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Discovery of Highly Potent and Selective $\alpha 4\beta 2$ -Nicotinic Acetylcholine Receptor (nAChR) Partial Agonists Containing an Isoxazolylpyridine Ether Scaffold that Demonstrate Antidepressant-like Activity. Part II

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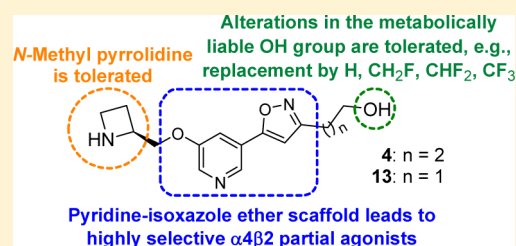
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ABSTRACT: In our continued efforts to develop $\alpha 4\beta 2$ -nicotinic acetylcholine receptor (nAChR) partial agonists as novel antidepressants having a unique mechanism of action, structure–activity relationship (SAR) exploration of certain isoxazolylpyridine ethers is presented. In particular, modifications to both the azetidione ring present in the starting structure 4 and its metabolically liable hydroxyl side chain substituent have been explored to improve compound druggability. The pharmacological characterization of all new compounds has been carried out using [³H]epibatidine binding studies together with functional assays based on ⁸⁶Rb⁺ ion flux measurements. We found that the deletion of the metabolically liable hydroxyl group or its replacement by a fluoromethyl group not only maintained potency and selectivity but also resulted in compounds showing antidepressant-like properties in the mouse forced swim test. These isoxazolylpyridine ethers appear to represent promising lead candidates in the design of innovative chemical tools containing reporter groups for imaging purposes and of possible therapeutics.



INTRODUCTION

Neuronal nAChRs are pentameric complexes assembled from varying combinations of subunits ($\alpha 2$ – $\alpha 10$, $\beta 2$ – $\beta 4$) that belong to the cys-loop superfamily of ligand-gated ion channels.^{1,2} Each nAChR subunit consists of a large amino-terminal extracellular domain (ECD), a transmembrane domain comprising four α -helices (M1–M4), and a variable cytoplasmic domain between M3 and M4. Acetylcholine (ACh) binding sites are formed between the subunit interfaces of the ECD bound by the cys-loop containing, primary face of an α -type subunit and the adjacent, complementary face of the neighboring subunit. When acutely activated by endogenous acetylcholine (ACh) or exogenous nicotinic ligands, nAChR channels transiently open, allowing Na⁺, K⁺, and Ca²⁺ cations to flow across the plasma membrane to induce cellular responses. Prolonged exposure to ACh or nicotinic agonists causes a gradual decrease in the rate of this ionic response, leading to a high affinity, longer-lasting, functionally inactive state, via a process referred to as desensitization. nAChRs are broadly distributed in both the central and peripheral nervous systems, and they can mediate excitatory neurotransmission and modulate actions of other neurotransmitters, including the monoamines dopamine (DA), norepinephrine (NE), and serotonin (5-HT).³ Previous studies have shown that nAChRs

are promising targets for the treatment of depression, especially for the patients who do not respond to selective serotonin reuptake inhibitors (SSRIs).^{4–6}

Among the nAChRs, $\beta 2$ subunit-containing complexes ($\beta 2^*$ -nAChRs, where the * indicates that other nAChR subunits are known or possible partners in the receptor assembly) are believed to be involved in the antidepressant effects of some nicotinic ligands and the tricyclic antidepressant (TCA) amitriptyline. For example, $\beta 2$ subunit-knockout mice show decreased immobility compared to the wild-type mice in the Porsolt forced swim test (FST), indicating that the absence of $\beta 2^*$ -nAChR-mediated signaling could manifest itself in an antidepressant-like phenotype *in vivo*.⁷ Additionally, the antidepressant-like effect of the nAChR antagonist mecamylamine in the FST was diminished when the $\beta 2$ -subunit was knocked out.⁸ Similarly, the antidepressant-like effects of the nAChR agonist sazetidine-A and of the TCA amitriptyline were absent in mice lacking the $\beta 2$ -subunit.^{7,9} Moreover, the activity of citalopram and reboxetine in the mouse FST was enhanced by agonists of $\alpha 4\beta 2^*$ -nAChRs, suggesting the involvement of $\beta 2$ -subunits in mediating these beneficial effects.¹⁰ Despite

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studies indicating that the *S*-enantiomer of mecamylamine (TC-5214) that shows antidepressant-like effects in animal models,¹¹ recent phase III human trial findings assessing efficacy of TC-5214 when used as an adjunct therapy in patients that are nonresponders to treatment using the traditional antidepressant citalopram failed to confirm earlier indications of the ligand's antidepressant potential.¹² This suggests that simple nAChR inactivation is not sufficient to produce antidepressant effects in treatment-resistant patients, although the lack of nAChR subtype specificity may also limit TC-5214's antidepressant efficacy. For example, although the main ganglionic nAChR subtype ($\alpha3\beta4^*$ -nAChRs) is expressed at a relatively low level in the brain, blockade of $\alpha3\beta4^*$ -nAChRs may result in unwanted side effects *in vivo*, including dysregulation of the autonomic nervous system, and $\alpha3\beta4^*$ -nAChRs also are sensitive to antagonism by mecamylamine. Given these considerations, we continue to pursue ligands highly selective for $\beta2^*$ -nAChRs as potential novel antidepressants, as do other groups in academia or the pharmaceutical industry.

A-85380 (**1**) is a 3-pyridyl ether that exhibits high potency and selectivity for $\alpha4\beta2$ -nAChRs ($K_i = 0.05$ nM for rat brain receptors, 0.04 nM for human brain receptors) relative to human $\alpha7$ -nAChRs ($K_i = 148$ nM) or muscle-type $\alpha1\beta1\gamma\delta$ -nAChRs ($K_i = 314$ nM) (Figure 1).¹³ Functionally, compound

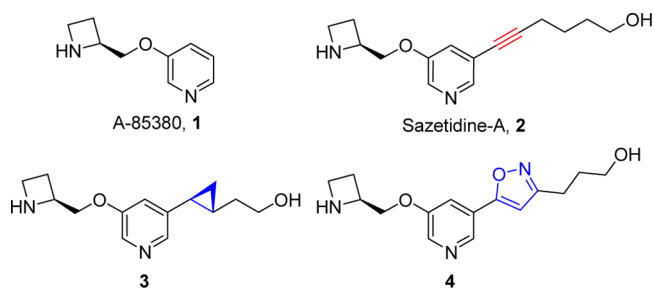


Figure 1. Structures of A-85380 and some of its analogues.

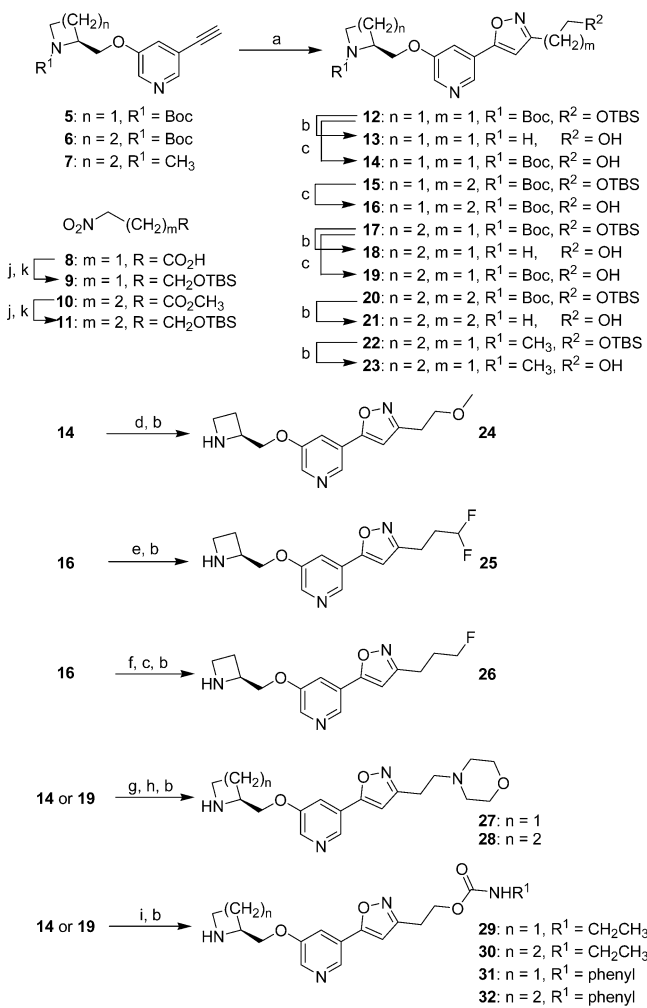
1 acts as a potent activator of both the human $\alpha4\beta2$ -nAChRs ($EC_{50} = 0.7$ μ M) and ganglionic nAChRs ($EC_{50} = 0.8$ μ M). It is also a full agonist of human $\alpha7$ -nAChRs ($EC_{50} = 8.9$ μ M) compared to nicotine. Sazetidine-A (**2**), an analogue of compound **1** having an alkynyl substituent attached to the 5-position of the pyridine ring, was identified as a highly potent partial $\alpha4\beta2$ -nAChR agonist. Compared to compound **1**, its selectivity relative to the ganglionic $\alpha3\beta4^*$ - and homomeric $\alpha7$ -nAChRs is highly improved. The robust antidepressant-like effects of compound **2** were confirmed in the forced swim, tail suspension, and novelty-induced hypophagia tests.^{9,14,15} However, the acetylenic bond in compound **2** may be oxidized to generate a labile, highly reactive oxirene. This metabolic transformation is a potential source of toxicity and discouraged further advancement of compound **2**. Novel analogues were designed to avoid the acetylene functional group while maintaining the important pharmacophoric elements of compound **2**. Previous work in our group has described the discovery of a novel series of $\alpha4\beta2$ -nAChRs partial agonists via replacing the metabolically unstable acetylene bond of compound **2** with a substituted cyclopropane or isoxazole ring, resulting in compounds **3** or **4**, which exhibited antidepressant-like efficacy in the mouse FST.^{16,17} Compound **4** is a highly selective $\alpha4\beta2$ -nAChR partial agonist with a K_i value of 0.7 nM and an EC_{50} value of 36 nM. The favorable *in vitro* and *in vivo* pharmacological profiles of these ligands

warrant further molecular optimization to create new antidepressant drug candidates. In this paper, we describe the synthesis, structure–activity relationship studies, and pharmacological evaluation of the isoxazolylopyridine ether series of nAChR ligands. Selected compounds were further assessed in behavioral tests relevant for the treatment of depression.

Rational Design and Synthesis of Isoxazolylopyridine Ether nAChR Ligands. In our previous studies of analogues of compound **4**, we found that a side chain length of two or three carbon atoms was optimal for biological activity.¹⁷ We thus chose to synthesize the isoxazolylopyridine ether ligands bearing a two or three carbon side chain counting from the isoxazole ring to the hydroxyl group or other functional group at the chain terminus. As compounds that contain hydroxyl groups are generally prone to metabolic clearance via glucuronidation and sulfation, we explored several approaches to lower this probability of metabolism-related liabilities. Our initial effort was to replace the hydroxyl group with a methoxy group. Given that the length of the C–F bond (1.41 Å) is similar to that of the C–O bond (1.43 Å), we next designed analogues in which the hydroxyl group was replaced by a fluoromethyl or difluoromethyl group. To better understand the SAR of the right-hand chain, analogues featuring a terminal morpholine ring, carbamate function, or alkyl group were designed.

The isoxazolylopyridine ether ligands **13**, **18**, **21**, and **23–32** were synthesized utilizing the routes shown in Scheme 1. The nitro compounds **9** and **11** were obtained in two steps from commercially available 3-nitropropionic acid or 4-nitrobutyric acid methyl ester. The desired isoxazoles **12**, **15**, **17**, **20**, and **22** were synthesized via 1,3-dipolar cycloaddition of nitrile oxides, derived from the nitro compounds, to the previously reported alkynes **5–7**. Subsequent deprotection of the precursors **12**, **17**, **20**, and **22** with TFA yielded the final compounds **13**, **18**, **21**, and **23**. The preparation of compounds **24–32** proceeded through the common intermediates **14**, **16**, and **19**, which were obtained by removal of the TBS group from **12**, **15**, or **17**. The methoxy analogue **24** was obtained from the alcohol **14** via methylation and subsequent deprotection. The aldehyde prepared from the alcohol **16** by Swern oxidation was converted to a *gem*-difluoride using the stable and mild reagent, (diethylamino)difluorosulfonium tetrafluoroborate (XtalFluor-E), in conjunction with $Et_3N \cdot 3HF$ as a promoter. After removal of the Boc group, the *gem*-difluoride **25** was obtained as its trifluoroacetate salt. The primary alcohols **14**, **16**, and **19** were transformed to the corresponding tosylates or iodides, followed by nucleophilic substitution with tetrabutylammonium fluoride (TBAF) or morpholine and removal of the Boc protecting group to afford the derivatives **26**, **27**, and **28**. The carbamate analogues **29–32** were prepared by reaction of **14** or **19** with the corresponding isocyanates. All the final compounds were obtained as trifluoroacetates and were purified by HPLC. The number of equivalents of TFA in these nonstoichiometric compounds was determined by elemental analysis.

The syntheses of the isoxazolylopyridine ethers **38**, **39**, **42**, and **43** are described in Scheme 2. Intermediate **34** was formed via the Sonogashira coupling of 3-(benzyloxy)-5-bromopyridine (**33**) and ethynyl(trimethyl)silane followed by the removal of the TMS group. The alkyne **34** underwent 1,3-dipolar cycloaddition with nitrile oxides to form the corresponding isoxazoles **35–37**. Sequential removal of the benzyl group and Mitsunobu reaction with Boc-protected 2(*S*)-azetidylmethanol afforded the trifluoroacetates **38** and **39** after removal of the

Scheme 1^a

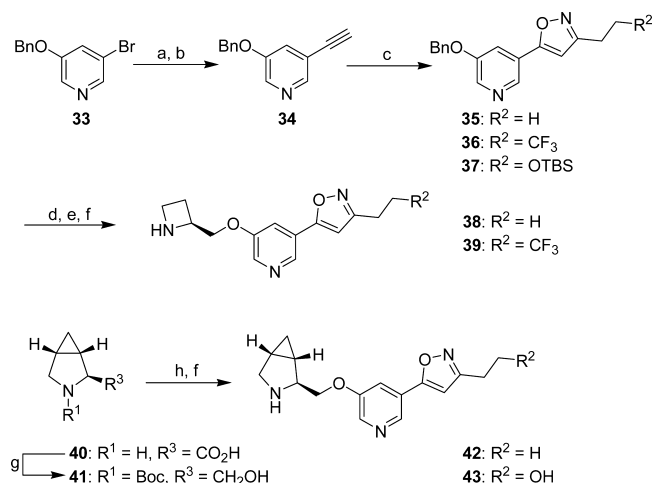
^aReagents and conditions: (a) **9** or **11**, PhNCO, Et₃N, PhMe; (b) TFA, CH₂Cl₂; (c) TBAF, THF; (d) NaH, CH₃I, DMF; (e) (i) (COCl)₂, DMSO, CH₂Cl₂, (ii) XtalFluor-E, Et₃N·3HF; (f) TsCl, Et₃N, DMAP, CH₂Cl₂; (g) I₂, PPh₃, imidazole, CH₂Cl₂; (h) morpholine, CH₃CN; (i) H₂NR¹, PhNCO; (j) BH₃·THF, THF; (k) TBSCl, DMAP, Et₃N, CH₂Cl₂.

Boc group under acidic conditions. Compounds **42** and **43** were synthesized by employing the same strategy starting from the commercially available amino acid **40** as shown in Scheme 2.

RESULTS AND DISCUSSION

In Vitro Characterization: Radioligand Binding Studies

In vitro binding affinities of the 17 compounds **13**, **18**, **21**, **23–32**, **38**, **39**, **42**, and **43** were determined by the standard [³H]epibatidine binding assay at seven rat nAChR subtypes.¹⁸ As shown in Table 1, these compounds generally demonstrated good selectivity for the β₂*-nAChRs (α₂β₂-, α₃β₂-, α₄β₂-, and α₄β₂*-nAChRs) over the β₄*-nAChRs (α₃β₄-, α₂β₄-, and α₄β₄-nAChRs) compared to nicotine and varenicline. Most of the compounds exhibited nanomolar or subnanomolar binding affinities for both the α₄β₂- and α₄β₂*-nAChRs while showing very weak binding affinity for α₃β₄-nAChRs with K_i values of greater than 7000 nM, indicating that unwanted side effects associated with the ganglionic α₃β₄-nAChR subtype would be unlikely. The previously reported azetidines **13** is very potent at

Scheme 2^a

^aReagents and conditions: (a) ethynyl(trimethyl)silane, CuI, PdCl₂(PPh₃)₂, PPh₃, Et₃N, 60 °C; (b) TBAF, THF; (c) O₂N-(CH₂)₃CF₃, O₂N(CH₂)₂CH₃, or **11**, PhNCO, Et₃N, PhMe; (d) 10% Pd/C, H₂; (e) 2-(hydroxymethyl)azetidines-1-carboxylic acid *tert*-butyl ester, azodicarbonyldipiperidine (ADDP), ^tBu₃P, PhMe; (f) TFA, CH₂Cl₂; (g) (i) Boc₂O, NaHCO₃, THF, H₂O, (ii) BH₃·THF, THF; (h) ADDP, ^tBu₃P, 5-[3-[2-[*tert*-butyl(dimethyl)silyloxy]ethyl]-isoxazol-5-yl]pyridin-3-ol, PhMe.

β₂*-nAChRs, with K_i values ranging from 0.6 to 15.4 nM. Ring expansion of the azetidines ring to a pyrrolidines ring (compound **13** vs compound **18**) resulted in at least 30-fold less potency at all the β₂*-nAChRs tested, including α₂β₂- (31.6 nM), α₄β₂- (23.1 nM), and α₄β₂*-nAChRs (176 nM). *N*-Methylation on the pyrrolidines ring (compound **23**) restored the binding affinities to those displayed by compound **13**. Similarly, chain elongation at the right-hand side alkoxy group of pyrrolidines **21** also gave low single-digit nanomolar binding affinities. Replacement of the azetidines ring with the 3-azabicyclo[3.1.0]hex-2-yl group as in **42** and **43** resulted in more than a 300-fold decrease in binding affinities at β₂*-nAChRs (213 and 296 nM at α₄β₂-nAChR). Taken together, the smaller azetidines ring was found to give more potent binding at β₂*-nAChRs compared to the larger pyrrolidines or bicyclic amine rings, with the exception of the *N*-methyl pyrrolidines **23**.

In reference to the isoxazolyl side chain, the *O*-methylation of compound **13** did not significantly alter binding affinities at the β₂*-nAChRs. Replacement of the terminal hydroxyl group with mono-, di-, or trifluoro substituents (compounds **25**, **26**, and **39**) in general maintained binding affinities. Deletion of the hydroxyl group (compound **38**) also resulted in similar binding affinities, indicating that the hydroxyl group does not comprise an essential pharmacophoric element for receptor recognition. However, substitution of the hydroxyl group with an ionizable morpholine ring (compound **27** vs **13**, **28** vs **18**) decreased the binding affinities. Ring expansion of the azetidines ring to the pyrrolidines **28** gave a further decrease (approximately 3-fold) in binding affinities, consistent with the previous observations recorded for the pyrrolidines **18**. The morpholine analogues **27** and **28** were inactive at all β₄*-nAChRs (α₂β₄-, α₃β₄-, and α₄β₄-). Attachment of a carbamoyl group at the terminal hydroxyl group (compounds **29–32**) maintained, in general, the subnanomolar to nanomolar binding affinities at the β₂*-nAChRs. In this carbamate series, the pyrrolidines **30** and **32** were again found to be approximately 5-fold less potent than

Table 1. Affinities of 17 Ligands for Rat nAChR Subtypes Defined by Competition for [³H]Epibatidine Binding

compd	K_i (nM) ^a						
	$\alpha 2\beta 2$	$\alpha 2\beta 4$	$\alpha 3\beta 2$	$\alpha 3\beta 4$	$\alpha 4\beta 2$	$\alpha 4\beta 2^*^b$	$\alpha 4\beta 4$
13	1.0 ± 0.2	935	15.4 ± 4.1	>10 ⁴	0.6 ± 0.1	3.0 ± 0.4	1790
18	31.6 ± 4.0	2820	1080	NA ^d	23.1 ± 2.8	176	NA
21	4.8 ± 0.8	4266	72.4 ± 13	NA	6.8 ± 2.5	10.2 ± 2.7	1288
23	3.2 ± 0.7	2399	93 ± 22	NA	1.3 ± 0.4	16.2 ± 2.8	912
24	1.7 ± 0.2	3631	22.9 ± 4.6	NA	1.1 ± 0.2	4.9 ± 0.6	637
25	0.9 ± 0.1	1023	11.7 ± 2.4	>10 ⁴	0.5 ± 0.1	4.0 ± 0.5	18.6 ± 2.8
26	1.0 ± 0.1	1445	19.5 ± 3.9	NA	0.4 ± 0.1	2.7 ± 0.3	170
27	12.3 ± 2.1	NA	117 ± 21	NA	11.0 ± 4.1	26.3 ± 6.8	NA
28	74 ± 13	NA	575	NA	32 ± 12	132	NA
29	1.4 ± 0.4	2042	12.3 ± 3.2	NA	0.5 ± 0.1	2.3 ± 0.3	355
30	5.8 ± 1.3	9550	87 ± 22	NA	2.3 ± 0.2	15.1 ± 2.3	1905
31	0.8 ± 0.2	513	9.8 ± 1.7	NA	0.4 ± 0.2	3.7 ± 1.0	102
32	6.2 ± 1.0	1413	60 ± 11	NA	2.0 ± 0.8	21.4 ± 5.5	513
38	0.5 ± 0.1	631	8.9 ± 1.8	7079	0.4 ± 0.1	1.7 ± 0.2	98 ± 14
39	1.1 ± 0.1	1188	33 ± 12	NA	0.6 ± 0.1	5.1 ± 0.6	300
42	285	NA	5967	NA	213	993	NA
43	449	NA	6433	NA	296	1313	NA
nicotine ^e	5.5	70	29	260	4.9	9.8	23
varenicline ^e				86	0.4		110

^aSee Experimental Section. SEM values are not provided for K_i values >100 nM. ^b $\alpha 4\beta 2^*$, endogenous receptors prepared from rat forebrain. Besides $\alpha 4$ and $\beta 2$, other unidentified subunits may also be present. Details are provided in the Experimental Section. ^c K_i values for nicotine are taken from the PDSP Assay Protocol Book. ^dNA: not active, defined as <50% inhibition of binding in the primary assay at 10 μ M. ^e K_i values for varenicline are from the literature.¹⁹

Table 2. Potencies and Efficacies of Ligand Agonism and Inactivation of Human $\alpha 4\beta 2$ -nAChRs^a

compd	EC ₅₀ (nM)	agonism			inactivation		
		pEC ₅₀	HS- $\alpha 4\beta 2$ efficacy (%)	LS- $\alpha 4\beta 2$ efficacy (%)	IC ₅₀ (nM)	pIC ₅₀	inactivation efficacy (%)
13	43	7.37 ± 0.19	69 ± 5.6	-7.8 ± 3.8	32	7.50 ± 0.26	68 ± 4.9
18	>100	<7	ND ^b	ND	>100	ND	ND
21	>100	<7	ND	ND	>100	ND	ND
23	98	7.01 ± 0.08	65 ± 7.9	-3.1 ± 5.1	110	6.96 ± 0.04	76 ± 1.4
24	95	7.02 ± 0.42	60 ± 4.9	-1.5 ± 3.1	98	7.01 ± 0.18	79 ± 5.6
25	66	7.18 ± 0.28	56 ± 6.6	-2.4 ± 4.2	79	7.10 ± 0.19	80 ± 5.7
26	38	7.42 ± 0.38	61 ± 6.2	-3.6 ± 3.9	50	7.30 ± 0.19	79 ± 4.8
27	>100	<7	ND	ND	>100	ND	ND
28	>100	<7	ND	ND	>100	ND	ND
29	28	7.55 ± 0.08	30 ± 6.5	1.2 ± 2.9	50	7.30 ± 0.19	81 ± 4.9
30	>100	<7	ND	ND	>100	ND	ND
31	35	7.45 ± 0.12	12 ± 6.1	4.8 ± 2.7	117	6.93 ± 0.21	85 ± 9
32	>100	<7	ND	ND	>100	ND	ND
38	51	7.29 ± 0.34	84 ± 4.1	-2.7 ± 2.6	32	7.50 ± 0.15	74 ± 3.1
39	54	7.27 ± 0.11	29 ± 4.7	0.14 ± 1.5	83	7.08 ± 0.09	92 ± 2.7
42	>100	<7	ND	ND	>100	ND	ND
43	>100	<7	ND	ND	>100	ND	ND
nicotine	295	6.53 ± 0.05	124 ± 8.5	70 ± 5.9	427	6.37 ± 0.06	92 ± 2.1
varenicline	1096	5.96 ± 0.18	106 ± 5.9	34 ± 3.3	39	7.41 ± 0.08	83 ± 0.95

^aSee Experimental Section for details. The term "inactivation" is used because compounds may be acting to desensitize receptors or as competitive or noncompetitive antagonists, and further work is needed to make such a distinction. Potencies (EC₅₀ or IC₅₀ values) and efficacies were measured for actions at a mixture of high-sensitivity (HS) and low-sensitivity (LS) $\alpha 4\beta 2$ -nAChRs. Reported errors are the standard error of the mean (SEM) for all values. ^bND: not determined. The efficacy was not determined if the EC₅₀ or the IC₅₀ value was greater than 100 nM.

the corresponding azetidines **29** and **31**. Additionally, three of the most potent $\alpha 4\beta 2$ - and $\alpha 4\beta 2^*$ -nAChR ligands, **13**, **25**, and **38**, were tested at $\alpha 7$ - and $\alpha 7^*$ -nAChRs ($\alpha 7^*$, endogenous receptors prepared from rat forebrain), where they were found to show no activity.

In Vitro Functional Characterization. In functional studies, all compounds were tested using ⁸⁶Rb⁺ ion efflux

assays and SH-EP1-h $\alpha 4\beta 2$ (expressing human $\alpha 4\beta 2$ -nAChRs), SH-SY5Y ($\alpha 3\beta 4^*$ -nAChRs), or TE671/RD ($\alpha 1\beta 1\gamma\delta$ -nAChRs) cells. Abilities of ligands to stimulate ion flux were characterized as EC₅₀ values, and inhibition by ligands following 10 min preincubation on ion flux responses to a challenge concentration of a nicotinic full agonist were characterized as inactivation IC₅₀ values. In general, the observed trends in

the binding affinities presented in Table 1 carried over to the functional assay data (Table 2). First, none of the ligands was active at $\alpha 3\beta 4^*$ - or $\alpha 1\beta 1\gamma\delta$ -nAChRs (EC_{50} or IC_{50} values were >1000 nM; data not shown because of this lack of activity). Acting at human $\alpha 4\beta 2$ -nAChRs (Table 2), azetidine **13** possesses an agonist EC_{50} value of 43 nM and an inactivation IC_{50} value of 32 nM. Compound **18**, which is the pyrrolidine analogue of compound **13**, was found to possess lower functional activities (EC_{50} and IC_{50} values greater than 100 nM). However, in contrast to the trends observed in the binding affinities for the pyrrolidines **21** and **23**, which are similar to those of compound **13**, the functional activities of these two compounds are lower than those of compound **13**. The methoxy analogue **24** was found to be 2-fold less active in the EC_{50} agonism assay and 3-fold less active in the IC_{50} inactivation assay. The mono-, di- and trifluoro analogues **25**, **26**, and **39** all gave very similar EC_{50} values to that of the azetidine **13**, but with approximately 2-fold less active IC_{50} values. The morpholine analogues **27** and **28** were found to be less active in the functional assays, consistent with the binding data. For the carbamate series (compounds **29–32**), the azetidines **29** and **31** have EC_{50} values similar to that of compound **13**, whereas the corresponding pyrrolidines **30** and **32** have lower functional activities. The bicyclic ring substituted analogues **42** and **43** were also found to be less active functionally in comparison to compound **13**, also consistent with their binding profiles. Interestingly, the simple ethyl analogue **38** showed the highest HS efficacy value of 84%, while the carbamates exhibited the lowest HS efficacies (**31**, 12%; **29**, 30%). None of the compounds displayed significant agonist or antagonist activity at ganglionic $\alpha 3\beta 4^*$ - or muscle-type $\alpha 1\beta 1\gamma\delta$ -nAChRs, even at the highest concentration tested (10 μ M).

It is informative to compare the SAR profile for the present isoxazolylpyridine ether series (Tables 1 and 2) to that reported previously for analogues of epibatidine (Figure 2). It has been

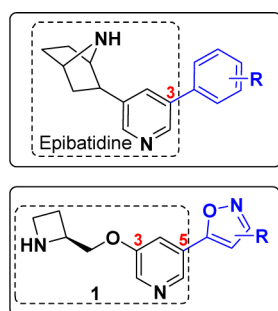


Figure 2. Structural comparison of phenyl-bearing epibatidine analogues and the isoxazolylpyridine ethers.

shown that the attachment of a phenyl ring to the 3-position of the pyridine ring present in epibatidine maintains approximately the same binding affinity at male rat cerebral cortex nAChRs.²⁰ This observed trend is consistent with our findings showing that the incorporation of an isoxazole ring at the 5-position of the isoxazolylpyridine scaffold does not dramatically alter the binding affinities at $\alpha 4\beta 2$ -nAChRs (Table 1) compared to the parent compound **1**,¹³ with the exception of the bicyclic amines **42** and **43**. Table 2 shows that the majority of the isoxazole-substituted analogues are potent partial agonists at $\alpha 4\beta 2$ -nAChRs, while the parent compound **1** was reported to be a potent, full agonist.²¹ Therefore, in the pyridine–ether series, it can be concluded that the appendage

of an isoxazole ring to the 5-position of the pyridine ring maintains binding affinities at $\alpha 4\beta 2$ -nAChRs while lowering the functional efficacy.

In Vivo Behavioral Studies: Mouse Forced Swim Test.

Antidepressant efficacy was assessed with the mouse forced swim test, an assay in which a mouse is placed into a beaker of water, and the time spent passively floating in the water (immobility) is recorded (Figure 3). Most traditional antidepressants decrease the amount of time the mouse spends immobile. Mice were administered compounds **25**, **26**, and **38** (5, 10, 30 mg/kg ip) or the SSRI antidepressant, sertraline, as a positive control (20 mg/kg). Drug administration produced a reduction in immobility, although it was not consistently dose-dependent. Compound **38** was next tested at lower doses (1 and 3 mg/kg). Fisher's post hoc tests showed that compounds **25**, **26**, and **38** reduced immobility at all three tested doses, suggestive of antidepressant-like effects in vivo.

CONCLUSIONS

In this paper, we describe the synthesis, pharmacological evaluation, and structure–activity relationships of a isoxazolylpyridine ether series of nAChR ligands. The majority of compounds belonging to this structural class were found to bind to both $\alpha 4\beta 2$ - and $\alpha 4\beta 2^*$ -nAChRs of rats with significantly higher affinity (K_i values ranging from 0.4 to 176 nM) than to rat $\alpha 3\beta 4$ -nAChRs ($K_i > 8000$ nM). In functional studies, these ligands acted as potent partial agonists at human $\alpha 4\beta 2$ -nAChRs and were totally inactive at both ganglionic $\alpha 3\beta 4^*$ - or muscle-type $\alpha 1\beta 1\gamma\delta$ -nAChRs. SAR analyses suggest that: (1) The appendage of an isoxazole ring to the 5-position of the pyridine nucleus of compound **1** maintained the binding affinities at $\alpha 4\beta 2$ -nAChRs while lowering functional agonist efficacy, consistent with observations made in studies of some epibatidine analogues. (2) The unsubstituted azetidine ring is superior to the unsubstituted pyrrolidine ring for both binding affinity and functional activity, which is consistent with our previous findings. In the previously reported piperidylpyridine ether series, no agonist activity was observed for either the *N*-methyl or NH pyrrolidine analogues.²² On the contrary, in the present isoxazolylpyridine ether series, the *N*-methyl pyrrolidine analogue **23** showed reasonable activity in the functional studies although its NH analogue **18** did not. (3) The unfunctionalized ethyl analogue **38** maintained biological activity comparable to the alcohol **13**, thus suggesting that the hydroxyl group does not play an essential role in receptor recognition. Moreover, methoxy, morpholine, carbamate, and mono-, di-, or trifluoro analogues were also found to be tolerated. In the mouse forced swim model, the difluoromethyl group compound **25**, fluoromethyl compound **26**, and ethyl compound **38** all demonstrated potent antidepressant-like properties at all tested doses. Thus, the isoxazolylpyridine ether scaffold functions as a versatile building block for achieving selectivity for the $\alpha 4\beta 2$ - over the $\alpha 3\beta 4$ -nAChRs. Various types of functionality can be built into this assembly in order to alter ADMET parameters as well as to potentially prepare compounds containing reporter groups for imaging purposes. On the basis of this advantageous selectivity profile, these $\alpha 4\beta 2$ -nAChR partial agonists warrant further study directed toward the design of a host of innovative chemical tools and potentially therapeutics.

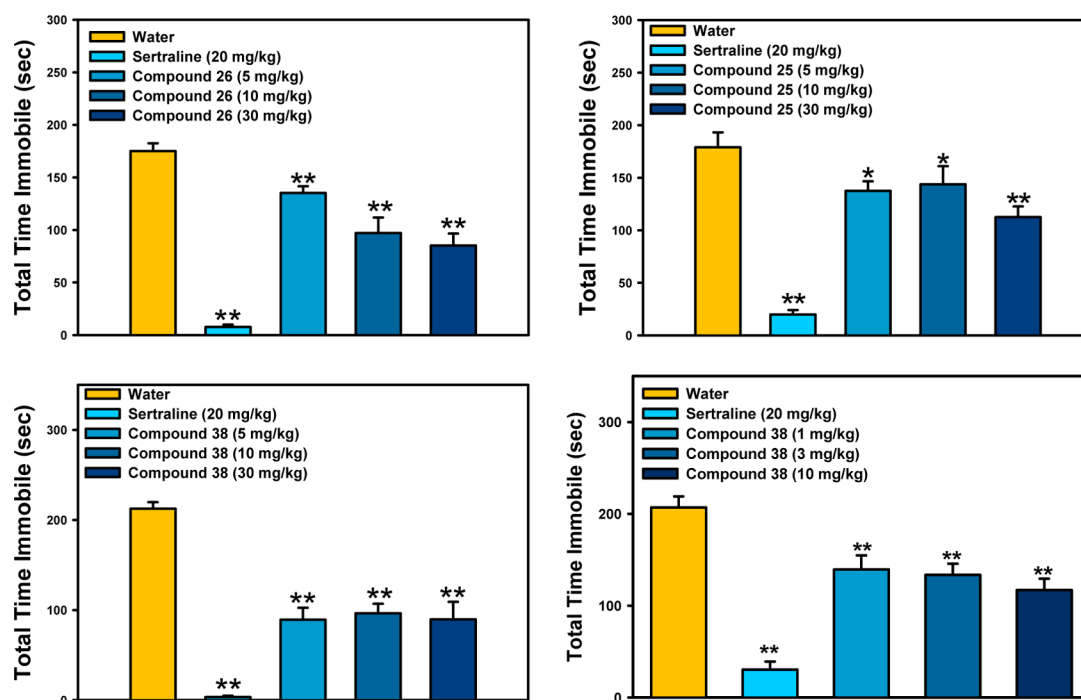


Figure 3. Compounds 25, 26, and 38 reduced immobility in the mouse forced swim test at all tested doses (5, 10, and 30 mg/kg for compounds 25 and 26; 1, 3, 5, 10, and 30 mg/kg for compound 38). The SSRI, sertraline, produced the expected decrease in immobility. ANOVAs: $F(4, 41) = 44.49$, $p < 0.0001$ (top left); $F(4, 42) = 23.40$, $p < 0.0001$ (top right); $F(4, 44) = 35.99$, $p < 0.0001$ (bottom left); $F(4, 44) = 32.57$, $p < 0.0001$ (bottom right). Fisher's PLSD posthoc test: ** p s < 0.0001 vs vehicle; * p s < 0.05 vs vehicle). $n = 9$ –10/group.

EXPERIMENTAL SECTION

Chemical Synthesis. General Methods. Starting materials, reagents, and solvents were purchased from commercial suppliers and used without further purification unless otherwise stated. Anhydrous THF and CH_2Cl_2 were obtained by distillation over sodium wire or CaH_2 , respectively. All nonaqueous reactions were run under an argon atmosphere with exclusion of moisture from reagents, and all reaction vessels were oven-dried. The progress of reactions was monitored by TLC on SiO_2 . Spots were visualized by their quenching of the fluorescence of an indicator admixed to the SiO_2 layer or by dipping into KMnO_4 solution followed by heating. SiO_2 for column chromatography (CC) was of 230–400 mesh particle size, and an EtOAc/hexane mixture or gradient was used for elution unless stated otherwise. ^1H NMR spectra were recorded at a spectrometer frequency of 300 or 400 MHz, ^{13}C NMR spectra at 75 or 100 MHz, and ^{19}F NMR spectra at 376 MHz. CDCl_3 , ^1H chemical shifts are reported in δ (ppm) using the δ 7.26 signal of CDCl_3 or the δ 4.80 signal of D_2O as internal standards. ^{13}C chemical shifts are reported in δ (ppm) using the δ 77.23 signal of CDCl_3 as internal standard. ^{13}C NMR spectra in D_2O were not adjusted. Purities of all final compounds (>98%) were established by analytical HPLC, which was carried out on an Agilent 1100 HPLC system with a Synergi 4 μ Hydro-RP 80A column, with detection at 280 or 254 nm on a variable wavelength detector G1314A; flow rate = 1.4 mL/min; gradient of 0–100% methanol in water (both containing 0.05 vol % of TFA) in 18 min. Final products were purified by preparative HPLC under the following conditions: column, ACE AQ, 250 mm \times 20 mm; flow, 17 mL/min; all solvents containing 0.05 vol % TFA; UV detection at 254 and 280 nm. Gradient I: 0–50% MeOH in water in 20 min, to 100% in 5 min, 100% for another 5 min, return to 0% in 5 min, and equilibration at 0% for 1 min. Gradient II: 8–100% MeOH in water in 30 min, 100% for 5 min, return to 25% in 4 min, and equilibration at 8% for 1 min.

General Procedure for the [3 + 2] Cycloaddition to Form Isoxazoles (Method A). To a solution of nitro compound (2.0–3.0 equiv) and alkyne (1.0 equiv) in anhydrous toluene were added phenyl isocyanate (1.1 equiv) and triethylamine (1.1 equiv). The reaction

mixture was stirred at 60 °C for 12–48 h. The reaction mixture was cooled to room temperature. After removal of the solvent, the residue was purified by CC on SiO_2 with hexane/acetone (2:1) to give the isoxazoles.

General Procedure for the Deprotection of *N*-Boc-Amines to Afford TFA Salts (Method B). To a solution of the *N*-Boc protected precursor (1 mmol) in CH_2Cl_2 (10 mL) was added TFA (1 mL) under argon with ice cooling. The mixture was stirred overnight at rt. After the solvent was evaporated, the residue was dissolved in distilled water (5 mL). The solution was filtered over a syringe filter (polytetrafluoroethylene, 17 mm diameter, 0.45 μm pore size) and then concentrated to 2–3 mL under reduced pressure at 30 °C bath temperature. The crude product was purified by preparative HPLC. After the solvent was evaporated, the residue was dissolved in distilled water (about 2–3 mL). The solution was lyophilized to obtain the TFA salt.

General Procedure for the Deprotection of TBS Ethers to Afford Free Alcohols (Method C). To a solution of the TBS-protected alcohol precursor (1 mmol) in THF (10 mL) was added 1 M tetrabutylammonium fluoride (TBAF) in THF (1 mL) under argon with ice cooling. After the mixture was stirred for 30 min at rt, it was diluted with water (20 mL) under ice cooling. The mixture was extracted with EtOAc (2 \times 30 mL), dried over anhydrous Na_2SO_4 , filtered, and concentrated under vacuum. The residue was purified by CC on SiO_2 to give the alcohol.

General Procedure for the Preparation of Iodides from Alcohols (Method D). To a stirred solution of an alcohol (1 mmol), imidazole (1.5 mmol), and PPh_3 (1.5 mmol) in anhydrous PhMe (8 mL) was added I_2 (1.5 mmol) with ice cooling under argon. After stirring overnight at rt, the solvent was evaporated. The residue was purified by CC on SiO_2 to give the iodide.

General Procedure for the Preparation of Morpholines from Iodides (Method E). To a solution of an iodide (1 mmol) in anhydrous CH_3CN (10 mL) was added morpholine (4 mmol) at rt. After stirring overnight, the reaction mixture was concentrated. The residue was purified by CC on SiO_2 ($\text{CH}_2\text{Cl}_2/\text{MeOH}$) to obtain the product.

General Procedure for the Preparation of Carbamates from Alcohols 14 and 19 (Method F). A solution of 14 or 19 (1 mmol), isocyanate (2 mmol), and 4-(dimethylamino)pyridine (DMAP; 0.1 mmol) in anhydrous toluene (5 mL) was stirred at 80 °C under Ar for 5 h. The solvent was removed under reduced pressure, and the residue was purified by CC on SiO₂ (acetone/hexane) to obtain the product.

General Procedure for O-Debenzylation to Afford Hydroxypyridines (Method G). To a solution of the Bn-protected hydroxypyridine (1 mmol) in MeOH (10 mL) was added 10% Pd/C (10%). A H₂ balloon was connected after the atmosphere was exchanged three times with Ar. The reaction was allowed to proceed at rt for 2 h, then the catalyst was filtered off over Celite. The filtrate was evaporated, and the residue was purified by CC on SiO₂ (CHCl₃/MeOH 30:1) to furnish the hydroxypyridine as a colorless oil.

General Procedure for the Mitsunobu Reaction of 2-(Hydroxymethyl)azetidine-1-carboxylic Acid tert-Butyl Ester or Compound 41 with Hydroxypyridines to Afford Alkoxyalpyridines (Method H). In a 100 mL side arm flask with stir bar and argon balloon, tri-*n*-butylphosphine (1 mmol) was added dropwise in 5 min to a solution of *N,N'*-azodicarbonyldipiperidine (1 mmol) in anhydrous toluene (5 mL). Stirring was continued at rt for 30 min to complete the formation of the Mitsunobu reagent. A solution of the hydroxypyridine intermediate (0.6 mmol) and 2-(hydroxymethyl)azetidine-1-carboxylic acid tert-butyl ester or 41 (1 mmol) in anhydrous toluene (10 mL) was added to the Mitsunobu reagent at 0 °C within 45 min. The mixture was warmed to rt and stirred overnight. After removal of the solvent, the residue was purified by CC on SiO₂ (hexane/EtOAc 2:1 to 1:1) to furnish the desired product.

General Procedure for the Reduction of Acids or Acid Esters to Alcohols (Method I). To a solution of acid or acid ester (4 mmol) in 20 mL anhydrous THF was added under argon with ice cooling a 1 M solution of borane in THF (5 mL). The reaction mixture was stirred overnight at rt and then quenched with satd NH₄Cl solution followed by extraction with EtOAc (2 × 20 mL). The combined organic layers were washed with water and brine, dried over Na₂SO₄, and evaporated to obtain the alcohol, which was used without further purification.

General Procedure for the Protection of Alcohols with TBS (Method J). To a solution of alcohol (3 mmol) and TBSCl (3 mmol) in 20 mL of anhydrous CH₂Cl₂ were added DMAP (0.1 mmol) and Et₃N (3 mmol). The reaction mixture was stirred overnight at rt. After removal of the solvent, the residue was purified by CC on SiO₂ (hexane/EtOAc 4:1) to obtain the desired product.

2(S)-[[5-(5-Ethynylpyridin-3-yloxy)methyl]pyrrolidine-1-carboxylic Acid tert-Butyl Ester (6) and 3-Ethynyl-5-[(1-methyl-2(S)-pyrrolidinyl)methoxy]pyridine (7). These two compounds were synthesized according to the previously reported methods for compound 5.¹⁷ Compound 6: ¹H NMR (400 MHz, CDCl₃) δ 8.01 (br, 2H), 7.11 (br, 1H), 3.93–3.67 (m, 3H), 3.13 (m, 3H), 1.80–1.65 (m, 4H), 1.26 (s, 9H). Compound 7: ¹H NMR (400 MHz, CDCl₃) δ 8.26 (s, 1H), 8.23 (s, 1H), 7.24 (s, 1H), 3.95 (m, 1H), 3.88 (m, 1H), 3.17 (s, 1H), 3.07 (t, 1H, J = 8.0 Hz), 2.62 (m, 1H), 2.42 (s, 3H), 2.27 (m, 1H), 1.98 (m, 1H), 1.78–1.52 (m, 3H). ¹³C NMR (100 MHz, CDCl₃): δ 154.6, 145.1, 138.3, 123.8, 119.5, 80.6, 80.3, 71.3, 64.2, 57.8, 41.8, 28.6, 23.1.

tert-Butyl(dimethyl)(3-nitropropoxy)silane (9). This compound was obtained from 8 employing methods I and J; pale-yellow oil; yield 56% over two steps. ¹H NMR (400 MHz, CDCl₃) δ 4.50 (t, 2H, J = 6.8 Hz), 3.71 (t, 2H, J = 5.6 Hz), 2.20 (m, 2H), 0.89 (s, 9H), 0.05 (s, 6H).

tert-Butyl(dimethyl)(4-nitrobutoxy)silane (11). This compound was obtained from 10 employing methods I and J; pale-yellow oil; yield 62% in two steps. ¹H NMR (400 MHz, CDCl₃) δ 4.43 (t, 2H, J = 6.0 Hz), 3.66 (t, 2H, J = 4.0 Hz), 2.10 (m, 2H), 1.60 (m, 2H), 0.90 (s, 9H), 0.06 (s, 6H).

2-[5-[5-(S)Azetidin-2-ylmethoxy]-3-pyridinyl]-3-isoxazoly]ethanol (13). This compound was obtained from 12¹⁷ employing method B and gradient I; colorless oil; yield 36% over two steps; purity 99.7%; [α]_D²⁰ –3.6 (c = 0.72, MeOH). ¹H NMR (400 MHz, D₂O) δ 8.94 (s, 1H), 8.67 (s, 1H), 8.59 (s, 1H), 7.12 (s, 1H), 5.03–5.01 (m, 1H), 4.65 (d, 2H, J = 2.0 Hz), 4.17–4.08 (m, 1H), 3.94 (t, 2H, J = 6.0 Hz), 3.00 (t, 2H, J = 6.4 Hz), 2.75–2.69 (m, 2H). ¹³C

NMR (100 MHz, D₂O) δ 163.4, 162.6, 162.3 (TFA), 156.3, 131.7, 130.0, 127.5, 127.4, 115.8 (TFA), 104.2, 67.7, 58.9, 58.1, 43.3, 28.0, 19.8. Anal. Calcd for C₁₄H₁₇N₃O₃·1.65TFA·0.7H₂O (FW 476): C, 43.65; H, 4.25; F, 19.75; N, 8.83. Found: C, 43.28; H, 3.87; F, 19.53; N, 8.56.

2(S)-[[5-[3-(2-Hydroxyethyl)-5-isoxazoly]-3-pyridinyl]oxy]methyl]azetidine-1-carboxylic Acid tert-Butyl Ester (14). ¹H NMR (400 MHz, CDCl₃) δ 8.48 (s, 1H), 8.28 (s, 1H), 7.53 (s, 1H), 6.55 (s, 1H), 4.48 (m, 1H), 4.32 (m, 1H), 4.11 (dd, 2H, J = 2.8, 10.0 Hz), 3.92 (t, 2H, J = 6.0 Hz), 3.83 (t, 2H, J = 7.6 Hz), 3.65 (br, 1H), 2.91 (t, 2H, J = 6.2 Hz), 2.33–2.21 (m, 2H), 1.33 (s, 9H).

2(S)-[[5-[3-[3-(tert-Butyl(dimethyl)silyloxy]propyl)-5-isoxazoly]pyridin-3-yloxy]methyl]azetidine-1-carboxylic Acid tert-butyl Ester (16). This was obtained from 15¹⁷ employing method C; colorless oil; yield 62%. ¹H NMR (400 MHz, CDCl₃) δ 8.48 (s, 1H), 8.27 (s, 1H), 7.49 (s, 1H), 6.44 (s, 1H), 4.42 (m, 1H), 4.28 (m, 1H), 4.07 (m, 1H), 3.76 (t, 2H, J = 7.0 Hz), 3.66 (br, 1H), 3.60 (m, 2H), 2.72 (t, 2H, J = 7.8 Hz), 2.29–2.18 (m, 2H), 1.85 (m, 2H), 1.26 (s, 9H). ¹³C NMR (100 MHz, CDCl₃) δ 171.0, 166.4, 164.4, 156.1, 139.3, 117.4, 100.8, 79.7, 68.8, 61.1, 60.2, 47.2, 30.9, 28.2, 22.5, 20.9, 18.9, 14.0.

2(S)-[[5-[3-[2-(tert-Butyl(dimethyl)silyloxy]ethyl)-5-isoxazoly]pyridin-3-yloxy]methyl]pyrrolidine-1-carboxylic Acid tert-Butyl Ester (17). ¹H NMR (400 MHz, CDCl₃) δ 8.53 (s, 1H), 8.30 (s, 1H), 7.66–7.50 (m, 1H), 6.56 (s, 1H), 4.40–3.98 (m, 3H), 3.89 (t, 2H, J = 6.0 Hz), 3.38–3.29 (m, 2H), 2.89 (t, 2H, J = 6.0 Hz), 1.66–1.59 (m, 4H), 1.46 (s, 9H), 0.84 (s, 9H), 0.00 (s, 6H).

2-[5-[5-(2(S)Pyrrolidinylmethoxy)-3-pyridinyl]-3-isoxazoly]ethanol (18). This compound was obtained from 17 employing method B and gradient I; colorless oil; yield 31% over two steps; purity 99.6%; [α]_D²⁰ 4.8 (c = 0.99, MeOH). ¹H NMR (400 MHz, D₂O) δ 8.94 (s, 1H), 8.64 (s, 1H), 8.54 (s, 1H), 7.13 (s, 1H), 4.70 (dd, 2H, J = 10.4, 3.2 Hz), 4.49 (dd, 2H, J = 10.0, 7.6 Hz), 4.20 (m, 1H), 3.96 (t, 2H, J = 8.0 Hz), 3.44 (t, 2H, J = 7.2 Hz), 3.02 (t, 2H, J = 6.2 Hz), 2.33 (m, 1H), 2.17–2.11 (m, 2H), 2.02–1.97 (m, 1H). ¹³C NMR (100 MHz, D₂O) δ 163.4, 162.7, 162.4 (TFA), 156.2, 131.9, 127.4, 127.2, 115.8 (TFA), 104.1, 67.8, 58.9, 57.9, 45.6, 28.0, 25.4, 23.0. Anal. Calcd for C₁₅H₁₉N₃O₃·2.35TFA (FW 557): C, 42.46; H, 3.86; F, 24.03; N, 7.54. Found: C, 42.66; H, 3.85; F, 24.17; N, 7.68.

2(S)-[[5-[3-(2-Hydroxyethyl)-5-isoxazoly]-3-pyridinyl]oxy]methyl]pyrrolidine-1-carboxylic Acid tert-Butyl Ester (19). ¹H NMR (400 MHz, CDCl₃) δ 8.52 (s, 1H), 8.29 (s, 1H), 7.70–7.57 (m, 1H), 6.62–6.57 (m, 1H), 4.22–4.12 (m, 3H), 3.97–3.89 (m, 3H), 3.36–3.31 (m, 3H), 2.72 (t, 2H, J = 6.2 Hz), 1.95–1.84 (m, 2H), 1.42 (s, 9H).

3-[5-[5-(2(S)Pyrrolidinylmethoxy)-3-pyridinyl]-3-isoxazoly]-1-propanol (21). This compound was obtained from 6 and 11 employing methods A and B and gradient I; colorless oil; yield 31% over two steps; purity 99.7%; [α]_D²⁰ 5.4 (c = 0.5, MeOH). ¹H NMR (400 MHz, D₂O) δ 8.92 (s, 1H), 8.63 (s, 1H), 8.53 (s, 1H), 7.08 (s, 1H), 4.69 (dd, 2H, J = 10.8, 3.2 Hz), 4.49 (dd, 2H, J = 10.4, 7.6 Hz), 4.20 (m, 1H), 3.66 (t, 2H, J = 6.4 Hz), 3.44 (t, 2H, J = 7.2 Hz), 2.84 (t, 2H, J = 7.6 Hz), 2.32 (m, 1H), 2.11–2.00 (m, 2H), 2.00–1.94 (m, 1H). ¹³C NMR (100 MHz, D₂O) δ 165.4, 162.5, 162.2 (TFA), 156.2, 131.7, 129.9, 127.5, 127.2, 117.3 (TFA), 104.0, 67.8, 60.2, 57.9, 45.6, 41.2, 29.2, 25.4, 23.0, 21.5. Anal. Calcd for C₁₆H₂₁N₃O₃·1.8TFA·0.8H₂O (FW 523): C, 45.01; H, 4.70; F, 19.62; N, 8.03. Found: C, 45.10; H, 4.47; F, 19.76; N, 7.81.

2-[5-[5-[(1-Methyl-2(S)-pyrrolidinyl)methoxy]-3-pyridinyl]-3-isoxazoly]ethanol (23). This compound was obtained from 7 and 9 employing methods A and B and gradient I; colorless oil; yield 14% in two steps; purity 99.5%; [α]_D²⁰ –2.3 (c = 0.61, MeOH). ¹H NMR (400 MHz, D₂O) δ 8.91 (s, 1H), 8.61 (s, 1H), 8.53 (s, 1H), 7.08 (s, 1H), 4.70 (dd, 1H, J = 2.6, 11.2 Hz), 4.54 (dd, 1H, J = 6.0, 10.8 Hz), 3.96 (m, 1H), 3.90 (t, 2H, J = 6.0 Hz), 3.73 (m, 1H), 3.22 (m, 1H), 3.02 (s, 3H), 2.97 (t, 2H, J = 6.2 Hz), 2.39 (m, 1H), 2.18 (m, 1H), 2.08 (m, 2H). ¹³C NMR (100 MHz, D₂O) δ 163.4, 162.4, 162.3 (TFA), 156.1, 131.8, 129.8, 127.5, 127.4, 115.7 (TFA), 104.2, 66.8, 66.5, 58.9, 56.8, 40.2, 28.0, 25.4, 21.7. Anal. Calcd for C₁₆H₂₁N₃O₃·2.6TFA (FW 556): C, 42.45; H, 3.97; N, 7.01. Found: C, 42.47; H, 3.78; N, 7.01.

3-(2(*S*)-Azetidinylmethoxy)-5-[3-(2-methoxyethyl)-5-isoxazolyl]pyridine (**24**). To a solution of compound **14** (150 mg, 0.4 mmol) in anhydrous DMF (3 mL) were added NaH (32 mg, 0.8 mmol, 60% in oil) under argon with ice cooling. After stirring at rt for 30 min, CH₃I was added. The reaction mixture was quenched with satd NH₄Cl and extracted with EtOAc (20 mL). The organic phase was washed with water (2 × 15 mL), dried over Na₂SO₄, and concentrated. The residue was purified by CC on SiO₂ (hexane/EtOAc) to obtain 2-[[5-[3-(2-methoxyethyl)isoxazol-5-yl]pyridin-3-yloxy]methyl]azetidone-1-carboxylic acid *tert*-butyl ester (130 mg, 84%) as a colorless oil. ¹H NMR (400 MHz, CDCl₃) δ 8.56 (s, 1H), 8.33 (s, 1H), 7.56 (s, 1H), 6.54 (s, 1H), 4.50 (m, 1H), 4.35 (m, 1H), 4.15 (dd, 1H, *J* = 2.4, 10.0 Hz), 3.85 (t, 2H, *J* = 7.6 Hz), 3.66 (t, 2H, *J* = 6.2 Hz), 3.34 (s, 3H), 2.95 (t, 2H, *J* = 6.4 Hz), 2.35–2.22 (m, 2H), 1.36 (s, 9H). ¹³C NMR (100 MHz, CDCl₃) δ 166.7, 152.5, 156.3, 155.2, 139.7, 139.5, 124.3, 117.6, 101.3, 79.9, 70.5, 58.8, 47.4, 28.5, 26.8, 19.2.

The title compound was obtained from the preceding intermediate employing method B and gradient I; colorless oil; yield 45%; purity 99.7%; [α]_D²⁰ –3.5 (*c* = 0.97, MeOH). ¹H NMR (400 MHz, D₂O) δ 8.91 (s, 1H), 8.67 (d, 1H, *J* = 1.6 Hz), 8.56 (s, 1H), 7.09 (s, 1H), 5.01 (m, 1H), 4.64 (d, 2H, *J* = 3.6 Hz), 4.12 (m, 2H), 3.79 (t, 2H, *J* = 6.0 Hz), 3.34 (s, 3H), 3.02 (t, 2H, *J* = 6.0 Hz), 2.71 (q, 2H, *J* = 8.4 Hz). ¹³C NMR (100 MHz, D₂O) δ 163.3, 162.6, 161.7 (TFA), 156.3, 131.7, 130.1, 127.4, 127.3, 115.9 (TFA), 104.1, 69.1, 67.7, 58.2, 57.4, 43.3, 40.5, 25.3, 19.8. Anal. Calcd for C₁₅H₁₆N₃O₃·2.05TFA·1H₂O (FW 541): C, 42.40; H, 4.29; F, 21.59; N, 7.77. Found: C, 42.21; H, 4.09; F, 21.41; N, 7.82.

3-(2(*S*)-Azetidinylmethoxy)-5-[3-(3,3-difluoropropyl)-5-isoxazolyl]pyridine (**25**). To a solution of oxalyl chloride (54 μL, 0.61 mmol) in anhydrous CH₂Cl₂ (5 mL) was added dropwise at –78 °C, a solution of anhydrous DMSO (54 μL, 0.72 mmol) in anhydrous CH₂Cl₂ (2 mL). This Swern reagent solution was stirred for another 15 min at the same temperature. A solution of alcohol **16** (140 mg, 0.36 mmol) in anhydrous CH₂Cl₂ (2 mL) was added in 10 min. After stirring for 20 min, Et₃N (310 μL, 2.2 mmol) was added within 5 min. After maintaining the temperature for another 20 min, the reaction mixture was warmed slowly to rt. The mixture was washed with water (2 × 5 mL), dried over Na₂SO₄, and concentrated to obtain the aldehyde, which was carried on without further purification.

To a solution of triethylamine trihydrofluoride (120 μL, 0.72 mmol) in CH₂Cl₂ (2 mL) was successively added XtalFluor-E (125 mg, 0.54 mmol). A solution of the crude aldehyde in anhydrous CH₂Cl₂ (2 mL) was slowly added, and the resulting mixture was stirred at rt for 2 h under argon. The reaction mixture was quenched with 5% aqueous Na₂CO₃ solution and extracted with CH₂Cl₂ (20 mL). The combined organic layers were washed with H₂O, brine, dried over Na₂SO₄, and evaporated. The residue was purified by CC on SiO₂ (hexane/acetone 4:1) to afford the desired product (105 mg, 71%, two steps) as a colorless oil. ¹H NMR (400 MHz, CDCl₃) δ 8.56 (s, 1H), 8.36 (s, 1H), 7.59 (s, 1H), 6.50 (s, 1H), 5.94 (tt, 1H, *J*_{H–H} = 4.2 Hz, *J*_{H–F} = 56.4 Hz), 4.51 (m, 1H), 4.38 (m, 1H), 4.17 (dd, *J* = 2.8, 10.0 Hz), 3.87 (t, 2H, *J* = 7.6 Hz), 2.89 (t, 2H, *J* = 8.4 Hz), 2.37–2.20 (m, 4H), 1.38 (s, 9H). ¹³C NMR (100 MHz, CDCl₃) δ 167.3, 162.8, 156.4, 155.3, 140.0, 139.5, 124.1, 117.7, 116.2 (*t*, *J*_{C–F} = 240.0 Hz), 100.6, 80.0, 69.1, 60.1, 47.3, 32.3 (*t*, *J*_{C–F} = 21.8 Hz), 28.5, 19.3 (*t*, *J*_{C–F} = 6.4 Hz), 19.2. ¹⁹F NMR (376 MHz, CDCl₃) δ –117.4 (dt, 2F, *J*_{H–F} = 56.6, 17.1 Hz).

The title compound was obtained from the preceding intermediate employing method B and gradient II; colorless oil; yield 65%; purity 99.7%; [α]_D²⁰ –2.8 (*c* = 1.2, MeOH). ¹H NMR (400 MHz, D₂O) δ 8.90 (s, 1H), 8.68 (s, 1H), 8.52 (s, 1H), 6.05 (t, 1H, *J*_{H–F} = 56.2 Hz), 5.04 (m, 1H), 4.65 (d, 2H, *J* = 3.2 Hz), 4.15 (m, 2H), 2.95 (t, 2H, *J* = 5.8 Hz), 2.74 (q, 2H, *J* = 8.4 Hz), 2.30 (m, 2H). ¹³C NMR (100 MHz, D₂O) δ 164.3, 162.8, 162.3 (TFA), 132.2, 130.6, 127.1, 126.7, 116.6 (*t*, *J*_{C–F} = 240.0 Hz), 115.8 (TFA), 103.8, 67.6, 58.1, 43.3, 30.8 (*t*, *J*_{C–F} = 21.4 Hz), 19.9, 18.0 (*t*, *J*_{C–F} = 6.6 Hz). ¹⁹F NMR (376 MHz, D₂O) δ –75.6 (TFA), –117.3 (dt, 2F, *J*_{H–F} = 56.4, 18.8 Hz). Anal. Calcd for C₁₅H₁₇F₂N₃O₂·1.9TFA·0.75H₂O (FW 539): C, 41.86; H, 3.81; F, 27.12; N, 7.79. Found: C, 42.01; H, 3.84; F, 27.23; N, 7.61.

3-(2(*S*)-Azetidinylmethoxy)-5-[3-(3-fluoropropyl)-5-isoxazolyl]pyridine (**26**). To the solution of **16** (200 mg, 0.5 mmol), DMAP (6

mg, 0.05 mmol), and Et₃N (0.17 mL, 1.25 mmol) in 10 mL of anhydrous CH₂Cl₂ was added under argon with ice cooling *p*-TsCl (130 mg, 0.65 mmol). The reaction mixture was stirred overnight at rt. Purification by CC on SiO₂ (hexane/EtOAc) afforded the intermediate *p*-toluenesulfonate (240 mg, 86%) as a colorless oil. ¹H NMR (400 MHz, CDCl₃) δ 8.46 (s, 1H), 8.28 (s, 1H), 7.66 (d, 2H, *J* = 8.0 Hz), 7.49 (s, 1H), 7.21 (d, 2H, *J* = 7.6 Hz), 6.37 (s, 1H), 4.44 (m, 1H), 4.31 (m, 1H), 4.09 (dd, 1H, *J* = 1.2, 10.0 Hz), 4.02 (t, 2H, *J* = 6.0 Hz), 3.79 (t, 2H, *J* = 7.4 Hz), 2.67 (t, 2H, *J* = 7.4 Hz), 2.30 (s, 3H), 2.26–2.21 (m, 2H), 1.99 (m, 2H), 1.30 (s, 9H). ¹³C NMR (100 MHz, CDCl₃) δ 166.7, 163.0, 156.1, 155.1, 144.8, 139.6, 139.2, 132.7, 129.8, 127.7, 117.3, 100.6, 79.6, 69.2, 68.8, 60.0, 53.5, 47.1, 43.9, 28.3, 27.1, 22.1, 21.5, 19.0. To a solution of the above *p*-toluenesulfonate (240 mg, 0.44 mmol) in 2 mL of anhydrous THF was added at rt 1 M TBAF in THF (3 mL, 3 mmol). After stirring for 3 h, the reaction mixture was quenched with satd NH₄Cl and extracted with EtOAc (2 × 15 mL). The combined organic phases were washed with brine, dried over Na₂SO₄, and evaporated. The residue was purified by CC on SiO₂ (CH₂Cl₂/MeOH 98:2) to afford the desired product (110 mg, 64%) as a colorless oil. ¹H NMR (400 MHz, CDCl₃) δ 8.51 (s, 1H), 8.30 (s, 1H), 7.54 (s, 1H), 6.45 (s, 1H), 4.45 (dt, 1H, *J*_{H–F} = 46.8 Hz, *J*_{H–H} = 5.8 Hz), 4.46 (m, 1H), 4.33 (m, 1H), 4.12 (dd, 1H, *J* = 2.8, 10.0 Hz), 3.81 (t, 2H, *J* = 7.6 Hz), 2.79 (t, 2H, *J* = 7.6 Hz), 2.30 (m, 2H), 2.04 (m, 2H), 1.32 (s, 9H). ¹³C NMR (100 MHz, CDCl₃) δ 166.8, 163.6, 156.2, 155.2, 139.7, 139.3, 124.1, 117.5, 100.7, 82.7 (d, *J*_{C–F} = 164.8 Hz), 79.7, 68.9, 60.2, 53.6, 43.9, 28.8 (d, *J*_{C–F} = 20.1 Hz), 28.4, 22.1 (d, *J*_{C–F} = 5.5 Hz), 19.0.

The title compound was obtained from the preceding intermediate employing method B and gradient II; colorless oil; yield 97%; purity 99.4%; [α]_D²⁰ –3.2 (*c* = 1.99, MeOH). ¹H NMR (400 MHz, D₂O) δ 8.89 (s, 1H), 8.67 (s, 1H), 8.52 (s, 1H), 7.07 (s, 1H), 5.03 (m, 1H), 4.66–4.60 (m, 3H), 4.50 (t, 1H, *J* = 5.4 Hz), 4.15 (m, 2H), 2.87 (t, 2H, *J* = 7.4 Hz), 2.74 (t, 2H, *J* = 8.4 Hz), 2.17–2.04 (m, 2H). ¹³C NMR (100 MHz, D₂O) δ 165.4, 163.0, 162.3 (TFA), 156.5, 132.4, 130.9, 127.6, 127.1, 115.5 (TFA), 104.3, 83.9 (d, *J*_{C–F} = 158.4 Hz), 68.0, 58.5, 43.7, 27.9 (d, *J*_{C–F} = 19.5 Hz), 21.4 (d, *J*_{C–F} = 5.6 Hz), 20.2. ¹⁹F NMR (376 MHz, D₂O) δ –74.2 (TFA), –217.8. Anal. Calcd for C₁₅H₁₈FN₃O₂·1.65TFA·1.5H₂O (FW 506): C, 43.40; H, 4.51; F, 22.32; N, 8.30. Found: C, 43.36; H, 4.13; F, 22.05; N, 7.91.

4-[2-[5-[5-(2(*S*)-Azetidinylmethoxy)-3-pyridinyl]-3-isoxazolyl]ethyl]morpholine (**27**). This compound was obtained from **14** employing methods D, E, and B and gradient II; colorless oil; yield 63% over three steps; purity 99.4%; [α]_D²⁰ –4.5 (*c* = 0.62, MeOH). ¹H NMR (400 MHz, D₂O) δ 8.95 (s, 1H), 8.70 (s, 1H), 8.58 (s, 1H), 7.16 (s, 1H), 5.04–5.02 (m, 1H), 4.65 (d, 2H, *J* = 2.0 Hz), 4.18–4.10 (m, 1H), 3.85 (t, 2H, *J* = 12.4 Hz), 3.68–3.62 (m, 4H), 3.37–3.26 (m, 4H), 2.73 (q, 2H, *J* = 12.4 Hz). ¹³C NMR (100 MHz, D₂O) δ 163.4, 162.2 (TFA), 160.8, 156.3, 131.9, 130.4, 127.3, 127.1, 115.8 (TFA), 103.9, 63.2, 58.1, 53.9, 51.4, 43.3, 20.2, 19.8. Anal. Calcd for C₁₈H₂₄N₄O₃·2.95TFA·0.65H₂O (FW 692): C, 41.45; H, 4.11; F, 24.28; N, 8.09. Found: C, 41.07; H, 3.73; F, 23.91; N, 7.90.

4-[2-[5-[5-(2(*S*)-Pyrrolidinylmethoxy)-3-pyridinyl]-3-isoxazolyl]ethyl]morpholine (**28**). This compound was obtained from **19** employing methods D, E, and B and gradient II; colorless oil; yield 57% over three steps; purity 98.8%; [α]_D²⁰ –1.4 (*c* = 0.36, MeOH). ¹H NMR (400 MHz, D₂O) δ 8.89 (s, 1H, *J* = 2.0 Hz), 8.64 (d, 1H, *J* = 2.0 Hz), 8.54 (s, 1H), 7.14 (s, 1H), 4.68–4.66 (m, 1H), 4.47 (m, 1H), 4.15–4.11 (m, 3H), 3.83 (t, 2H, *J* = 12.4 Hz), 3.66–3.60 (m, 4H), 3.42 (t, 2H, *J* = 7.2 Hz), 3.35–3.28 (m, 4H), 2.32–2.30 (m, 1H), 2.17–2.09 (m, 1H), 2.00–1.98 (m, 1H). ¹³C NMR (100 MHz, D₂O) δ 163.3, 162.2 (TFA), 160.8, 156.2, 131.7, 130.2, 127.3, 127.1, 115.6 (TFA), 103.9, 99.6, 67.8, 63.2, 57.9, 53.9, 51.4, 45.6, 25.3, 23.0, 20.2. Anal. Calcd for C₁₉H₂₆N₄O₃·3TFA·0.5H₂O: C, 42.32; H, 4.26; F, 24.1; N, 7.90. Found: C, 42.34; H, 4.15; F, 24.07; N, 7.79.

Ethylcarbamate 2-[5-[5-(2(*S*)-Azetidinylmethoxy)-3-pyridinyl]-3-isoxazolylethyl Ester (**29**). This compound was obtained from **14** and ethyl isocyanate employing methods F and B and gradient I; colorless oil; yield 77% over two steps; purity 99.5%; [α]_D²⁰ –3.6 (*c* = 0.39, MeOH). ¹H NMR (400 MHz, D₂O) δ 8.87 (s, 1H), 8.70 (s, 1H), 8.50 (s, 1H), 7.11 (s, 1H), 5.03–4.99 (m, 1H), 4.63 (d, 2H, *J* =

3.6 Hz), 4.29 (t, 2H, $J = 5.6$ Hz), 4.19–4.06 (m, 2H), 3.05–2.96 (m, 4H), 2.71 (q, 2H, $J = 8.4$ Hz), 0.95 (t, 2H, $J = 7.2$ Hz). ^{13}C NMR (100 MHz, D_2O) δ 162.7, 161.8 (TFA), 157.4, 156.2, 131.9, 130.5, 127.1, 126.8, 115.8 (TFA), 104.1, 67.6, 61.9, 58.1, 43.3, 35.0, 25.2, 19.8, 13.6. Anal. Calcd for $\text{C}_{17}\text{H}_{22}\text{N}_4\text{O}_4 \cdot 2.05\text{TFA} \cdot 0.15\text{H}_2\text{O}$ (FW 583): C, 43.48; H, 4.21; F, 20.05; N, 9.61. Found: C, 43.43; H, 4.03; F, 19.87; N, 9.67.

Ethylcarbamic Acid 2-[5-[5-(2*S*)-Pyrrolidinylmethoxy]-3-pyridinyl]-3-isoxazolylethyl Ester (30). This compound was obtained from **19** and ethyl isocyanate employing methods F and B and gradient I; colorless oil; yield 42% over two steps; purity 99.8%; $[\alpha]_{\text{D}}^{20}$ 2.2 ($c = 0.36$, MeOH). ^1H NMR (400 MHz, D_2O) δ 8.76 (s, 1H), 8.54 (d, 1H, $J = 2.0$ Hz), 8.41 (s, 1H), 7.03 (s, 1H), 4.59–4.55 (m, 1H), 4.38 (t, 1H, $J = 7.6$ Hz), 4.21 (t, 2H, $J = 6.0$ Hz), 4.10–4.08 (m, 2H), 3.32 (t, 2H, $J = 6.8$ Hz), 2.97–2.87 (m, 4H), 2.22 (m, 1H), 2.06–1.90 (m, 2H), 1.89–1.84 (m, 1H), 0.86 (t, 3H, $J = 7.2$ Hz). ^{13}C NMR (100 MHz, D_2O) δ 162.6, 162.5, 161.8 (TFA), 157.4, 156.2, 131.4, 129.9, 127.3, 127.2, 115.7 (TFA), 104.2, 67.8, 61.9, 57.8, 45.5, 35.0, 25.3, 25.1, 22.9, 13.5. Anal. Calcd for $\text{C}_{18}\text{H}_{24}\text{N}_4\text{O}_4 \cdot 2.65\text{TFA} \cdot 0.95\text{H}_2\text{O}$ (FW 680): C, 41.17; H, 4.23; F, 22.22; N, 8.24. Found: C, 40.89; H, 3.94; F, 22; N, 8.12.

Phenylcarbamic Acid 2-[5-[5-(2*S*)-Azetidylmethoxy]-3-pyridinyl]-3-isoxazolylethyl Ester (31). This compound was obtained from **14** and phenyl isocyanate employing methods F and B and gradient I; colorless oil; yield 31% over two steps; purity 99.2%; $[\alpha]_{\text{D}}^{20}$ –2.5 ($c = 0.28$, MeOH). ^1H NMR (400 MHz, D_2O) δ 8.71 (s, 1H), 8.55 (d, 1H, $J = 2.0$ Hz), 8.30 (s, 1H), 7.17–7.10 (m, 4H), 7.00 (s, 1H), 6.95 (t, 1H, $J = 6.8$ Hz), 5.00–4.97 (m, 1H), 4.50 (d, 2H, $J = 3.6$ Hz), 4.38 (t, 2H, $J = 6.0$ Hz), 4.17–4.07 (m, 2H), 3.09 (t, 2H, $J = 6.0$ Hz), 2.71–2.66 (m, 2H). ^{13}C NMR (100 MHz, D_2O) δ 162.7, 162.5, 162.2 (TFA), 155.9, 154.6, 136.9, 131.9, 130.4, 128.6, 126.9, 126.3, 123.3, 118.7, 115.8 (TFA), 104.0, 67.5, 62.2, 58.1, 43.3, 25.1, 19.9. Anal. Calcd for $\text{C}_{21}\text{H}_{22}\text{N}_4\text{O}_4 \cdot 1.8\text{TFA} \cdot 0.05\text{H}_2\text{O}$ (FW 600): C, 49.20; H, 4.01; F, 17.08; N, 9.33. Found: C, 49.23; H, 3.98; F, 17.07; N, 9.31.

Phenylcarbamic Acid 2-[5-[5-(2*S*)-Pyrrolidinylmethoxy]-3-pyridinyl]-3-isoxazolylethyl Ester (32). This compound was obtained from **19** and phenyl isocyanate employing methods F and B and gradient I; colorless oil; yield 88% over two steps; purity 99.0%; $[\alpha]_{\text{D}}^{20}$ 2.7 ($c = 0.22$, MeOH). ^1H NMR (400 MHz, D_2O) δ 8.72 (s, 1H), 8.52 (s, 1H), 8.13 (s, 1H), 7.05–6.95 (m, 4H), 6.88 (s, 1H), 6.78–6.76 (m, 1H), 4.42–4.39 (m, 1H), 4.28–4.23 (m, 3H), 4.04–4.02 (m, 1H), 3.34 (t, 2H, $J = 7.2$ Hz), 2.95 (t, 2H, $J = 5.6$ Hz), 2.22–2.18 (m, 1H), 2.07–1.99 (m, 2H), 1.86–1.81 (m, 2H). ^{13}C NMR (100 MHz, D_2O) δ 162.4, 162.3, 162.4 (TFA), 155.9, 154.1, 137.0, 131.3, 129.9, 128.4, 126.8, 126.3, 123.0, 118.3, 115.8 (TFA), 104.1, 67.6, 62.0, 57.8, 45.5, 25.4, 25.0, 22.9. Anal. Calcd for $\text{C}_{22}\text{H}_{24}\text{N}_4\text{O}_4 \cdot 2\text{TFA}$ (FW 636): C, 49.06; H, 4.12; F, 17.91; N, 8.80. Found: C, 49.15; H, 4.12; F, 18.18; N, 8.97.

3-(Benzyloxy)-5-ethynylpyridine (34). To a stirred solution/suspension of **33** (1.85 g, 7.0 mmol), PPh_3 (605 mg, 2.3 mmol), and CuI (530 mg, 2.8 mmol) in Et_3N (30 mL) was added $\text{PdCl}_2(\text{PPh}_3)_2$ (490 mg, 0.70 mmol). The mixture was stirred at room temperature for 30 min under argon, and then ethynyl-(trimethyl)silane (3.0 mL, 21.3 mmol) was added. After stirring at 60 °C for 24 h, the reaction mixture was concentrated. The residue was purified by CC on SiO_2 (hexane/acetone 3:1) to give the trimethylsilylalkyne (1.60 g, 81%) as a pale-brown solid. ^1H NMR (400 MHz, CDCl_3) δ 8.31 (s, 1H), 8.30 (s, 1H), 7.43–7.31 (m, 6H), 5.03 (s, 2H), 3.21 (s, 1H).

To a solution of the above trimethylsilylalkyne (1.40 g, 5.0 mmol) in 10 mL of THF, 1 M TBAF in THF (8 mL) was added under argon with ice cooling. After stirring at rt for 1 h, the reaction mixture was quenched with water and extracted with EtOAc (2×30 mL). The combined organic layers were washed with brine, dried over Na_2SO_4 , and evaporated. The residue was purified by CC on SiO_2 (hexane/ EtOAc 4:1) to afford the desired product (750 mg, 72%) as white solid. ^1H NMR (400 MHz, CDCl_3) δ 8.34 (s, 1H), 8.33 (s, 1H), 7.39–7.24 (m, 6H), 5.03 (s, 2H), 3.21 (s, 1H). ^{13}C NMR (100 MHz, CDCl_3) δ 154.1, 145.3, 138.5, 135.7, 128.8, 128.4, 127.5, 124.0, 119.4, 80.7, 80.2, 70.3.

3-(Benzyloxy)-5-(3-ethyl-5-isoxazolyl)pyridine (35). This compound was obtained from **34** and 1-nitropropane employing method A; pale-yellow oil; yield 72%. ^1H NMR (400 MHz, CDCl_3) δ 8.56 (s, 1H), 8.37 (s, 1H), 7.59 (s, 1H), 7.42–7.18 (m, 5H), 6.44 (s, 1H), 5.11 (s, 2H), 2.57 (q, 2H, $J = 7.6$ Hz), 1.29 (t, 3H, $J = 7.6$ Hz). ^{13}C NMR (100 MHz, CDCl_3) δ 166.1, 165.5, 154.5, 139.3, 139.0, 135.3, 128.4, 128.0, 127.2, 117.3, 99.8, 70.1, 19.2, 12.2.

3-(Benzyloxy)-5-[3-(3,3,3-trifluoropropyl)-5-isoxazolyl]pyridine (36). This compound was obtained from **30** and 1,1,1-trifluoro-4-nitrobutane employing method A; pale-yellow oil; yield 72%. ^1H NMR (400 MHz, CDCl_3) δ 8.59 (s, 1H), 8.44 (d, 1H, $J = 2.8$ Hz), 7.63 (t, 1H, $J = 2.2$ Hz), 7.46–7.26 (m, 5H), 6.49 (s, 1H), 5.18 (s, 2H), 3.01 (m, 2H), 2.59 (m, 2H).

3-(Benzyloxy)-5-[3-[2-[tert-Butyl(dimethyl)silyloxy]ethyl]-5-isoxazolyl]pyridine (37). This compound was obtained from **30** and **9** employing method A; pale-yellow oil; yield 86%. ^1H NMR (400 MHz, CDCl_3) δ 8.56 (s, 1H), 8.38 (s, 1H), 7.59 (s, 1H), 7.42–7.29 (m, 5H), 6.54 (s, 1H), 5.07 (s, 2H), 3.91 (t, 2H, $J = 6.0$ Hz), 2.91 (t, 2H, $J = 6.0$ Hz), 0.88 (s, 9H), 0.03 (s, 6H).

3-(2*S*)-Azetidylmethoxy)-5-(3-ethyl-5-isoxazolyl)pyridine (38). This compound was obtained from **35** and 2(*S*)-(hydroxymethyl)-azetidine-1-carboxylic acid *tert*-butyl ester employing methods G, H, and B and gradient I; colorless oil; yield 11% over three steps; purity 99.4%; $[\alpha]_{\text{D}}^{20}$ –4.2 ($c = 1.11$, MeOH). ^1H NMR (400 MHz, D_2O) δ 8.88 (s, 1H), 8.65 (s, 1H), 8.53 (s, 1H), 7.03 (s, 1H), 5.00 (m, 1H), 4.62 (d, 2H, $J = 4.0$ Hz), 4.17–4.04 (m, 2H), 2.75–2.67 (m, 4H), 1.23 (t, 3H, $J = 7.6$ Hz). ^{13}C NMR (100 MHz, D_2O) δ 167.6, 162.5, 162.6 (TFA), 156.6, 132.0, 130.3, 127.9, 127.6, 116.1 (TFA), 104.2, 68.0, 58.5, 43.6, 20.2, 19.0, 11.5. Anal. Calcd for $\text{C}_{14}\text{H}_{17}\text{N}_3\text{O}_2 \cdot 2.3\text{TFA} \cdot 0.9\text{H}_2\text{O}$ (FW 538): C, 41.54; H, 3.95; F, 24.38; N, 7.81. Found: C, 41.73; H, 3.84; F, 24.39; N, 7.62.

3-(2*S*)-Azetidylmethoxy)-5-[3-(3,3,3-trifluoropropyl)-5-isoxazolyl]pyridine (39). This compound was obtained from **36** and 2(*S*)-(hydroxymethyl)azetidine-1-carboxylic acid *tert*-butyl ester employing methods G, H, and B and gradient I; colorless oil; yield 12.5% over three steps; purity 99.7%; $[\alpha]_{\text{D}}^{20}$ –3.3 ($c = 0.81$, MeOH). ^1H NMR (400 MHz, D_2O) δ 8.84 (s, 1H), 8.60 (s, 1H), 8.49 (s, 1H), 7.03 (s, 1H), 4.93 (m, 1H), 4.56 (m, 2H), 4.04 (m, 2H), 2.95 (t, 2H, $J = 7.4$ Hz), 2.63 (m, 2H), 2.54 (m, 2H). ^{13}C NMR (100 MHz, D_2O) δ 163.7, 163.0, 162.2 (TFA), 156.7, 131.9, 130.7, 127.4, 126.1 (q, $J_{\text{C-F}} = 229.0$ Hz), 116.0 (TFA), 104.3, 68.0, 58.4, 43.6, 30.9 (q, $J_{\text{C-F}} = 29.0$ Hz), 20.2, 18.6. Anal. Calcd for $\text{C}_{15}\text{H}_{16}\text{F}_3\text{N}_3\text{O}_2 \cdot 2.75\text{TFA} \cdot 1.05\text{H}_2\text{O}$ (FW 613): C, 37.32; H, 3.19; F, 32.39; N, 6.37. Found: C, 37.25; H, 2.96; F, 32.18; N, 6.35.

2-(Hydroxymethyl)-3-azabicyclo[3.1.0]hexane-3-carboxylic acid *tert*-Butyl Ester (41). To a solution of **40** (250 mg, 2 mmol) in 40 mL of half-saturated NaHCO_3 solution was added dropwise with ice cooling Boc_2O (870 mg, 4 mmol) in H_2O (11 mL) and THF (5 mL). After stirring overnight at rt, the solvent was evaporated. HCl (2 M, 20 mL) was added with ice cooling, and then the mixture was extracted with CH_2Cl_2 (3×10 mL). The combined CH_2Cl_2 phases were dried over Na_2SO_4 and concentrated to obtain 3-azabicyclo[3.1.0]hexane-2,3-dicarboxylic acid 3-*tert*-butyl ester, which was carried on without further purification.

To the solution of this intermediate in anhydrous THF (20 mL), $\text{BH}_3 \cdot \text{THF}$ in THF (1 M, 3 mL) was added under argon with ice cooling. After stirring at rt for 2 h, the reaction mixture was quenched with satd NH_4Cl . The phases were separated, and the organic phase was washed with water and brine, dried over Na_2SO_4 , and concentrated to obtain the title compound; colorless oil; yield 310 mg (73%) over two steps. ^1H NMR (400 MHz, CDCl_3) δ 5.52 (br, 1H), 3.88 (m, 1H), 3.74 (m, 1H), 3.56 (m, 1H), 3.42–3.37 (m, 3H), 1.55 (m, 1H), 1.37 (s, 9H), 0.48 (m, 1H), 0.27 (m, 1H).

2-[5-[5-[(3*S*,*R*)-Azabicyclo[3.1.0]-2(*S*)-hexyl]methoxy]-3-pyridinyl]-3-isoxazolylethane (42). This compound was obtained from **35** and **41** employing methods G, H, and B and gradient I; colorless oil; yield 20% over three steps; purity 99.6%; $[\alpha]_{\text{D}}^{20}$ 0 ($c = 0.48$, MeOH). ^1H NMR (400 MHz, D_2O) δ 8.89 (s, 1H), 8.63 (s, 1H), 8.50 (s, 1H), 7.06 (s, 1H), 4.50–4.33 (m, 2H), 3.61–3.52 (m, 2H), 2.77 (q, 2H, $J = 7.6$ Hz), 1.95 (m, 2H), 1.26 (t, 3H, $J = 7.6$ Hz), 0.92 (m, 1H), 0.65 (m,

1H). ¹³C NMR (100 MHz, D₂O) δ 167.3, 162.4 (TFA), 162.3, 156.1, 131.9, 130.1, 127.4, 126.9, 117.4 (TFA), 103.7, 67.8, 58.9, 47.4, 18.6, 15.6, 13.7, 11.1, 3.5. Anal. Calcd for C₁₆H₁₉N₃O₂·2.6TFA·0.9H₂O (FW 554): C, 42.58; H, 3.94; F, 24.78; N, 7.03. Found: C, 42.25; H, 3.59; F, 24.50; N, 6.79.

2-[5-[5-[(3*S*,*R*)-Azabicyclo[3.1.0]-2(*S*)-hexyl)methoxy]-3-pyridin-yl]-3-isoxazolyl]ethanol (**43**). This compound was obtained from **37** and **41** employing methods G, H, and B and gradient I; colorless oil; yields of individual steps 72%, 61%, and 64%; purity 99.8%; [α]_D²⁰ -0.4 (*c* = 0.48, MeOH). ¹H NMR (400 MHz, D₂O) δ 8.88 (s, 1H), 8.71 (s, 1H), 8.50 (s, 1H), 7.08 (s, 1H), 4.47–4.39 (m, 2H), 3.91 (t, 2H, *J* = 6.0 Hz), 3.57–3.48 (m, 2H), 2.96 (t, 2H, *J* = 5.8 Hz), 1.91 (m, 2H), 0.87 (m, 2H), 0.63 (s, 1H). ¹³C NMR (100 MHz, D₂O) δ 163.7, 162.9, 162.5 (TFA), 156.6, 132.1, 130.4, 127.7, 127.4, 116.2 (TFA), 104.5, 68.2, 59.3, 47.7, 28.4, 15.9, 14.1, 3.9. Anal. Calcd for C₁₆H₁₉N₃O₃·2.5TFA·0.3H₂O (FW 549): C, 42.29; H, 3.76; F, 24.08; N, 7.10. Found: C, 42.29; H, 3.36; F, 23.68; N, 6.92.

General Procedures for Binding and Functional Studies. *In Vitro Binding Studies.* [³H]Epibatidine competition studies and broad-range screening were carried out by the National Institute of Mental Health's Psychoactive Drug Screening Program, contract no. HHSN-271-2008-00025-C (NIMH PDSP). For experimental details, please refer to the PDSP Web site <http://pdsp.med.unc.edu/>.

Cell Lines and Culture. Cell lines naturally or heterologously expressing specific, functional, human nAChR subtypes were used. The human clonal cell line TE671/RD naturally expresses human muscle-type $\alpha 1^*$ -nAChRs, containing $\alpha 1$, $\beta 1$, γ , and δ subunits, with function detectable using ⁸⁶Rb⁺ efflux assays.²³ The human neuroblastoma cell line SH-SY5Y naturally expresses autonomic $\alpha 3\beta 4^*$ -nAChRs, containing $\alpha 3$, $\beta 4$, probably $\alpha 5$, and sometimes $\beta 2$ subunits and also displays function detectable using ⁸⁶Rb⁺ efflux assays.²⁴ SH-SY5Y cells also express homopentameric $\alpha 7$ -nAChRs. However, their function is not detected in the ⁸⁶Rb⁺ efflux assay under the conditions used. SH-EP1 human epithelial cells stably transfected with human $\alpha 4$ and $\beta 2$ subunits (SH-EP1- $\alpha 4\beta 2$ cells) have been established and the human $\alpha 4\beta 2$ -nAChRs that they stably and heterologously express have been characterized with both ion flux and radioligand binding assays.²⁵

TE671/RD, SH-SY5Y, and transfected SH-EP1 cell lines were maintained as low passage number (1–26 from our frozen stocks) cultures to ensure stable expression of native or heterologously expressed nAChRs as previously described.²³ Cells were passaged once a week by splitting just-confluent cultures 1/300 (TE671/RD), 1/10 (SH-SY5Y), or 1/40 (transfected SH-EP1) in serum-supplemented medium to maintain log-phase growth.

⁸⁶Rb⁺ Efflux Assays. Function of nAChR subtypes was investigated using an established ⁸⁶Rb⁺ efflux assay protocol.²³ The assay is specific for nAChR function under the conditions used, for example, giving identical results in the presence of 100 nM atropine to exclude possible contributions of muscarinic acetylcholine receptors. Cells harvested at confluence from 100 mm plates under a stream of fresh medium only (SH-SY5Y cells) or after mild trypsinization (Irvine Scientific, USA; for TE671/RD or transfected SH-EP1 cells) were then suspended in complete medium and evenly seeded at a density of 1.25–2 confluent 100 mm plates per 24-well plate (Falcon; ~100–125 mg of total cell protein per well in a 500 μ L volume; poly L-lysine-coated for SH-SY5Y cells). After cells had adhered generally overnight, but no sooner than 4 h later, the medium was removed and replaced with 250 μ L per well of complete medium supplemented with ~350000 cpm of ⁸⁶Rb⁺ (NEN; counted at 40% efficiency using Cerenkov counting and the Packard TriCarb 1900 liquid scintillation analyzer). After at least 4 h and typically overnight, ⁸⁶Rb⁺ efflux was measured using the “flip-plate” technique. Briefly, after aspiration of the bulk of ⁸⁶Rb⁺ loading medium from each well of the “cell plate,” each well containing cells was rinsed with 2 mL of fresh ⁸⁶Rb⁺ efflux buffer (130 mM NaCl, 5.4 mM KCl, 2 mM CaCl₂, 5 mM glucose, and 50 mM HEPES; pH 7.4) to remove extracellular ⁸⁶Rb⁺. Following removal of residual rinse buffer by aspiration, the flip-plate technique was used again to simultaneously introduce 1.5 mL of fresh efflux buffer containing drugs of choice at indicated final concentrations from a 24-well “efflux/drug plate” into the wells of the cell plate. After a 9.5 min incubation, the

solution was “flipped” back into the efflux/drug plate, and any remaining buffer in the cell plate was removed by aspiration. Ten min after the initiation of the first drug treatment, a second efflux/drug plate was used to reintroduce the same concentrations of drugs of choice with the addition of an ~EC₉₀ concentration of the full agonist carbamylcholine for 5 min (~EC₉₀ concentrations were 200 μ M for SH-EP1- $\alpha 4\beta 2$ cells, 2 mM for SHSY5Y cells, and 464 mM for TE671/RD cells). The second drug treatment was then flipped back into its drug plate, and the remaining cells in the cell plate were lysed and suspended by addition of 1.5 mL of 0.1 M NaOH with 0.1% sodium dodecyl sulfate to each well. Suspensions in each well were then subjected to Cerenkov counting (Wallac Micobeta Trilux 1450; 25% efficiency) after placement of inserts (Wallac 1450–109) into each well to minimize cross-talk between wells.

For quality control and normalization purposes, the sum of ⁸⁶Rb⁺ in cell plates and efflux/drug plates was defined to confirm material balance (i.e., that the sum of ⁸⁶Rb⁺ released into the efflux/drug plates and ⁸⁶Rb⁺ remaining in the cell plate were the same for each well). Similarly, the sum of ⁸⁶Rb⁺ in cell plates and efflux/drug plates also determined the efficiency of ⁸⁶Rb⁺ loading (the percentage of applied ⁸⁶Rb⁺ actually loaded into cells). Furthermore, the sum of ⁸⁶Rb⁺ in cell plates and the second efflux/drug plates defined the amount of intracellular ⁸⁶Rb⁺ available at the start of the second 5 min assay and were used to normalize nAChR function assessed.

For each experiment, in one set of control samples, total ⁸⁶Rb⁺ efflux was assessed in the presence of a fully efficacious concentration of carbamylcholine alone (1 mM for SH-EP1- $\alpha 4\beta 2$ and TE671/RD cells, or 3 mM for SH-SY5Y cells). Nonspecific ⁸⁶Rb⁺ efflux in another set of control samples was measured either in the presence of the fully efficacious concentration of carbamylcholine plus 100 μ M mecamylamine, which gave full block of agonist-induced and spontaneous nAChR-mediated ion flux, or in the presence of efflux buffer alone. Both determinations of nonspecific efflux were equivalent. Specific efflux was then taken as the difference in control samples between total and nonspecific ⁸⁶Rb⁺ efflux. The same approaches were used to define total, nonspecific, and specific ion flux responses in samples subjected to the second 5 min exposure to test drug with or without carbamylcholine at its ~EC₉₀ concentration.

Intrinsic agonist activity of test drugs was ascertained during the first 9.5 min of the initial 10 min exposure period using samples containing test drug only at different concentrations and was normalized, after subtraction of nonspecific efflux, to specific efflux in carbamylcholin control samples. Specific ⁸⁶Rb⁺ efflux elicited by test drug as a percentage of specific efflux in carbamylcholine controls was the same in these samples whether measured in absolute terms or as a percentage of loaded ⁸⁶Rb⁺. Even in samples previously giving an efflux response during the initial 10 min exposure to a partial or full agonist, residual intracellular ⁸⁶Rb⁺ was adequate to allow assessment of nAChR function in the secondary 5 min assay. However, care was needed to ensure that data were normalized to the amount of intracellular ⁸⁶Rb⁺ available at the time of the assay, as absolute levels of total, nonspecific, or specific efflux varied in cells partially depleted of intracellular ⁸⁶Rb⁺ due to action of any agonist present during the 10 min drug exposure period. That is, calculations of specific efflux as a percentage of loaded ⁸⁶Rb⁺ typically were corrected for any variation in the electrochemical gradient of ⁸⁶Rb⁺ created by intracellular ion depletion after the first (agonism/pretreatment) drug treatment.

Ion flux assays (*n* ≥ 3 separate studies for each drug and cell line combination) were fit to the Hill equation, $F = F_{\max}/(1 + (X/EC_{50})^n)$, where *F* is the percentage of control, *F*_{max} for EC₅₀ (*n* > 0 for agonists) or IC₅₀ (*n* < 0 for antagonists) values using Prism 4 (GraphPad, San Diego, USA). Most ion flux data were fit allowing maximum and minimum ion flux values to be determined by curve fitting but in some cases, where antagonists or agonists had weak functional potency, minimum ion flux was set at 0% of control or maximum ion flux was set at 100% of control, respectively.

Determinations of ligand agonist efficacy at HS or LS $\alpha 4\beta 2$ -nAChR were made by extrapolations of results in each of several experiments plotting the maximal efficacy of the test ligand against the efficacy of 1 μ M compound **2**, which is a full agonist at HS $\alpha 4\beta 2$ -nAChRs but

inactive at LS $\alpha 4\beta 2$ -nAChRs. These two $\alpha 4\beta 2$ -nAChR isoforms are spontaneously expressed in different ratios in cells assayed in different experiments performed with different cell batches. However, determinations of compound 2 efficacy relative to that of carbamylcholine, which is the reference full agonist at both receptor isoforms, can be used to define the response proportion due to compound 2-activated HS $\alpha 4\beta 2$ -nAChRs in the cells being studied (typically varying from 25 to 60%). Thus, extrapolation of results for efficacy of the test ligand on one axis to the origin (0% compound 2 response or HS $\alpha 4\beta 2$ -nAChR expression) or the maximum extreme (100% compound 2 response or HS $\alpha 4\beta 2$ -nAChR expression) on the other axis allows separate determination of the test ligand's activity if it were acting a pure populations of LS or HS $\alpha 4\beta 2$ -nAChRs. Just as is the case for compound 2, the test ligands are partial agonists at the mixture of $\alpha 4\beta 2$ -nAChR isoforms because they are inactive (efficacy $\sim 0\%$ of that of a full agonist) at LS $\alpha 4\beta 2$ -nAChR but active at HS $\alpha 4\beta 2$ -nAChR, usually with an efficacy at the latter that is less than or comparable to that of compound 2 (Table 2).

General Procedures for Behavioral Studies. *Animals.* BALB/cJ male mice (8–10 weeks old at testing) were obtained from Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed four to a cage in a colony room maintained at 22 ± 2 °C on a 12 h light–dark cycle. All animal experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the PsychoGenics Animal Care and Use Committee.

Drugs. Compounds 25, 26, and 38 were synthesized as described above, and sertraline was purchased from Toronto Research Chemicals (Ontario, Canada). All compounds were dissolved in water and administered by intraperitoneal (IP) injection in a volume of 10 mL/kg.

Mouse Forced Swim Test. Procedures were based on those previously described.²⁵ Mice were individually placed into clear glass cylinders (15 cm tall \times 10 cm wide, 1 L beakers) containing 23 ± 1 °C water 12 cm deep (approximately 800 mL). Mice were administered vehicle, the SSRI sertraline (20 mg/kg) as a positive control, or test compound (25, 26, or 38; 5, 10, 30 mg/kg). Compound 38 was also tested at 1 and 3 mg/kg.) Thirty minutes following IP administration, mice were placed in the water, and the time the animal spent immobile was recorded over a 6 min trial. Immobility was defined as the postural position of floating in the water.

Statistical Analysis. Data were analyzed with Analysis of Variance (ANOVA) with treatment group (vehicle, sertraline, or compound 25, 26, or 38) as the between-group variable and total time immobile (seconds over the 6 min trial) as the dependent variable. Significant main effects were followed up with the post hoc Fisher's LSD test.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

CNS, central nervous system; AD, Alzheimer's disease; ADHD, attention deficit hyperactivity disorder; NIMH-PDSP, National Institute of Mental Health Psychoactive Drug Screening Program; nAChR(s), nicotinic acetylcholine receptor(s); TCA, tricyclic antidepressant; FST, forced swim test; SAR, structure–activity relationship; SSRI, selective serotonin reuptake inhibitor; CC, column chromatography; rt, room temperature; TFA, trifluoroacetic acid; HS, high-sensitivity; LS, low-sensitivity

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