for smoking cessation (George and O'Malley 2004). The mechanism of action is not known but Bupropion is a nicotinic antagonist at $\alpha 3\beta 2$, $\alpha 4\beta 2$, and $\alpha 7$
receptor subtypes expressed in *Xenopus* oocytes (IC$_{50}$ = 1.3, 8.0, and 60 µM, respectively) (Slemmer et al. 2000). However, given its limited effectiveness and cost, Bupropion is not considered a long term solution (Sweet 2003).
Chapter 3: Synthesis and Evaluation of mono- and bis-Alkylated Quaternary Ammonium Salts

3.1. Introduction

The neuronal nicotinic acetylcholine receptor (nAChR) belongs to a superfamily of ligand gated ion channel receptors, and has several diverse and complicated substructures. Each receptor is composed of five individual subtypes either heterologously or homologously expressed. Selected heterogeneous subtypes include combinations of α3, α4, α5, α6 and β2 or β4 (e.g. α4β2*) subunits, while the predominant homologous subtype is composed of α7 subunits (α7*) (Holladay et al. 1997). The endogenous ligand, acetylcholine (ACh), as well as NIC are agonists at the receptor binding site. NIC has been studied as a potential treatment in several pathologies (e.g. Alzheimer’s and Parkinson’s disease, schizophrenia, and analgesia) and has been used as a treatment in smoking cessation programs (Lindstrom 1997). However, NIC is a nonspecific agonist interacting with several receptor subtypes, both in the periphery, as well as in the central nervous system (CNS). Therefore, a large amount of research has been devoted to the discovery of agonists that may increase the potential beneficial therapeutic effects and decrease the side effects of NIC (Holladay et al. 1997). These efforts have afforded a number of agonist compounds that are both potent and selective for nAChR subtypes. Surprisingly, very little research has focused on structure activity relationships (SARs) involving nAChR antagonists that are subtype selective (Dwoskin et al. 2000; Dwoskin and Crooks 2001).
**mono-Substituted Quaternary Ammonium Molecules**

Crooks *et al.* (1995) synthesized a small series of *N*-alkylated nicotinium compounds that demonstrated selective antagonist activities at different neuronal nAChR subtypes. Alkylating the pyridino *N*-atom of the NIC molecule with an *n*-octyl carbon chain afforded a compound that was both potent and selective for the nAChRs mediating dopamine (DA) release, a purported *α*6 containing receptor (Crooks *et al.* 1995). Incorporating an *n*-decyl carbon chain was later shown to afford an analog that was a selective antagonist at the *α*4*β*2* sub receptor subtype (Dwoskin *et al.* 1999). Thus, subtle changes in the alkyl chain length of these *N*-*n*-alkynicotinium analogs produced a surprising difference in the antagonist activities of the compounds. Conformationally restricted analogs of the NIC molecule were also *N*-*n*-alkylated and these compounds retained antagonist activity at the nAChRs mediating DA release, with chain lengths of 8-12 carbons in the *syn*-conformation (24a-e) and 8-11 carbons in the *anti*-conformation (25a-d) affording various degrees of antagonist activity (Xu *et al.* 2001). Unlike the *N*-*n*-alkynicotinium analogs, none of these conformationally restricted molecules antagonized the *α*4*β*2* nAChR subtype. Therefore, structural modification of the nicotine molecule can result in compounds with potent and selective nAChR antagonist characteristics.

**bis-Substituted Quaternary Ammonium Molecules**

*bis*-Quaternary ammonium salts have also been used as ligands in several receptor systems. For example, *bis*-quinolinium cyclophans have been used to block Apamin-sensitive *Ca*2+-activated *K*+ channels (Chen *et al.* 2000). Investigators determined that altering the chain lengths of alkane spacers between the quaternary ammonium centers
could effectively afford analogs that inhibit receptor function. Additionally, like the
*mono*-substituted *N*-alkyl quaternized ammonium salts, *bis*-substituted quaternized
ammonium salts have demonstrated selective inhibition of peripheral nicotinic
acetylcholine receptor subtypes. Peripheral nicotinic receptor antagonists, such as
hexamethonium (HEX, 46) and decamethonium (DEC), were originally prepared as
simple analogs of the neuromuscular blocking agent, *d*-tubocurarine (*d*-Tub, 16). The ten
carbon analog, DEC, demonstrated competitive inhibition of nicotinic receptors at the
neuromuscular junction, while HEX primarily antagonized nerve-nerve terminals
(ganglionic receptors). These two *bis*-quaternary ammonium compounds were among
the first to be used to differentiate between the different subtypes of peripheral nicotinic
receptors, muscle and ganglionic. Although their mechanisms of action are quite
different, DEC and HEX have been shown to have the optimum chain lengths for binding
to their respective receptor subtypes in the periphery. In the CNS, HEX and DEC have
been evaluated for NIC binding at αβ2* receptors and found to exhibit weak affinities
(Ki = 31.9 and 4.27, respectively) for the receptor subtype (Ayers *et al.* 2005). *d*-Tub, a
more complex *bis*-quaternary structure, also has activity at the muscle-type peripheral
nAChR subtype, but it also has activities at the ganglionic receptor subtypes and at αβ2*
subtypes (Ascher *et al.* 1979; Colquhoun *et al.* 1979; Ogden and Colquhoun 1985;
Bertrand *et al.* 1990). Therefore, *bis*-quaternary ammonium structures have been used
mainly as antagonists peripherally, and have the potential to differentiate between these
nAChR subtypes by both altering the chain length and the quaternary ammonium
headgroup.
In order to assess the pyrrolidine moiety’s importance to the antagonist activity of these interesting series of compounds, a series of quaternized aza-aromatic compounds (e.g. pyridine, 3-picoline, nicotinamide, and NIC) were evaluated (53-56). In addition to the assessment of the importance of the pyrrolidine ring, alkyl chain lengths of between one to twenty carbon units were attached to the pyridino functionality to evaluate the relationship between alkyl chain length and inhibition of and selectivity for various nAChR subtypes.

Figure 3.1 mono-Alkylated Quaternary Ammonium Salts

Additionally, bis-quaternary ammonium salts (57-61) were evaluated for their effect on various nAChR subtypes as analogs of the existing peripheral antagonists HEX, DEC, and d-Tub to determine if such analogs antagonized neuronal nicotine receptors. Varying
chain lengths and different pyridinium moieties (e.g. pyridinium, 3-picolinium, quinolinium, isoquinolinium, and nicotinum) were utilized to determine if selective nicotinic receptor subtype antagonism could be achieved.

Figure 3.2 *bis*-Alkylated Quaternary Ammonium Salts

![Figure 3.2 bis-Alkylated Quaternary Ammonium Salts](image)

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64
3.2. Experimental

3.2.1. Chemicals

S-(-)-Nicotine, pyridine, 3-picoline, nicotinamide, quinoline, isoquinoline, iodomethane, iodoethane, 1-iodopropane, 1-iodobutane, 1-iodopentane, 1-iodohexane, 1-iodoheptane, 1-iodooctane, 1-iodononane, 1-iododecane, 1-iodoundecane, 1-iodododecane, 1-bromopentadecane, 1-bromoeicosane, 1,5-diiodopentane, 1,6-diiodohexane, 1,8-diiodooctane, 1,9-dibromononane, 1,10-diiododecane, 1,11-dibromoundecane, 1,12-dibromododecane, diethylether, chloroform, deuterium oxide (100% atom D), deuterium chloride solution (20% W/W solution in D$_2$O, 99.5% atom D), 3-(trimethylsilyl)-2,2,3,3-$d_4$-propionate, and $\alpha$-D-glucose were purchased from Aldrich Chemical Company Inc. (Milwaukee, WI). Acetic acid, was obtained from Fisher Scientific (Pittsburgh, PA). S-(-)-Nicotine ditartrate, nomifensine maleate, and mecamylamine hydrochloride (MEC) were purchased from Research Biochemicals, Inc. (Natick, MA). S-(-)-Cytisine, pargyline hydrochloride, N-[2-hydroxyethyl]piperazine-$N'$-[2-ethanesulfonic acid] (HEPES), Tris[hydroxymethyl]aminomethane hydrochloride (Trizma HCl), Tris[hydroxymethyl]aminomethane (Trizma base), polyethylenimine (PEI), and dihydroxy-β-erythroidine hydrobromide (DHβE) were purchased from Sigma Chemical Co. (St. Louis, MO). Ascorbic acid, and TS-2 tissue solubilizer were purchased from Fluka (Ronkonkona, NY), AnalAr (BHD Ltd., Poole, U.K.), and Research Products International (Mount Prospect, IL), respectively. $[^{3}\text{H}]$Dopamine ($[^{3}\text{H}]$DA); 3,4-ethyl-2-[N-$^{3}$H]dihydroxyphenylethylamine; specific activity, 25.6 Ci/mmol) and $[^{3}\text{H}]$nicotine (L-(-)-[N-methyl-$^{3}$H]; specific activity, 78.4 Ci/mmol) were obtained from New England Nuclear (Boston, MA).
3.2.2. **Synthetic Procedures**

3.2.3. **Synthetic Procedures for the Preparation of mono-Substituted Pyridinium Derivatives**

3.2.3.1. **Pyridinium Salts**

The appropriate 1-iodoalkane or 1-bromoalkane (5.01-16.1 mmol) was added to a solution (30 mL) of dry pyridine and the solution was heated for 24 h at 65°C. The resulting solid precipitates were filtered and washed five times with dry diethyl ether. For liquid products, the excess pyridine was decanted off, the resulting liquid precipitates were washed with diethyl ether five times, and the remaining liquid was dried under vacuum. The resulting solids or liquids were isolated in 56-95% yields.

*N*-Methylpyridinium Iodide (NMPI, **53a**)

$^1$H NMR (300 MHz, DMSO-D$_6$), δ 9.01 (2H, d, C2&C6-H), 8.58 (1H, t, C4-H), 8.13 (2H, t, C3&C5-H), 4.37 (3H, s, C’1-CH$_3$); $^{13}$C NMR (75 MHz, DMSO-D$_6$), δ 145.3, 144.8, 127.5, 48.0. The resulting tan solid was isolated in 95% yield, with a melting point of 117°C. Combustion Analysis: calculated for C$_6$H$_8$IN, C, 32.60; H, 3.65; N, 6.35. Found: C, 32.51; H, 3.62; N, 6.34.

*N*-Ethylpyridinium Iodide (NEPI, **53b**)

$^1$H NMR (300 MHz, DMSO-D$_6$), δ 9.14 (2H, d, C2&C6-H), 8.60 (1H, t, C4-H), 8.16 (2H, t, C3&C5-H), 4.67 (2H, q, C’1-CH$_2$), 1.53 (3H, t, C’2-CH$_3$); $^{13}$C NMR (75 MHz,
DMSO-D$_6$), $\delta$ 145.5, 144.0, 127.8, 56.0, 16.0. The resulting yellow solid was isolated in 92% yield, with a melting point of 88-89$^\circ$C. Combustion Analysis: calculated for C$_7$H$_{10}$IN, C, 35.77; H, 4.29; N, 5.96. Found: C, 35.86; H, 4.33; N, 5.94.

$N$-$n$-Propylpyridinium Iodide (NPrPI, 53c)

$^1$H NMR (300 MHz, DMSO-D$_6$), $\delta$ 9.19 (2H, d, C2&C6-H), 8.63 (1H, t, C4-H), 8.17 (2H, t, C3&C5-H), 4.64 (2H, t, C’1-CH$_2$), 1.89 (2H, m, C’2-CH$_2$), 0.80 (3H, t, C’3-CH$_3$);

$^{13}$C NMR (75 MHz, DMSO-D$_6$), $\delta$ 144.7, 143.8, 127.1, 61.1, 23.4, 9.4. The resulting brown liquid was isolated in 87% yield. Combustion Analysis: calculated for C$_8$H$_{12}$IN, C, 38.57; H, 4.86; N, 5.62. Found: C, 38.73; H, 4.92; N, 5.61.

$N$-$n$-Butylpyridinium Iodide (NBuPI, 53d)

$^1$H NMR (300 MHz, DMSO-D$_6$), $\delta$ 9.19 (2H, d, C2&C6-H), 8.64 (1H, t, C4-H), 8.18 (2H, t, C3&C5-H), 4.67 (2H, t, C’1-CH$_2$), 1.90 (2H, p, C’2-CH$_2$), 1.25 (2H, m, C’3-CH$_2$), 0.86 (3H, t, C’4-CH$_3$); $^{13}$C NMR (75 MHz, DMSO-D$_6$), $\delta$ 146.2, 144.5, 127.9, 60.4, 32.4, 18.4, 13.1. The resulting orange liquid was isolated in 90% yield, with a melting point of 59-62$^\circ$C. Combustion Analysis: calculated for C$_9$H$_{14}$IN, C, 41.08; H, 5.36; N, 5.32. Found: C, 41.14; H, 5.41; N, 5.32.

$N$-$n$-Pentylpyridinium Iodide (NPePI, 53e)

$^1$H NMR (300 MHz, DMSO-D$_6$), $\delta$ 9.16 (2H, d, C2&C6-H), 8.62 (1H, t, C4-H), 8.17 (2H, t, C3&C5-H), 4.83 (2H, t, C’1-CH$_2$), 1.89 (2H, p, C’2-CH$_2$), 1.23 (4H, m, C’3&C’4-CH$_2$), 0.80 (3H, t, C’5-CH$_3$); $^{13}$C NMR (75 MHz, DMSO-D$_6$), $\delta$ 146.2, 144.5, 127.9,
60.4, 30.0, 27.2, 21.3, 13.7. The resulting orange solid was isolated in 93% yield, with a melting point of 62°C. Combustion Analysis: calculated for C\textsubscript{10}H\textsubscript{16}IN, C, 43.34; H, 5.82; N, 5.05. Found: C, 43.47; H, 5.87; N, 5.04.

**N-n-Hexylpyridinium Iodide (NHxPI, 53f)**

\(^1\)H NMR (300 MHz, DMSO-D\textsubscript{6}), δ 9.17 (2H, d, C2&C6-H), 8.63 (1H, t, C4-H), 8.19 (2H, t, C3&C5-H), 4.85 (2H, t, C’1-CH\textsubscript{2}), 2.00 (2H, p, C’2-CH\textsubscript{2}), 1.24 (6H, m, C’3-C’5-CH\textsubscript{2}), 0.80 (3H, t, C’6-CH\textsubscript{3}); \(^13\)C NMR (75 MHz, DMSO-D\textsubscript{6}), δ 145.5, 144.6, 128.1, 60.6, 30.6, 30.4, 24.9, 21.8, 13.8. The resulting yellow solid was isolated in 94% yield, with a melting point of 62°C. Combustion Analysis: calculated for C\textsubscript{11}H\textsubscript{18}IN, C, 45.37; H, 6.23; N, 4.81. Found: C, 45.30; H, 6.20; N, 4.85.

**N-n-Heptylpyridinium Iodide (NHpPI, 53g)**

\(^1\)H NMR (300 MHz, DMSO-D\textsubscript{6}), δ 9.17 (2H, d, C2&C6-H), 8.63 (1H, t, C4-H), 8.18 (2H, t, C3&C5-H), 4.65 (2H, t, C’1-CH\textsubscript{2}), 1.90 (2H, p, C’2-CH\textsubscript{2}), 1.22 (8H, m, C’3-C’6-CH\textsubscript{2}), 0.80 (3H, t, C’7-CH\textsubscript{3}); \(^13\)C NMR (75 MHz, DMSO-D\textsubscript{6}), δ 145.5, 144.6, 128.1, 60.6, 31.1, 30.6, 27.9, 25.2, 21.8, 13.9. The resulting yellow solid was isolated in 89% yield, with a melting point of 63°C. Combustion Analysis: calculated for C\textsubscript{12}H\textsubscript{20}IN, C, 47.22; H, 6.61; N, 4.59. Found: C, 47.40; H, 6.72; N, 4.58.

**N-n-Octylpyridinium Iodide (NOPI, 53h)**

\(^1\)H NMR (300 MHz, DMSO-D\textsubscript{6}), δ 9.16 (2H, d, C2&C6-H), 8.62 (1H, t, C4-H), 8.17 (2H, t, C3&C5-H), 4.83 (2H, t, C’1-CH\textsubscript{2}), 1.89 (2H, p, C’2-CH\textsubscript{2}), 1.22 (10H, m, C’3-C’7-
CH\(_2\), 0.80 (3H, t, C'8-CH\(_3\)); \(^{13}\)C NMR (75 MHz, DMSO-D\(_6\)), \(\delta\) 145.0, 144.2, 127.6, 60.1, 30.6, 30.2, 27.9, 27.8, 24.8, 21.4, 13.4. The resulting brown liquid was isolated in 87% yield. Combustion Analysis: calculated for C\(_{13}\)H\(_{22}\)IN, C, 48.91; H, 6.95; N, 4.39. Found: C, 49.01; H, 7.08; N, 4.39.

\(N\)-\(n\)-Nonylpyridinium Iodide (NNPI, 53i)

\(^1\)H NMR (300 MHz, DMSO-D\(_6\)), \(\delta\) 9.14 (2H, d, C2&C6-H), 8.62 (1H, t, C4-H), 8.17 (2H, t, C3&C5-H), 4.62 (2H, t, C'1-CH\(_2\)), 1.90 (2H, p, C'2-CH\(_2\)), 1.22 (12H, m, C'3-C'8-CH\(_2\)), 0.82 (3H, t, C'9-CH\(_3\)); \(^{13}\)C NMR (75 MHz, DMSO-D\(_6\)), \(\delta\) 145.5, 144.6, 128.1, 60.7, 31.3, 30.6, 28.6, 28.5, 28.3, 25.3, 22.0, 13.9. The resulting yellow solid was isolated in 88% yield, with a melting point of 76°C. Combustion Analysis: calculated for C\(_{14}\)H\(_{24}\)IN, C, 50.46; H, 7.26; N, 4.20. Found: C, 50.42; H, 7.34; N, 4.21.

\(N\)-\(n\)-Decylpyridinium Iodide (NDPI, 53j)

\(^1\)H NMR (300 MHz, DMSO-D\(_6\)), \(\delta\) 9.15 (2H, d, C2&C6-H), 8.62 (1H, t, C4-H), 8.17 (2H, t, C3&C5-H), 4.82 (2H, t, C'1-CH\(_2\)), 1.92 (2H, p, C'2-CH\(_2\)), 1.22 (14H, m, C'3-C'9-CH\(_2\)), 0.82 (3H, t, C'10-CH\(_3\)); \(^{13}\)C NMR (75 MHz, DMSO-D\(_6\)), \(\delta\) 145.5, 144.7, 128.1, 60.8, 31.2, 30.7, 28.8, 28.7, 28.6, 28.4, 25.3, 22.0, 13.9. The resulting pale yellow solid was isolated in 84% yield, with a melting point of 71°C. Combustion Analysis: calculated for C\(_{15}\)H\(_{26}\)IN, C, 51.88; H, 7.55; N, 4.03. Found: C, 51.91; H, 7.54; N, 4.00.
N-\textit{n}-Undecylpyridinium Iodide (NUPI, 53k)

$^1$H NMR (300 MHz, DMSO-$D_6$), $\delta$ 9.12 (2H, d, C2&C6-H), 8.61 (1H, t, C4-H), 8.17 (2H, t, C3&C5-H), 4.61 (2H, t, C’1-CH$_2$), 2.00 (2H, p, C’2-CH$_2$), 1.24 (16H, m, C’3-C’10-CH$_2$), 0.83 (3H, t, C’11-CH$_3$); $^{13}$C NMR (75 MHz, DMSO-$D_6$), $\delta$ 145.2, 144.4, 127.8, 60.1, 31.0, 30.3, 28.6 (x2), 28.4, 28.3, 28.1, 25.0, 21.7, 13.6. The resulting pale yellow solid was isolated in 82% yield, with a melting point of 91°C. Combustion Analysis: calculated for C$_{16}$H$_{28}$IN, C, 53.19; H, 7.81; N, 3.88. Found: C, 53.24; H, 7.80; N, 3.89.

N-\textit{n}-Dodecylpyridinium Iodide (NDDPI, 53l)

$^1$H NMR (300 MHz, DMSO-$D_6$), $\delta$ 9.13 (2H, d, C2&C6-H), 8.62 (1H, t, C4-H), 8.18 (2H, t, C3&C5-H), 4.73 (2H, t, C’1-CH$_2$), 1.89 (2H, p, C’2-CH$_2$), 1.24 (18H, m, C’3-C’11-CH$_2$), 0.84 (3H, t, C’12-CH$_3$); $^{13}$C NMR (75 MHz, DMSO-$D_6$), $\delta$ 145.4, 144.6, 128.0, 60.7, 31.2, 30.8, 28.9 (x3), 28.8, 28.7, 28.6, 25.4, 22.0, 13.9. The resulting yellow solid was isolated in 85% yield, with a melting point of 84°C. Combustion Analysis: calculated for C$_{17}$H$_{30}$IN, C, 54.40; H, 8.06; N, 3.73. Found: C, 54.59; H, 7.97; N, 3.73.

N-\textit{n}-Pentadecylpyridinium Bromide (NPeDPB, 53m)

$^1$H NMR (300 MHz, CDCl$_3$), $\delta$ 9.44 (2H, d, C2&C6-H), 8.49 (1H, t, C4-H), 8.11 (2H, t, C3&C5-H), 5.02 (2H, t, C’1-CH$_2$), 2.01 (2H, p, C’2-CH$_2$), 1.35 (24H, m, C’3-C’14-CH$_2$), 0.86 (3H, t, C’15-CH$_3$); $^{13}$C NMR (75 MHz, CDCl$_3$), $\delta$ 145.2, 145.1, 128.4, 62.6, 32.3, 32.2, 30.0, 29.9(x3), 29.8, 29.7, 29.6, 29.3, 26.4, 22.1, 14.5. The resulting tan solid was
isolated in 65% yield, with a melting point of 76°C. Combustion Analysis: calculated for C₂₀H₃₆BrN.1.0H₂O, C, 61.84; H, 9.86; N, 3.61. Found: C, 61.73; H, 9.62; N, 3.60.

*N-Ecosylpyridinium Bromide (NEcPB, 53n)*

¹H NMR (300 MHz, DMSO-D₆), δ 9.13 (2H, d, C2&C6-H), 8.62 (1H, t, C4-H), 8.17 (2H, t, C3&C5-H), 4.61 (2H, t, C’1-CH₂), 1.80 (2H, p, C’2-CH₂), 1.25 (34H, m, C’3-C’19-CH₂), 0.85 (3H, t, C’20-CH₃); ¹³C NMR (75 MHz, DMSO-D₆), δ 145.3, 144.6, 127.9, 60.7, 31.3, 30.7, 29.0 (x10), 28.9, 28.8, 28.7, 28.4, 25.4, 22.1, 14.0. The resulting white solid was isolated in 56% yield, with a melting point of 73°C. Combustion Analysis: calculated for C₂₅H₄₆BrN.1.75H₂O, C, 63.61; H, 10.57; N, 2.97. Found: C, 63.45; H, 10.27; N, 3.00.

3.2.3.2. **Picolinium Salts**

The appropriate 1-iodoalkane (5.01-16.1 mmol) was added to a solution (30 mL) of 3-picoline, and the solution heated for 24 h at 65°C. The resulting liquid precipitates had the excess picoline decanted off and the resulting oil precipitates were washed with diethyl ether five times, and the remaining oil was dried under vacuum. The resulting oil was isolated in yields ranging from 89-94%.

*N-n-Octylpicolinium Iodide (NOPiI, 54a)*

¹H NMR (300 MHz, DMSO-D₆), δ 9.04 (1H, s, C2 -H), 8.94 (1H, s, C6-H), 8.45 (1H, d, C4-H), 8.06 (1H, t, C5-H), 4.55 (2H, t, C’1-CH₂), 2.50 (3H, s, C3-CH₃), 1.91 (2H, p, C’2-CH₂), 1.25 (10H, m, C’3-C’7-CH₂), 0.85 (3H, t, C’8-CH₃); ¹³C NMR (75 MHz,
DMSO-D$_6$), $\delta$ 145.5, 144.0, 141.8, 138.5, 127.2, 60.5, 31.1, 30.6, 28.4 (x2), 25.4, 22.1, 17.9, 14.0. The resulting dark brown oil was isolated in 92% yield. Combustion Analysis: calculated for C$_{14}$H$_{24}$IN, C, 50.46; H, 7.26; N, 4.20. Found: C, 50.26; H, 7.28; N, 4.18.

**N-n-Nonylpicolinium Iodide (NNPiI, 54b)**

$^1$H NMR (300 MHz, DMSO-D$_6$), $\delta$ 9.00 (1H, s, C2 -H), 8.94 (1H, s, C6-H), 8.45 (1H, d, C4-H), 8.06 (H, t, C5-H), 4.55 (2H, t, C’1-CH$_2$), 2.50 (3H, s, C3-CH$_3$), 1.91 (2H, p, C’2-CH$_2$), 1.25 (10H, m, C’3-C’7-CH$_2$), 0.85 (3H, t, C’8-CH$_3$); $^{13}$C NMR (75 MHz, DMSO-D$_6$), $\delta$ 145.5, 144.0, 141.8, 138.5, 127.2, 60.5, 31.2, 30.6, 28.7, 28.6, 28.4, 25.4, 22.1, 17.9, 14.0. The resulting dark brown oil was isolated in 94% yield. Combustion Analysis: calculated for C$_{15}$H$_{26}$IN, C, 51.88; H, 7.55; N, 4.03. Found: C, 51.99; H, 7.54; N, 4.04.

**N-n-Decylpicolinium Iodide (NDPiI, 54c)**

$^1$H NMR (300 MHz, DMSO-D$_6$), $\delta$ 9.02 (1H, s, C2 -H), 8.93 (1H, s, C6-H), 8.45 (1H, d, C4-H), 8.06 (1H, t, C5-H), 4.53 (2H, t, C’1-CH$_2$), 2.50 (3H, s, C3-CH$_3$), 1.91 (2H, p, C’2-CH$_2$), 1.23 (14H, m, C’3-C’9-CH$_2$), 0.84 (3H, t, C’10-CH$_3$); $^{13}$C NMR (75 MHz, DMSO-D$_6$), $\delta$ 145.5, 144.0, 141.8, 138.5, 127.2, 60.5, 31.3, 30.6, 28.9, 28.8, 28.7, 28.4, 25.4, 22.1, 17.9, 14.0. The resulting dark brown oil was isolated in 90% yield. Combustion Analysis: calculated for C$_{16}$H$_{28}$IN, C, 53.19; H, 7.81; N, 3.88. Found: C, 53.24; H, 7.79; N, 3.89.
**N-n-Undecylpicolinium Iodide (NUPiI, 54d)**

$^1$H NMR (300 MHz, DMSO-$D_6$), $\delta$ 9.04 (1H, s, C2 -H), 8.94 (1H, s, C6-H), 8.46 (1H, d, C4-H), 8.07 (1H, t, C5-H), 4.55 (2H, t, C’1-CH$_2$), 2.50 (3H, s, C3-CH$_3$), 1.91 (2H, p, C’2-CH$_2$), 1.24 (18H, m, C’3-C’11-CH$_2$), 0.85 (3H, t, C’12-CH$_3$); $^{13}$C NMR (75 MHz, DMSO-$D_6$), $\delta$ 145.5, 144.0, 141.7, 138.5, 127.2, 60.5, 31.3, 30.6, 29.0, 28.9, 28.8, 28.7, 28.4, 25.4, 22.1, 17.9, 14.0. The resulting dark brown oil was isolated in 89% yield.

Combustion Analysis: calculated for C$_{17}$H$_{30}$IN, C, 54.40; H, 8.06; N, 3.73. Found: C, 54.53; H, 7.99; N, 3.75.

**N-n-Dodecylpicolinium Iodide (NDDPiI, 54e)**

$^1$H NMR (300 MHz, DMSO-$D_6$), $\delta$ 9.03 (1H, s, C2 -H), 8.94 (1H, s, C6-H), 8.46 (1H, d, C4-H), 8.17 (1H, t, C5-H), 4.54 (2H, t, C’1-CH$_2$), 2.50 (3H, s, C3-CH$_3$), 1.92 (2H, p, C’2-CH$_2$), 1.25 (18H, m, C’3-C’11-CH$_2$), 0.86 (3H, t, C’12-CH$_3$); $^{13}$C NMR (75 MHz, DMSO-$D_6$), $\delta$ 145.7, 144.1, 141.9, 138.7, 127.3, 60.6, 31.2, 30.5, 28.9 (x2), 28.8, 28.7, 28.6, 28.3, 25.3, 22.0, 17.8, 13.9. The resulting dark brown oil was isolated in 91% yield.

Combustion Analysis: calculated for C$_{18}$H$_{32}$IN, C, 55.53; H, 8.28; N, 3.60. Found: C, 55.70; H, 8.20; N, 3.59.

**3.2.3.3. N-Alkylated Nicotinamide Salts**

The appropriate 1-iodoalkane (5.01-16.1 mmol) was added to a solution (30 mL) of nicotinamide (40.94 mmol) in methanol. The solution was heated for 24 h at 65°C. The resulting precipitate was filtered, and the product washed five times with dry diethyl ether. The remaining solid was then dissolved in water and extracted with chloroform (3
x 25 mL). The organic layer was dried over anhydrous magnesium sulfate, filtered, and the solvent removed. The resulting solid was isolated in yields ranging from 70-83%.

_N-n-Octynicotinamide Iodide (NONAI, 55a)_

$^1$H NMR (300 MHz, DMSO-D$_6$), δ 9.47 (1H, s, C2 -H), 9.20 (1H, d, C6-H), 8.92 (1H, d, C4-H), 8.54 (1H, s, N1’-H), 8.27 (2H, t, C5-H), 8.18 (1H, s, N1’-H), 4.64 (2H, t, C’1-CH$_2$), 1.93 (2H, p, C’2-CH$_2$), 1.27 (10H, m, C’3-C’7-CH$_2$), 0.87 (3H, t, C’8-CH$_3$); $^{13}$C NMR (75 MHz, DMSO-D$_6$), δ 162.6, 146.2, 144.6, 143.0, 133.7, 127.7, 61.1, 31.1, 30.6, 28.4 (x2), 25.4, 22.1, 14.0. The resulting golden solid was isolated in 83% yield, with a melting point of 154-155°C. Combustion Analysis: calculated for C$_{14}$H$_{23}$I$_2$N$_2$O 0.25 H$_2$O, C, 45.85; H, 6.46; N, 7.64. Found: C, 45.60; H, 6.36; N, 7.71.

_N-n-Nonylnicotinamide Iodide (NNNAI, 55b)_

$^1$H NMR (300 MHz, DMSO-D$_6$), δ 9.48 (1H, s, C2 -H), 9.22 (1H, d, C6-H), 8.92 (1H, d, C4-H), 8.54 (1H, s, N1’-H), 8.28 (2H, t, C5-H), 8.17 (1H, s, N1’-H), 4.63 (2H, t, C’1-CH$_2$), 1.93 (2H, p, C’2-CH$_2$), 1.25 (12H, m, C’3-C’7-CH$_2$), 0.85 (3H, t, C’8-CH$_3$); $^{13}$C NMR (75 MHz, DMSO-D$_6$), δ 162.6, 146.2, 144.6, 143.0, 133.7, 127.7, 61.1, 31.2, 30.6, 28.7, 28.6, 28.4, 25.4, 22.1, 14.0. The resulting yellow solid was isolated in 80% yield, with a melting point of 164°C. Combustion Analysis: calculated for C$_{15}$H$_{25}$I$_2$N$_2$O 0.5 H$_2$O, C, 46.76; H, 6.80; N, 7.27. Found: C, 46.55; H, 6.69; N, 7.30.
N-n-Decynicotinamide Iodide (NDNAI, 55c)

\(^1\)H NMR (300 MHz, DMSO-D\(_6\)), \(\delta\) 9.48 (1H, s, C2 -H), 9.22 (1H, d, C6-H), 8.92 (1H, d, C4-H), 8.54 (1H, s, N1’-H), 8.28 (2H, t, C5-H), 8.18 (1H, s, N1’-H), 4.65 (2H, t, C’1-CH\(_2\)), 1.92 (2H, p, C’2-CH\(_2\)), 1.24 (14H, m, C’3-C’9-CH\(_2\)), 0.86 (3H, t, C’10-CH\(_3\)); \(^{13}\)C NMR (75 MHz, DMSO-D\(_6\)), \(\delta\) 162.6, 146.1, 144.5, 143.1, 133.7, 127.7, 61.1, 31.3, 30.6, 28.9, 28.8, 28.7, 28.4, 25.4, 22.1, 14.0. The resulting yellow solid was isolated in 77% yield, with a melting point of 170\(^\circ\)C. Combustion Analysis: calculated for C\(_{16}\)H\(_{27}\)IN\(_2\)O\(_0.33\)H\(_2\)O, C, 48.49; H, 7.04; N, 7.07. Found: C, 48.38; H, 6.85; N, 6.95.

N-n-Undecynicotinamide Iodide (NUNAI, 55d)

\(^1\)H NMR (300 MHz, DMSO-D\(_6\)), \(\delta\) 9.48 (1H, s, C2 -H), 9.21 (1H, d, C6-H), 8.92 (1H, d, C4-H), 8.54 (1H, s, N1’-H), 8.28 (2H, t, C5-H), 8.18 (1H, s, N1’-H), 4.64 (2H, t, C’1-CH\(_2\)), 1.93 (2H, p, C’2-CH\(_2\)), 1.25 (16H, m, C’3-C’10-CH\(_2\)), 0.85 (3H, t, C’11-CH\(_3\)); \(^{13}\)C NMR (75 MHz, DMSO-D\(_6\)), \(\delta\) 162.6, 146.1, 144.6, 143.0, 133.7, 127.7, 61.1, 31.3, 30.6, 29.0, 28.9, 28.8, 28.7, 28.4, 25.4, 22.1, 14.0. The resulting dark yellow solid was isolated in 70% yield, with a melting point of 175\(^\circ\)C. Combustion Analysis: calculated for C\(_{17}\)H\(_{29}\)IN\(_2\)O \(0.25\)H\(_2\)O, C, 49.94; H, 7.27; N, 6.85. Found: C, 49.81; H, 7.33; N, 6.83.

N-n-Dodecylnicotinamide Iodide (NDDNAI, 55e)

\(^1\)H NMR (300 MHz, DMSO-D\(_6\)), \(\delta\) 9.47 (1H, s, C2 -H), 9.21 (1H, d, C6-H), 8.92 (1H, d, C4-H), 8.54 (1H, s, N1’-H), 8.27 (2H, t, C5-H), 8.18 (1H, s, N1’-H), 4.64 (2H, t, C’1-CH\(_2\)), 1.92 (2H, p, C’2-CH\(_2\)), 1.23 (18H, m, C’3-C’11-CH\(_2\)), 0.84 (3H, t, C’12-CH\(_3\)); \(^{13}\)C NMR (75 MHz, DMSO-D\(_6\)), \(\delta\) 162.6, 146.1, 144.5, 143.1, 133.7, 127.7, 61.2, 31.3, 30.6,
29.0 (x2), 28.9, 28.8, 28.7, 28.4, 25.4, 22.1, 14.0. The resulting golden solid was isolated in 72% yield, with a melting point of 181°C. Combustion Analysis: calculated for C_{18}H_{31}N_{2}O 0.33 H_{2}O, C, 50.95; H, 7.52; N, 6.60. Found: C, 50.73; H, 7.48; N, 6.43.

3.2.3.4. **Nicotinium Salts**

S-(-)-Nicotine (30.8 mmol) was dissolved in glacial acetic acid (35 mL) and the solution was stirred at ambient temperature for five minutes. The appropriate 1-iodoalkane (30.8 mmol) was added to the solution, and the mixture was refluxed for three days. The mixture was then evaporated under reduced pressure to remove the solvent, and the resulting oil was treated with an aqueous solution of sodium bicarbonate. Diethyl ether (3 x 50 mL) and chloroform (3 x 50 mL) were added successively to the resulting mixture to extract the unreacted NIC and quaternized analog, respectively. The chloroform layers were collected, combined, and dried over magnesium sulfate. The chloroform was then filtered and evaporated to dryness on a rotary evaporator. The resulting oil was triturated with dry diethyl ether as needed, and the ether washings discarded. The remaining residue was utilized as a homogenous viscous oil in the pharmacological assays. The resulting oil was isolated in yields ranging from 60-72%.

**N-n-Butynicotinium Iodide (NnBNI, 56d)**

$^1$H NMR (300 MHz, DMSO-D$_6$) $\delta$ 9.05 (1H, s, C2-H), 8.99 (1H, d, C6-H), 8.54 (1H, d, C4-H), 8.12 (1H, t, C3-H), 4.61 (2H, t, C’’1-CH$_2$), 3.43 (1H, t, pyrrolidine CH$_2$), 3.18 (1H, t, pyrrolidine CH$_2$), 2.34 (2H, m, C’’2-CH$_2$), 2.15 (3H, s, pyrrolidine N-CH$_3$), 1.88 (4H, m, pyrrolidine CH$_2$CH$_2$), 1.65 (1H, m, pyrrolidine CH), 1.30 (2H, m, C’’3-CH$_2$), 0.92 (3H, t, C”'-CH$_3$); $^{13}$C NMR (75 MHz, DMSO-D$_6$) $\delta$ 144.3, 143.9, 143.3, 127.8, 66.5,
The resulting dark brown oil was isolated in 60% yield. Combustion Analysis: calculated for C_{14}H_{23}IN_2 0.125 H_2O, C, 48.25; H, 6.72; N, 8.04. Found: C, 48.02; H, 6.58; N, 8.02.

\textbf{N-n-Pentynicotinium Iodide (NPeNI, 56e)}

\textsuperscript{1}H NMR (300 MHz, CDCl\textsubscript{3}) \(\delta\) 9.37 (1H, d, C6-H), 9.18 (1H, s, C2-H), 8.50 (1H, d, C4-H), 8.11 (1H, t, C3-H), 4.92 (2H, t, C’’1-CH\textsubscript{2}), 3.64 (1H, t, pyrrolidine CH\textsubscript{2}), 3.25 (1H, t, pyrrolidine CH\textsubscript{2}), 2.46 (2H, m, C’’2-CH\textsubscript{2}), 2.25 (3H, s, pyrrolidine N-CH\textsubscript{3}), 1.80-2.02 (4H, m, pyrrolidine CH\textsubscript{2}CH\textsubscript{2}), 1.67 (1H, m, pyrrolidine CH), 1.36 (4H, m, C’’3&’’4-CH\textsubscript{2}), 0.87 (3H, t, C’’-CH\textsubscript{3}); \textsuperscript{13}C NMR (75 MHz, CDCl\textsubscript{3}) \(\delta\) 146.7, 144.3, 143.7, 143.2, 128.6, 67.1, 62.4, 57.0, 40.9, 36.1, 31.9, 28.4, 23.6, 22.5, 14.2. The resulting light brown oil was isolated in 72% yield. Combustion Analysis: calculated for C_{15}H_{25}IN\textsubscript{2} 0.25 H_2O, C, 45.93; H, 6.49; N, 7.14. Found: C, 45.76; H, 6.34; N, 7.15.

\textbf{N-n-Dodecylnicotinium Iodide (NDDNI, 56l)}

\textsuperscript{1}H NMR (300 MHz, CDCl\textsubscript{3}) \(\delta\) 9.30 (1H, d, C6-H), 9.08 (1H, s, C2-H), 8.52 (1H, d, C4-H), 8.11 (1H, t, C5-H), 4.90 (2H, t, C’’1-CH\textsubscript{2}), 3.69 (1H, t, pyrrolidine CH\textsubscript{2}), 3.30 (1H, t, pyrrolidine CH\textsubscript{2}), 2.50 (2H, m, C’’2-CH\textsubscript{2}), 2.30 (3H, s, pyrrolidine N-CH\textsubscript{3}), 1.65-2.12 (5H, m, pyrrolidine CH\textsubscript{2}CH\textsubscript{2}CH), 1.18-1.50 (18H, m, C’’3-11-CH\textsubscript{2}), 0.89 (3H, t, C’’12-CH\textsubscript{3}); \textsuperscript{13}C NMR (75 MHz, CDCl\textsubscript{3}) \(\delta\) 146.7, 144.3, 143.6, 143.0, 128.6, 67.1, 62.6, 56.9, 40.9, 36.1, 32.1 (x2), 29.8 (x2), 29.7, 29.6 (x2), 29.3, 26.3, 23.5, 23.0, 14.4. The resulting dark brown oil was isolated in 65% yield. Combustion Analysis: calculated for C_{22}H_{39}IN\textsubscript{2} 0.25 HI, C, 53.88; H, 8.07; N, 5.71. Found: C, 53.74; H, 8.04; N, 5.58.
3.2.4. Synthetic Procedures for the Preparation of bis-Pyridinium Derivatives

3.2.4.1. bis-Pyridinium Salts

1,X-dihalogenoalkane, where X denotes the appropriate carbon length, (6.09-12.13 mmol) was added to a solution (30 mL) of dry pyridine, and the solution heated for 24 h at 65°C. The resulting solid precipitates were filtered and washed five times with dry diethyl ether. For liquid products, the excess pyridine was decanted off, the resulting liquid precipitates were washed with diethyl ether five times, and the remaining liquid was dried under vacuum. The resulting solid or liquid was isolated yields ranging from 87-93%.

\[ \text{N,N'}-\text{Pentane-1,5-diyl-bis-pyridinium Diiodide (bPPeI, 57a)} \]

\[ \text{H} \text{NMR (300 MHz, DMSO-D}_6\text{)} \delta 9.14 (2H, d, C_2&C_6-H), 8.62 (1H, t, C_4-H), 8.19 (2H, t, C_3&C_5-H), 4.62 (2H, t, C'_1-CH_2), 1.92 (2H, m, C'_2-CH_2), 1.25 (1H, m, C'_3-CH_2); \]

\[ \text{C NMR (75 MHz, DMSO-D}_6\text{)} \delta 145.3, 144.5, 127.9, 60.1, 29.9, 21.7. \] The resulting yellow solid was isolated in 90% yield, with a melting point of 146-147°C. Combustion Analysis: calculated for C_{15}H_{20}I_2N_2, C, 37.37; H, 4.18; N, 5.81. Found: C, 37.51; H, 4.20; N, 5.73.

\[ \text{N,N'}-\text{Hexane-1,6-diyl-bis-pyridinium Diiodide (bPHxI, 57b)} \]

\[ \text{H} \text{NMR (300 MHz, DMSO-D}_6\text{)} \delta 9.13 (2H, d, C_2&C_6-H), 8.63 (1H, t, C_4-H), 8.18 (2H, t, C_3&C_5-H), 4.52 (2H, t, C'_1-CH_2), 1.93 (2H, m, C'_2-CH_2), 1.34 (2H, m, C'_3-CH_2); \]

\[ \text{C NMR (75 MHz, DMSO-D}_6\text{)} \delta 145.3, 144.5, 127.9, 60.1, 29.9, 21.7. \]
NMR (75 MHz, DMSO-D$_6$) $\delta$ 145.3, 144.5, 127.9, 60.5, 30.7, 24.8. The resulting pale yellow solid was isolated in 92% yield, with a melting point of 242-243$^\circ$C. Combustion Analysis: calculated for C$_{16}$H$_{22}$I$_2$N$_2$, C, 38.73; H, 4.47; N, 5.65. Found: C, 38.62; H, 4.53; N, 5.87.

$N,N'$-Octane-1,8-diyl-bis-pyridinium Diiodide (bPOI, 57c)

$^1$H NMR (300 MHz, DMSO-D$_6$) $\delta$ 9.11 (2H, d, C2&C6-H), 8.63 (1H, t, C4-H), 8.18 (2H, t, C3&C5-H), 4.59 (2H, t, C'1-CH$_2$), 1.89 (2H, m, C'2-CH$_2$), 1.28 (4H, m, C'3&4-CH$_2$); $^{13}$C NMR (75 MHz, DMSO-D$_6$) $\delta$ 145.3, 144.5, 127.9, 60.5, 30.6, 28.1, 25.2. The resulting pale yellow solid was isolated in 93% yield, with a melting point of 203-204$^\circ$C. Combustion Analysis: calculated for C$_{18}$H$_{26}$I$_2$N$_2$, C, 41.24; H, 5.00; N, 5.43. Found: C, 41.20; H, 5.08; N, 5.68.

$N,N'$-Nonane-1,9-diyl-bis-pyridinium Dibromide (bPNB, 57e)

$^1$H NMR (300 MHz, DMSO-D$_6$) $\delta$ 9.17 (2H, d, C2&C6-H), 8.61 (1H, t, C4-H), 8.18 (2H, t, C3&C5-H), 4.63 (2H, t, C'1-CH$_2$), 1.89 (2H, m, C'2-CH$_2$), 1.22 (5H, m, C'3-5-CH$_2$); $^{13}$C NMR (75 MHz, DMSO-D$_6$) $\delta$ 145.3, 144.5, 127.9, 60.5, 30.7, 28.4, 28.2, 25.3, C$_{19}$H$_{28}$Br$_2$N$_2$. The resulting clear liquid was isolated in 87% yield. Combustion Analysis: calculated for C$_{19}$H$_{28}$Br$_2$N$_2$, C, 51.37; H, 6.35; N, 6.31. Found: C, 51.42; H, 6.31; N, 6.25.

$N,N'$-Decane-1,10-diyl-bis-pyridinium Diiodide (bPDI, 57f)
$^1$H NMR (300 MHz, DMSO-D$_6$) $\delta$ 9.10 (2H, d, C2&C6-H), 8.63 (1H, t, C4-H), 8.19 (2H, t, C3&C5-H), 4.60 (2H, t, C’1-CH$_2$), 1.89 (2H, m, C’2-CH$_2$), 1.24 (6H, m, C’3-5-CH$_2$); $^{13}$C NMR (75 MHz, DMSO-D$_6$) $\delta$ 145.3, 144.5, 127.9, 60.6, 30.6, 28.8, 28.1, 25.2. The resulting pale yellow solid was isolated in 90% yield, with a melting point of 162-163$^\circ$C. Combustion Analysis: calculated for C$_{20}$H$_{30}$I$_2$N$_2$, C, 43.50; H, 5.48; N, 5.07. Found: C, 43.53; H, 5.67; N, 5.29.

$N,N'$-Undecane-1,11-diyl-bis-pyridinium Dibromide (bPUB, 57g)

$^1$H NMR (300 MHz, DMSO-D$_6$) $\delta$ 9.18 (2H, d, C2&C6-H), 8.62 (1H, t, C4-H), 8.19 (2H, t, C3&C5-H), 4.63 (2H, t, C’1-CH$_2$), 1.88 (2H, m, C’2-CH$_2$), 1.22 (7H, m, C’3-6-CH$_2$); $^{13}$C NMR (75 MHz, DMSO-D$_6$) $\delta$ 145.3, 144.6, 127.9, 60.6, 30.8, 28.8 (x2), 28.4, 25.4. The resulting amber liquid was isolated in 91% yield. Combustion Analysis: calculated for C$_{21}$H$_{32}$Br$_2$N$_2$, C, 53.40; H, 6.83; N, 5.93. Found: C, 53.39; H, 6.91; N, 5.88.

$N,N'$-Dodecane-1,12-diyl-bis-pyridinium Dibromide (bPDDB, 57h)

$^1$H NMR (300 MHz, DMSO-D$_6$) $\delta$ 9.15 (2H, d, C2&C6-H), 8.62 (1H, t, C4-H), 8.19 (2H, t, C3&C5-H), 4.61 (2H, t, C’1-CH$_2$), 1.88 (2H, m, C’2-CH$_2$), 1.22 (8H, m, C’3-6-CH$_2$); $^{13}$C NMR (75 MHz, DMSO-D$_6$) $\delta$ 145.3, 144.6, 127.9, 60.7, 30.8, 28.9, 28.8, 28.5, 25.4. The resulting white solid was isolated in 93% yield, with a melting point of 222-223$^\circ$C. Combustion Analysis: calculated for C$_{22}$H$_{34}$Br$_2$N$_2$, C, 54.33; H, 7.05; N, 5.76. Found: C, 54.40; H, 7.00; N, 5.86.
3.2.4.2. *bis*-Picolinium Salts

1,X-dihalogenoalkane, where X denotes the appropriate carbon length, (6.09-12.13 mmol) was added to a solution (30 mL) of 3-picoline, and the solution heated for 24 h at 65°C. The resulting precipitate was filtered, and the product washed five times with dry diethyl ether. The precipitate was then dissolved in water (30 mL) and extracted with chloroform (3 x 30 mL). The organic layer was collected and evaporated to dryness on a rotary evaporator. The resulting solid or liquid was isolated in yields ranging from 90-94%.

*N,N*-Hexane-1,6-diyl-*bis*-picolinium Diiodide (bPiHxI, 58a)

$^1$H NMR (300 MHz, DMSO-$D_6$) δ 9.06 (1H, s, C2-H), 8.94 (1H, d, C6-H), 8.46 (1H, d, C4-H), 8.07 (1H, t, C5-H), 4.56 (2H, t, C’1-CH₂), 2.50 (3H, s, C3-CH₃), 1.86 (2H, m, C’2-CH₂), 1.33 (2H, m, C’3-CH₂); $^{13}$C NMR (75 MHz, DMSO-$D_6$) δ 145.6, 144.0, 141.7, 138.5, 127.1, 60.3, 30.3, 24.8, 17.9. The resulting pale yellow solid was isolated in 91% yield, with a melting point of 214-215°C. Combustion Analysis: calculated for C₁₈H₂₆I₂N₂, C, 41.24; H, 5.00; N, 5.34. Found: 41.03; H, 5.32; N, 5.22.

*N,N*-Octane-1,8-diyl-*bis*-picolinium Diiodide (bPiOI, 58b)

$^1$H NMR (300 MHz, DMSO-$D_6$) δ 9.18 (1H, s, C2-H), 8.96 (1H, d, C6-H), 8.46 (1H, d, C4-H), 8.07 (1H, t, C5-H), 4.62 (2H, t, C’1-CH₂), 2.50 (3H, s, C3-CH₃), 1.94 (2H, m, C’2-CH₂), 1.27 (4H, m, C’3&4-CH₂); $^{13}$C NMR (75 MHz, DMSO-$D_6$) δ 145.5, 143.9, 141.7, 138.5, 127.1, 60.4, 30.5, 28.1, 25.2, 17.9. The resulting canary yellow solid was
isolated in 94% yield, with a melting point of 161-162°C. Combustion Analysis: calculated for C\textsubscript{20}H\textsubscript{30}I\textsubscript{2}N\textsubscript{2}, C, 43.50; H, 5.48; N, 5.07. Found: C, 43.32; H, 5.47; N, 4.98.

\( N,N' \)-Nonane-1,9-diyl-\textit{bis}-picolinium Dibromide (bPiNB, 58c)

\( ^1 \)H NMR (300 MHz, DMSO-\textsubscript{D\textsubscript{6}}) \( \delta \) 9.17 (1H, s, C2-H), 9.04 (1H, d, C6-H), 8.47 (1H, d, C4-H), 8.08 (1H, t, C5-H), 4.60 (2H, t, C’1-CH\textsubscript{2}), 2.50 (3H, s, C3-CH\textsubscript{3}), 1.92 (2H, m, C’2-CH\textsubscript{2}), 1.27 (5H, m, C’3-5-CH\textsubscript{2}); \( ^{13} \)C NMR (75 MHz, DMSO-\textsubscript{D\textsubscript{6}}) \( \delta \) 145.5, 144.1, 141.8, 138.5, 127.1, 60.4, 30.7, 28.5, 28.3, 25.4, 17.9. The resulting tan liquid was isolated in 90% yield. Combustion Analysis: calculated for C\textsubscript{21}H\textsubscript{32}Br\textsubscript{2}N\textsubscript{2}. 0.5 H\textsubscript{2}O, C, 52.40; H, 6.91; N, 5.82. Found: C, 52.26; H, 6.89; N, 5.70.

\( N,N' \)-Decane-1,10-diyl-\textit{bis}-picolinium Diiodide (bPiDI, 58d)

\( ^1 \)H NMR (300 MHz, CDCl\textsubscript{3}) \( \delta \) 9.04 (1H, s, C2-H), 8.94 (1H, d, C6-H), 8.46 (1H, d, C4-H), 8.06 (1H, t, C5-H), 4.58 (2H, t, C’1-CH\textsubscript{2}), 2.50 (3H, s, C3-CH\textsubscript{3}), 1.90 (2H, m, C’2-CH\textsubscript{2}), 1.27 (6H, m, C’3-5-CH\textsubscript{2}); \( ^{13} \)C NMR (75 MHz, DMSO-\textsubscript{D\textsubscript{6}}) \( \delta \) 145.6, 144.0, 141.7, 138.6, 127.2, 60.5, 30.6, 28.7, 28.4, 25.4, 18.0. The resulting tan solid was isolated in 92% yield, with a melting point of 161-162°C. Combustion Analysis: calculated for C\textsubscript{22}H\textsubscript{34}I\textsubscript{2}N\textsubscript{2}. 1.5 H\textsubscript{2}O, C, 43.51; H, 6.14; N, 4.61. Found: C, 43.82; H, 6.02; N, 4.61.

\( N,N' \)-Undecane-1,11-diyl-\textit{bis}-picolinium Dibromide (bPiUB, 58e)

\( ^1 \)H NMR (300 MHz, DMSO-\textsubscript{D\textsubscript{6}}) \( \delta \) 9.10 (1H, s, C2-H), 8.98 (1H, d, C6-H), 8.46 (1H, d, C4-H), 8.07 (1H, t, C5-H), 4.58 (2H, t, C’1-CH\textsubscript{2}), 2.50 (3H, s, C3-CH\textsubscript{3}), 1.90 (2H, m, C’2-CH\textsubscript{2}), 1.26 (7H, m, C’3-6-CH\textsubscript{2}); \( ^{13} \)C NMR (75 MHz, DMSO-\textsubscript{D\textsubscript{6}}) \( \delta \) 145.5, 144.0,
The resulting tan liquid was isolated in 90% yield. Combustion Analysis: calculated for \(\text{C}_{23}\text{H}_{36}\text{Br}_2\text{N}_2\). 0.33 \(\text{H}_2\text{O}\), C, 54.56; H, 7.30; N, 5.53. Found: C, 54.23; H, 7.43; N, 5.41.

\(\text{N,N'}\)-Dodecane-1,12-diyl-\(\text{bis}\)-picolinium Dibromide (bPiDB, 58f)

\(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 9.04 (1H, s, C2-H), 8.94 (1H, d, C6-H), 8.46 (1H, d, C4-H), 8.16 (1H, t, C5-H), 4.55 (2H, t, C’1-CH\(_2\)), 2.50 (3H, s, C3-CH\(_3\)), 1.90 (2H, m, C’2-CH\(_2\)), 1.25 (8H, m, C’3-6-CH\(_2\)); \(^{13}\)C NMR (75 MHz, DMSO-D\(_6\)) \(\delta\) 145.5, 144.1, 141.8, 138.5, 127.2, 60.4, 30.7, 28.9, 28.8, 28.4, 25.4, 17.9. The resulting tan liquid was isolated in 92% yield. Combustion Analysis: calculated for \(\text{C}_{24}\text{H}_{38}\text{Br}_2\text{N}_2\), C, 56.04; H, 7.45; N, 5.45. Found: C, 55.89; H, 7.62; N, 5.32.

3.2.4.3. \textit{bis-Quinolinium Salts}

1,X-dihalogenoalkane, where \(X\) denotes the appropriate carbon length, (6.09-12.13 mmol) was added to a solution (30 mL) of dry quinoline, and the solution heated for 24 h at 65\(^\circ\)C. The resulting precipitate was filtered, and the product washed five times with dry diethyl ether. The resulting solid was isolated in yields ranging from 87-92%.

\(\text{N,N'}\)-Hexane-1,6-diyl-\(\text{bis}\)-quinolinium Diiodide (bQHxI, 59a)

\(^1\)H NMR (300 MHz, DMSO-D\(_6\)) \(\delta\) 9.53 (1H, d, C2-H), 9.29 (1H, d, C3-H), 8.61 (1H, d, C8-H), 8.50 (1H, d, C4-H), 8.29 (1H, t, C7-H), 8.18 (1H, t, C5-H), 8.07 (1H, d, C6-H), 5.09 (2H, t, C’1-CH\(_2\)), 1.96 (2H, m, C’2-CH\(_2\)), 1.25 (2H, m, C’3-CH\(_2\)); \(^{13}\)C NMR (75 MHz, DMSO-D\(_6\)) \(\delta\) 149.4, 147.2, 137.2, 135.5, 130.6, 129.7, 129.6, 122.0, 118.8, 57.2,
The resulting mustard yellow solid was isolated in 89% yield, with a melting point of 234-235°C. Combustion Analysis: calculated for C_{24}H_{26}I_{2}N_{2}, C, 48.34; H, 4.39; N, 4.70. Found: C, 48.29; H, 4.43; N, 4.85.

\(N,N'\)-Octane-1,8-diyl-bis-quinolinium Diiodide (bQOI, 59b)

\(^1\)H NMR (300 MHz, DMSO-D\(_6\)) \(\delta\) 9.53 (1H, d, C2-H), 9.29 (1H, d, C3-H), 8.61 (1H, d, C8-H), 8.50 (1H, d, C4-H), 8.29 (1H, t, C7-H), 8.18 (1H, t, C5-H), 8.07 (1H, d, C6-H), 5.09 (2H, t, C’1-CH\(_2\)), 1.96 (2H, m, C’2-CH\(_2\)), 1.25 (4H, m, C’3&4-CH\(_2\)); \(^{13}\)C NMR (75 MHz, DMSO-D\(_6\)) \(\delta\) 149.5, 147.4, 137.4, 135.6, 130.7, 129.9, 129.7, 122.1, 118.9, 57.3, 29.5, 28.5, 25.7. The resulting canary yellow solid was isolated in 89% yield, with a melting point of 222-223°C. Combustion Analysis: calculated for C_{26}H_{30}I_{2}N_{2}. 0.25 H\(_2\)O, C, 49.54; H, 4.90; N, 4.44. Found: C, 49.41; H, 5.19; N, 4.11.

\(N,N'\)-Nonane-1,9-diyl-bis-quinolinium Dibromide (bQNB, 59c)

\(^1\)H NMR (300 MHz, DMSO-D\(_6\)) \(\delta\) 9.68 (1H, d, C2-H), 9.34 (1H, d, C3-H), 8.67 (1H, d, C8-H), 8.55 (1H, d, C4-H), 8.27 (2H, m, C5&C7-H), 8.07 (1H, d, C6-H), 5.09 (2H, t, C’1-CH\(_2\)), 1.95 (2H, m, C’2-CH\(_2\)), 1.19-1.45 (5H, m, C’3-5-CH\(_2\)); \(^{13}\)C NMR (75 MHz, DMSO-D\(_6\)) \(\delta\) 149.5, 147.2, 137.2, 135.5, 130.6, 129.7, 129.5, 122.0, 118.9, 57.2, 29.6, 28.6, 28.4, 25.7. The resulting dark purple solid was isolated in 92% yield, with a melting point of 185-186°C. Combustion Analysis: calculated for C_{27}H_{32}Br_{2}N_{2}. 0.25 H\(_2\)O, C, 59.08; H, 5.97; N, 5.10. Found: C, 59.01; H, 6.09; N, 5.05.
**N,N’-Decane-1,10-diyl-bis-quinolinium Diiodide (bQDI, 59d)**

$^1$H NMR (300 MHz, DMSO-D$_6$) $\delta$ 9.53 (1H, d, C2-H), 9.29 (1H, d, C3-H), 8.61 (1H, d, C8-H), 8.50 (1H, d, C4-H), 8.29 (1H, t, C7-H), 8.18 (1H, t, C5-H), 8.06 (1H, d, C6-H), 5.04 (2H, t, C’1-CH$_2$), 1.96 (2H, m, C’2-CH$_2$), 1.32 (6H, m, C’3-5-CH$_2$); $^{13}$C NMR (75 MHz, DMSO-D$_6$) $\delta$ 149.5, 147.4, 137.4, 135.6, 130.7, 129.9, 129.7, 122.1, 118.9, 57.3, 29.5, 28.9, 28.5, 25.7. The resulting canary yellow solid was isolated in 91% yield, with a melting point of 235-236°C. Combustion Analysis: calculated for C$_{28}$H$_{34}$I$_2$N$_2$.0.66 H$_2$O, C, 50.62; H, 5.36; N, 4.22. Found: C, 50.48; H, 5.52; N, 4.19.

**N,N’-Undecane-1,11-diyl-bis-quinolinium Dibromide (bQUB, 59e)**

$^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 9.54 (1H, d, C2-H), 9.28 (1H, d, C3-H), 8.48 (1H, d, C8-H), 8.48 (1H, d, C8-H), 8.27 (1H, t, C7-H), 8.16 (1H, t, C5-H), 8.05 (1H, d, C6-H), 5.03 (2H, t, C’1-CH$_2$), 1.95 (2H, m, C’2-CH$_2$), 1.15-1.48 (7H, m, C’3-6-CH$_2$); $^{13}$C NMR (75 MHz, DMSO-d$_6$) $\delta$ 149.5, 147.4, 137.4, 135.6, 130.7, 129.9, 129.7, 122.1, 118.9, 57.3, 29.5, 28.9, 28.8, 28.5, 25.7. The resulting dark purple solid was isolated in 87% yield, with a melting point of 188-189°C. Combustion Analysis: calculated for C$_{29}$H$_{36}$Br$_2$N$_2$.1.0 H$_2$O, C, 58.99; H, 6.49; N, 4.75. Found: C, 59.23; H, 6.56; N, 4.62.

**N,N’-Dodecane-1,12-diyl-bis-quinolinium Dibromide (bQDDB, 59f)**

$^1$H NMR (300 MHz, DMSO-D$_6$) $\delta$ 9.58 (1H, d, C2-H), 9.29 (1H, d, C3-H), 8.61 (1H, d, C8-H), 8.50 (1H, d, C4-H), 8.29 (1H, t, C7-H), 8.18 (1H, t, C5-H), 8.04 (1H, d, C6-H), 5.04 (2H, t, C’1-CH$_2$), 1.95 (2H, m, C’2-CH$_2$), 1.2-1.4 (8H, m, C’3-6-CH$_2$); $^{13}$C NMR (75 MHz, DMSO-D$_6$) $\delta$ 149.5, 147.4, 137.4, 135.6, 130.7, 129.9, 129.7, 122.1, 118.9,
57.3, 29.5, 28.9, 28.8, 28.5, 25.7. The resulting dark purple solid was isolated in 92% yield, with a melting point of 187-188°C. Combustion Analysis: calculated for C\textsubscript{38}H\textsubscript{38}Br\textsubscript{2}N\textsubscript{2}, 0.33 H\textsubscript{2}O, C, 60.82; H, 6.58; N, 4.73. Found: C, 61.01; H, 6.63; N, 4.64.

### 3.2.4.4. *bis*-Isoquinolinium Salts

1,X-dihalogenoalkane, where X denotes the appropriate carbon length, (6.09-12.13 mmol) was added to a solution (30 mL) of isoquinoline, and the solution heated for 24 h at 65°C. The resulting precipitate was filtered, and the product washed five times with dry diethyl ether. The resulting solid was isolated in yields ranging from 89-92%.

#### \(N,N'\)-Hexane-1,6-divyl-*bis*-isoquinolinium Diiodide (bIQHxI, 60a)

\(^1\text{H} \text{NMR} (300 \text{ MHz, DMSO-D}_6) \delta 10.09 (1\text{H, d, C1-H}), 8.80 (1\text{H, d, C3-H}), 8.61 (1\text{H, d, C8-H}), 8.48 (1\text{H, d, C4-H}), 8.36 (1\text{H, d, C7-H}), 8.27 (1\text{H, t, C5-H}), 8.08 (1\text{H, t, C6-H}), 4.71 (2\text{H, t, C'1-CH}_2), 2.03 (2\text{H, m, C'2-CH}_2), 1.40 (4\text{H, m, C'3&4-CH}_2); {^{13}\text{C}} \text{NMR (75 MHz, DMSO-D}_6) \delta 149.7, 136.7 (x2), 134.7, 131.0, 130.1, 127.1, 127.0, 125.7, 60.6, 30.2, 25.0. The resulting pale yellow solid was isolated in 87% yield, with a melting point of 233°C. Combustion Analysis: calculated for C\textsubscript{24}H\textsubscript{26}I\textsubscript{2}N\textsubscript{2}, C, 48.34; H, 4.39; N, 4.70. Found: C, 48.46; H, 4.57; N, 4.98.

#### \(N,N'\)-Octane-1,8-divyl-*bis*-isoquinolinium Diiodide (bIQOI, 60b)

\(^1\text{H} \text{NMR} (300 \text{ MHz, DMSO-D}_6) \delta 10.05 (1\text{H, d, C1-H}), 8.77 (1\text{H, d, C3-H}), 8.58 (1\text{H, d, C8-H}), 8.48 (1\text{H, d, C4-H}), 8.35 (1\text{H, d, C7-H}), 8.26 (1\text{H, t, C5-H}), 8.07 (1\text{H, t, C6-H}), 4.68 (2\text{H, t, C'1-CH}_2), 2.00 (2\text{H, m, C'2-CH}_2), 1.30 (4\text{H, m, C'3&4-CH}_2); {^{13}\text{C}} \text{NMR (75 MHz, DMSO-D}_6) \delta 149.7, 136.7 (x2), 134.7, 131.0, 130.1, 127.1, 127.0, 125.7, 60.6, 30.2, 25.0. The resulting pale yellow solid was isolated in 87% yield, with a melting point of 233°C. Combustion Analysis: calculated for C\textsubscript{24}H\textsubscript{26}I\textsubscript{2}N\textsubscript{2}, C, 48.34; H, 4.39; N, 4.70. Found: C, 48.46; H, 4.57; N, 4.98.
MHz, DMSO-D$_6$) $\delta$ 149.7, 136.9 (x2), 134.8, 131.3, 130.3, 127.3, 127.2, 125.9, 60.9, 30.6, 28.4, 25.6. The resulting yellow solid was isolated in 92% yield, with decomposition at 250°C. Combustion Analysis: calculated for C$_{26}$H$_{30}$I$_2$N$_2$, C, 50.02; H, 4.84; N, 4.49. Found: C, 50.22; H, 4.61; N, 4.53.

$N,N'$-Nonane-1,9-diyl-bis-isoquinolinium Dibromide (bIQNB, 60c)

$^1$H NMR (300 MHz, DMSO-D$_6$) $\delta$ 10.17 (1H, d, C1-H), 8.85 (1H, d, C3-H), 8.62 (1H, d, C8-H), 8.49 (1H, d, C4-H), 8.36 (2H, d, C7-H), 8.26 (2H, t, C5-H), 8.08 (1H, t, C6-H), 4.72 (2H, t, C’1-CH$_2$), 2.02 (2H, m, C’2-CH$_2$), 1.11-1.45 (5H, m, C’3-5-CH$_2$); $^{13}$C NMR (75 MHz, DMSO-D$_6$) $\delta$ 149.8, 136.7 (x2), 134.8, 131.0, 130.1, 127.1, 127.1, 125.7, 60.7, 30.5, 28.6, 28.4, 25.5. The resulting light purple solid was isolated in 92% yield, with a melting point of 162°C. Combustion Analysis: calculated for C$_{27}$H$_{32}$Br$_2$N$_2$. 0.33 H$_2$O C, 58.92; H, 5.98; N, 5.09. Found: C, 58.76; H, 5.92; N, 4.90.

$N,N'$-Decane-1,10-diyl-bis-isoquinolinium Diiodide (bIQDI, 60d)

$^1$H NMR (300 MHz, DMSO-D$_6$) $\delta$ 10.07 (1H, d, C1-H), 8.78 (1H, d, C3-H), 8.58 (1H, d, C8-H), 8.48 (1H, d, C4-H), 8.36 (1H, d, C7-H), 8.26 (1H, t, C5-H), 8.07 (1H, t, C6-H), 4.69 (2H, t, C’1-CH$_2$), 2.0 (2H, m, C’2-CH$_2$), 1.15-1.50 (6H, m, C’3-5-CH$_2$); $^{13}$C NMR (75 MHz, DMSO-D$_6$) $\delta$ 149.7, 136.9 (x2), 134.9, 131.2, 130.0, 127.3, 127.2, 125.9, 60.9, 30.7, 28.9, 28.6, 25.7. The resulting dark yellow solid was isolated in 92% yield, with a melting point of 192°C. Combustion Analysis: calculated for C$_{28}$H$_{34}$I$_2$N$_2$. 0.33 H$_2$O C, 51.08; H, 5.31; N, 4.25. Found: C, 50.88; H, 5.42; N, 4.13.
N,N'-Undecane-1,11-diyl-bis-isoquinolinium Dibromide (bIQUB, 60e)

$^1$H NMR (300 MHz, DMSO-D$_6$) δ 10.07 (1H, d, C1-H), 8.78 (1H, d, C3-H), 8.57 (1H, d, C8-H), 8.48 (1H, d, C4-H), 8.35 (1H, d, C7-H), 8.25 (1H, t, C5-H), 8.07 (1H, t, C6-H), 4.69 (2H, t, C’1-CH$_2$), 2.0 (2H, m, C’2-CH$_2$), 1.05-1.45 (7H, m, C’3-6-CH$_2$); $^{13}$C NMR (75 MHz, DMSO-D$_6$) δ 149.8, 136.7 (x2), 134.8, 131.0, 130.2, 127.1 (x2), 125.7, 60.7, 30.5, 28.9, 28.8, 28.5, 25.6. The resulting purple solid was isolated in 89% yield, with a melting point of 166$^\circ$C. Combustion Analysis: calculated for C$_{29}$H$_{36}$Br$_2$N$_2$, C, 60.85; H, 6.34; N, 4.89. Found: C, 60.65; H, 6.43; N, 4.92.

N,N'-Dodecane-1,12-diyl-bis-isoquinolinium Dibromide (bIQDDB, 60f)

$^1$H NMR (300 MHz, DMSO-D$_6$) δ 10.06 (1H, d, C1-H), 8.78 (1H, d, C3-H), 8.58 (1H, d, C8-H), 8.48 (1H, d, C4-H), 8.35 (1H, d, C7-H), 8.25 (1H, t, C5-H), 8.07 (1H, t, C6-H), 4.70 (2H, t, C’1-CH$_2$), 2.0 (2H, m, C’2-CH$_2$), 1.15-1.40 (8H, m, C’3-6-CH$_2$); $^{13}$C NMR (75 MHz, DMSO-D$_6$) δ 149.8, 136.7 (x2), 134.8, 131.0, 130.1, 127.1 (x2), 125.7, 60.6, 30.5, 28.9, 28.8, 28.5, 25.5. The resulting gray-purple solid was isolated in 91% yield, with a melting point of 225$^\circ$C. Combustion Analysis: calculated for C$_{30}$H$_{38}$Br$_2$N$_2$.0.5 H$_2$O, C, 60.51; H, 6.60; N, 4.70. Found: C, 60.75; H, 6.56; N, 4.54.

3.2.4.5. *bis*-Nicotinium Salts

S-(-)-Nicotine (30.8 mmol) was dissolved in glacial acetic acid (35 mL) and the solution was stirred at ambient temperature for five minutes. The 1,X-dihalogenoalkane, where X denotes the appropriate carbon length, (15.4 mmol) was added to the solution and the mixture stirred with refluxing for three days. The mixture was then evaporated under
reduced pressure to remove the solvent, and the resulting oil was treated with an aqueous solution of sodium bicarbonate. The resulting aqueous mixture was extracted with diethyl ether (3 x 50 mL), and then chloroform (3 x 50 mL) and the remaining aqueous layer separated and evaporated to dryness on a rotary evaporator. The resulting solid was dissolved in chloroform (200 mL) and filtered. The filtrate was collected and evaporated to dryness under pressure. The resulting oil was isolated in yields ranging from 15-35%.

\[ N,N'-\text{Hexane-1,6-diyi-bis-nicotinium Diiodide (bNHxI, 61a)} \]

1H NMR (300 MHz, CDCl₃), δ 9.44 (1H, s, C2-H), 9.38 (1H, d, C6-H), 8.44 (1H, d, C4-H), 8.13 (1H, m, C5-H), 4.95 (2H, t, C’’1-CH₂), 4.03 (1H, t, pyrrolidine CH₂), 3.25 (1H, t, pyrrolidine CH₂), 2.49 (2H, m, C’’2-CH₂), 2.23 (3H, s, pyrrolidine N-CH₃), 1.60-2.19 (5H, m, pyrrolidine CH₂CH₂), 1.30-1.55 (2H, m, C’’3-CH₃); 13C NMR (75 MHz, CDCl₃) δ 146.8, 143.9, 143.7 (x2), 128.2, 66.8, 61.1, 57.0, 40.8, 36.0, 31.3, 24.7, 23.5. The resulting brown viscous oil was isolated in 25% yield. Combustion Analysis: calculated for C₂₆H₄₀I₂N₄.0.75H₂O, C, 46.20; H, 6.19; N, 8.29. Found: C, 46.08; H, 6.27; N, 8.23.

\[ N,N'-\text{Octane-1,8-diyi-bis-nicotinium Diiodide (bNOI, 61b)} \]

1H NMR (300 MHz, CDCl₃), δ 9.58 (1H, s, C2-H), 9.50 (1H, d, C6-H), 8.47 (1H, d, C4-H), 8.03 (1H, m, C5-H), 4.96 (2H, t, C’’1-CH₂), 3.65 (1H, t, pyrrolidine CH₂), 3.24 (1H, t, pyrrolidine CH₂), 2.49 (2H, m, C’’2-CH₂), 2.25 (3H, s, pyrrolidine N-CH₃), 1.60-2.19 (5H, m, pyrrolidine CH₂CH₂), 1.40-1.60 (4H, m, C’’3&4-CH₂); 13C NMR (75 MHz, CDCl₃) δ 146.9, 143.8, 143.6, 143.5, 128.3, 66.8, 61.7, 57.0, 40.8, 36.1, 32.0, 27.7, 25.2, 23.5. The resulting yellow viscous oil was isolated in 15% yield. Combustion Analysis:
calculated for C$_{28}$H$_{44}$I$_2$N$_4$. 0.25 H$_2$O, C, 48.39; H, 6.45; N, 8.06. Found: C, 48.13; H, 6.61; N, 7.98.

$N,N'$-Nonane-1,9-diyl-bis-nicotinium Dibromide (bNNB, 61c)

$^1$H NMR (300 MHz, CDCl$_3$), $\delta$ 9.75 (1H, s, C2-H), 9.56 (1H, d, C6-H), 8.49 (1H, d, C4-H), 8.08 (1H, m, C5-H), 5.05 (2H, t, C’’1-CH$_2$), 3.65 (1H, t, pyrrolidine CH$_2$), 3.25 (1H, t, pyrrolidine CH$_2$), 2.48 (2H, m, C’’2-CH$_2$), 2.25 (3H, s, pyrrolidine N-CH$_3$), 1.60-2.23 (5H, m, pyrrolidine CH$_2$CH$_2$), 1.30-1.60 (5H, m, C’’3-5-CH$_2$); $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 146.5, 144.2, 143.7, 143.6, 128.3, 66.9, 61.7, 56.9, 40.7, 36.0, 32.1, 28.2, 28.0, 25.5, 23.4. The resulting yellow viscous oil was isolated in 27% yield. Combustion Analysis: calculated for C$_{29}$H$_{46}$Br$_2$N$_4$. 0.33 H$_2$O, C, 56.50; H, 7.63; N, 9.09. Found: C, 56.18; H, 7.81; N, 9.01.

$N,N'$-Decane-1,10-diyl-bis-nicotinium Diiodide (bNDI, 61d)

$^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 9.55 (1H, s, C2-H), 9.44 (1H, d, C6-H), 8.53 (1H, d, C4-H), 8.09 (1H, m, C5-H), 4.98 (2H, t, C’’1-CH$_2$), 3.74 (1H, t, pyrrolidine CH$_2$), 3.30 (1H, t, pyrrolidine CH$_2$), 2.50 (2H, m, C’’2-CH$_2$), 2.29 (3H, s, pyrrolidine N-CH$_3$), 1.65-2.20 (5H, m, pyrrolidine CH$_2$CH$_2$), 1.30-1.57 (6H, m, C’’3-5-CH$_2$); $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 146.6, 143.9 x 2, 143.4, 128.4, 66.9, 61.9, 57.0, 40.8, 36.1, 32.1, 28.5, 28.2, 25.8, 23.5. The resulting yellow viscous oil was isolated in 27% yield. Combustion Analysis: calculated for C$_{30}$H$_{48}$I$_2$N$_4$. 1.0 H$_2$O, C, 48.92; H, 6.84; N, 7.61. Found: C, 48.64; H, 6.58; N, 7.48.
N,N'-Undecane-1,11-diyl-bis-nicotinium Dibromide (bNUB, 61e)

$^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 9.70 (1H, s, C2-H), 9.47 (1H, d, C6-H), 8.49 (1H, d, C4-H), 8.12 (1H, m, C5-H), 5.02 (2H, t, C''1-CH$_2$), 3.65 (1H, t, pyrrolidine CH$_2$), 3.26 (1H, t, pyrrolidine CH$_2$), 2.49 (2H, m, C''2-CH$_2$), 2.25 (3H, s, pyrrolidine N-CH$_3$), 1.60-2.20 (5H, m, pyrrolidine CH$_2$CH$_2$), 1.20-1.50 (8H, m, C''3-6-CH$_2$); $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 146.5, 144.2, 143.7, 143.5, 128.4, 66.9, 61.9, 56.9, 40.7, 36.1, 32.2, 28.8, 28.7, 28.6, 25.9, 23.4. The resulting yellow viscous oil was isolated in 30% yield. Combustion Analysis: calculated for C$_{31}$H$_{50}$Br$_2$N$_4$. 0.33 H$_2$O, C, 57.76; H, 7.92; N, 8.69. Found: C, 57.55; H, 7.99; N, 8.61.

N,N'-Dodecane-1,12-diyl-bis-nicotinium Dibromide (bNDDB, 61f)

$^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 9.66 (1H, s, C2-H), 9.38 (1H, d, C6-H), 8.46 (1H, d, C4-H), 8.10 (1H, t, C5-H), 5.01 (2H, t, C''1-CH$_2$), 3.61 (1H, t, pyrrolidine CH$_2$), 3.25 (1H, t, pyrrolidine CH$_2$), 2.45 (2H, m, C''2-CH$_2$), 2.23 (3H, s, pyrrolidine N-CH$_3$), 1.60-2.19 (5H, m, pyrrolidine CH$_2$CH$_2$), 1.20-1.50 (8H, m, C''3-6-CH$_2$); $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 146.6, 144.2, 143.7, 143.4, 128.5, 67.0, 62.0, 60.0, 40.8, 36.1, 32.4, 29.0, 28.8, 28.7, 26.0, 23.5. The resulting yellow viscous oil was isolated in 35% yield. Combustion Analysis: calculated for C$_{32}$H$_{52}$Br$_2$N$_4$. 0.5 H$_2$O, C, 58.09; H, 8.07; N, 8.47. Found: C, 57.86; H, 8.12; N, 8.39.
3.2.5. Binding Assays for $\alpha_4\beta_2^*$ and $\alpha_7^*$ Nicotinic Receptor Subtypes

$[^3H]$Nicotine Binding Assay ($\alpha_4\beta_2^*$)

The interaction of the compounds with the high affinity $[^3H]$NIC binding site of the $\alpha_4\beta_2^*$ nAChR was studied utilizing a modification of the methodology of Marks et al. (1986). Rat striata were dissected, pooled, and homogenized in 10 vol ice-cold modified Krebs-HEPES buffer (in mM: 20 HEPES, 118 NaCl, 4.8 KCl, 2.5 CaCl$_2$, 1.2 MgSO$_4$, pH 7.5). Homogenates were incubated at 37°C for 5 min, and then centrifuged at 27,000 g for 20 min at 4°C. The pellets were resuspended in 10 vol ice-cold MilliQ water and incubated for 5 min at 37°C, followed by centrifugation at 27,000 g for 20 min at 4°C. The second and third pellets were resuspended in 10 vol fresh, ice-cold 10% KRH buffer, incubated for 5 min at 37°C, and centrifuged as above. The final pellets were resuspended in 20 vol of ice-cold MilliQ water, which afforded ~200 µg membrane protein/100 µL aliquot [Bradford, 1976], using BSA as the protein standard. Binding assays were performed in duplicate in a final vol of 200 µL Krebs-HEPES buffer containing 200 mM Tris (pH 7.5 at 4°C). Assays were initiated by the addition of 100 µL of membrane suspension to assay tubes containing final concentrations of 3 nM $[^3H]$NIC (50 µL) and one of 9 concentrations of compound being evaluated. Nonspecific binding was defined in the presence of 10 µM NIC. After a 90 min incubation at 4°C, reactions were terminated by dilution with 3 mL ice-cold Krebs-HEPES buffer, followed immediately by filtration through Schleicher and Schuell #32 glass fiber filters (pre-soaked in 0.5% polyethyleneimine) using a Brandel cell harvester (Biomedical Research...
Filters were rinsed three times with 3 mL of ice-cold Krebs-HEPES buffer and transferred to scintillation vials, 3 mL of scintillation cocktail was added, and radioactivity was determined by liquid scintillation spectroscopy (Marks et al. 1986).

### [3H]MLA Binding Assay (α7*)

[3H]MLA binding probes the α7 nAChR subtype, and assays were carried out using a modification of the method of Davies et al. (1999). Whole brain minus cortex, striatum and cerebellum was homogenized in 20 vol of ice-cold hypotonic buffer (in mM: 2 HEPES, 14.4 NaCl, 0.15 KCl, 0.2 CaCl₂ and 0.1 MgSO₄, pH 7.5). Homogenates were incubated at 37°C for 10 min and centrifuged at 29,000 g for 17 minutes at 4°C. Pellets were washed three times by resuspension in 20 vol of the same buffer followed by centrifugation as described above. Final pellets were resuspended in the incubation buffer to yield ~150 µg membrane protein/100 µL membrane suspension. Binding assays were performed in duplicate, in a final volume of 250 µL of incubation buffer (in mM: 20 HEPES, 144 NaCl, 1.5 KCl, 2 CaCl₂ and 1 MgSO₄, pH 7.5). Binding assays were initiated by the addition of 100 µL of membrane suspension to 150 µL of sample containing 2.5 nM [3H]MLA and the desired concentration of the test compound, and incubated for 2 hrs at ambient temperature. Nonspecific binding was determined in the presence of 1 mM nicotine. Assays were terminated by dilution of samples with 3 mL of ice-cold incubation buffer, followed by immediate filtration through Schleicher and Schuell #32 glass fiber filters (pre-soaked in 0.5% polyethyleneimine) using a Brandel
cell harvester (Davies et al. 1999). Filters were processed as described above for the $[^{3}\text{H}]$NIC binding assay.

3.2.6. **Functional Assays for the Nicotinic Receptors Modulating DA Release and $\alpha_{4}\beta_{2}^{*}$ Nicotinic Receptor Subtype**

$[^{3}\text{H}]$**Dopamine Overflow Assay**

Quaternary ammonium analog effects on $[^{3}\text{H}]$overflow from rat striatal slices preloaded with $[^{3}\text{H}]$DA, were determined using a modification of a previously published method (Dwoskin and Zahniser 1986; Wilkins et al. 2002). Briefly, rat striata were dissected and coronal slices (500 µm, 6-8 mg) were obtained using a McIlwain tissue chopper. Slices were incubated for 30 min in Krebs buffer (in mM: 118 NaCl, 4.7 KCl, 1.3 CaCl$_2$, 1.2 MgCl$_2$, 1.0 NaH$_2$PO$_4$, 11.1 α-D-glucose, 25 NaHCO$_3$, 0.11 L-ascorbic acid and 0.004 disodium EDTA; pH 7.4, saturated with 95% O$_2$ / 5% CO$_2$) at 34°C in a metabolic shaker. Slices were then incubated for an additional 30 min in fresh buffer containing 0.1 µM $[^{3}\text{H}]$DA, after rinsing, each slice was transferred to a glass superfusion chamber, maintained at 34 °C and superfused (1.0 mL/min) with oxygenated Krebs’ buffer containing nomifensine (10 µM), a DA reuptake inhibitor), and pargyline (10 µM), a monoamine oxidase inhibitor, to ensure that $[^{3}\text{H}]$overflow represented $[^{3}\text{H}]$DA, rather than $[^{3}\text{H}]$DA metabolites.

Following superfusion for 60 min, three 5-min samples (5 mL/sample) were collected to determine basal $[^{3}\text{H}]$outflow. After collection of the third basal sample, slices from an
individual rat were superfused for 60 min in the absence (0 nM; control) or presence of analog (1 nM-10 µM) to determine the intrinsic activity of the analog, e.g., ability of the analog to evoke \(^{3}\text{H}\)overflow. Each slice was exposed to only one concentration of analog, which remained in the buffer through the completion of the experiment. After a 60 min superfusion in the absence or presence of analog, NIC (10 µM) was added to the superfusion buffer and samples were collected for an additional 60 min to determine the ability of the analog to inhibit NIC-evoked \(^{3}\text{H}\)DA overflow. At least one striatal slice in each experiment was superfused for 60 min in the absence of analog, followed by superfusion with only NIC (10 µM) to determine a NIC control for NIC-evoked \(^{3}\text{H}\)DA overflow in the absence of analog. Using a repeated-measures design, striata from a single rat were used to determine the ability of an analog to evoke \(^{3}\text{H}\)DA release (intrinsic activity) and to determine analog-induced inhibition of NIC-evoked \(^{3}\text{H}\)DA overflow (antagonist activity).

After collection of the superfusate samples, slices were retrieved from the chambers and solubilized using 1 mL TS-2 tissue solubilizer. Scintillation cocktail (10 mL) was added to the superfusate samples. The pH and volume of the solubilized tissue samples were adjusted to those of the superfusate samples. Radioactivity in the superfusate and tissue samples was determined by liquid scintillation spectroscopy (Packard model B1600 TR Scintillation Counter, Downer’s Grove, IL).
Quaternary ammonium analog effects on $^{86}$Rb$^+$ efflux were determined using a previously published method (Marks et al. 1995). Rat thalamus was homogenized and centrifuged at 1000xg for 10 min at $4^\circ$C. The supernatant fraction was centrifuged at 12,000xg for 20 min at $4^\circ$C to obtain the synaptosomal fraction. Synaptosomes were incubated for 30 min in 35 µl of uptake buffer (140 mM NaCl, 1.5 mM KCl, 2.0 mM CaCl$_2$, 1.0 mM MgSO$_4$, 20 mM α-D-glucose; pH 7.5) containing 4 µCi of $^{86}$Rb$^+$. $^{86}$Rb$^+$ uptake was terminated by filtration of the synaptosomes onto glass fiber filters (6 mm; Type A/E, Gelman Sciences, Ann Arbor, MI) under gentle vacuum (0.2 atm), followed by three washes with superfusion buffer (0.5 ml each). Subsequently, each filter containing the $^{86}$Rb$^+$-loaded synaptosomes (39 ± 4.8 µg protein/µl) was placed on a 13 mm glass fiber filter (Type A/E) mounted on a polypropylene platform. $^{86}$Rb$^+$ efflux assay buffer (125 mM NaCl, 5 mM CsCl, 1.5 mM KCl; 2 mM CaCl$_2$, 1 mM MgSO$_4$, 25 mM HEPES, 20 mM α-D-glucose, 0.1 µM TTX, 1.0 g/l bovine serum albumin; pH 7.5) was superfused onto the synaptosomes at a rate of 2.5 ml/min. TTX and CsCl were included in the buffer to block voltage-gated Na$^+$ and K$^+$ channels, respectively, and to reduce the rate of basal $^{86}$Rb$^+$ efflux (Marks et al. 1995).

The intrinsic activity of the quaternary ammonium analog as well as the ability to inhibit $^{86}$Rb$^+$ efflux evoked by 1 µM NIC was determined. After 8 min of superfusion, samples were collected (sample/18 s) for 5 min to determine basal $^{86}$Rb$^+$ efflux. Subsequently, synaptosomes were superfused for 3 min with the quaternary ammonium analog to determine the intrinsic properties of the analog. The exposure to the analog was followed
by superfusion with buffer containing both the quaternary ammonium analog and NIC for an additional 3 min, to determine the ability of the analog to inhibit NIC-evoked $^{86}$Rb$^+$ efflux. Each aliquot part of thalamic synaptosomes was exposed to only one concentration of quaternary ammonium analog. In each experiment, one synaptosomal aliquot part was superfused in the absence of either compound to determine basal $^{86}$Rb$^+$ efflux over the course of the superfusion period, and another aliquot was superfused with NIC (1 µM) to determine the effect of NIC on $^{86}$Rb$^+$ efflux in the absence of the quaternary ammonium analog. Samples were analyzed by liquid scintillation spectroscopy (Packard model B1600 TR Scintillation Counter).

3.2.7. **Data Analysis**

For analog induced inhibition of [$^3$H]NIC and [$^3$H]MLA binding, data were expressed as specific [$^3$H]NIC or [$^3$H]MLA bound as a percentage of control and plotted as a function of log analog concentration. The concentration (IC$_{50}$) of analog that inhibited specific [$^3$H]NIC or [$^3$H]MLA binding by 50% were fit by a one-site binding competition model, using nonlinear least-squares regression, such that $Y = B_t + (T_p - B_t)/[1 + 10^{(\log IC_{50} - X)\cdot n}]$, where $Y$ is the specific [$^3$H]NIC or [$^3$H]MLA binding expressed as a percentage, $X$ is the logarithm of analog concentration, $B_t$ and $T_p$ are the minimum and maximum specific [$^3$H]NIC and [$^3$H]MLA binding densities, respectively, and $n$ is the pseudo-Hill coefficient. The inhibition constant (Ki) for each analog was calculated from IC$_{50}$ values using the Cheng-Prusoff equation [$K_i = IC_{50}/(1 + \text{ligand}/K_d)$], where $c$ is the concentration of free radioligand and $K_d$ is the equilibrium dissociation constant of the ligand.
In [\textsuperscript{3}H]DA overflow assays, fractional release of each superfusate sample was calculated by dividing the total [\textsuperscript{3}H] collected in each sample by the total [\textsuperscript{3}H] present in the tissue at the time of sample collection and was expressed as a percentage of total tissue tritium. Basal [\textsuperscript{3}H]outflow was calculated from the average of the fractional release in three five minute samples collected prior to the addition of analog to the superfusion buffer. Total [\textsuperscript{3}H]overflow was calculated by summing the increases of [\textsuperscript{3}H] above the basal [\textsuperscript{3}H]outflow, resulting from exposure to the analog, NIC, or both and subtracting the basal [\textsuperscript{3}H]outflow for an equivalent period of analog exposure. The data were expressed as percent of control and were fitted by non-linear, non-weighted least-squares regression using the sigmoidal function, \( Y = B_t + (T_p - B_t)/(1 + 10^{(\log IC_{50} - X)}) \), where \( Y \) is the total [\textsuperscript{3}H]overflow expressed as a percentage of NIC-evoked response, \( X \) is the logarithm of analog concentration, \( B_t \) and \( T_p \) are the minimum response (held constant at 0%) and maximum response (held constant at 100%), respectively, and \( \log IC_{50} \) value represents the logarithm of analog concentration required to decrease NIC (10 \( \mu \)M)-evoked [\textsuperscript{3}H]DA overflow by 50% of control levels. The effect of each analog on [\textsuperscript{3}H]DA overflow (intrinsic activity) and its ability to inhibit NIC-evoked [\textsuperscript{3}H]DA overflow (inhibition effect) was analyzed by one-way repeated-measure analysis of variance (ANOVA), where analog concentration was a within subject factor (SPSS version 9.0, Chicago, IL); where appropriate, Dunnett’s \textit{post hoc} tests were performed to determine the concentration of analog that significantly evoked or inhibited [\textsuperscript{3}H]DA overflow relative to the control (buffer or NIC-evoked [\textsuperscript{3}H]DA overflow, respectively). A two-way repeated-measures ANOVA was performed to analyze the time course of the NIC-
induced increase in the fractional release (within-subjects factor) for different concentrations of the analog (within-subject factor). IC_{50} values for analog-induced inhibition of NIC-evoked [{^3}H]DA overflow were determined using an iterative non-linear least squares curve-fitting program, (PRISM version 3.0, Graph PAD Software, Inc., San Diego, CA).

3.3. Results and Discussion

Chemistry

Several N-n-alkyl quaternized ammonium salts were successfully synthesized. mono-Substituted N-n-alkylpyridinium, N-n-alkylpicolinium, and N-n-alkynicotinamide salts were prepared in similar manners, however, their purification work-up varied. All three precipitated from solution upon reaction and were filtered to afford the crude salts. The N-n-alkylpyridinium and N-n-alkylpicolinium salts were triturated with diethyl ether to afford the pure quaternary ammonium salts. The nicotinamide series were isolated differently, due to the low solubility of the nicotinamide starting material in the diethyl ether. These salts were isolated using both an initial diethyl ether wash, followed by an aqueous dissolution and subsequent extraction with chloroform. The N-n-alkynicotininium salts were synthesized in a different manner to ensure that the pyridino nitrogen was selectively alkylated over the pyrrolidino nitrogen. Previous research by Menshutkin demonstrated that tertiary amines react with alkyl halides resulting in quaternary ammonium salt (Figure 3.3a) (Menshutkin 1890). In the nicotine molecule, the pyrrolidine nitrogen is more reactive than the pyridine nitrogen. Therefore, the pyridine nitrogen would be quaternized only after, or at the same time as, the pyrrolidine nitrogen, causing a doubly quaternized species (Figure 3.3b). Due to the reactive nature
of both nitrogens and to avoid the double quaternization, a protection of the pyrrolidino nitrogen was necessary. Using the higher reactivity of the pyrrolidino nitrogen, a simple acid protection step may be utilized to direct the Menshutkin towards the pyridine nitrogen. Acetic acid was used by Crooks et al. (1995) to achieve this selective alkylation of the pyridino nitrogen with excellent results (Figure 3.3c).

Figure 3.3  The Menshutkin reaction and its use in quaternizing nicotine

However, the resulting work-up was modified in the current synthesis to increase the yield and decrease the labor-intensive work-up. The existing synthetic route called for the protonated NIC molecule and the iodoalkane reactant to be stirred for three days at room temperature. The increase in the reaction temperature due to the refluxing increased the yield of the reaction in the present synthesis. The previous synthesis also involved a time-consuming basification and subsequent removal of water at increased
temperature followed by a 24-hour reflux in chloroform to remove the excess NIC. This resulted in a loss of the \( N\)-alkylpicolinium salt due to the salt having some solubility in chloroform. In the current synthesis, after the basification step, the mixture was sequentially treated with diethyl ether and chloroform to remove the excess NIC and the corresponding \( N\)-alkylnicotinium salt, respectively, without evaporating the aqueous layer. This procedure removed the two previous time-consuming steps, and increased the overall yield of the product.

The \( bis-N,N'\)-alkyl quaternary ammonium salts were prepared similarly to their corresponding \( mono\)-alkyl quaternized ammonium salts. \( bis-N,N'\)-Alkylpyridinium salts and \( bis-N,N'\)-alkylpicolinium salts, as well as \( bis-N,N'\)-alkylquinolinium salts and \( bis-N,N'\)-alkylisoquinolinium salts, were isolated in high yields when diethyl ether was utilized to remove the excess reactant. The \( bis-N,N'\)-alkylnicotinium salts were synthesized via a similar synthetic route to that utilized for the corresponding \( mono\)-alkylnicotinium salts, although the final purification step was modified. The diethyl ether extraction step removed the unreacted NIC, but the subsequent chloroform extraction revealed the presence of a product from an unexpected side reaction; a \( mono\)-substituted \( N\)-alkylnicotinium salt with an acetate terminating the carbon chain instead of another NIC molecule was synthesized and had greater solubility than the \( bis-N,N'\)-alkylnicotinium salt in chloroform. During the reaction, it is possible that the acetate ion displaced the iodide or bromide causing the impurity. Alternatively, the \( bis-N,N'\)-alkylnicotinium salt may have formed but over the course of the reaction the a carbon to the quaternized pyridine nitrogen could have been attacked by the acetate ion, resulting in
the mono-substituted $N$-alkynicotinium salt with the terminal acetate moiety (Figure 3.4). The aqueous layer containing the bis-$N,N'$-alkynicotinium salt was evaporated under reduced pressure and chloroform was added to extract the bis-$N,N'$-alkynicotinium salt. The chloroform layer was dried using magnesium sulfate, filtered, and evaporated under reduced pressure to afford the final product.

Figure 3.4 Formation of the acetate impurity

![Formation of the acetate impurity](image)

It is important to note, that despite several different functionalities and charges on the molecules, the mono- and bis-quaternary ammonium salts all demonstrated chloroform solubility.

**Pharmacology of the mono-Substituted Quaternary Ammonium Salts**

*N-n*-Alkylpyridinium salts

*N-n*-Alkylpyridinium salts with chain lengths ranging from one to twenty carbon units weakly inhibited $[^3]H$NIC binding to rat striatal membranes (see Table 3.1 for affinity values). Salts with chain lengths less than 15 carbons completely inhibited $[^3]H$NIC
binding. The C\textsubscript{20} analog NEcPB inhibited binding by only 25%. The binding affinities (Ki) at the a4β2* nAChR subtypes ranged from 9-20 μM for the C\textsubscript{1}-C\textsubscript{11} and C\textsubscript{15} analogs. The C\textsubscript{12} and C\textsubscript{20} chain length salts were not active (Ki > 100). No significant linear relationship was found between compound affinity and carbon chain length. The N-n-alkylpyridinium salts did not inhibit [\textsuperscript{3}H]MLA binding, and thus had no significant affinity at the α7* nAChR subtype (Table 3.1).

Initial screens at two concentrations, i.e. 0.1 and 1.0 μM, of analog, C\textsubscript{1}-C\textsubscript{12}, were determined for the N-n-alkylpyridinium series in the [\textsuperscript{3}H]DA overflow assay. Chain lengths of nine or less carbons were not active; however, carbon chain lengths greater than nine were found to significantly inhibit NIC-evoked [\textsuperscript{3}H]DA overflow by 70-90%. Evaluation of the activities of these compounds, for both intrinsic and inhibitory activity, over the full concentration range (0.01-100 μM) was performed. NUPI (C\textsubscript{11}), NDDPI (C\textsubscript{12}), and NPDPB (C\textsubscript{15}) increased [\textsuperscript{3}H]DA overflow at 10 μM and NDPI (C\textsubscript{10}), NEcPB (C\textsubscript{20}) increase overflow at 100 μM. Concentrations that elicited intrinsic activity were not included in the analysis of inhibition of NIC-evoked DA overflow. Carbon chain lengths greater than nine carbons, i.e. NDPI, NUPI, NDDPI, NPDPB, and NEcPB all inhibited NIC-evoked [\textsuperscript{3}H]DA overflow in the high nanomolar concentration range (IC\textsubscript{50} values = 120 – 490 nM, Table 3.1). For each analog, repeated measures one-way ANOVA revealed significant concentration-dependent inhibition of nicotine-evoked [\textsuperscript{3}H]DA overflow: NDPI (C\textsubscript{10}), \textit{F}_{5,35} = 29.44, p < 0.0001; NUPI (C\textsubscript{11}), \textit{F}_{4,29} = 24.58, p < 0.0001; NDDPI (C\textsubscript{12}), \textit{F}_{3,23} = 11.07, p < 0.001; NPDPB (C\textsubscript{15}), \textit{F}_{5,18} = 24.31, p < 0.001; NEcPB (C\textsubscript{20}), \textit{F}_{5,15} = 9.29, p < 0.001. NDPI, NUPI, and NDDPI all inhibited overflow
completely ($I_{\text{max}} = 100\%$), whereas NPDPB and NEcPB only inhibited overflow by a maximum of 45-55%.

**N-\textit{n}-Alkylpicolinium salts**

\textit{N-\textit{n}}-Alkylpicolinium salts exhibited similar properties to the \textit{N-\textit{n}}-alkylpyridinium salts in their binding affinities at $\alpha4\beta2^*$ and $\alpha7^*$ nAChR subtypes. Analogs with chain lengths of C$_8$-C$_{12}$ had weak or no affinity in the [$^3$H]NIC binding assay, and had no affinity in the [$^3$H]MLA binding assay (Table 3.2). In the NIC-evoked [$^3$H]DA overflow assay, NOPiI (C$_8$) and NDPiI (C$_{10}$) did not exhibit intrinsic activity up to 1.0 $\mu$M, but NDDPiI (C$_{12}$) did evoke overflow at $\geq$ 1.0 $\mu$M. Due to the intrinsic activity, the concentration 1.0 $\mu$M of NDDPiI was not used in determining the inhibitory concentration of the compound. In this series of compounds, NDDPiI was the most potent of the \textit{N-\textit{n}}-alkylpicolinium salts tested at the nAChR subtypes mediating NIC-evoked DA overflow (IC$_{50}$ = 30 nM) and there was an increase of inhibitory activity in this series as the carbon chain increased (Table 3.2). Interestingly, NDDPiI only inhibited overflow by 63%.

**\textit{N-\textit{n}}-alkynicotinamide salts**

\textit{N-\textit{n}}-alkynicotinamide salts were not active in either of the binding assays and were not tested in the NIC-evoked DA release assay (Table 3.3).
Table 3.1 Pharmacological Data Derived from the *mono*-Alkylated Pyridinium Salts

<table>
<thead>
<tr>
<th>Compound</th>
<th>Name</th>
<th>R</th>
<th>[³H]NIC Binding Assay, Ki (µM)</th>
<th>[³H]MLA Binding Assay, Ki (µM)</th>
<th>[³H]DA Release Assay, IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>53a</td>
<td>NMPI</td>
<td>Methyl</td>
<td>20.1 (15.6 – 25.9)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;100</td>
<td>&gt;1.0</td>
</tr>
<tr>
<td>53b</td>
<td>NEPI</td>
<td>Ethyl</td>
<td>28.7 (22.3 – 37.0)</td>
<td>&gt;100</td>
<td>&gt;1.0</td>
</tr>
<tr>
<td>53c</td>
<td>NPrPI</td>
<td>Propyl</td>
<td>42.3 (30.2 – 59.3)</td>
<td>&gt;100</td>
<td>&gt;1.0</td>
</tr>
<tr>
<td>53d</td>
<td>NBuPI</td>
<td>Butyl</td>
<td>9.65 (7.57 – 12.3)</td>
<td>&gt;100</td>
<td>&gt;1.0</td>
</tr>
<tr>
<td>53e</td>
<td>NPePI</td>
<td>Pentyl</td>
<td>8.72 (5.68 – 13.4)</td>
<td>67.7 (2.31 – 1980)</td>
<td>&gt;1.0</td>
</tr>
<tr>
<td>53f</td>
<td>NHxPI</td>
<td>Hexyl</td>
<td>9.07 (6.12 – 13.5)</td>
<td>&gt;100</td>
<td>&gt;1.0</td>
</tr>
<tr>
<td>53g</td>
<td>NHpPI</td>
<td>Heptyl</td>
<td>15.0 (13.0 – 17.2)</td>
<td>&gt;100</td>
<td>&gt;1.0</td>
</tr>
<tr>
<td>53h</td>
<td>NOPI</td>
<td>Octyl</td>
<td>20.1 (16.3 – 24.8)</td>
<td>&gt;100</td>
<td>&gt;1.0</td>
</tr>
<tr>
<td>53i</td>
<td>NNPI</td>
<td>Nonyl</td>
<td>19.0 (19.0 – 22.1)</td>
<td>61.8 (3.35 – 1142)</td>
<td>&gt;1.0</td>
</tr>
</tbody>
</table>
Table 3.1 (continued) Pharmacological Data Derived from the *mono*-Alkylated Pyridinium Salts

<table>
<thead>
<tr>
<th>Compound</th>
<th>Pharmacological Assays</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Assay, Ki (µM)</td>
</tr>
<tr>
<td>X [N^+ ] [R ]</td>
<td>Name</td>
</tr>
<tr>
<td>53j</td>
<td>NDPI</td>
</tr>
<tr>
<td>53k</td>
<td>NUPI</td>
</tr>
<tr>
<td>53l</td>
<td>NDDPI</td>
</tr>
<tr>
<td>53m</td>
<td>NPeDPB</td>
</tr>
<tr>
<td>53n</td>
<td>NEcPB</td>
</tr>
</tbody>
</table>

* Data represent mean (95% confidence intervals)
Table 3.2 Pharmacological Data Derived from the *mono*-Alkylated 3-Picolinium Salts

<table>
<thead>
<tr>
<th>Compound</th>
<th>Pharmacological Assays</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Name</strong></td>
<td><strong>R</strong></td>
</tr>
<tr>
<td>NOPiI</td>
<td>Octyl</td>
</tr>
<tr>
<td>NNPiI</td>
<td>Nonyl</td>
</tr>
<tr>
<td>NDPiI</td>
<td>Decyl</td>
</tr>
<tr>
<td>NUPiI</td>
<td>Undecyl</td>
</tr>
<tr>
<td>NDDPiI</td>
<td>Dodecyl</td>
</tr>
</tbody>
</table>

\(^a\) Data represent mean ± S.E.M \(^b\) From 3 concentrations, \(^c\) ND = Not Determined
Table 3.3 Pharmacological Data Derived from the *mono*-Alkylated Nicotinamide Salts

<table>
<thead>
<tr>
<th>Name</th>
<th>R</th>
<th>[^3H]NIC Binding Assay, Ki (µM)</th>
<th>[^3H]MLA Binding Assay, Ki (µM)</th>
<th>[^3H]DA Release Assay, IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>55a</td>
<td>NONAI</td>
<td>Octyl</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>55b</td>
<td>NNNAI</td>
<td>Nonyl</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>55c</td>
<td>NDNAI</td>
<td>Decyl</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>55d</td>
<td>NUNAI</td>
<td>Undecyl</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>55e</td>
<td>NDDNAI</td>
<td>Dodecyl</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

ᵃ ND = Not Determined
Table 3.4 Pharmacological Data Derived from the *mono*-Alkylated Nicotinium Salts

<table>
<thead>
<tr>
<th>Compound</th>
<th>Pharmacological Assays</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[\textsuperscript{3}H]NIC Binding</td>
</tr>
<tr>
<td></td>
<td>Assay, Ki (\mu M)</td>
</tr>
<tr>
<td>56a</td>
<td>3.53 (2.77 – 4.48)</td>
</tr>
<tr>
<td>56b</td>
<td>1.04 (0.76 – 1.43)</td>
</tr>
<tr>
<td>56c</td>
<td>21.5 (18.2 – 25.3)</td>
</tr>
<tr>
<td>56d</td>
<td>10.9 (9.70 – 12.3)</td>
</tr>
<tr>
<td>56e</td>
<td>ND\textsuperscript{a}</td>
</tr>
<tr>
<td>56f</td>
<td>0.53 (0.46 – 0.60)</td>
</tr>
<tr>
<td>56g</td>
<td>2.06 (1.85 – 2.28)</td>
</tr>
<tr>
<td>56h</td>
<td>19.7 (15.4 – 25.1)</td>
</tr>
<tr>
<td>56i</td>
<td>0.84 (0.73 – 0.95)</td>
</tr>
</tbody>
</table>

\textsuperscript{a}ND: Not determined.
Table 3.4 continued Pharmacological Data Derived from the *mono*-Alkylated Nicotinium Salts

<table>
<thead>
<tr>
<th>Compound</th>
<th>Pharmacological Assays</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[³H]NIC Binding Assay, Ki (µM)</td>
<td>[³H]MLA Binding Assay, Ki (µM)</td>
<td>[³H]DA Release Assay, IC&lt;sub&gt;50&lt;/sub&gt; (µM)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Name</td>
<td>R</td>
<td>0.09 (0.08 – 0.11)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>~100</td>
</tr>
<tr>
<td>56j</td>
<td>NDNI</td>
<td>Decyl</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>≤100</td>
</tr>
<tr>
<td>56k</td>
<td>NUNI</td>
<td>Undecyl</td>
<td>0.14 (0.11 – 0.17)</td>
<td>&gt;100</td>
</tr>
<tr>
<td>56l</td>
<td>NDDNI</td>
<td>Dodecyl</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Data represent mean (95% confidence intervals)  
<sup>b</sup> ND = Not Determined
N-n-Alkylnicotinium salts

N-n-Alkylnicotinium salts demonstrated a wide range of affinities in the [³H]NIC binding assay (90 nM – 20 µM, Table 3.4). The C₁₀ and C₁₂ analogs (NDNI and NDDNI) had the highest affinities, Ki = 90 nm and 140 nm, respectively. A linear relationship between N-n-alkyl chain length and analog affinity was determined, with the exception of the N-n-octyl compound, NONI, which had one of the lowest affinities in the series. The N-n-alkylnicotinium salts all demonstrated low affinity in the [³H]MLA binding assay (Table 3.4).

In the evaluation of the N-n-alkylnicotinium salts to inhibit NIC-evoked [³H]DA overflow, analogs with chain lengths greater than six carbons demonstrated intrinsic activity at concentrations greater than 1 µM. Longer chain compounds were more likely to evoke significant amounts of [³H]overflow. NDNI (C₁₀) had the lowest concentration necessary for evoking [³H]overflow at 1 µM, followed by NDDNI (C₁₂) at 10 µM, and NONI (C₈), NHpNI (C₇), and NHxNI (C₆) at 100 µM. NNNI (C₉) did not evoke [³H]overflow at any concentration tested. For each analog, repeated measures one-way ANOVA revealed significant concentration-dependent inhibition of nicotine-evoked [³H]DA overflow: NMNI (C₁), F₃,₁₅ = 5.02, p < 0.05; NPNI (C₃), F₆,₃₈ = 4.33, p < 0.005; NnBNI (C₄), F₃,₁₉ = 8.83, p < 0.005; NHxNI (C₆), F₅,₂₀ = 3.81, p < 0.05; NHpNI (C₇), F₅,₂₄ = 26.3, p < 0.0001; NONI (C₈), F₅,₂₇ = 10.5, p < 0.0001; NNNI (C₉), F₅,₂₃ = 4.58, p < 0.01; and NDDNI (C₁₂), F₄,₁₈ = 9.00, p < 0.001. Evaluation of the inhibition of NIC-evoked [³H]DA overflow by the N-n-alkylnicotinium salts revealed that NPNI was the least potent of the N-n-alkylnicotinium analogs (IC₅₀ = 37 µM), while NnBNI and
NHxNI also exhibited low micromolar activity (IC\textsubscript{50} = 9 and 3 µM, respectively). NHpNI, NONI, and NNNI had IC\textsubscript{50} values in the range 0.2 - 0.8 µM. NDDNI, the C\textsubscript{12} analog, had the highest inhibitory potency with an IC\textsubscript{50} value of 9 nM (Table 3.4).

**Structure Activity Relationship of the mono-Substituted Quaternary Ammonium Salts**

The affinities of \textit{N}-\textit{n}-alkylnicotinium compounds in the [\textsuperscript{3}H]NIC binding assay revealed that as the carbon chain was increased so was the affinity for the \(\alpha_4\beta_2^*\) receptor. Subsequent evaluation of these compounds in the functional Rb\textsuperscript{86+} efflux assay demonstrated that \textit{N}-\textit{n}-alkylnicotinium compounds with good affinity in the [\textsuperscript{3}H]NIC binding assay, were receptor antagonists at the \(\alpha_4\beta_2^*\) nAChR subtype. Analog with longer \(n\)-alkyl chain lengths of nine, ten, and twelve all had nanomolar affinities for the \(\alpha_4\beta_2^*\) nAChR subtype, with the \(N-n\)-decylnicotinium (NDNI) and \(N-n\)-dodecylnicotinium (NDDNI) having the highest affinities for the receptor with Ki values of 90 nM and 140 nM, respectively. These Ki values are comparable to that of the classical nAChR antagonist DH\textbeta\textsubscript{E}, which has a Ki value of 149 nM at the \(\alpha_4\beta_2^*\) receptor subtype (Wilkins \textit{et al.} 2003). Interestingly, a one-carbon deletion from the nine-carbon chain analog, to afford the \(N-n\)-nonylnicotinium salt (NNNI), results in a twenty-fold decrease in affinity for the \(\alpha_4\beta_2^*\) receptor subtype. Therefore, a subtle change to the length of the carbon chain may cause a large difference in activity. This is further supported by the inactivity of the C\textsubscript{8} compound, \(N-n\)-octylnicotinium, which had low affinity in the [\textsuperscript{3}H]NIC binding assay. The higher affinities in the longer chain compounds may be due to an increase in the lipophilic interaction of the carbon chain and
the hydrophobic regions of the binding site. This interaction may have a stabilizing effect on the compound-receptor complex, increasing the affinity for the C₉-C₁₂ compounds of this series of compounds.

In addition to the carbon chain length, the pyrrolidine ring of this series of compounds also appears to play an important role in the interaction at the binding site. The N-n-alkynicotinium salts with increased numbers of carbon units all demonstrated a greater affinity for the α4β2* binding site over their corresponding N-n-alkylpyridinium, N-n-alkylpicolinium, and N-n-alkylnicotinamide salts. Complete removal of the pyrrolidine ring in the N-n-alkylpyridinium series resulted in a complete loss in affinity for the α4β2* receptor. In the N-n-alkylpicolinium series, the importance of a 3-substituent was explored. However, the low affinity of these salts in the [³H]NIC binding assay indicates that a simple methyl group in the three position of the pyridine ring does not afford compounds with high affinity for the α4β2* binding site. The nicotinamide moiety is a somewhat larger and more polar substituent that incorporates a second nitrogen atom into the receptor-ligand model. Due to the lack of activity of these molecules at the α4β2* binding site, and since the nicotinamide nitrogen is not basic in nature, it can be assumed that it is not necessarily the size of the 3-substituent that promotes interaction with the receptor but rather the incorporation of an appropriate functional moiety. Therefore, a basic nitrogen, such as that in the pyrrolidine ring may be important for recognition by the high affinity [³H]NIC binding site on the α4β2* receptor subtype.
Several of the quaternized ammonium salts were potent antagonists in the NIC-evoked \[^{3}H\]DA overflow assay, with the \(N\)-\(n\)-dodecynicotinium analog (NDDNI) exhibiting the greatest activity at the nAChR subtypes mediating DA release (IC\(_{50}\) = 9 nM); this inhibition is significantly greater than that exhibited by DH\(\beta\)E (IC\(_{50}\) = 1.6 \(\mu\)M). However, the \(N\)-\(n\)-alkylpyridinium and \(N\)-\(n\)-alkylpicolinium salts with similar \(N\)-\(n\)-alkyl chain lengths also antagonized the receptor subtype at higher nanomolar concentrations, with IC\(_{50}\) values of 260 nM (NNDPI) and 30 nM (NDDPiI). The \(N\)-\(n\)-alkylpicolininium and \(N\)-\(n\)-alkylnicotinium salts both demonstrate a linear relationship between antagonist activity and carbon chain length at the nAChR subtypes that mediate DA release. The removal of the pyrrolidine ring did not completely eliminate the ability of these \(N\)-\(n\)-alkylpyridinium salts to interact with this class of nAChR subtypes.

The quaternary ammonium salts demonstrated low activities in the \[^{3}H\]MLA binding assay. The \(N\)-\(n\)-alkylnicotinium molecules only inhibited binding with carbon chain lengths of \(C_6\), \(C_7\), \(C_8\) (Ki = 28.3-92.4 \(\mu\)M). These values are at least ten fold less active at the receptor than NIC, a weak agonist. The \(N\)-\(n\)-alkylpyridinium compounds also did not exhibit affinity for \(\alpha7^*\) nAChRs, with only two compounds, NPePI (\(C_6\)) and NNPI (\(C_9\)), having Ki values below 100 \(\mu\)M (67.7 and 61.8 \(\mu\)M, respectively). Therefore, these compounds are not active at the \(\alpha7^*\) receptor subtype.

**Pharmacology of the bis-Substituted Quaternary Ammonium Salts**

\(N,N'\)-bis-alkylpyridinium salts
**N,N’-bis-Alkylpyridinium salts** with *n*-alkyl chain lengths ranging from six to twelve carbon atoms were evaluated in the [³H]NIC binding assay and had weak affinities for the α4β2* nAChR subtype binding site (Table 3.5). These compounds did show a general trend of increasing affinity as the *n*-alkyl carbon chain length was increased. Compound bPDDDB, had the highest affinity for the α4β2* receptor subtype with a Ki value of 9.15 µM. The *N,N’-bis*-alkylpyridinium salts with chain lengths ranging from C₆-C₁₂ demonstrated low affinity or no affinity with respect to [³H]MLA binding, and thus exhibited no significant interaction with the α7* nAChR subtype (Table 3.5).

The C₁₂ analog, bPDDDB, had no intrinsic activity at any of the concentrations tested (0.001-10 µM) in the [³H]DA overflow assay. bPDDDB inhibited NIC-evoked [³H]DA overflow with an IC₅₀ value of 1.0 µM. No other *N,N’-bis*-alkylpyridinium compounds were evaluated at nAChR subtypes mediating DA release (Table 3.5).

**N,N’-bis-Alkynicotinium salts**

*N,N’-bis*-Alkynicotinium salts were also evaluated in the [³H]NIC binding assay. The C₁₀ (bNDI) and C₁₁ (bNUB) analogs had the highest affinities for the α4β2* receptor subtype, with Ki values of 330 and 430 nM, respectively (Table 3.6). Moreover, bNDI and bNUB inhibited NIC-evoked ⁸⁶Rb⁺ efflux from rat thalamic synaptosomes (IC₅₀ = 3.76 ± 1.1 µM and 5.22 ± 1.4 µM, respectively), without producing intrinsic activity. Several other compounds, i.e. the C₆-C₉ and C₁₂ analogs, had low micromolar affinities (Ki = 1.54 - 20.0 µM). None of the *N,N’-bis*-alkynicotinium salts were active in the [³H]MLA binding assay (Table 3.6).
In addition to the $\alpha$4$\beta$2* and $\alpha$7 receptor subtype assays, the $N,N'$-bis-alkynicotinium $C_{12}$ analog, bNDDB, was evaluated for both intrinsic and inhibitory activity in the $[^3H]$DA overflow assay. bNDDB had intrinsic activity at 10 µM. The C$_{12}$ salt afforded an IC$_{50}$ value of 3.30 µM in the NIC-evoked $[^3H]$DA overflow assay (Table 3.6).

$N,N'$-bis-Alkylpicolinium salts

$N,N'$-bis-Alkylpicolinium salts generally did not exhibit high affinities at the $\alpha$4$\beta$2* nAChR subtype, with Ki values for the C$_9$, C$_{11}$, C$_{12}$ analogs in the high micromolar range (48.6-79.9 µM) and the C$_6$, C$_{10}$ and C$_{11}$ compounds had no affinity at this nAChR subtype (>100 µM). The $N,N'$-bis-alkylpicolinium salts were not active in the $[^3H]$MLA binding assay (Table 3.7).

The C$_{12}$ $N,N'$-bis-alkylpicolinium analog, bPiDDB, was evaluated for its intrinsic and inhibitory behavior in the $[^3H]$DA overflow assay. bPiDDB had no intrinsic activity in $[^3H]$DA overflow assay at any of the concentrations tested (0.001-10 µM). The compound was very potent in inhibiting NIC-evoked $[^3H]$DA overflow. With an IC$_{50}$ value of 2 nM, this compound is one of the most potent compounds in inhibiting NIC-evoked DA release (Table 3.7). Interestingly, bPiDDB inhibited NIC-evoked $[^3H]$DA overflow by a maximum of only 50%.

Due to the high potency of bPiDDB to inhibit NIC-evoked $[^3H]$DA overflow, all the $N,N'$-bis-alkylpicolinium analogs were tested for intrinsic activity and for their ability to
inhibit [3H]DA overflow. Generally, \(N,N'-\text{bis-}\)-alkylpicolinium analogs did not exhibit intrinsic activity in this assay. One-way ANOVAs revealed significant concentration-dependent inhibition for each analog: bPiOI (C\(_8\)), \(F_{5,19} = 4.441, p = 0.007\); bPiNB (C\(_9\)), \(F_{5,19} = 4.053, p = 0.011\); bPiDI (C\(_{10}\)), \(F_{5,23} = 7.346, p < 0.001\); bPiUB (C\(_{11}\)), \(F_{5,28} = 11.750, p < 0.001\); bPiDDB (C\(_{12}\)), \(F_{5,23} = 13.282, p < 0.001\). Contrary to what was observed in the pyridinium series, the shorter chain length compounds, i.e. C\(_8\) and C\(_{10}\), were potent antagonists at the nAChR subtype responsible for mediating DA release. However, the antagonism pattern did not seem to increase as carbon chain length increased, as has been observed in several of the other series. The C\(_8\) (bPiOI) and C\(_{10}\) (bPiDI), analogs had low nanomolar activities (IC\(_{50}\) = 10 and 30 nM, respectively), while the C\(_9\) (bPiNB) and C\(_{11}\) (bPiUB), analogs had low micromolar activities (IC\(_{50}\) = 1.5 - 1.6 \(\mu\)M) and the C\(_6\) analog (bPiHxI), was inactive (Table 3.7). All the active \(N,N'-\text{bis-}\)-alkylpicolinium salts inhibited NIC-evoked [3H]DA overflow similar to bPiDDB, with a maximum of \(-50\%\) (I\(_{\text{max}}\) = 50\%).

\(N,N'-\text{bis-}\)-Alkylisoquinolinium and \(N,N'-\text{bis-}\)-alkylquinolinium salts

\(N,N'-\text{bis-}\)-Alkylisoquinolinium and \(N,N'-\text{bis-}\)-alkylquinolinium salts were evaluated in the [3H]NIC binding assay and generally had low affinities for the \(\alpha 4\beta 2^*\) nAChR (Tables 3.8 and 3.9). The C\(_6\), C\(_8\), C\(_9\), C\(_{11}\), and C\(_{12}\) \(N,N'-\text{bis-}\)-alkylisoquinolinium analogs had low to moderate micromolar affinities (Ki = 6.10-65.5 \(\mu\)M) for the \(\alpha 4\beta 2^*\) nAChR subtype, while the C\(_{10}\) analog had no affinity (Ki > 100 \(\mu\)M). The pharmacological profile of the \(N,N'-\text{bis-}\)-alkylquinolinium salts was similar to that of the \(N,N'-\text{bis-}\)-alkylisoquinolinium salts in the [3H]NIC binding assay, with the C\(_6\), C\(_9\), and C\(_{11}\) analogs exhibiting only
moderate micromolar affinities (Ki = 22.5-39.3 µM) for the α4β2* nAChR subtype, while the C8, C10, and C12 analogs had no affinity (Ki > 100 µM). Interestingly, analogs in both the N,N′-bis-alkylisoquinolinium series and the N,N′-bis-alkylquinolinium series were all active in the [3H]MLA binding assay, with Ki values in the low micromolar range (1.61-16.0 µM, Tables 3.8 and 3.9); the two most potent analogs in these two groups were the C12 analogs, bQDDB and bIQDDB (Ki = 1.61 and 2.21 µM, respectively).
Table 3.5 Pharmacological Data Derived from the *bis*-Alkylpyridinium Salts

<table>
<thead>
<tr>
<th>Compound</th>
<th>Pharmacological Assays</th>
<th>[(^3\text{H})]NIC Binding Assay, Ki (µM)</th>
<th>[(^3\text{H})]MLA Binding Assay, Ki (µM)</th>
<th>[(^3\text{H})]DA Release Assay, IC(_{50}) (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name</td>
<td>n</td>
<td>57(^a)</td>
<td>57(^b)</td>
<td>57(^c)</td>
</tr>
<tr>
<td>bPPeI</td>
<td>5</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;1.0</td>
</tr>
<tr>
<td>bPHxI</td>
<td>6</td>
<td>97.8 ± 17.5(^a)</td>
<td>&gt;100</td>
<td>ND(^b)</td>
</tr>
<tr>
<td>bPOI</td>
<td>8</td>
<td>34.3 ± 2.74</td>
<td>&gt;100</td>
<td>&gt;1.0</td>
</tr>
<tr>
<td>bPNB</td>
<td>9</td>
<td>24.2 ± 1.19</td>
<td>&gt;100</td>
<td>ND</td>
</tr>
<tr>
<td>bPDI</td>
<td>10</td>
<td>18.7 ± 3.09</td>
<td>&gt;100</td>
<td>&gt;1.0</td>
</tr>
<tr>
<td>bPUB</td>
<td>11</td>
<td>14.0 ± 3.21</td>
<td>57.0 ± 4.89</td>
<td>ND</td>
</tr>
<tr>
<td>bPDDDB</td>
<td>12</td>
<td>9.15 ± 0.17</td>
<td>33 ± 4.6</td>
<td>1.0 ± 0.38</td>
</tr>
</tbody>
</table>

\(^a\) Data represent mean ± S.E.M  \(^b\) ND = Not Determined
<table>
<thead>
<tr>
<th>Compound Name</th>
<th>Pharmacological Assays</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[(^3)H]NIC Binding, Ki (µM)</td>
</tr>
<tr>
<td>61a bNHxI 6</td>
<td>20.0 ± 2.84&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>61b bNOI 8</td>
<td>1.54 ± 0.17</td>
</tr>
<tr>
<td>61c bNNB 9</td>
<td>5.03 ± 0.80</td>
</tr>
<tr>
<td>61d bNDI 10</td>
<td>0.33 ± 0.01</td>
</tr>
<tr>
<td>61e bNUB 11</td>
<td>0.43 ± 0.06</td>
</tr>
<tr>
<td>61f bNDDN 12</td>
<td>1.95 ± 0.19</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data represent mean ± S.E.M  
<sup>b</sup> ND = Not Determined
Table 3.7 Pharmacological Data Derived from the *bis*-Alkylpicolinium Salts

<table>
<thead>
<tr>
<th>Compound</th>
<th>Name</th>
<th>n</th>
<th>[³H]NIC Binding Assay, Ki (µM)</th>
<th>[³H]MLA Binding Assay, Ki (µM)</th>
<th>[³H]DA Release Assay, IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X⁺CH₃NH₃⁺</td>
<td>bPiHxI</td>
<td>6</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>2X⁺CH₃NH₃⁺</td>
<td>bPiOI</td>
<td>8</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>0.30 ± 0.18</td>
</tr>
<tr>
<td>2X⁺CH₃NH₃⁺</td>
<td>bPiNB</td>
<td>9</td>
<td>79.9 ± 16.6ᵃ</td>
<td>&gt;100</td>
<td>5.81 ± 5.07</td>
</tr>
<tr>
<td>2X⁺CH₃NH₃⁺</td>
<td>bPiDI</td>
<td>10</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>0.18 ± 0.11</td>
</tr>
<tr>
<td>2X⁺CH₃NH₃⁺</td>
<td>bPiUB</td>
<td>11</td>
<td>69.2 ± 28.9</td>
<td>&gt;100</td>
<td>1.12 ± 0.65</td>
</tr>
<tr>
<td>2X⁺CH₃NH₃⁺</td>
<td>bPiDDB</td>
<td>12</td>
<td>48.6 ± 17.2</td>
<td>&gt;100</td>
<td>0.002 ± 0.001</td>
</tr>
</tbody>
</table>

ᵃ Data represent mean ± S.E.M
Table 3.8 Pharmacological Data Derived from the *bis*-Alkylisoquinolinium Salts

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>Pharmacological Assays</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[(^3)H]NIC Binding Assay, Ki (µM)</td>
</tr>
<tr>
<td></td>
<td>(17.9 \pm 3.59^a)</td>
</tr>
<tr>
<td>60a</td>
<td>(65.5 \pm 14.5)</td>
</tr>
<tr>
<td>60b</td>
<td>(7.89 \pm 0.92)</td>
</tr>
<tr>
<td>60c</td>
<td>(&gt;100)</td>
</tr>
<tr>
<td>60d</td>
<td>(9.02 \pm 1.56)</td>
</tr>
<tr>
<td>60e</td>
<td>(6.10 \pm 0.73)</td>
</tr>
<tr>
<td>60f</td>
<td>(bIQDDB)</td>
</tr>
</tbody>
</table>

\(^a\) Data represent mean ± S.E.M
Table 3.9 Pharmacological Data Derived from the \textit{bis}-Alkylquinolinium Salts

<table>
<thead>
<tr>
<th>Compound</th>
<th>Name</th>
<th>n</th>
<th>[(^3)H]NIC Binding Assay, Ki (µM)</th>
<th>[(^3)H]MLA Binding Assay, Ki (µM)</th>
<th>[(^3)H]DA Release Assay, IC\textsubscript{50} (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>59a</td>
<td>bQHxI</td>
<td>Hexyl</td>
<td>39.3 ± 5.59\textsuperscript{a}</td>
<td>13.3 ± 2.12</td>
<td>0.50 ± 0.46</td>
</tr>
<tr>
<td>59b</td>
<td>bQOI</td>
<td>Octyl</td>
<td>&gt;100</td>
<td>2.39 ± 0.69</td>
<td>0.06 ± 0.03</td>
</tr>
<tr>
<td>59c</td>
<td>bQNB</td>
<td>Nonyl</td>
<td>22.5 ± 1.30</td>
<td>3.58 ± 0.45</td>
<td>0.24 ± 0.17</td>
</tr>
<tr>
<td>59d</td>
<td>bQDI</td>
<td>Decyl</td>
<td>&gt;100</td>
<td>7.81 ± 1.90</td>
<td>&gt;1</td>
</tr>
<tr>
<td>59e</td>
<td>bQUB</td>
<td>Undecyl</td>
<td>37.8 ± 8.65</td>
<td>2.58 ± 0.46</td>
<td>0.21 ± 0.18</td>
</tr>
<tr>
<td>59f</td>
<td>bQDDB</td>
<td>Dodecyl</td>
<td>&gt;100</td>
<td>1.61 ± 0.25</td>
<td>0.02 ± 0.008</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Data represent mean ± S.E.M
The compounds in the $N,N'$-bis-alkylosoquinolinium and $N,N'$-bis-alkylquinolinium series were evaluated for both intrinsic activity and their ability to inhibit NIC-evoked $[^3H]$DA overflow. The C$_8$ and C$_{12}$ $N,N'$-bis-alkylquinolinium salts, bQOI and bQDDB, and the C$_{10}$ $N,N'$-bis-alkylosoquinolinium salt, bIQDDB, elicited $[^3H]$DA overflow at 10 µM. The other $N,N'$-bis-alkylquinolinium and $N,N'$-bis-alkylosoquinolinium salts, did not exhibit intrinsic activity at any concentration tested $N,N'$-bis-alkylquinolinium. One-way ANOVA revealed significant concentration-dependent inhibition for bQHxI (C$_6$), $F_{5,24} = 5.055$, $p = 0.002$; bIQOI (C$_8$), $F_{4,14} = 6.428$, $p = 0.003$; bQNB (C$_9$), $F_{5,19} = 4.872$, $p = 0.004$; bQDI (C$_{10}$), $F_{4,14} = 3.294$, $p = 0.033$; bQUB (C$_{11}$), $F_{5,19} = 7.873$, $p < 0.001$; bQDDB (C$_{12}$), $F_{5,24} = 9.16$, $p < 0.001$; bIQOI (C$_8$), $F_{5,20} = 4.141$, $p = 0.01$; bIQDI (C$_{10}$), $F_{4,16} = 4.835$, $p < 0.001$; bIQUB (C$_{11}$), $F_{5,20} = 4.157$, $p < 0.001$; bIQDDB (C$_{12}$), $F_{5,22} = 3.827$, $p < 0.05$. $N,N'$-bis-alkylosoquinolinium salts exhibited antagonist activity at nAChR subtypes that mediate DA release ($IC_{50} = 20-90$ nM). In addition, the analogs only partially inhibited NIC-evoked $[^3H]$DA overflow ($I_{\text{max}} = 62-78\%$).

$F_{5,19} = 4.441$, $p = 0.007$; bPiNB (C$_9$), $F_{5,19} = 4.053$, $p = 0.011$; bPiDI (C$_{10}$), $F_{5,23} = 7.346$, $p < 0.001$; bPiUB (C$_{11}$), $F_{5,28} = 11.750$, $p < 0.001$, bPiDDB (C$_{12}$).

**Structure Activity Relationship of the bis-Substituted Quaternary Ammonium Salts**

At muscle-type peripheral receptors, the compounds with only a trimethylammonium headgroup, were found to require an optimum chain length of ten carbon units (DEC). Although DEC had three orders of magnitude lower affinity for the $\alpha4\beta2^*$ subtype compared to NIC ($Ki = 5.73 \pm 0.76$ µM versus $0.0014 \pm 0.0001$ µM) for the $\alpha4\beta2^*$ subtype and had no affinity for the $\alpha7^*$ subtype, it is apparent that replacing the $N,N'$-
trimethylammonium moieties of DEC with azaaromatic moieties, such as nicotinium moieties (α4β2*) and quinolinium and isoquinolinium moieties (α7*), affords molecules with good affinity for nAChR subtypes.

Of particular interest is the finding that two compounds in this study exhibited high selectivity at the α4β2* nAChR subtype. In this respect, the N,N’-bis-alkylnicotinium analogs, bNDI and bNUB exhibit low nanomolar affinities (330 and 430 nM, respectively) for the α4β2* nAChR subtype, and functionally inhibited NIC-evoked 86Rb+ efflux, while demonstrating no affinity for the α7* nAChR subtype, these data indicate that these two analogs are selective α4β2* nAChR subtype antagonists. As seen in the mono-quaternary ammonium series, the importance of the pyrrolidino moiety to achieve activity at the α4β2* subtype is also seen in the bis-quaternary ammonium series. The N,N’-bis-alkylnicotinium series, when compared to the N,N’-bis-alkylpyridinium and N,N’-bis-alkylpicolinium series, demonstrates a two-fold decrease in affinity in the [3H]NIC binding assay for the most potent compounds. Additionally, the N,N’-bis-alkylisoquinolinium and N,N’-bis-alkylquinolinium series had low affinity for the α4β2* subtype, indicating that increased aromaticity, i.e. 6π system to 10π system, is not necessarily an important requirement for binding.

In contrast, replacing the bis-nicotinium headgroups with bis-quinolinium and bis-isoquinolinium moieties afforded compounds with no affinity for the α4β2* nAChR, but which had similar affinity to NIC and d-TBC (Ki values = 0.45 ± 0.09 µM and 4.79 ± 0.20 µM, respectively) at the α7* nAChR subtype. Although no correlation between
carbon chain length and affinity was observed in either series, the C_{12} analogs in the \(N,N'-\text{bis-alkylisoquinolinium}\) (bIQDDB) and \(N,N'-\text{bis-alkylquinolininium}\) (bQDDB) series had the highest affinities, and when the \textit{bis-quinolinium} and \textit{bis-isoquinolininium} headgroups were replaced with simple \textit{bis-pyridinium} moieties, only the longer chain molecules were active (C_{11} and C_{12} analogs). The presence of a 3-position methyl substituent in the pyridinium ring in the \(N,N'-\text{bis-alkylpicolinium}\) series appears to decrease the affinity for the \(\alpha7^*\) nAChR subtype, as is seen in the loss of \(\alpha7^*\) affinity in the \textit{bis-nicotinium} and \textit{bis-picolinium} series. The \textit{bis-quinolinium} analog, bQDDB exhibited a Ki value of 1.61 \(\mu\text{M}\) for the \(\alpha7^*\) nAChR subtype and this compound had no affinity for the \(\alpha4\beta2^*\) nAChR subtype. Therefore, it appears that increased aromaticity is an important structural requirements for the \(\alpha7^*\) nAChR subtype and not for the \(\alpha4\beta2^*\) nAChR subtype. The \(N,N'-\text{bis-alkylisoquinolinium}\) and \(N,N'-\text{bis-alkylquinolininium}\) series is the first active compound in both the \textit{mono-} and \textit{bis-azaaromatic} series for the \(\alpha7^*\) nAChR subtype.

In initial experiments, the C_{12} analogs of the \textit{bis-} azaaromatic series were evaluated in the NIC-evoked [\(^3\text{H}\)]DA overflow, because the C_{12} \textit{N-n-alkylnicotinium} analog, NDDNI, was the most potent antagonist at the nAChR subtypes mediating DA release. In all of the \textit{bis-azaaromatic} series, the C_{12} analogs were able to inhibit NIC-evoked [\(^3\text{H}\)]DA overflow at low micromolar or low nanomolar concentrations. The most potent antagonist in the series was the C_{12} analog in the \(N,N'-\text{bis-alkylpicolinium}\) series. Taken together with the data from the \(\alpha4\beta2^*\) and \(\alpha7^*\) assays, this compound is highly selective as an antagonist at the nAChR subtype mediating DA release. Four to five orders of
magnitude separate the affinity for the nAChR subtypes mediating DA release with those for the α4β2* and α7* nAChR subtypes. Additionally, other analogs in the N,N’-bis-alkylpicolinium series of compounds were evaluated and all were found to have inhibitory activity in the NIC-evoked [3H]DA overflow assay, although none were as potent as bPiDDB. The simple C₈ and C₁₀ analogs N,N’-bis-alkylpyridinium series were also tested to determine their activity and surprisingly, none of the N,N’-bis-alkylpyridinium analogs had any activity at concentrations below 1 μM. This represents a 10-fold decrease in potency for the corresponding C₈ and C₁₀ N,N’-bis-alkylpicolinium analogs and a 1000-fold decrease for the C₁₂ analogs. Surprisingly, the C₁₂ N,N’-bis-alkynicotinium analog was not as active as bPiDDB, indicating that the bulky pyrrolidino moiety maybe hindering binding at the nAChR subtypes mediating DA release and therefore decreasing the activity. The increased aromaticity of the N,N’-bis-alkylisoquinolinium and N,N’-bis-alkylquinolinium series also led to an increase in antagonist activity at nAChR subtypes mediating NIC-evoked DA release but none of the analogs in these series of compounds approached the inhibitory activity of bPiDDB. Thus, the 3-methyl substituent on the picolinium ring is important for determining NIC-evoked [3H]DA overflow at these receptor subtypes.

As stated previously, there is currently considerable debate as to the exact subunit composition of nAChR subtypes mediating NIC-evoked DA release (Klink et al. 2001; Azam et al. 2002; Champtiaux et al. 2002; Whiteaker et al. 2002; Zoli et al. 2002). The discovery and use of the α-conotoxins (α-ctx), particularly the α-ctx MII, has greatly increased our understanding of this receptor class. Two receptor subtypes, α-ctx MII
sensitive and α-ctx MII resistant, have been determined as the nAChR subtypes responsible for NIC-evoked DA release. Using knockout mice data coupled with the α-ctx MII data, Luetje (2004) has identified four distinct DA releasing subtypes, α-ctx MII sensitive subtypes, α6β2β3 and α6α4β2β3, where α6 and α4 form with the β2 subunit to form a functional receptor; and α-ctx MII resistant subtypes consist of α4β2 and α4α5β2 with the α4 and β2 subunits forming the agonist binding sites (Luetje 2004). Interestingly, bPiDDB only partially blocks the effects of NIC-evoked [3H]DA overflow (I_{max}~50%), indicating that it is only antagonizing select subtypes that mediate DA release, similar to α-ctx MII. The observed low affinity of bPiDDB for the α4β2* nAChR subtype, in addition to the partial antagonism activity demonstrated by bPiDDB in the NIC-evoked [3H]DA overflow assay, indicates that bPiDDB most likely antagonizes the α-ctx MII sensitive subtypes, α6β2β3 and α6α4β2β3 and not the ctx MII resistant subtypes, α4β2 and α4α5β2.

**Binding Model of Quaternized Azaaromatic Compounds at the nAChR subtypes mediating DA release**

In order to propose a binding model for these quaternary ammonium compounds, it is important to identify if these antagonists are competing for the same receptor binding site. It was determined that the C₈ N-n-alkynicotinium salt, NONI, was a competitive antagonist in the NIC-evoked [3H]DA overflow assay (Wilkins *et al.* 2002). Crooks *et al.* (1995) reported that N-n-alkylation of the pyridyl nitrogen of NIC resulted in a change in the physical properties of both the pyridyl nitrogen atom as well as the pyrrolidine nitrogen atom of the molecule. The pyridyl alkylation caused the pKa of the pyrrolidine
nitrogen to fall 2 pH units, resulting in it being mostly unprotonated at physiological pH. Therefore, a nAChR binding model was hypothesized for the nAChR subtypes mediating DA release, wherein the nAChR recognition points of the quaternized molecule were reversed from those in the protonated NIC molecule, resulting in a novel interaction of such molecules at the nAChR binding site (Crooks et al. 1995). In this model, the cationic binding area of the nAChR receptor recognizes the quaternary ammonium moiety of the N-n-alkynicotinium compound, while the hydrogen-bonding site accommodates the unprotonated pyrrolidine nitrogen (lone pair) (Figure 3.5).

Figure 3.5 Illustration of the different modes of interaction of protonated NIC and NDDNI at the nAChR

Many compounds belonging to the mono- and bis-azaaromatic series were active in the NIC-evoked [³H]DA release assay. Therefore, a flexible model should be examined for the nAChR subtypes mediating DA release. Because of the fact that the quaternized pyridinium moiety is a constant structural feature in many compounds of the mono-azaaromatic series, there must still be a cationic binding site that interacts with these types of molecules. This interaction is not sufficient to inhibit release, as evidenced by
the lack inhibition exhibited by the smaller chain \( N-n \)-alkyl analogs; therefore, the carbon chain length is also an important structural feature. The length of the carbon chain in active molecules is found to be variable, depending on the substituent in the three position of the pyridyl moiety. It is hypothesized that the \( N-n \)-alkylnicotinium molecules fit into the receptor binding site at a unique angle, which allows the pyrrolidino moiety to interact with a nearby H-bonding site, and an \( n \)-alkyl moiety with critical carbon chain length is necessary to complete the strong interaction of the molecule with the DA-releasing nAChR at the \( N-n \)-alkyl binding site. The \( n \)-alkyl chain would likely interact with residues adjacent to the binding site itself that are of a hydrophobic nature. This hydrophobic interaction may also explain the observed decrease in activity seen with \( C_{10} \) \( N-n \)-alkylnicotinium salt, NDNI. In this case, the ten-carbon alkyl chain may not fit the binding area due to a negative interaction of the chain with the receptor protein, leading to a decrease in affinity. The \( C_{12} \) \( n \)-alkyl analog was a potent antagonist in the NIC-evoked DA release assay. This may be due to an interaction that overcomes the disruption that NDNI encounters. The longer alkyl chain may be able to bend to a conformation that minimizes the interaction or that moves around the amino acid causing the issue.

The recognition site also allows the \( N-n \)-alkylpyridinium and \( N-n \)-alkylpicolinium molecules to interact with the binding site. However, the \( N-n \)-alkylpyridinium and \( N-n \)-alkylpicolinium molecules still have a decreased affinity when compared to the \( N-n \)-alkylnicotinium series. This points to the importance of the absence of the pyrrolidine ring, decreasing the strength of the interaction with the binding site. Without the
pyrrolidine ring, these series of compounds may lack the ability to seat into the cationic binding site as strongly as the \( N-n \)-alkylnicotinium molecules. Therefore, they require longer chain lengths to create a more favorable ionic interaction at the cationic site while maintaining the ability to interact at the \( N-n \)-alkyl binding site. However, the effect of the increase in carbon chain length does not fully compensate for the loss of the pyrrolidine ring, as the \( N-n \)-alkylpyridinium and \( N-n \)-alkylpicolinium series demonstrated at least 10 fold decreased affinities (0.12 and 0.03 µM, respectively) when compared to the \( N-n \)-alkylnicotinium series (0.009 µM). Taken overall, these data underline the importance of the \( N-n \)-alkyl binding site, the H-binding site, and the cationic binding site in the binding of this series of compounds.

The activity of compounds in the \( \text{bis-azaaromatic} \) series provides additional information on the interaction of these quaternary ammonium compounds with nAChR subtypes mediating DA release. Due to the presence of the second quaternized headgroup, the carbon chain length and the quaternary ammonium center are not the only factors in determining interaction between the molecule and the receptor. The inclusion of a second quaternary ammonium headgroup increases antagonist activity at the nAChR subtypes mediating DA release for the \( N,N'\text{-bis} \)-alkylpicolinium series. This increased activity is likely due to interaction of these molecules with a second quaternary ammonium binding area remote from the first quaternary ammonium binding site. Due to the poor activity of the \( C_{12} \) \( N,N'\text{-bis} \)-alkylnicotinium analog in the DA release assay, the second quaternary ammonium binding site may be different than the first quaternary ammonium binding site. It is possible that site has a smaller area that cannot
accommodate the entire nicotine molecule, but a smaller 3-position substituent may be fit into the second binding site (Figure 3.6C).
Even though the exact sub-unit composition is not known, the heterologously assembled DA-releasing nAChR subtype binding sites utilize an α-β interface (Kuo et al. 2005). The main binding pocket of the mostly resides in the α subunit of the receptor site (Karlin 2002; Unwin 2005). Recently, it was determined that the β subunits may play a dominant role in receptor inactivation (Fenster et al. 1997; Gentry et al. 2003). Therefore, the β-subunit must be integral to the strong inhibitory activity of this series of compounds at the nAChR subtypes mediating DA release. If the second binding pocket, as well as, some of the hydrophobic residues that interact with the carbon chain of quaternized molecule, resides on the β-subunit interface, the interaction of the molecule
could prevent a conformational shift of the binding pocket and not allow the ionic pore to open, inactivating the receptor.

### 3.4. Conclusions

Several interesting *mono-* and *bis-*azaaromatic quaternary ammonium compounds have emerged from this structure-activity study. bPiDDB was found to be a potent and selective antagonist at the nAChRs mediating DA release, indicating it to be a possible $\alpha_6\beta_2\beta_3$ or $\alpha_6\alpha_4\beta_2\beta_3$ nAChR subtype antagonist. The $C_{10}$ *mono-* and *bis-*nicotinium analogs, NDNI and bNDI, both had good antagonist activity and selectivity for $\alpha_4\beta_2^*$ nAChR subtypes. Additionally, bQDDB and bIQDDB have been shown to be the first members of these series of quaternary ammonium molecules that have affinity for $\alpha_7^*$ nAChR subtypes. The selectivity of these series of compounds for nAChR subtypes demonstrates that these agents may be useful for both basic and clinical research aimed at determining the pharmacological role of nAChR subtypes and their potential use as therapeutic agents.
4.1. Introduction

Over the past decade, the nAChR has become a target for drug development. The most prevalent nAChR subtype in the mammalian brain is the heterologously expressed α4β2* receptor subtype, which is the primary high affinity [3H]nicotine binding site. Ligands that specifically interact with this nAChR subtype are of interest in a number of pathologies, including tobacco dependence and drug addiction, Alzheimer’s disease, Parkinson's disease, Tourette’s syndrome, schizophrenia, and pain (Lindstrom 1997; Dwoskin et al. 2000; Lloyd and Williams 2000; Dwoskin and Crooks 2001; Dani et al. 2004; Hogg and Bertrand 2004). NIC and other nAChR agonists (e.g., epibatidine) are not selective for α4β2* receptor subtypes, and produce significant side effects, making these compounds less attractive as drug candidates. Consequently, previous studies have generated quantitative structure-activity relationships (QSAR) in an attempt to understand the interactions of these agonists with their recognition sites on nAChRs (Barlow and McLeod 1969; Barlow and Johnson 1989; Dukat et al. 1996; Nielsen et al. 2000; Nicolotti et al. 2001).

To date, several of these QSAR studies have utilized linear regression analysis of ligand-receptor binding or function, in order to elucidate the nature of the ligand interactions within several nAChR receptor subtypes (Barlow and McLeod 1969; Dukat et al. 1996; Nielsen et al. 2000). Of note, QSAR studies have been used to investigate ligand interactions at the α4β2* nAChR subtype (Dukat et al. 1996; Tonder and Olesen 2001;
Tonder et al. 2001). Though generalities for the ligand interactions were drawn from the SARs generated, insufficient model validation limited the ability of the previously used models to recognize the training set of compounds. As such, generation of a nAChR pharmacophore has proven difficult. In this respect the earlier pharmacophore model generated by Sheridan et al. (1986), despite being based on crude pharmacological data, still constitutes an approach worthy of further exploration (Sheridan et al. 1986; Schmitt 2000; Glennon et al. 2004).

Another model surmises that a water molecule may mediate binding of a ligand to the receptor, perhaps through an interaction with the protonated nitrogen atom of the ligand or the hydrogen bonding acceptor moiety in the ligand molecule (Glennon et al. 2004). The crystallization of an acetylcholine binding protein (AChBP) from snail glia has also provided insight into the binding domain of the nAChR. Brejc et al. (2001) have determined that the loop C (Changeux’s nomenclature) of the acetylcholine binding site was crystallographically less well resolved than the rest of the binding site (Brejc et al. 2001), leading to the possibility that part of the binding site may take on different conformations, depending on ligand shape and size. This represents a plausible challenge to the notion of a static pharmacophore. Due to the complexity of the nAChR molecular recognition process and the difficulty in obtaining high-resolution (e.g. microscopic kinetics) data for specific receptor subtypes, unambiguous models defining specific nAChR subtype pharmacophores do not yet exist.
It has been established previously that QSAR descriptor data has non-linear characteristics (Zupan and Gasteiger 1993; Rogers and Hopfinger 1994; Kohonen 2001). Therefore, alternative approaches such as self-organizing maps (SOMs) and the genetic functional approximation (GFA) algorithm have been utilized to generate QSAR for several biological systems (Gokhale and Kulkarni 2000; So et al. 2000; Yuan and Parrill 2002; Schneider and Nettekoven 2003; von Korff and Steger 2004), and may be better suited for analysis of ligand interactions with \( \alpha 4\beta 2^* \) nACHRs. Regression analysis typically employed in QSAR, such as Partial Least Squares (PLS) or Multiple Least Squares (MLS) are limited to relating linear descriptor trends to a given activity or property. Considering the complexity in the modeling of the mono- and bis-quaternary ammonium salt interactions with nACHRs described in this study, we elected to use SOMs and GFAs in the QSAR analysis described below as one valid modeling approach.

4.2. Experimental

4.2.1. Biological Data

Biological data were obtained from the \([^3]H\)NIC binding assay using rat striatal membranes, a well established assay reported to identify the \( \alpha 4\beta 2^* \) subtype (Xu et al. 2001; Ayers et al. 2002; Grinevich et al. 2003; Wilkins et al. 2003). The data set consisted of ninety-three mono- and bis-substituted quaternary ammonium compounds together with other related compounds developed in the Crooks laboratory, many of which are described in earlier section of this dissertation. The chemical structures are presented in Figure 1. Activities (e.g. \( \alpha 4\beta 2^* \) receptor affinities) are expressed as experimentally determined Ki values as explained in Chapter three.
4.2.2. **Descriptor Generation**

Descriptors were generated using the chemical modeling program Cerius2 (Accelrys, Inc. San Diego CA) and QSARIS (MDL Information Systems Inc., San Liandro CA). Descriptors were divided into one of several physical property categories: conformational, electronic, information, molecular shape analysis (MSA), quantum mechanical, receptor, spatial, structural, thermodynamic, and topological. The descriptors were used for the generation of both the SOMs and GFAs.

4.2.3. **Self-Organizing Maps with Descriptor Selection**

In order to relate bioactivity and molecular features, a set of computational models were developed using a custom-built stepwise descriptor selection algorithm coupled to a supervised SOM (Bayram et al. 2004). The algorithm was developed using Matlab 6.1 with the SOM Toolbox 2.0 (http://www.cis.hut.fi/projects/somtoolbox/). Table 4.1 contains a description of the descriptors employed in the various models.
Table 4.1 Descriptors utilized in the generation of SOM and GFA models

<table>
<thead>
<tr>
<th>Descriptors</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area</td>
<td>Molecular area</td>
</tr>
<tr>
<td>CIC</td>
<td>Complementary informational content</td>
</tr>
<tr>
<td>E-DIST-mag</td>
<td>Entropy of the matrix</td>
</tr>
<tr>
<td>Fh2o</td>
<td>Desolvation free energy for water</td>
</tr>
<tr>
<td>Foct</td>
<td>Desolvation free energy for octanol</td>
</tr>
<tr>
<td>IC</td>
<td>Informational content</td>
</tr>
<tr>
<td>Jurs-FNSA-3</td>
<td>Fractional negatively charged partial surface areas</td>
</tr>
<tr>
<td>Jurs-PNSA-1</td>
<td>Partial negative surface area</td>
</tr>
<tr>
<td>Jurs-RASA</td>
<td>Relative hydrophobic surface area</td>
</tr>
<tr>
<td>Jurs-RPCS</td>
<td>Relative positive charge surface area</td>
</tr>
<tr>
<td>Jurs-WNSA-2</td>
<td>Surface weighted, partial negative charge</td>
</tr>
<tr>
<td>Jurs-WNSA-3</td>
<td>Partial weighting of the surface accessible portions of the molecule</td>
</tr>
<tr>
<td>Jurs-WPSA-1</td>
<td>Surface-weighted charged partial surface areas</td>
</tr>
<tr>
<td>LogP</td>
<td>Octanol/water partition coefficient</td>
</tr>
<tr>
<td>Log Z</td>
<td>Logarithm of the Hosoya index</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>PHI</td>
<td>Kier flexibility index</td>
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<tr>
<td>S_aaCH</td>
<td>Energy states of aromatic CH bonds</td>
</tr>
<tr>
<td>S_aasC</td>
<td>Energy states of the C bonds</td>
</tr>
<tr>
<td>S_dCH2</td>
<td>Energy states of all CH₂ groups</td>
</tr>
<tr>
<td>S_dO</td>
<td>Energy states of all carbonyl moieties</td>
</tr>
<tr>
<td>S_sCH3</td>
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<td>S_ssCH2</td>
<td>Energy states of the CH₂ bonds</td>
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<tr>
<td>S_sssNH</td>
<td>Energy states of all NH bonds</td>
</tr>
<tr>
<td>S_tCH</td>
<td>Energy states of total CH bonds</td>
</tr>
<tr>
<td>SC-3_P</td>
<td>Molecular connectivity index of longer chain lengths</td>
</tr>
<tr>
<td>SIC</td>
<td>Structural informational content</td>
</tr>
<tr>
<td>Vm</td>
<td>Molecular volume</td>
</tr>
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</table>

Datasets

Three data sets were created: (1) both mono- and bis-salts (92 molecules; the ‘ALL’ data set); (2) mono-quaternary ammonium salts (69 molecules; the ‘MONO’ data set); and (3) bis-quaternary ammonium salts (23 molecules; the ‘BIS’ data set). A binning scheme was created to convert the numerical bioactivity into categorical data since our implementation of SOMs works on categorical data. The scheme assigned roughly the same number of molecules to each of three bins.
Figure 4.1 N-Alkyl quaternary ammonium salts and their affinities (Ki) at $\alpha_4\beta_2^*$ nAChRs used in SOM and GFA studies

**N-Alkylpyridinium Salts**

![Diagram of N-Alkylpyridinium Salts]

<table>
<thead>
<tr>
<th>R</th>
<th>Ki</th>
<th>R</th>
<th>Ki</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl</td>
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<td>Allyl</td>
<td>56.0</td>
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<tr>
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<tr>
<td>Propyl</td>
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<td>Butyl</td>
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<tr>
<td>Pentyl</td>
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<td>Oct-7-ynyl</td>
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<tr>
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<td>12.3</td>
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<tr>
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**N-Alkylpicolinium Salts**

![Diagram of N-Alkylpicolinium Salts]

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<th>R</th>
<th>Ki</th>
<th>R</th>
<th>Ki</th>
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</thead>
<tbody>
<tr>
<td>Octyl</td>
<td>&gt;100</td>
<td>Octyl</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Dodecyl</td>
<td>&gt;100</td>
<td>Nonyl</td>
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</tr>
<tr>
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</tr>
<tr>
<td>Undecyl</td>
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<td>Undecyl</td>
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**N-Alkynicotinamide Salts**

![Diagram of N-Alkynicotinamide Salts]

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<th>R</th>
<th>Ki</th>
<th>R</th>
<th>Ki</th>
</tr>
</thead>
<tbody>
<tr>
<td>Octyl</td>
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<td>Octyl</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Nonyl</td>
<td>&gt;100</td>
<td>Nonyl</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Decyl</td>
<td>&gt;100</td>
<td>Decyl</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Undecyl</td>
<td>&gt;100</td>
<td>Undecyl</td>
<td>&gt;100</td>
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</table>

140
**N-Alkylnicotinium Salts**

![Structure](image)

<table>
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<tr>
<th>R</th>
<th>Ki</th>
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<tr>
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<table>
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<td>Octyl-cyclopentyl</td>
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**Conformationally Restricted N-Alkylnicotinium Salts**

![Structure](image)

<table>
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<tr>
<th>R</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Octyl</td>
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</tr>
<tr>
<td>Nonyl</td>
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</tr>
<tr>
<td>Decyl</td>
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</tr>
<tr>
<td>Undecyl</td>
<td>100</td>
</tr>
<tr>
<td>Dodecyl</td>
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</table>

<table>
<thead>
<tr>
<th>R</th>
<th>Ki</th>
</tr>
</thead>
<tbody>
<tr>
<td>Octyl</td>
<td>100</td>
</tr>
<tr>
<td>Nonyl</td>
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</tr>
<tr>
<td>Decyl</td>
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<tr>
<td>Undecyl</td>
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</tr>
<tr>
<td>Dodecyl</td>
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</tr>
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</table>
**bis-Quaternary Ammonium Salts**

![Structure 1](image1.png)

**45** HEX  \(K_i = 31.9\)

![Structure 2](image2.png)

**63** DEC  \(K_i = 4.27\)

**bis-N,N'-Alkylpyridinium Salts**

![Structure 3](image3.png)

**bis-N,N'-Alkylpicolinium Salts**

![Structure 4](image4.png)

**bis-N,N'-Alkylanilidinium Salts**

![Structure 5](image5.png)

**bis-N,N'-Alkynicotinium Salts**

![Structure 6](image6.png)

**56a** bPPeI  \(n = 1; X = I\) \(K_i > 100\)

**56c** bPOI  \(n = 4; X = I\) 33.9

**56d** bPNB  \(n = 5; X = Br\) 24.1

**56e** bPDl  \(n = 6; X = I\) 18.6

**56f** bPUB  \(n = 7; X = Br\) 14.4

**56g** bPDDDB  \(n = 8; X = Br\) 9.14

**57b** bPPOI  \(n = 4; X = I\) \(K_i > 100\)

**57c** bPNNB  \(n = 5; X = Br\) 66.7

**57d** bPNDI  \(n = 6; X = I\) \(K_i > 100\)

**57e** bPUB  \(n = 7; X = Br\) 82.0

**57f** bPDDDB  \(n = 8; X = Br\) 33.0

**58b** bQOI  \(n = 4; X = I\) \(K_i > 100\)

**58c** bQNB  \(n = 5; X = Br\) 20.1

**58d** bQDI  \(n = 6; X = I\) \(K_i > 100\)

**58e** bQUB  \(n = 7; X = Br\) 38.3

**58f** bQDDDB  \(n = 8; X = Br\) \(K_i > 100\)

**60b** bNOI  \(n = 4; X = I\) 1.50

**60c** bNNB  \(n = 5; X = Br\) 4.31

**60d** bNDI  \(n = 6; X = I\) 0.28

**60e** bNUB  \(n = 7; X = Br\) 0.37

**60f** bNDDDB  \(n = 8; X = Br\) 1.43

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In using the supervised SOM learning algorithm provided in the toolbox, this binning scheme allowed for a more accurate clustering of the data sets and for extracting the underlying commonalities of the molecules.

Bin 1: $0 < x = 5$
Bin 2: $5 < x = 50$
Bin 3: $50 < x = 100$

The data set being modeled was partitioned into a set of test molecules (20%) and training molecules (80%). Membership in the test or training set was random, with the one requirement that the proportion of molecular population in each bin be identical in both test and training sets. The descriptor blocks for each data set were normalized to unit variance and mapped to a logistic function. Descriptors with zero variance were removed, as were pairs with linear correlation above 0.90, retaining the most representative of the pair.

**The SOM-Stepwise Feature Reduction Algorithm**

Finally, a SOM-oriented variation of a forward entry stepwise regression algorithm was employed, to select a small number of independent variables from which the SOM model was constructed. The algorithm used is identical to the forward entry stepwise method with the following variations:

1. the entry statistic was defined to be the percent of training set molecules correctly classified by the model, any increase of which resulted in the inclusion of the descriptors being tested;
(2) the entry statistic was calculated by constructing a SOM using a supervised learning algorithm; and,
(3) descriptors to be entered into the model were chosen in pairs, one from each of two randomized lists of the full set of descriptors.

Algorithm training was conducted iteratively until some convergence criteria were met.

**Experimental Design**

For each of the three data sets, ten independent runs were conducted, each with distinct training set/test set samplings. At the end of each training process, the test set molecules were predicted with the trained SOM model, providing the benchmark statistic, e.g., ‘percentage correctly classified’. Averaging these statistics over the ten runs produced an estimate of the algorithm’s success in both learning the training data and generalizing to the test data. Conducting multiple runs to some extent minimizes sampling bias, as well as bias arising from the stochastic SOM initialization process. The best model for each of the three data sets was further examined using custom visualization tools, as shown in the Figures 4.2-4.4.

**4.2.4. Genetic Functional Activity Algorithms**

Genetic functional algorithms (GFAs) as described by Rogers and Hopfinger (Rogers and Hopfinger 1994) were implemented using Cerius² (version 4.6) software (Accelrys Inc., San Diego, CA). GFAs were conducted using static 100 member populations; linear, quadratic, and offset quadratic basis functions; the mutation probability for both additions
and deletions was set at 0.2%. Lack of fit criterion was set at 1.0 and initial equation length was set to three. Models were evolved over 50,000-80,000 generations using standard convergence criterion.

4.3. Results and Discussion

Self-Organizing Maps

U-matrix maps represent the descriptor-based relationships of the training set of molecules by the SOM, and are constructed by projecting all selected descriptor’s contributions to the mapping.

D-matrix maps indicate the classification mapping of the SOM, as learned from the corresponding training sets. All three maps illustrate strong clustering.

The color level, in all the maps seen below (see Figures 4.2-4.4) describes the relative clustering in the data; the darker areas indicate small distances, while the lighter areas indicate larger distances. The colored hexagons indicate the points on the map corresponding to training molecules, and are colored by the affinity classification and labeled with the corresponding training molecule names.

SOM models of the ALL data set

The ten models learned the training set with high classification accuracy (93% ± 0.02) and were able to correctly classify the test compounds with a percent accuracy of 68% ± 0.08. The average number of descriptors chosen via the stepwise SOM was 11, which
were selected from a total number of 47 descriptors. False negative values, where the model placed the compound two bins removed from its actual value, were low, accounting for less than 2% of the failures generated by the model. There were no false positives reported for any of the models. Figure 4.2 is a graphical representation of the best model of the ten models generated from the ALL data set. The descriptors selected by the best model were, S_dO, S_aaCH, IC, S_sCH3, S_sssNH, S_tCH, and PHI.
Figure 4.2 (A) D-matrix generated from the ALL data set, red numbers indicate the classification mapping of each node; (B) U-matrix SOM generated from the ALL data set. Key: red- class 1, green- class 2, blue- class 3. Inter-node distances are shown in grey-scale coloring, the closer the distance the darker the shading.
The D-matrix (Figure 4.2A) shows that there are three general clusters for the full complement of compounds in the ALL data set. The descriptor, IC (informational content), is an information-theoretic topological index and may be identifying steric qualities of the molecules. The descriptors utilized, S_dO, S_aaCH, IC, S_sCH3, S_sssNH, S_tCH, and PHI, by the model mostly correspond to the electrotopological (E-Top) indices. S_dO describes the sum of the energies of carbonyl moieties in the set from the \( N-n \)-alkynicotinamide series. As the carbonyl-containing compounds of this family are inactive, S_dO may be acting largely as a binary classifier. Additionally, the best model used descriptors for the energy states of all CH\(_3\), aromatic CH, total CH, and NH bonds, e.g. S_sCH\(_3\), S_aaCH, S_tCH, and S_sssNH, respectively. These descriptors may differentiate the C3-functionality of the pyridine ring; the S_dO and S_sssNH descriptors together likely map the SAR relative to the inclusion of the amide functionality at the C3-position of the pyridine ring, leading to a decrease in affinity. Another descriptor that may demonstrate the importance of the C3-position functionality is the Kier flexibility index, PHI. The compounds in the \( N-n \)-alkylpicolinium series, which contain a methyl group at the C3-position of the pyridine ring, were also inactive and grouped with the \( N-n \)-alkynicotinamide salts. The active \( N-n \)-alkylnicotinium salts, with a 2-N-methylpyrrolidino functionality at the C3-position of the pyridine ring, however, were separated from the inactive compounds. The rigidity of the methyl and amide functionalities when compared to the more flexible pyrrolidine ring may also be key to the observed affinity of these compounds at the a4\( \beta \)2* nAChR.
The U-matrix (Figure 4.2B) reflects chemically intuitive trends; the longer saturated \(N-n\)-alkylnicotinium salts were clustered together. Additionally, the unsaturated \(N-n\)-alkylnicotinium compounds, NONB-3y, NDNB-4t, and NDNB-3y, were similar in bioactivity and clustered near the saturated \(N-n\)-alkylnicotinium compound NNNI. However, NONI, the less active eight-carbon analog, was clustered among the active compounds, pointing to a limitation in the SOM or the descriptors. Shorter carbon chain \(N-n\)-alkylnicotinium compounds and \(N-n\)-alkylpyridinium compounds were likewise clustered together. Interestingly, rotationally restricted analogs were not found in the freely rotating \(N-n\)-alkylnicotinium compound cluster. This reflects a bias toward their different affinities, as the model does not recognize the two classes as structurally similar, but actually classifies the restricted rotation analogs as being structurally similar to the longer chain \(N-n\)-alkylpyridinium analogs (NEcPB and NDDPI).

A strong distinction is observed between the different structural classes of \textit{bis}-substituted quaternary ammonium compounds. The active \textit{bis}-quaternary ammonium salts are closely associated with the \(N-n\)-alkylnicotinium compounds, as well as the classical muscle nAChR antagonist, decamethonium bromide (DEC). The \textit{bis}-quaternary ammonium compounds were clustered nearer to the mono-substituted \(N-n\)-alkylpyridinium salts. This clustering may indicate that the \textit{bis}-quaternary ammonium compounds bind to the receptor in similar fashion as the \(N-n\)-alkylnicotinium compounds.
SOM models of the MONO data set

The ten models learned the training set with high accuracy (95% ± 0.03) and were able to predict test compound affinities with 72% ± 0.10 accuracy. The average number of descriptors chosen via the stepwise SOM regression method was 7.8 descriptors, selected from a total number of 42. A 7.2 percentile of the missed assignments by the model were false positives. False negatives accounted for less than 1% of the failures generated by the model. Figure 4.3 is a graphical demonstration of the best model generated from the mono-substituted series of compounds. From the model generated, the descriptors used to create the model were S_\text{aaCH}, Jurs-RASA, Jurs-PNSA-1, Foct, S_dCH2, Fh2o, and LogP.
Figure 4.3 (A) D-matrix generated from the MONO data set; (B) the U-matrix representation.
The mono-substituted quaternary ammonium salt model based on the MONO data set also demonstrated three distinct areas of clustering (Figure 4.3A) and used different descriptors, \( S_{\text{aaCH}} \), Jurs-RASA, Jurs-PNSA-1, Foct, \( S_{\text{dCH2}} \), Fh2o, and LogP, than those used in the ALL compound set, with only one similar descriptor, e.g. \( S_{\text{aaCH}} \). The model did use a similar descriptor, \( S_{\text{dCH2}} \), to describe the importance of the energy states of all of the \( \text{CH}_2 \) groups in the molecule. One of the new descriptors, Jurs-PNSA-1 describes the partial negative surface area (e.g. the sum of the solvent-accessible surface areas of all negatively charged atoms). Also included in the model is the related Jurs-RASA descriptor, which indicates the relative hydrophobic surface area, defined as the total hydrophobic surface area divided by the total molecular solvent-accessible surface area. The hydrophobic character of compounds in this series appears to be a major determining factor for their affinity, and is supported by the three additional related descriptors, e.g. Foct, Fh2o, and LogP. These descriptors are thermodynamic descriptors that estimate the desolvation free energy for octanol, water, and the octanol/water partition coefficient, respectively.

The long chain \( N-n \)-alkylnicotinium salts and the unsaturated \( N-n \)-alkenylnicotinium salts were clustered together in the SOM model (Figure 4.3B). These compounds share similar structures and similar \( \alpha_4\beta_2^* \) nAChR affinities. The unsaturated and saturated \( N \)-alkylpyridinium salts were grouped together with the shorter chained \( N-n \)-alkylnicotinium compounds, which indicates, according to the descriptors chosen, that both the \( N \)-alkyl chain and the pyridine C3-position substituent are important for determining \( \alpha_4\beta_2^* \) nAChR affinity. The longer chain \( N-n \)-alkylnicotinium salts
exhibited the highest affinities, which correlate with a larger number of lipophilic group (e.g. CH$_2$ groups in the alkyl chains and in the N-methylpyrrolidine ring), whereas the longer chain N-n-alkylpyridinium salts and the shorter chain N-n-alkylnicotinium salts have fewer numbers of CH$_2$ groups and therefore, lower affinities for the a4β2* nAChR receptor.

The third cluster contains two interesting compound families: N-n-alkylnicotinamide salts, which were inactive in the $[^3]$HNIC binding assay, and the conformationally restricted N-n-alkylnicotinium analogs, which were also inactive. The N-n-alkylnicotinamide salts possess a hydrophilic moiety at the 3-position of the pyridinium ring. Similar to many nAChR ligands, affinity at the α4β2* nAChR subtype is positively influenced by a hydrophobic interaction, whereas a hydrophilic moiety at the 3-position of the pyridinium ring serves to decrease affinity. The low-affinity, conformationally-restricted N-n-alkylnicotinium analogs have a high degree of hydrophobicity, and the model was able to differentiate them from other nicotinium molecules. Although it is unclear exactly which descriptor or descriptors may map this phenomenon, it is clear that the SOMs can differentiate in a manner that takes into account both distributed (hydropathy) and localized (functional groups) determinants of the observed SAR.

**SOM models of the BIS data set**

The ten models learned the training set with high accuracy (98% ± 0.03) and predicted the test compounds with a percentage accuracy of 58% ± 0.15. The average number of descriptors chosen via the stepwise SOM was 4.8 selected from a total number of 30
descriptors. False positive and negative values were not observed in this model. Figure 4.4 is a graphical representation of the best model generated from the *bis*-substituted series of compounds. From the model generated, the descriptors used to create the model were $S_{ssCH2}$, CIC, $S_{aasC}$, Jurs-RPCS, and Jurs-WNSA-3.
Figure 4.4 (A) D-matrix generated from the BIS dataset; (B) the U-matrix representation.
The model developed for the *bis*-quaternary ammonium salts based on the BIS data set also shows three distinct clustering areas (Figure 4.4A and 4.4B). This model contains the descriptors S_ssCH2 (energy states of the CH2 bonds) and S_aasC (energy states of the C bonds), again stressing the importance of the lipophilic nature of these compounds. The model also contained the descriptor, CIC (complementary informational content), which is an information-theoretic topological index similar to IC and may be identifying steric interactions of the molecules. The *bis*-quaternary ammonium salt model utilized the Jurs spatial descriptors to generate the model. However, unlike the *mono*-N-n-alkylated quaternary ammonium salt model, which uses a hydrophobic interaction descriptor, the *bis*-quaternary ammonium salt SOM used a positive charge surface area descriptor, Jurs-RPCS. This descriptor represents the most positively charged atom and its relative accessible surface area. Since these *bis*-molecules possess two positive charges, the selected descriptors may indicate an increased importance of charge distribution within the series. The model also uses the Jurs-WNSA-3 descriptor, which is similar to the Jurs-PNSA-1 descriptor used in the *mono*-N-n-alkylated quaternary ammonium salt model, except that it represents only a partial weighting of the surface accessible portions of the molecule. The highest affinity members of this data set, the *bis*-quaternary ammonium salts, were clustered together. DEC was also clustered in the high affinity bin, whereas the classical ganglionic nAChR antagonist, hexamethonium bromide (HEX), was clustered with the lower affinity compounds, *viz*. the *bis*-quinolinium and the *bis*-pyridinium salts. Additionally, the *bis*-picolinium compounds were grouped with two of the *bis*-quinolinium salts in the weakest affinity set. Therefore, the presence of the 2-N-methylpyrrolidine ring of the *bis*-nicotinium salts plays an
important role in determining the affinities of this series of compounds for the a4β2* nAChR receptor. It was demonstrated that a pKa shift occurs in the pyrrolidino nitrogen when the pyridino functionality in the NIC molecule is quaternized (Crooks et al. 1995). Therefore, the importance of the 2-N-methylpyrrolidine may be due to increased lipophilicity and/or to the possibility that the less basic nitrogen may act as a hydrogen bond acceptor.

**Genetic Functional Activity Algorithms**

**Genetic Functional Algorithm Models**

*N*-Alkylpyridinium and *N*-Alkyl-3-substituted pyridinium salts

The resulting equation was derived using the GFA methods:

\[
K_i = 161.582 + 0.004244 \times \text{(Area)}^2 - 2.7 \times 10^{-5} \times \text{(E-DIST-mag)}^2 - 5.12623 \times \text{(Vm)} + 36.4862 \times \text{(SC-3_P)} + 0.001581 \times \text{(Jurs-WPSA-1)}^2
\]  

(1)

\[r^2: 0.835734\]

\[q^2: 0.720\]

The GFA methodology was applied to generate three equations for the prediction of the quaternary ammonium compound’s affinity at the α4β2* nAChR subtype. Equation (1) was generated to correlate the descriptors to the bioactivity of all of the *N*-n-alkylpyridinium salts, *N*-n-alkylpicolinium salts and *N*-n-alkylnicotinamide salts (MONO dataset). The equation reveals a positive correlation with increased molecular volume (Vm) and area (Area), while the molecular connectivity index of longer chain lengths (SC-3_P) has a negative correlation. Interestingly, the compounds in the *N*-n-alkylnicotinamide series, which have larger molecular volumes than their corresponding
pyridinium cousins, possess less affinity toward this nAChR subtype. Additionally, the affinities of the N-n-alkyl nicotinamide salts were also decreased when compared to the simpler N-n-alkylpyridinium salts. The GFA also had a negative correlation with the E-DIST-mag descriptor, which describes the entropy of the matrix. The Jurs-WPSA-1 descriptor describes the surface-weighted charged partial surface areas; an indication that the positive charge distribution plays a role in receptor recognition (note that the converse descriptor, Jurs-PNSA-1 was used in the corresponding SOM model).

N-Alkynicotinium salts

The resulting equation was derived using the GFA methods:

$$K_i = 43.5762 + 1.0e-6 \text{ (E-DIST-mag)}^2 - 3.03532 \text{ (Jurs-RPCS)}^2 - 6862.5 \text{ (Jurs-FNSA-3)}^2 + 0.000602 \text{ (MW)}^2$$  \hspace{1cm} (2)

$$r^2: 0.592152$$

$$q^2: 0.369$$

Equation (2) models the SAR of the N-n-alkynicotinium salts only. As can be seen in equation (1), the E-DIST-mag descriptor was utilized, however, a positive correlation was found with this set of data. Jurs-RPCS, a measurement of the relative positive surface charge, and Jurs-FNSA-3, fractional negatively charged partial surface areas, positively correlate with affinity for the $\alpha4\beta2^*$ nAChR binding site, with the latter descriptor having the most weight. Interestingly, molecular weight (MW) is also negatively correlated. Since the highest affinity analogs NDDNI and NDNI ($C_{12}$ and the $C_{10}$ N-n-alkynicotinium compounds, respectively) possess the highest molecular weight, the spatial partial charge interactions described by the Jurs descriptors must be more
heavily weighted in this case. Additionally, this data set did not include NONI, the \(C_8\) \(N-n\)-alkylnicotinium analog. The GFA was unable to generate a reliable model with NONI included, indicating that the descriptor set was likely insufficient to fully describe the SAR of this series.

**bis-Quaternary Ammonium salts**

The resulting equation was derived using the GFA methods:

\[
K_i = -141.363 + 1449.55 (\text{SIC})^2 + 0.000602 (\text{Jurs-WNSA-2})^2 - 47.5526 (\text{IC})^2 + 2.43134 (\text{Log} \ Z)
\]  

\(r^2: 0.810622\)

\(q^2: 0.657\)

The affinity of the \(bis\)-quaternary ammonium compounds is modeled by equation (3). Similar to the \(N-n\)-alkylnicotinium salts and the SOM maps, the \(bis\)-quaternary ammonium salts utilized a surface weighted, partial charge descriptor. Again, the information-theoretic topological indices are found in the model equation. IC and SIC (structural informational content) are present and may be identifying steric qualities of the molecules and \(\text{Log} \ Z\) (logarithm of the Hosoya index), which deals with connectivity of the molecule. It also appears that the Jurs descriptors are effective in describing the SAR of the quaternary ammonium salts. The GFA selected a surface weighted, partial negative charge descriptor, Jurs-WNSA, which is most likely related to a portion of the molecule, possibly the aromatic pyridinium ring.
It is important to note that although the SOM and GFA methodologies were provided the same set of descriptors at the outset of training, not surprisingly, models resulting from the two methods utilize different subsets of these descriptors. A combination of three factors is thought to underlie this observation: 1) GFAs and SOMs model nonlinearity in fundamentally different ways; 2) each method approaches descriptor selection in fundamentally different ways and; 3) each have different susceptibilities to getting stuck in local minima.

4.4. Conclusion

Two different computational modeling methodologies were used to model the SAR of the interaction of quaternary ammonium compounds with the $\alpha 4\beta 2^* \text{nAChR}$ subtype. The SOM methodology was effective in appropriately grouping compounds with diverse structures and activities. GFAs were also able to predict compound activities from a set of physical descriptions. Taken together, the charge distribution and the hydrophobic free energies seem to be important indicators of bioactivity for this particular class of molecules. However, both methodologies exhibited limitations when compounds with similar physical properties, but vastly different biological activities were evaluated. Nevertheless, the current work demonstrates that non-linear in silico methodologies can be used to screen large series of compounds, and successful models can be generated to aide in the selection of active compounds from larger compound libraries to be further evaluated.

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Chapter 5: Enantioselective Synthesis of Nicotine Analogs as Precursors for the Preparation of Novel Quaternary Ammonium Analogs

5.1. Introduction

The principal tobacco alkaloid, NIC, is a potent agonist at nAChRs, and interacts with all known nAChR subtypes. nAChR ligands modulate a number of functions within both the peripheral and the central nervous systems, mediated by a diversity of nAChR subtypes containing α2-α10 and β2-β4 subunits. Interestingly, leaves from *Nicotiana tobaccum* contain a number of other minor alkaloids structurally related to nicotine, e.g., nornicotine, anabasine, and anatabine. S-(−)-Nicotine is biosynthesized in the tobacco plant from nicotinic acid and L-ornithine or L-arginine. Nornicotine, the second most abundant tobacco alkaloid, is biosynthesized via enzymatic N-demethylation of S-(−)-nicotine; the minor alkaloid, anabasine, is generally believed to be biosynthesized from nicotinic acid and lysine, via a similar pathway to that described above for nicotine (Bush *et al.* 1999). Anatabine is derived biosynthetically solely from nicotinic acid, which is decarboxylated to 1, 2-dihydropyridine and 2, 5-dihydropyridine followed by condensation of these two molecules to form 3, 6-dihydroanatabine (Bush *et al.* 1999). Aromatization of 3, 6-dihydroanatabine then affords anatabine.

While nicotine in tobacco is found almost exclusively in the S-(−)-enantiomeric form (Crooks *et al.* 1992; Armstrong *et al.* 1999), the minor tobacco alkaloids are present as mixtures of their respective optical isomers (Armstrong *et al.* 1999). In this respect, unlike nicotine, nornicotine exists as a mixture of its S-(−) and R-(+) enantiomeric forms in various ratios, depending on the species of *Nicotiana* from which it was isolated. Even
different sources of tobacco, e.g. Burley, Turkish, or Virginia, contain different ratios of nornicotine enantiomers (Armstrong et al. 1999). The presence of both enantiomeric forms of nornicotine in the tobacco plant results from the enzymatic N-demethylation of S-(-)-nicotine in the plant. Biosynthetic studies with 2’-[3H]-nicotine have shown that the 2’-[3H]-label is lost during formation of R-(+)-nornicotine, but is retained in the formation of S-(-)-nornicotine (Bush et al. 1999). These observations suggest the formation of a planar intermediate in the formation of the R-(+)-isomer, and the likely existence of two distinct N-demethylase enzymes.

The minor tobacco alkaloids are also known to possess nAChR agonist activity, and may contribute to the neuropharmacological effects of smoking, although they are generally relatively less pharmacologically active than S-(-)-nicotine (Crooks and Dwoskin 1997). Pharmacological investigations and structure-activity studies with these minor tobacco alkaloids have been somewhat limited, due to the lack of availability of their optically pure forms. Previous structure-activity studies have demonstrated that the optical isomers of nornicotine show significant differences in their ability to bind to nicotinic receptors in rat brain and generally are less potent compared with nicotine (Reavill et al. 1988; Copeland et al. 1991). For example, S-(-)-nornicotine inhibits [3H]-nicotine binding to rat brain membranes with a 50-fold lower affinity (Ki= 47 nM) compared with nicotine (Ki = 1.0 nM) (Xu et al. 2001). In contrast, S-(-)-nicotine and S-(-)-nornicotine exhibit similar affinities for the [3H]methyllycaconitine binding site (Ki = 770 nM and 1340 nM, respectively) in brain (Xu et al. 2001). These results indicate interaction of nornicotine with both a4ß2* and a7* nAChR subtypes. Evidence has also accumulated
demonstrating that nornicotine enantiomers differ in their neurochemical and behavioral effects (Bardo et al. 1997; Crooks and Dwoskin 1997; Green et al. 2000; Green et al. 2001). Similar to nicotine, both enantiomers of nornicotine evoke a concentration-dependent, Ca\(^{2+}\)-dependent and mecamylamine-sensitive increase in DA release from rat striatal and nucleus accumbens slices (Dwoskin et al. 1993; Teng et al. 1997; Green et al. 2001), indicating that nornicotine acts as an agonist at nAChR subtypes modulating DA release. Interestingly, \(R\)-(+)nornicotine was more potent than the \(S\)-(−)-enantiomer in evoking \[^{3}H\]dopamine overflow from rat nucleus accumbens slices, whereas both isomers were equipotent in this respect in experiments with rat striatal slices, suggesting the involvement of different nicotinic receptor subtypes in these brain regions. Whereas, \(S\)-(−)-nicotine and \(S\)-(−)-nornicotine were equipotent in releasing DA from striatal slices, nicotine was 43-fold more potent than nornicotine (EC\(_{50}\) = 70 nM and 3.0 \(\mu\)M, respectively) in releasing DA from nucleus accumbens slices (Green et al. 2001). Interestingly, nornicotine has a longer half-life than nicotine in both rodent and non-human primate plasma and brain (Kyerematen et al. 1990; Crooks et al. 1997; Ghosheh et al. 1999; Valette et al. 2003); and following chronic treatment with \(S\)-(−)-nicotine, nornicotine (of unknown chirality) accumulates in rodent brain reaching pharmacologically relevant concentrations (Ghosheh et al. 2001).

Behavioral studies using animal models also provide support for the use of nornicotine as a tobacco use cessation agent. Nornicotine produces nicotine-like discriminative stimulus effects (Bardo et al. 1997), as well as nicotine-like effects on schedule-controlled operant responding (Risner et al. 1988). Recent results indicate that
nornicotine functions as a positive reinforcer (Bardo et al. 1999); however, under similar experimental conditions, nornicotine is associated with a lower rate of responding in comparison with nicotine (Corrigall and Coen 1989; Bardo et al. 1999), suggesting that nornicotine has a lower reinforcing efficacy. Moreover, nornicotine has been shown to decrease nicotine self-administration in rats (Green et al. 2000). Furthermore, across repeated nornicotine pretreatments, tolerance did not develop to nornicotine-induced decrease in nicotine self-administration. Importantly, nornicotine has been recently found to inhibit dopamine transporter (DAT) function (Middleton et al. 2005), and thus, acts similarly to bupropion, which is a currently available smoking cessation agent. In summary, nornicotine increases DA release and inhibits DAT function, both mechanisms being beneficial in the treatment of tobacco use. Thus, a simple structural change, e.g., removal of the N-methyl moiety from the pyrrolidine ring of nicotine, which affords nornicotine, may be beneficial with respect to its pharmacological profile. Taken together, these current preclinical neurochemical and behavioral results suggest that nornicotine could be a promising candidate for development as a tobacco cessation agent (Middleton et al. 2005). In this respect, it should be noted that R- (+)-nornicotine is currently being investigated as a potential smoking cessation agent (Crooks et al. 1998).

Dwoskin et al. (1995) have shown that S-(-)-anabasine increases fractional $[^3]$H] release in a concentration-dependent manner from rat striatal slices preloaded with $[^3]$H]-dopamine (EC$_{50} = 19.3 \pm 3.2$ µM), but was less potent than S-(-)-nicotine (EC$_{50} = 3.0 \pm 2.2$ µM) (Dwoskin et al. 1995). Similar results have been reported by Grady et al. (1992), who demonstrated that $\pm$-anabasine stimulates $[^3]$H]-dopamine release from mouse striatal
synaptosomes, and $\pm$-anabasine has been reported to inhibit high affinity binding of $[^3H]$-S-(-)-nicotine to rat and mouse striatal membranes (Reavill et al. 1988; Grady et al. 1992). Thus far, direct comparisons of these pharmacological properties between the enantiomers of anabasine and anatabine have not been determined due to difficulties in obtaining the pure optical isomers of each compound.

Early efforts to develop synthetic routes to racemic minor tobacco alkaloids have utilized a variety of methods (for a comprehensive review, see Crooks, 1999). However, few examples of general preparative methods for these alkaloids have been reported. Deo and Crooks (1996) have utilized a benzophenone imine intermediate for the general synthesis of racemic nornicotine, anabasine, and anatabine, while a number of strategies have been developed for the enantioselective synthesis of nornicotine, anabasine and anatabine optical isomers (Deo and Crooks 1996; Crooks 1999; Loh et al. 1999; Yus et al. 2001), few of these are of general utility. Recently, the $S$-(-)-isomers of nornicotine, anabasine and anatabine have been synthesized via a 5-step enantioselective synthesis that utilizes a ring-closing metathesis route (Felpin et al. 2001); also, Amat et al. (2002) have reported a chiral synthesis of $S$-(-)-anabasine from a chiral lactam intermediate (Amat et al. 2002). Additionally, $S$-(-)-anatabine has been synthesized by reacting an allylsulfone with an azaaromatic chiral sulfinimine (Balasubramanian and Hassner 1998).

Aiqiao et al. (1991) have reported a rapid and convenient use of a 2-hydroxy-3-pinane intermediate for the enantioselective synthesis of 2-pyridyl methylamines; this method was subsequently adapted by Swango et al. (1999) for the efficient synthesis of $S$-(−) and $R$-(+) nornicotine isomers in three steps, with a reported ee of 91% and 81%, respectively.
This current study describes a general synthetic procedure for the facile preparation of the optical isomers of nornicotine, anabasine, and anatabine, utilizing modifications of the Swango et al. (1999) synthetic methodology (Figure 5.1), and more importantly, establishes this synthetic route as a convenient method for the preparation of the enantiomerically pure minor tobacco alkaloids.

5.2. Experimental

5.2.1. Materials

The chemicals utilized in the synthesis of the enantiomers of nornicotine, anabasine, and anatabine were obtained from Aldrich Chemical Company (Milwaukee, WI), or from Acros Organics (Somerville, NJ), and were used without further purification. Flash column chromatography was carried out using ICN SILITECH 32-63, 60Å silica gel. Melting points were determined on a Fisher Scientific melting point apparatus and are uncorrected. $^{1}$H-NMR and $^{13}$C-NMR spectra were recorded on a Varian 300 MHz NMR. All Spectra were referenced, and chemical shifts were determined using tetramethylsilane (TMS) as the internal standard. Mass spectra were recorded on a JEOL JMS-700T Mstation, or on a Bruker Autoflex MALDI-TOF mass spectrometer. Specific rotation measurements were carried out on a Perkin-Elmer Model 241 Polarimeter. Microanalyses were carried out by Atlantic Microlabs, Inc. (Atlanta, GE).
5.2.2. Synthetic Procedures

Enantioselective NIC Alkaloid Synthesis

(1R,2R,5R)- and (1S,2S,5S)- 2,6,6-Trimethyl-3-(pyridin-3-ylmethylimino)-

bicyclo[3.1.1]heptan-2-ol (67 and 67R)

Boron trifluoride diethyl etherate (0.3 mL) was added to a solution of 3-(aminomethyl)
pyridine (3.30 g, 30.6 mmol) and 1R, 2R, 5R-(+)-2-hydroxy-3-pinanone (5.00 g, 29.8
mmol). The reaction mixture was refluxed for 2.5 h under N\textsubscript{2} using a Dean-Stark
apparatus. After cooling, the solvent was evaporated under reduced pressure. The
residue was purified by flash silica gel column chromatography using

CHCl\textsubscript{3}:hexane:methanol, 12:2:1 as the elution solvent to afford the (+)-2-hydroxy-3-

pinanone ketimine 67 (6.24 g, 81%) as white crystals after removal of solvent: [a\textsubscript{D}] =
+3.8° (c = 1.0, methanol); \textsuperscript{1}H NMR (300 MHz, CDCl\textsubscript{3}) \(\delta\) 8.58 (1H, d, J = 2.4 Hz), 8.48
(1H, dd, J = 4.8, 1.8 Hz), 7.70 (1H, d, J = 7.8 Hz), 7.25 (1H, dd, J = 7.8, 4.8 Hz), 4.48
(2H, s), 2.80 (1H, br s), 2.58 (2H, m), 2.34 (1H, m), 2.06 (2H, m), 1.56 (1H, d, J = 10.8
Hz), 1.50 (3H, s), 1.31 (3H, s), 0.84 (3H, s) ppm.; \textsuperscript{13}C NMR (75 MHz, CDCl\textsubscript{3}) \(\delta\) 178.19,
149.33, 148.28, 135.81, 135.52, 123.60, 51.83, 50.46, 38.75, 38.50, 34.12, 28.56, 28.37,
27.51, 23.11 ppm. m/z 258 (M\textsuperscript{+}, base peak).

The same procedure was utilized for the synthesis of 1S, 2S, 5S-(−)-2-hydroxy-3-pinanone
ketimine (6.58 g, 85%), which was obtained as white crystals from the reaction of 1S, 2S,
5S-(−)-2-hydroxy-3-pinanone with 3-(aminomethyl)pyridine; [a\textsubscript{D}] = -3.6° (c = 1.0,
methanol).
Figure 5.1 Enantioselective Synthesis of S-(-)-Nornicotine, S-(-)-Anabasine, and S-(-)-Anatabine

(S)-(-)-Nornicotine and (R)-nornicotine (3 and 3R)

To a stirred solution of ketimine 67 (258 mg, 1.00 mmol) in THF (8 mL) was added dropwise LDA (1.50 mL, 2.0 M solution in THF, 3.00 mmol) at 0°C. The purple solution was stirred at 0°C for 15 min then cooled to –78°C. 1-Chloro-3-iodopropane (0.32 mL, 3.00 mmol) in THF (2 mL) was then added dropwise. The reaction mixture was stirred for an additional 30 min at –78°C, and then quenched with saturated aqueous
NH$_4$Cl solution. The solution was extracted with diethyl ether (3x10 mL). The combined organic layers were washed with brine, dried over anhydrous magnesium sulfate, filtered, and the solvent evaporated. The residue was passed through a short silica gel column, eluting with hexane, followed by CHCl$_3$:MeOH (20:1) to give the C-alkylated intermediate as a yellow oil. The crude product was not purified, but immediately added to a mixture of 95% EtOH (6 mL), hydrazine monohydrate (4 mL) and acetic acid (2 mL). The mixture was stirred overnight at room temperature and 10% aqueous NaOH solution (10 mL) was added. The solution was then extracted with CHCl$_3$ (3x20 mL), the combined organic liquors were dried over anhydrous magnesium sulfate, filtered, and the solvent evaporated. The residue was purified by flash column chromatography on silica gel, eluting with CHCl$_3$:MeOH:Et$_3$N (100:10:1) to afford S-(-)-nornicotine (3) (118 mg, 79% overall) as a pale yellow oil; [a$_D$] $= -32.6^\circ$ (c = 1.0, methanol); $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 8.60 (1H, d, J = 1.8 Hz), 8.45 (1H, dd, J = 4.8, 1.7 Hz), 7.74 (1H, “dt”, J = 7.8, 1.9 Hz), 7.25 (1H, dd, J = 7.8, 4.8 Hz), 4.14 (1H, dd, J =7.3, 2.1 Hz), 3.11 (2H, m), 2.20 (1H, m), 2.11 (1H, m), 1.91 (2H, m), 1.66 (1H, m) ppm.; $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 148.3, 148.0, 140.1, 134.2, 123.2, 59.7, 46.7, 34.0, 25.2 ppm; m/z 148 (M$^+$).

The same procedure was utilized for the synthesis of R-(+)-nornicotine (110 mg, 76% overall) from 1$S$, 2$S$, 5$S$-(-)-2-hydroxy-3-pinanone ketimine and 1-chloro-3-iodopropane, and the product was obtained as a pale yellow oil; [a$_D$] $= +32.8^\circ$ (c = 1.0, methanol).
(S)-(−)-Anabasine and (R)-(−)-anabasine (5 and 5R)

A similar procedure used to synthesize 3 was also utilized for the synthesis of S-(−)-anabasine (5), which was obtained in 72% yield from 1R, 2R, 5R-(−)-2-hydroxy-3-pinanone ketimine (67) using 1-chloro-4-iodobutane as the alkylating agent instead of 1-chloro-3-iodopropane. The product was obtained as a light-yellow oil; [a_D] = −79.8° (c = 0.9, methanol); ^1H NMR (300 MHz, CDCl_3) δ 8.60 (1H, s), 8.50 (1H, dt, J = 4.8, 1.7 Hz), 7.74 (1H, dm, J = 7.8, 1.8 Hz), 7.25 (1H, dd, J = 7.8, 4.8 Hz), 3.65 (2H, dd, J = 11, 2.2 Hz), 3.22 (1H, dm, J = 11 Hz), 2.75-2.85 (1H, m), 2.00 (1H, s), 1.46-1.95 (6H, m) ppm.; ^13C NMR (75 MHz, CDCl_3) δ 148.0, 148.2, 139.9 133.7, 122.8, 59.2, 47.0, 34.00, 24.9, 24.6 ppm. m/z 163 (M^+).

Additionally, R-(+)-anabasine was synthesized in a similar manner, utilizing 1S, 2S, 5S-(−)-2-hydroxy-3-pinanone ketimine as the chiral intermediate, and was obtained as a pale yellow oil (75% yield); [a_D] = +80.2° (c = 1.0, methanol).

S-(−)-Anatabine and R-(+)-anatabine

cis-2-(4-Bromobut-2-enyloxy)tetrahydropyran (68)

p-Toluenesulfonic acid monohydrate (0.13 g) was added to a stirred solution of cis-but-2-ene-1,4-diol (2.64 g, 30.0 mmol) and THP (2.53 g, 30.0 mmol) in CH_2Cl_2 (10 mL) and THF (25 mL) at 0°C. After stirring at 0 °C for 2 h, the reaction mixture was stirred at room temperature for a further 20 min. Water was added, and the aqueous phase was extracted with diethyl ether (3x20 mL). The combined organic layers were dried over anhydrous magnesium sulfate, filtered, and the solvent evaporated. The residue was purified by flash column chromatography on silica gel, eluting with hexane:EtOAc, (2:1)
to give cis-4-(tetrahydropyran-2-yloxy)but-2-en-1-ol (2.66 g, 52%) as a colorless oil: $^1$H NMR (300 MHz, CDCl$_3$) δ 5.81 (1H, m), 5.67 (1H, m), 4.64 (1H, m), 4.15 (4H, m), 3.82 (1H, m), 3.49 (1H, m), 1.42-1.84 (6H, m) ppm; $^{13}$C NMR (75 MHz, CDCl$_3$) δ 132.54, 128.11, 97.68, 62.63, 62.31, 58.50, 30.71, 25.59, 19.49 ppm.

To a solution of cis-4-(tetrahydropyran-2-yloxy)but-2-en-1-ol (2.44 g, 14.2 mmol) in DMF (40 mL) was added triphenylphosphine (4.17 g, 15.9 mmol). The solution was cooled to 0°C and NBS (2.73 g, 15.5 mmol) was added in portions. After stirring for 30 min at room temperature, the reaction was quenched with methanol (2 mL). The solution was diluted with diethyl ether (200 mL), and washed with water, saturated aqueous NaHCO$_3$, and brine, successively. The organic layer was dried over anhydrous magnesium sulfate, filtered, and concentrated. The residue was purified by flash chromatography on silica gel, eluting with hexane to afford the bromide 68 (2.24 g, 67%) as a colorless oil: $^1$H NMR (300 MHz, CDCl$_3$) δ 5.86 (1H, m), 5.69 (1H, m), 4.61 (1H, dd, J = 4.2, 3.0 Hz), 4.31 (1H, m), 4.14 (1H, m), 4.01 (2H, d, J = 7.8 Hz), 3.84 (1H, m), 3.51 (1H, m), 1.40-1.90 (6H, m) ppm; $^{13}$C NMR (75 MHz, CDCl$_3$) δ 130.95, 128.41, 98.15, 62.49, 62.08, 30.80, 26.84, 25.65, 19.67 ppm. m/z: 155 (M$^+$-81), 133/135, 85, 53.

1R, 1'S, 2R, 5R-2,6,6-Trimethyl-3-[1'-pyridin-3''-yl-5'-(tetrahydro-pyran-2''-yloxy)pent-3'-enylimino]bicyclo[3.1.1]heptan-2-ol (69)

To a stirred solution of ketimine 67 (1.94 g, 7.50 mmol) in THF (60 mL) was added dropwise LDA (11.3 mL, 2.0 M solution in THF, 22.6 mmol) at 0°C. The purple solution was stirred at 0°C for 15 min then cooled to −78°C. The bromide 68 (2.12 g,
9.00 mmol) in THF (8 mL) was added dropwise. The reaction mixture was stirred for an additional 30 min at –78°C, and then quenched with saturated aqueous NH₄Cl solution. The aqueous phase was extracted with diethyl ether (3x20 mL). The combined organic layers were washed with brine, dried over anhydrous magnesium sulfate, filtered, and evaporated. The residue was flash chromatographed on silica gel, eluting with CHCl₃:hexane:MeOH (100:20:2) to give 69 (2.80 g, 91%) as a colorless oil: ¹H NMR (300 MHz, CDCl₃)  δ 8.56 (1H, d, J = 2.1 Hz), 8.48 (1H, dd, J = 5.1, 1.8 Hz), 7.72 (1H, d, J = 7.5 Hz), 7.25 (1H, dd, J = 7.5, 4.8 Hz), 5.61 (1H, m), 5.50 (1H, m), 4.59 (2H, m), 4.19 (1H, m), 4.01 (1H, m), 3.84 (1H, m), 3.49 (1H, m), 2.63 (3H, m), 2.44 (1H, m), 2.26 (1H, m), 2.06 (1H, t, J = 6.3 Hz), 1.99 (1H, m), 1.62-1.88 (2H, m), 1.52 (3H, s), 1.44-1.60 (4H, m), 1.39 (1H, d, J = 10.5 Hz), 1.31 (3H, s), 0.90 (3H, s) ppm; ¹³C NMR (75 MHz, CDCl₃) shows signals for two diastereomers δ 176.13, 148.77, 148.53, 138.73 (138.70), 134.69, 129.13 (128.98), 128.80 (128.74), 128.74, 99.81 (97.99), 62.94 (62.85), 62.46, 61.10 (61.06), 50.26, 38.63, 37.08, 33.91, 30.90, 28.79, 28.26, 27.60, 25.71, 23.32, 19.77 ppm. m/z: 412 (M⁺), 327, 311, 257, 162, 144, 85; Anal. Calcd for C₂₅H₃₆N₂O₃·H₂O: C, 69.74; H, 8.90; N, 6.51. Found: C, 69.80; H, 8.58; N, 6.33.

1R, 1’S, 2R, 5R-3-(5’-Hydroxy-1’-pyridin-3’-yl-pent-3’-enylimino)-2,6,6-trimethyl-bicyclo[3.1.1]heptan-2-ol (70)

To a solution of THP ether 69 (1.45 g, 3.52 mmol) in MeOH (90 mL) was added p-toluene sulfonic acid (1.37 g, 7.04 mmol). The mixture was stirred at room temperature for 3 h and then concentrated. Saturated aqueous NaHCO₃ solution (20 mL) was added, and the aqueous phase was extracted with diethyl ether (3x20 mL). The combined
organic layers were washed with brine, dried over anhydrous magnesium sulfate, and evaporated. The residue was flash chromatographed on silica gel, eluting with CHCl₃:MeOH (25:1) to give 70 (1.05 g, 90%) as a colorless oil: [αᵲ] = −33.1° (c = 1.0, MeOH); ¹H NMR (200 MHz, CDCl₃) δ 8.51 (1H, br s), 8.44 (1H, d, J = 4.0 Hz), 7.66 (1H, dt, J = 8.0, 2.0 Hz), 7.22 (1H, dd, J = 8.0, 5.0 Hz), 5.71 (1H, m), 5.42 (1H, m), 4.55 (1H, dd, J = 8.4, 4.8 Hz), 4.08 (2H, m), 2.42-2.84 (4H, m), 2.16-2.38 (2H, m), 20.1 (1H, t, J = 6.0 Hz), 1.94 (1H, m), 1.50 (3H, s), 1.33 (1H, d, J = 11.4 Hz), 1.27 (3H, s), 0.85 (3H, s) ppm; ¹³C NMR (75 MHz, CDCl₃) δ 177.81, 148.78, 148.69, 138.69, 134.68, 131.98, 128.25, 123.85, 76.79, 60.45, 58.64, 50.55, 38.52, 38.46, 37.23, 34.15, 28.52, 28.13, 27.41, 23.13 ppm. m/z: 329 (M⁺+1); Anal. Calcd for C₂₀H₂₈N₂O₂⋅0.3CHCl₃: C, 66.93; H, 7.83; N, 7.69. Found: C, 67.16; H, 7.94; N, 7.77.

S-5-Amino-5-pyridin-3-yl-pent-2-en-1-ol (71)

To a solution of 70 (0.86 g, 2.6 mmol) in MeOH (40 mL) was added hydroxylamine hydrochloride (1.39 g, 20.0 mmol). The mixture was stirred at room temperature for 18 h. Ammonia gas was bubbled into the solution to adjust the pH to 9. The MeOH was then evaporated and CHCl₃ (15 mL) was added to the residue. The resulting mixture was filtered and the filtrate was concentrated. The resulting residue was flash chromatographed on silica gel, eluting with CHCl₃:MeOH:Et₃N (100:10:1) to give 71 (0.34 g, 74%) as a colorless oil: [αᵲ] = −44.3° (c = 1.0, MeOH); ¹H NMR (200 MHz, CDCl₃) δ 8.53 (1H, d, J = 2.4 Hz), 8.46 (1H, dd, J = 4.8, 1.6 Hz), 7.68 (1H, dt, J = 7.6, 1.8 Hz), 7.24 (1H, dd, J = 7.4, 5.0 Hz), 5.85 (1H, m), 5.47 (1H, m), 3.90-4.20 (3H, m), 2.30-2.60 (5H, m) ppm; ¹³C NMR (50 MHz, CDCl₃) δ 149.00, 148.65, 140.70, 134.10,
133.01, 128.43, 123.80, 57.84, 52.73, 37.42 ppm. m/z: 179 (M$^+$+1); Anal. Calcd for C$_{10}$H$_{14}$N$_2$O·0.2H$_2$O: C, 66.05; H, 7.98; N, 15.41. Found: C, 66.23; H, 7.91; N, 15.37.

**S-(−)-Anatabine and R-(+)-anatabine (6 and 6R)**

Diethyl azodicarboxylate (DEAD, 0.23 mL, 1.40 mmol) was added dropwise to a stirred solution of triphenyl phosphine (365 mg, 1.40 mmol) in THF (6 mL) at 0°C under N$_2$. The reaction mixture was stirred at 0°C for 20 min. A solution of 71 (178 mg, 1.00 mmol) in THF (3 mL) was then added dropwise. The mixture was stirred at 0°C for 30 min, and then at room temperature for 2 h. Evaporation of the solvent followed by purification of the residue by flash column chromatography on silica gel (CHCl$_3$:MeOH, 20:1) gave S-(−)-anatabine (6) (124 mg, 77%) as a colorless oil: [a$_D$] = -180° (c = 1.0, methanol); $^1$H NMR (300 MHz, CDCl$_3$) δ 8.56 (1H, d, J = 1.6 Hz), 8.46 (1H, dd, J = 4.8, 1.6 Hz), 7.68 (1H, dt, J = 8.0, 1.8 Hz), 7.23 (1H, dd, J = 4.0, 2.8 Hz), 5.79 (2H, m), 3.84 (1H, t, J = 7.0 Hz), 3.33-3.65 (2H, m), 2.21 (2H, m), 2.00 (1H, br s) ppm; $^{13}$C NMR (75 MHz, CDCl$_3$) δ 148.93, 148.82, 140.05, 134.29, 126.46, 125.26, 123.71, 55.44, 46.12, 34.01 ppm. m/z: 160 (M$^+$, base peak), 159, 145, 131, 118, 105, 80, 54.

The same procedure was utilized for the synthesis of R-(+)-anatabine from 1S, 2S, 5S-(−)-2-hydroxy-3-pinanone ketimine and cis-2-(4-bromobut-2-enyloxy)tetrahydropyran, which was obtained as a pale yellow oil (70% yield); [a$_D$] = +177° (c = 1.0, methanol).
5.3. Results and Discussion

The $R$- and $S$-enantiomers of nornicotine, anabasine, and anatabine were successfully synthesized in good to moderate yields, and in good enantiomeric purity. The stereo-controlled C-alkylation of the 2-hydroxy-3-pinanone-3-(aminomethyl)pyridine ketimine intermediate is the key step in the reaction sequence. Experimental evidence from the crystal structure of the ketimine (Ayers et al., 2005) formed during the C-alkylation step supports the findings that the $(+)$-ketimine is C-alkylated from the $re$ face of the $(-)$-ketimine-lithium complex. This alkylation affords the $S$-isomer exclusively, while the C-alkylated product with the $R$-configuration is generated from the $(-)$-ketimine-lithium complex. LDA was chosen from a number of possible catalytic bases, including KtBuOH, due to the higher yields obtained, and because of the improvement in purity of the isolated products when using this base. As determined by Aiqiao et al., the base (LDA) forms a stable complex with the ketimine, effectively blocking the $si$ face of the molecule and opening the $re$ face of the molecule to addition of an alkyl halide (Figure 5.2) (Aiqiao et al. 1991).

Figure 5.2 Stable carbanion $(+)$-ketimine intermediate.
An advantage in the above procedure over previously reported literature syntheses of \( R-\) and \( S-\)-nornicotine, and \( R-\) and \( S-\)-anabasine isomers is the small number of overall steps to achieve the formation of the desired alkaloids. In an earlier approach to that outlined above, Swango et al. (1999) utilized the 2-hydroxy-3-pinanone-3-(aminomethyl)pyridine ketimine reagent in the synthesis of nornicotine isomers, but C-alkylation was carried out with a halogenoalcohol, to afford an intermediate \( N-\)alkylhydroxy analog, which had to be isolated and converted to the corresponding \( N-\)alkylbromo analog, prior to cyclization to afford the final product. The current synthesis outlined in this paper constitutes a two-step, general procedure for the synthesis of the optical isomers of both nornicotine and anabasine, by utilizing a 1, 4-dihalogeno alkane instead of a halogenoalcohol in the C-alkylation of the ketimine. This change reduces the number of steps in the reaction sequence from three to two, and significantly simplifies the work-up procedure for obtaining the final chiral product. Attempts at utilizing diiodopropane or diiodobutane in the reaction resulted in lower yields of reaction products. The use of a chloroiodoalkane reactant affords rapid and more efficient C-alkylation of the ketimine, but slow solvolytic degradation at the chloro-terminus. This allows for flash silica gel column purification to be performed, thereby obtaining a stable intermediate for the final cyclization event of the reaction sequence in the presence of hydrazine and water.

In addition to Swango et al., other groups have successfully synthesized nornicotine and anabasine enantiomerically pure (Felpin et al. 2001; Amat et al. 2002). However, both these syntheses are five steps and result in lower overall yields. Felpin et al., utilize a
novel approach, employing ring closuring metathesis. Though this synthesis resulted in high enantioselective yields the metal used in the final cyclization step is expensive and the final step is significantly longer than the ketimine synthesis.

The above procedure can also be utilized for the synthesis of anatabine enantiomers. However, three steps are required to introduce the C-hydroxy-cis-alkene substituent at the α-carbon of the 2-hydroxy-3-pinanone-3-(aminomethyl)pyridine ketimine, followed by a final cyclization step using a Mitsunobu reaction. Nevertheless, this synthetic route is very favorably comparable to previously reported enantioselective syntheses of anatabine described by Balasubramanian and Hassner (1998), and by Mehmandoust et al. (1989), both of which involve five steps overall (Mehmandoust et al. 1989; Balasubramanian and Hassner 1998). The methodology outlined by Balasubramanian and Hassner could not definitively rule out the presence of an enantiomer in the final steps. The resulting enantiomeric yields were only 70% and the overall yield was 60%.
5.4. Conclusions
Successful adaptation and improvement of an existing methodology has allowed for a streamlined approach in the stereoselective synthesis of the enantiomers of the nicotinic analogs, nornicotine, anabasine, and anatabine. A two-step synthetic procedure for the enantioselective synthesis of the optical isomers of nornicotine and anabasine, and a modified procedure for the synthesis of anatabine enantiomers have been successfully developed, and all compounds were synthesized in good yields and high enantiomeric purity. Due to this purity, these compounds are ideal to use as precursors for another generation of quaternary ammonium analogs.
Chapter 6: Summary and Future Directions

6.1. Summary

This research project successfully utilized the introductory phases of the drug discovery process in order to explore neuronal nAChR subtypes. Previous research indicated that NONI and NDNI were selective and potent antagonists for the nAChRs that mediate DA release and for \( \alpha 4\beta 2^* \) nAChR subtypes, respectively. Synthetic analogs of NONI and NDNI were synthesized and evaluated at \( \alpha 4\beta 2^* \), \( a7^* \) and nAChRs that mediate DA release to create a structure activity relationship and afforded potent and selective antagonists. The \([^3H]NIC\) binding data were also used to create predictive models for the binding of these series of compounds to \( \alpha 4\beta 2^* \) receptor subtypes. Additionally, new enantioselective synthetic routes were developed to afford optically pure NIC analogs.

6.1.1. Quaternary Ammonium Analogs

In order to begin the drug discovery process, a series of compounds were designed based on initial work on NONI and NDNI and synthesized. Many of the compounds were isolated in high yields, and all were obtained in high purity and fully characterized. In the \( N\)-n-alkynicotinium series, in order to selectively \( N\)-alkylate the pyridyl \( N\)-atom of \( S\)-(\( -\))-nicotine, a protonation of the pyrrolidine \( N\)-atom, using an adaptation on the Menshutkin reaction using acetic acid, was necessary. Purification of the resulting compounds was especially important, due to the need to eliminate nicotine completely from the product, as it would interfere with pharmacological evaluation in the binding and release assays. Additionally, the \( N-N\)-bis-compounds, although similar in structure,
proved difficult to isolate and purify, and several different work-up techniques had to be developed.

The mono-quaternary ammonium compounds evaluated had a variety of nAChR antagonist activities, depending on the length of the chain, as well as the nature of the 3-pyridyl substituent. N-n-Alkynicotinium salts exhibited antagonist activity at both $\alpha_4\beta_2^*$ receptor subtypes and nAChR subtypes that mediate DA release depending on alkyl chain length, while having little to no affinity for the $\alpha_7^*$ receptor subtype. From these studies, NDDNI has emerged as a potential tool for antagonism of the $\alpha_4\beta_2^*$ receptor subtype. The activities of the $N-n$-alkylnicotinium, $N-n$-alkylpicolinium, and $N-n$-alkynicotinamide salts have also led to a better understanding of how the $N-n$-nicotinium series of compounds may be interacting with the $\alpha_4\beta_2^*$ binding site, and has highlighted the importance of the pyrrolidine ring as a necessary component to obtain antagonist activity. The length of the $N-n$-alkyl substituent is also an important determinant of the antagonist activity of these molecules, as evidenced by the increased activities when the chain length increased for the $N-n$-alkylpicolinium and $N-n$-alkynicotininium salts.

In contrast to the $\alpha_4\beta_2^*$ receptor subtype, the pyrrolidine ring of the NONI molecule was found not to be critical for binding to and inactivation of nAChR subtypes that mediate DA release. However, the presence of the pyrrolidine ring did afford the most potent antagonist, NDDNI. In addition to the $N-n$-alkynicotinium compounds, the $N-n$-alkylnicotinium and $N-n$-alkylpicolinium compounds were generally active in inhibiting NIC-
evoked DA release from rat striatal slices, and had relatively lower affinity for $\alpha 4\beta 2^*$ and $\alpha 7^*$ nAChR subtypes. Thus, removal of the pyrrolidine ring from the $N$-$n$-alkylnicotinium series afforded a series of compounds which exhibited an increase in antagonist potency for the nAChR subtypes that mediate DA release, while having little or no activity in $\alpha 4\beta 2^*$ and $\alpha 7^*$ nAChR subtypes. This supports the idea that there is a difference in the binding domains of $\alpha 4\beta 2^*$ nAChR subtypes, $\alpha 7^*$ nAChR subtypes, and certain nAChRs that mediate DA release.

The $bis$-quaternary ammonium compounds also demonstrated activity at the various nAChR subtypes. $N,N$-$bis$-alkylnicotinium compounds were found to be selective antagonists at $a4\beta 2^*$ receptor subtypes, similar to the $N$-$n$-alkylnicotinium series. However, unlike the $N$-$n$-alkylnicotinium series, the $N,N$-$bis$-alkylnicotinium compounds were not the most potent antagonists at the receptors that mediate NIC-evoked DA release. $N,N$-$bis$-Dodecylpicolinium (bPiDDB) proved to be the most potent antagonist at the receptors that mediate NIC-evoked DA release. That, coupled with it inactivity at $a4\beta 2^*$ and $a7^*$ receptor subtypes, make this compound the most potent and selective compound in the all the series tested. Furthermore, the lack of $a4\beta 2^*$ activity and the partial antagonist effect of bPiDDB in the NIC-evoked DA overflow assay, allows for a putative assignment of the antagonist activity occurring at $\alpha 6\beta 2\beta 3$ and $\alpha 6\alpha 4\beta 2\beta 3$ nAChR subtypes. bPiDDB is an excellent lead candidate for exploring the these nAChR subtypes due to it pharmacological profile. In addition to its laboratory utility, acute dosing of bPiDDB has recently been shown to decrease nicotine self-administration in
rats and may be used as a lead compound for clinical development (Neugebauer et al. 2006).

The only compounds that were found to be active in the $\alpha 7*$ MLA binding assay were the heteroaromatic analogs (i.e. $N,N$-bis-isoquinolinium and $N,N$-bis-quinolinium salts). Within these series of compounds, the most potent analog was bQDDB, the twelve-carbon quinoline analog, suggesting that the position of the extended aromatic ring is an important aspect to the binding of these compounds.

6.1.2. **Modeling**

Using several different classes of quaternary ammonium salts, two different established molecular modeling techniques were applied to create a novel approach to SAR studies. Three self-organizing map (SOMs) models were generated from three different sets of compounds. The groups consisted of the mono-substituted compounds, the bis-substituted compounds, and both sets combined. The models were able to successfully "bin" the test set of compounds after developing a model from a similar set of training compounds. Additionally, using genetic functional activity (GFA) algorithms an evolutionary approach to generating predictive model equations was applied to the compounds. Three separate equations were generated in order to form a predictive method for evaluating affinities at the $\alpha 4\beta 2*$ receptor subtype.
6.1.3. **Synthesis of Enantiomerically Pure Nicotinic Analogs**

In addition to the modeling and SAR work of the quaternary ammonium compounds, novel synthetic methods were also employed to develop enantiomerically pure nicotine analogs. Efficient enantioselective syntheses of (S)- and R-(+)-nor nicotine, (S)-and R-(+)-anabasine, and (S)-and R-(+)-anatabine have been developed, affording isomers in high enantiomeric excess.

6.2. **Future Directions**

The three areas of focus of this project can all be expanded. Evaluation of the quaternary ammonium pyridinium analogs in the functional $[^3]$H$]DA$ release assay, as well as in the $^{86}$Rb$^+$ efflux assay, is necessary for a complete functional profile to be obtained on each compound in each of these series. Additionally, those compounds that had affinity for the $\alpha7^*$ nAChR subtype should be tested for their functional activities. Once the functional profile was complete for these compounds, further study of their mode of action would be important. Of considerable interest would also be the functional activity of the nicotinamide analogs at nAChRs that mediate DA release. The compounds provide a different functional 3-position substituent with different electronic properties. This would allow probing the H-bonding binding area of the nAChRs that mediate DA release.

In addition to further testing, novel pyridinium compounds could also be performed on those compounds that were synthesized in Chapter 5. S-(−)- and R-(+)-Nor nicotine (3 and 3R), S-(−)- and R-(+)-anabasine (5 and 5R), S-(−)- and R-(+)-anatabine (6 and 6R), as
well as their N-methylated derivatives, could be quaternized with various chain lengths, (Figure 6.1).

Figure 6.1 N-Alkylated quaternary ammonium salts of S-(−)- and R-(+)-Nornicotine (72 and 73), S-(−)- and R-(+)-anabasine (74 and 75), S-(−)- and R-(+)-anatabine (76 and 77), and their methylated derivatives (78, 79, 80, 81, and 82).

Novel synthetic compounds may also be synthesized using the techniques outlined in Chapter 5. Although the racemic forms of azetidine and azepine NIC analogs have been synthesized, no enantioselective syntheses of these compounds have so far been reported. Using the enantioselective synthetic route developed for nornicotine, anabasine, and anatabine, synthesis of enantiomerically pure forms of these compounds may be possible.
To better direct the next generation of compounds in these series, the computational models developed from the initial SAR study of the \( \alpha 4\beta 2^* \) may be used predictively to determine active compounds \textit{in silico}. Additionally, predictive models could be developed for nAChR subtypes that mediate DA release and \( \alpha 7^* \) nAChR subtypes, as more data become available.

One of the advantages of the peripheral antagonists, DEC and HEX, is that their quaternary ammonium cationic nature does not allow for their passage into the central nervous system (CNS), therefore isolating their antagonist activity to nAChRs in the periphery. Although several of these compounds have excellent chloroform solubility and could theoretically pass into the CNS, it is not certain if a quaternary ammonium molecule can passively enter the blood-brain barrier (BBB). Active transport into the CNS, via the choline transporter may allow the passage of such quaternary ammonium molecules. Allen et al. (2003) have investigated NONI, NDNI, NOPI, binding to the BBB choline transporter and found that these salts do have affinity (27 – 49 \( \mu \)M) for the transporter binding site. Research on the \textit{bis}-azaaromatic quaternary ammonium analogs, revealed that bPiDDB also had affinity for the BBB choline transporter (1.57 \( \mu \)M). Furthermore, it was determined that radiolabeled NONI and bPiDDB are both transported via the BBB choline transporter into the CNS (Allen et al. 2003; Geldenhuys et al. 2005). Another possible strategy for delivery of these pyridinium compounds across the BBB into the CNS involves the synthesis of a pyridinium head group designed to be a bioprecursor of these compounds. Bodor et al. (1990) have designed uncharged stable dihydropyridine analogs that do not rearomatize due to the presence of a \( C_3 \) or \( C_5 \)
electron withdrawing substitution. Once the reduced product passes the BBB, the CNS enzyme monoamine oxidase-B (MAO-B) oxidizes the dihydropyridine analog to the quaternary ammonium compound that is active at neuronal nAChRs. This could be important in establishing a delivery method for this class of compounds, and would further the potential use of these compounds in nAChR related pathologies.

6.3. Conclusion

There is much to explore regarding the pharmacology of the neuronal nAChRs and the interaction of potential drug molecules with these receptors. The studies described herein have provided initial useful information that may be helpful in determining the functional properties of the individual nAChR subtypes, as well as in developing new synthetic methods for the preparation of novel compounds to add to the library of ligands available for probing the function of the various nAChR subtypes. Additionally, using established modeling techniques, new predictive models could be developed and utilized to increase the probability of success in the discovery of biologically active compounds with clinical potential. Using these methodologies allows for the successful identification of potent and selective nAChR ligands, of utility in unraveling the complexities of neuronal nAChRs, as well as having potential therapeutic uses in the clinic.
List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>α-Btx</td>
<td>α-Bungarotoxin</td>
</tr>
<tr>
<td>α-CBT</td>
<td>α-Cobratoxin</td>
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<td>α-ctx</td>
<td>α-Conotoxins</td>
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<td>5-HT</td>
<td>Serotonin</td>
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<td>ACh</td>
<td>Acetylcholine</td>
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<td>AChBP</td>
<td>Acetylcholine binding protein</td>
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<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
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<tr>
<td>ADME</td>
<td>Absorption, distribution, metabolism, and elimination</td>
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<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate</td>
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<td>ANOVA</td>
<td>One-way repeated-measure analysis of variance</td>
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<td>Atx</td>
<td>(+)-Anatoxin-a</td>
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<td>BBB</td>
<td>Blood-brain barrier</td>
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<td>CHCl₃</td>
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<td>CNS</td>
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<td>CoMFA</td>
<td>Comparative molecular field analysis</td>
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<td>DCEH</td>
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<td>FDA</td>
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<td>GABAₐ</td>
<td>Gamma-aminobutyric acid</td>
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<td>GFA</td>
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<td>HEX</td>
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<td>KtBuOH</td>
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<td>LDA</td>
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<td>MLS</td>
<td>Multiple least squares</td>
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<td>MSA</td>
<td>Molecular shape analysis</td>
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<td>nAChR</td>
<td>Nicotinic acetylcholine receptor</td>
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<td>NaHCO₃</td>
<td>Sodium bicarbonate</td>
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<td>NBS</td>
<td>N-bromosuccinimide</td>
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<td>n-Btx</td>
<td>Neuronal bungarotoxin</td>
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<td>NMMDA</td>
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<td>NSAID</td>
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<td>NT</td>
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<td>Open channel blockers</td>
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<td>PEI</td>
<td>Polyethylenimine</td>
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<td>PLS</td>
<td>Partial least squares</td>
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<tr>
<td>R-NIC</td>
<td>R-(+)-Nicotine</td>
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<td>SAR</td>
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<td>Self-organizing map</td>
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<td>Tetrahydrofuran</td>
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<td>THP</td>
<td>Tetrahydropyran</td>
</tr>
<tr>
<td>Trizma HCl</td>
<td>Tris[hydroxymethyl]-aminomethane hydrochloride</td>
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PRESENTATIONS

- “The Pharmacology of the Nicotinic Acetylcholine Receptor: An Overview”
  Guest Lecture for Dr. George Digenis PHR 924 Drug Design, University of
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- “The Pharmacology of the Nicotinic Acetylcholine Receptor: An Overview”
  Guest Lecture for Dr. George Digenis PHR 924 Drug Design, University of
  Kentucky, College of Pharmacy, Lexington KY (2000)

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  Guest Lecture for Dr. George Digenis PHR 924 Drug Design, University of
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- "Chain-Modified Pyridino-N-Substituted Nicotine Compounds for Use In the
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- “N-Pyridinium Analogs for the Use in the Treatment of CNS Pathologies.” By Peter A. Crooks, Joshua T. Ayers, Vladimir P. Grinevich and Linda P. Dwoskin, Deposition for U.S. patent to be filed: August 2001

**PUBLISHED ABSTRACTS AND POSTER PRESENTATIONS**


**PUBLICATIONS**


• L. H. Wilkins, Jr., D. Miller, **J. T. Ayers**, P. A. Crooks, and L. P. Dwoskin. N-n-Alkylnicotinium Analogues, a Novel Class of Antagonists at a4β2* Nicotinic


Joshua T. L. Ayers

07 July 2006