Identification of Novel α4β2-Nicotinic Acetylcholine Receptor (nAChR) Agonists Based on an Isoxazole Ether Scaffold that Demonstrate Antidepressant-like Activity

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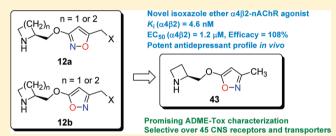
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ⓢ Supporting Information

ABSTRACT: There is considerable evidence to support the hypothesis that the blockade of nAChR is responsible for the antidepressant action of nicotinic ligands. The nicotinic acetylcholine receptor (nAChR) antagonist, mecamylamine, has been shown to be an effective add-on in patients that do not respond to selective serotonin reuptake inhibitors. This suggests that nAChR ligands may address an unmet clinical need by providing relief from depressive symptoms in refractory patients. In this study, a new series of nAChR ligands based on an isoxazole-ether scaffold have been designed and synthesized for binding and functional assays. Preliminary structure−activity relationship (SAR) efforts identified a lead compound **43**, which possesses potent antidepressant-like activity (1 mg/kg, IP; 5 mg/kg, PO) in the classical mouse forced swim test. Early stage absorption, distribution, metabolism, excretion, and toxicity (ADME-Tox) studies also suggested favorable drug-like properties, and broad screening toward other common neurotransmitter receptors indicated that compound **43** is highly selective for nAChRs over the other 45 neurotransmitter receptors and transporters tested.
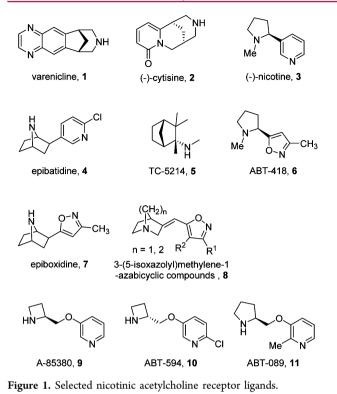
Novel isoxazole ether α4β2-nAChR agonist
K_i (α4β2) = 4.6 nM
EC_{50} (α4β2) = 1.2 μM, Efficacy = 108%
Potent antidepressant profile *in vivo*

Promising ADME-Tox characterization
Selective over 45 CNS receptors and transporters

INTRODUCTION

Neuronal nicotinic acetylcholine receptors (nAChRs) are pentamers assembled from varying combinations of subunits (α2−α10, β2−β4) and belong to the ligand-gated ion channel superfamily of neurotransmitter receptors.[1−3] These receptors are broadly distributed in the central and peripheral nervous systems, where they modulate many processes, such as ganglionic transmission regulated by α3β4*-nAChRs (the * indicates that subunits other than those specified are known or possible partners in the closed assembly), neuroprotection of dopaminergic pathways and nociception mediated by α4*-nAChRs, as well as learning, memory, and addiction by β2*-nAChR.[3−6] Over the past two decades, many compounds targeting nAChRs have been tested in various stages of clinical trials.[7] However, only one new chemical entity, varenicline (**1**), has been launched and marketed as a potent partial agonist at the α4β2-nAChR for smoking cessation (Figure 1).[8−10]

Given nAChR subtype diversity and their involvement in the modulation of a host of neurotransmitter systems, nicotinic ligands have the potential to treat a multitude of central nervous system (CNS)-related dysfunctions, including chronic depression.[8,11] There is considerable evidence to support the hypothesis that the blockade (antagonism or receptor desensitization) of nAChR is responsible for the antidepressant action of nicotinic ligands.[12−14] In particular, clinical studies have shown that the cholinesterase inhibitor, physostigmine, produces depressive symptoms in humans,[15] whereas mecamylamine[16] and the muscarinic antagonist scopolamine[17,18] relieve depressive symptoms in humans. Additionally, preclinical studies provide support for the hypothesis that increased cholinergic activity leads to depressed mood states. Flinders sensitive rats, a line selectively bred for increased cholinergic sensitivity, exhibit several depressive-like behaviors.[19,20] Moreover, administration of the nicotinic antagonist mecamylamine elicits an antidepressant-like effect in the mouse forced swim test, and this effect is reduced when the β2 subunit gene is knocked out.[11] The same effects were also observed in response to the tricyclic antidepressant amitriptyline, strongly suggesting that β2*-nAChRs are involved in the antidepressant efficacy of nicotinic ligands.[21] The α4β2-nAChR is the predominant subtype in the vertebrate CNS, and the α4β2 nicotinic agonists

Figure 1. Selected nicotinic acetylcholine receptor ligands.

cytisine (**2**),[22] A-85380 (**9**),[23] and compound **1**[24] induce antidepressant-like effects in mice that are similar to the effects of the antagonist mecamylamine. The *S*-enantiomer of mecamylamine (TC-5214, **5**) is an α4β2-nAChR modulator now in Phase II clinical trials for use in the treatment of depression.[25] Therefore, the α4β2-nAChR is an attractive target for the development of novel antidepressants, although it is unclear whether nAChR activation, desensitization, or some combination of both is essential. It is also known from clinical studies that α3β4*-nAChRs contribute to adverse side effects in vivo, although roles in mood control also are possible, as exemplified by mecamylamine.[26−28] Consequently, we chose to focus on developing potent agonists selective for the α4β2-nAChR, bearing in mind that activity at the α3β4*-nAChR subtype might be an attribute or a detriment.

Multiple modifications to the structure of natural nicotinic ligands, especially nicotine (**3**), epibatidine (**4**), and compound **2**, have already been explored over the past 20 years. Most of the reported nicotinic ligands bear a substituted pyridine ring as the core scaffold. Some compound classes involving replacement of the pyridine ring by isosteres have been investigated, as exemplified by substituted phenyl derivatives,[29] quinolines,[30] furopyridines,[31] structurally related chroman derivatives,[32] and most interestingly the five-membered heteroaromatic rings isoxazole and isothiazole.[33−37] The ability to replace the pyridine moiety of *S*-(−)-nicotine with an isoxazole ring was first investigated by Abbott Laboratories, leading to the clinical study of ABT-418 (**6**) for the treatment of both Alzheimer's disease (AD) and attention deficit hyperactivity disorder (ADHD). Compound **6** is a selective, full agonist at the α4β2-nAChR with a K_i value of 7.4 nM.[8,38] The complete subtype selectivity profile was not determined. In preclinical studies, compound **6** demonstrated efficacy and potency similar to that of compound **3** in animal models of cognition while exhibiting reduced toxicity.[38] Although compound **6** failed in clinical development due to the occurrence of nausea as a side

effect, isoxazole-containing nicotinic ligands remain an exciting area of investigation. Substituted isoxazoles were also successfully applied in optimization studies of compound **4**. Replacement of the chloropyridyl group in compound **4** with a methylisoxazolyl group led to epiboxidine (**7**),[39,40] which was approximately 10-fold less potent than compound **4** but 17-fold more potent than compound **6** in the displacement of [³H]nicotine at the α4β2-nAChR from rat cerebral cortical membranes. Epiboxidine retained analgesic activity in mice at 25 mg/kg compared to compound **4** at 10 mg/kg, with greatly reduced toxicity. Furthermore, a series of 3-(5-isoxazolyl)-methylene-1-azabicyclic compounds (**8**) were synthesized as potent nicotinic ligands.[41]

Pyridyl ethers, in which a CH₂O linker is inserted between compound **3**'s pyrrolidine (or in analogues, azetidine) ring and its pyridine ring, have attracted considerable interest as α4β2-nAChR agonists because of their high potency.[42,43] For example, compound **9** possesses a K_i value of ca. 50 pM and a high efficacy of 163% compared to compound **3** at the human α4β2-nAChR.[44] ABT-594 (**10**), a nAChR agonist, was advanced to a Phase II clinical trial for the treatment of chronic and neuropathic pain with a potency 50 times that of morphine but was discontinued due to an unacceptably narrow therapeutic index.[45,46] ABT-089 (**11**) is currently under investigation as a replacement for compound **6** that has an improved preclinical therapeutic index and a better pharmacokinetic profile. It has gone through Phase II clinical studies for cognitive dysfunction.[47,48] Upon the basis of the precedent quoted above, we anticipated that an isoxazole moiety could be used as a readily accessible replacement for the pyridine core in the design of new nicotinic ligands. Herein, we report the synthesis and pharmacological evaluation of a novel series of nAChR ligands based on an isoxazole core in combination with the CH₂O linker ("isoxazole ethers"; Figure 2). Selected
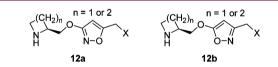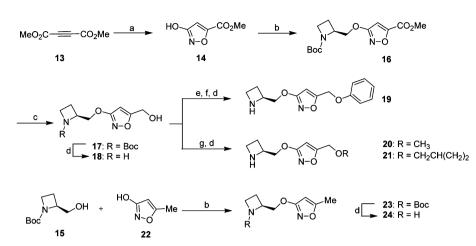

Figure 2. General structure of the present series of isoxazole ether nAChR ligands.

compounds were further assessed in behavioral tests, in a broad screening panel of common CNS neurotransmitter receptors and transporters, as well as in preliminary in vitro ADME-Tox studies.
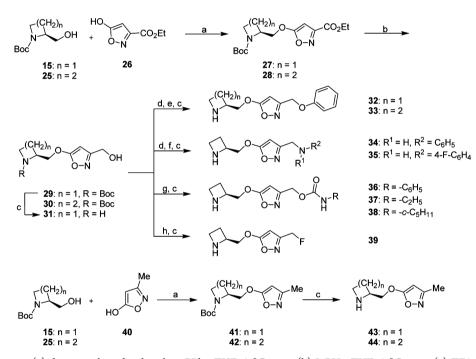
RESULTS AND DISCUSSION

Chemistry. First, we designed compounds that could be accessed from readily available starting materials to ascertain whether an isoxazole moiety could replace the pyridine core in the previously published pyridine ether nicotinics developed by Abbott. The 3-alkoxyisoxazoles **18−21** were synthesized in 3−6 steps utilizing the synthetic routes shown in Scheme 1. Intermediate **16** was formed via the Mitsunobu reaction of Boc-protected 2(*S*)-azetidinylmethanol (**15**) and 3-hydroxyisoxazole-5-carboxylic acid methyl ester (**14**), which was in turn prepared as described in the literature from dimethyl 2-butynedioate (**13**). The ester **16** was subsequently reduced with LiBH₄ to furnish the primary alcohol **17**, and this intermediate was carried on to the iodide. The phenyl ether **19** and the aliphatic ethers **20−21** were obtained through nucleophilic

Scheme 1[a]

[a]Reagents and conditions: (a) *N*-hydroxyurea, 1,5-diazabicyclo[5.4.0]undec-5-ene, MeOH, 0 °C, then HCl; (b) 1-(*tert*-butoxycarbonyl)-2(*S*)-azetidinylmethanol (15), diisopropyl azodicarboxylate, PPh₃, THF, 0 °C to rt; (c) LiBH₄, THF, 0 °C to rt; (d) TFA, CH₂Cl₂; (e) I₂, PPh₃, imidazole, CH₂Cl₂, 0 °C to rt; (f) phenol, K₂CO₃, DMF, rt; (g) NaH, DMF, RBr, 0 °C to rt.

Scheme 2[a]

[a]Reagents and conditions: (a) diisopropyl azodicarboxylate, PPh₃, THF, 0 °C to rt; (b) LiBH₄, THF, 0 °C to rt; (c) TFA, CH₂Cl₂; (d) I₂, PPh₃, imidazole, CH₂Cl₂, 0 °C to rt; (e) K₂CO₃, DMF, phenol, rt; (f) HNR¹R², CH₃CN, rt; (g) RN=C=O, 4-(dimethylamino)pyridine, PhMe, 80 °C; (h) Et₂NSF₃, CH₂Cl₂, −78 °C.

substitution following standard methods. After acidic deprotection and subsequent purification by HPLC, compounds 18–21 were obtained as trifluoro acetates. The number of equivalents of trifluoroacetic acid (TFA) in these nonstoichiometric compounds was determined by elemental analysis. The 5-methylated 3-alkoxyisoxazole 24 was synthesized in the same manner from commercially available 3-hydroxy-5-methylisoxazole 22 (Scheme 1).

The preparation of 5-alkoxyisoxazole ligands proceeded through the common intermediates 29 and 30, which were in turn prepared from the commercially available ester 26 (Scheme 2). Compounds 31, 32, 33, and 43 were synthesized by employing the same strategy as described in Scheme 1. The primary alcohol 29 was transformed to an iodide, followed by

nucleophilic substitution with aniline or 4-fluoroaniline to afford the precursors of amine derivatives 34 and 35. Carbamate analogues 34–38 were prepared by reaction of 29 with the corresponding isocyanates. The fluoromethyl derivative 39 was obtained by treatment of alcohol 29 with (diethylamino)sulfur trifluoride. Subsequent Boc deprotection of the precursors yielded the desired final compounds 34–39.

In Vitro Characterization—Radioligand Binding Studies. In vitro binding affinities of the five 3-alkoxyisoxazoles (18–21, 24) were determined by the standard [³H]epibatidine binding assay at seven rat nAChR subtypes (Table 1).[49] While this initial set of compounds showed weak binding to all seven nAChR subtypes tested, compound 18 exhibited a moderate affinity for α4β2- and α4β2*-nAChRs.

Table 1. Binding Affinities of 3-Alkoxyisoxazole Ligands at Seven Rat nAChR Subtypes

ID	X	K_i (nM)^a						
		$\alpha2\beta2$	$\alpha2\beta4$	$\alpha3\beta2$	$\alpha3\beta4$	$\alpha4\beta2$	$\alpha4\beta2*^b$	$\alpha4\beta4$
18	OH	337	>10^4	990	>10^4	176	405	7530
19	OC$_6$H$_5$	NA^d	NA	NA	NA	NA	NA	NA
20	OCH$_3$	NA	NA	4100	NA	NA	NA	NA
21	OCH$_2$CH(CH$_2$)$_2$	NA	NA	NA	NA	NA	NA	NA
24	H	3340	>10^4	6670	>10^4	1950	7380	>10^4
3^c		5.5	70.0	29.0	260	4.9	9.8	23.0

^{a}See Experimental Section. $^b\alpha4\beta2*$, endogenous receptors prepared from rat forebrain. Besides $\alpha4$ and $\beta2$, other unidentified subunits may also be present. Details are provided in the Experimental Section. ^{c}The K_i values for compound **3** are taken from the PDSP Assay Protocol Book. ^{d}Not active, defined as <50% binding in the primary assay at 10 μM.

Table 2. Binding Affinities of 5-Alkoxyisoxazole Ligands at Seven Rat nAChR Subtypes

ID	n	X	K_i (nM)^a								
			$\alpha2\beta2$	$\alpha2\beta4$	$\alpha3\beta2$	$\alpha3\beta4$	$\alpha4\beta2$	$\alpha4\beta2*^b$	$\alpha4\beta4$	$\alpha7$	$\alpha7*^b$
31	1	OH	197	>10^4	521	>10^4	137	637	4900	ND^d	ND
32	1	OC$_6$H$_5$	47.9	58.0	362	186	23.8	172	27.5	ND	ND
33	2	OC$_6$H$_5$	176	320	2040	809	160	2120	55.1	ND	ND
34	1	NHC$_6$H$_5$	150	144	462	771	75.9	386	33.2	ND	ND
35	1	NHC$_6$H$_4$F-p	201	30.3	ND	171	49.0	417	9.9	ND	ND
36	1	OC(O)NHC$_6$H$_5$	42.3	162	123	1760	19.7	157	60.0	ND	ND
37	1	OC(O)NHC$_2$H$_5$	157	6570	315	NA^e	31.2	207	1240	ND	ND
38	1	OC(O)NH-c-C$_5$H$_{11}$	126	9970	370	NA	13.1	149	3480	ND	ND
39	1	F	11.8	472	17.3	1270	7.3	11.9	163	ND	ND
43	1	H	4.3	311	8.7	692	4.6	12.0	86.0	2890	6790
44	2	H	616	5810	1030	8780	129	1100	4140	ND	ND
3^c			5.5	70.0	29.0	260	4.9	9.8	23.0	ND	ND
1^f						86	0.4		110	125	

^{a}See Experimental Section. $^b\alpha4\beta2*$ or $\alpha7*$, endogenous receptors prepared from rat forebrain. Besides $\alpha4$, $\beta2$, or $\alpha7$, other unidentified subunits may also be present. Details are provided in the Experimental Section. ^{c}The K_i values for compound **3** are taken from the PDSP Assay Protocol Book. ^{d}ND: not detected. ^{e}Not active, defined as <50% binding in the primary assay at 10 μM. ^{f}The K_i values for compound **1** are from ref 51.

It is commonly accepted that the essential pharmacophore of nicotinic ligands consists of a cationic center (e.g., quaternized or protonated nitrogen) and a hydrogen-bond acceptor (e.g., a nitrogen atom in the case of pyridine-containing ligands).[50] The inactivity of our first batch of isoxazole-ether compounds is possibly a result of misalignment of these two key elements. Therefore, to align these pharmacophoric elements differently, and hopefully more appropriately, isoxazoles with a reverse position of their N and O ring atoms were synthesized. As the alcohol **18** (Table 1) was the only congener that so far showed moderate affinity to the $\alpha4\beta2$-nAChR, the reverse 5-hydroxyisoxazole **31** (Table 2) and its phenyl ether **32** were synthesized. Encouragingly, the binding affinities of **32** were dramatically improved, while compound **31** maintained its affinity in comparison to **18**. Subsequently amines, carbamates, simple alkyl, and fluoromethyl derivatives were synthesized to further study the structure−activity relationship (SAR).

In general, most of the 5-alkoxyisoxazoles (Table 2) bind to $\alpha4\beta2$-nAChRs with nanomolar affinity. The binding affinities of all amines (**34**, **35**), carbamates (**36**, **37**, and **38**), the 3-methyl derivative **43**, and the 3-fluoromethyl derivative **39** were increased 1.8−30-fold compared to the parent alcohol **31**. Compound **43** carrying a 3-methyl substituent turned out to

have the best affinity for $\alpha4\beta2$- and $\alpha4\beta2*$-nAChRs with K_i values of 4.6 nM and 12.0 nM, respectively. All of these compounds have a similar subtype selectivity profile as compound **3** and compound **1**;[51] they display selectivity for the $\alpha4\beta2$- over the $\alpha3\beta4$-nAChR in radioligand binding competition assays. The alcohol **31**, methyl derivative **43**, fluoromethyl derivative **39**, and carbamates **36**−**38** actually exceed compound **3**'s $\alpha4\beta2$- over $\alpha3\beta4$- selectivity. Among these ligands, the cyclopentylcarbamate **38** exhibits the best selectivity with a K_i value of 13.1 nM at the $\alpha4\beta2$-nAChR and inactivity at the $\alpha3\beta4$-nAChR. The aforementioned six compounds also exhibit selectivity for all the other $\beta2$- over $\beta4$-containing subtypes. Their affinities at the $\alpha4\beta2*$-nAChR dropped 1.6−13.5- fold compared to those at the $\alpha4\beta2$-nAChR, probably due to the existence of additional subunits in the former.

To determine the influence of different heteroalicyclic ring sizes, the pyrrolidine analogues **33** and **44** were also synthesized. Pyrrolidines **33** and **44** retained all of the selectivity exhibited by the corresponding azetidines (**32** and **43**), but their affinities were reduced at all seven rat nAChR subtypes, which is consistent with previous studies in the pyridine ether series.[42]
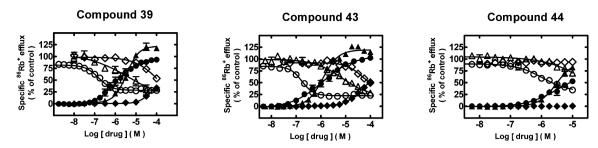
Figure 3. Functional activities of compounds 39, 43, and 44 at nAChR subtypes. Specific $^{86}Rb^+$ efflux (ordinate; percentage of control ± SEM) was determined as described in the Experimental Section for intrinsic agonist activity over a 9.5 min period for compounds **39** (left), **43** (middle), or **44** (right) at the indicated concentrations (abscissa; log molar scale) on human $\alpha4\beta2$-nAChR (●), $\alpha3\beta4^*$-nAChR (▲), or $\alpha1\beta1\gamma\delta$-nAChR (♦) naturally or heterologously expressed by SH-EP1-h$\alpha4\beta2$, SH-SY5Y, or TE671/RD cells, respectively. Also shown are functional inactivation effects of pretreatment for 10 min with the same agents at the indicated concentrations on subsequent agonist action of an EC_{90} concentration of the full agonist, carbamylcholine (applied in the continuing presence of the indicated agents), acting at $\alpha4\beta2$-nAChR (○), $\alpha3\beta4^*$-nAChR (△), or $\alpha1\beta1\gamma\delta$-nAChR (◇). Results are normalized to responses to a fully efficacious concentration of carbamylcholine (see Experimental Section for details). Nanomolar agonist EC_{50} values and inactivation IC_{50} values are provided in Tables 3 and 4, as are agonism and inactivation efficacies (normalized to those for a full agonist or antagonist, respectively). SEM values were determined for each parameter and although not presented here, typically are less than 15% for efficacy measures and no more than a factor of 2 for molar EC_{50} or IC_{50} values. Compounds **39** and **43** have full agonist activity at $\alpha4\beta2$-nAChRs and $\alpha3\beta4^*$-nAChRs (efficacies are similar to that of carbamylcholine) and little activity at $\alpha1^*$-nAChRs. Compound **44** was less potent than **39** and **43** by approximately 1 order of magnitude, so a full functional profile would have required 1 mM concentrations of the compound and was not pursued.

In Vitro Functional Characterization. The most potent $\alpha4\beta2$-nAChR ligands based on binding assays, **39** and **43**, as well as pyrrolidine analogue **44** were selected for evaluation of functional activity using the $^{86}Rb^+$ ion flux assay in SH-EP1-h$\alpha4\beta2$, SH-SY5Y ($\alpha3\beta4^*$) and TE671/RD ($\alpha1\beta1\gamma\delta$) cells (Figure 3; Tables 3 and 4). Consistent with the binding data, the azetidines **39** and **43** were found to be more potent than the pyrrolidine **44**, both in agonism and functional inactivation

Table 3. Potencies and Efficacies of Ligand Agonism and Inactivation of Human $\alpha4\beta2$-nAChRs[a]

	agonism		inactivation		K_i (nM)
compound	EC_{50} (nM)	efficacy (%)	IC_{50} (nM)	efficacy (%)	$\alpha4\beta2$
39	1090	93	151	72	7.3
43	1180	108	169	78	4.6
44	>3000	>50	1100	69	129
3	290	88	430	92	4.9
1	1400	53	~110	~85	0.05

[a]See 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. SEM values were determined for each parameter and, although not presented here, typically are less than 3% and frequently less than 1% of the maximal carbamylcholine response for efficacy measures for ligands potent enough to reach maximal efficacy at 10 μM. SEM values for EC_{50} and IC_{50} values were no more than a factor of 2. See Table 2 for structures. For compounds that were not potent enough to cause maximal inhibition at the highest concentration tested, inactivation efficacy was fixed at 100% to allow IC_{50} values to be fit during graphical analysis.

at the $\alpha4\beta2$-nAChR (Figure 3, Table 3). Compounds **39** and **43** had agonist efficacies at the $\alpha4\beta2$-nAChR comparable to compound **3** and higher than that of compound **1**. Compounds **39** and **43** have functional inactivation efficacies lower than those of compound **3** or compound **1**. They were both full agonists at the $\alpha3\beta4^*$-nAChR, with potencies similar to those seen at the $\alpha4\beta2$-nAChR, though they were less potent in the functional inactivation of the $\alpha3\beta4^*$-nAChR (Table 4, Figure 3). Whereas compounds **39** and **43** have high selectivity for

Table 4. Potencies and Efficacies of Ligand Agonism and Inactivation of $\alpha3\beta4^*$-nAChRs[a]

	agonism		inactivation		K_i (nM)
compound	EC_{50} (nM)	efficacy (%)	IC_{50} (nM)	efficacy (%)	$\alpha3\beta4$
39	3400	120	>3000	>70	1270
43	1200	120	>3000	>70	692
44	>3000	>80	>3000	>30	8780
3	30000	90	ND	ND	260
1	2200	110	ND	ND	ND

[a]See Experimental Section and the legend to Table 3 for details.

$\alpha4\beta2$- over $\alpha3\beta4^*$-nAChRs (174- and 150-fold) in the binding affinity assays (Table 2), this selectivity does not translate to the functional assay (Tables 3 and 4, Figure 3). None of the compounds displayed significant activity at the $\alpha1\beta1\gamma\delta$-nAChR. Compounds **31−38** were not potent in our preliminary functional screening.

In addition, compounds **39** and **43** are similar in potency at the $\alpha4\beta2$-nAChR (EC_{50} values of 1090 nM and 1180 nM, respectively) compared to compound **1** (EC_{50} value of 1400 nM). Both are inactive at the $\alpha1\beta1\gamma\delta$-nAChR and have activity similar to compound **1** at the $\alpha3\beta4^*$-nAChR. Whereas in vitro binding and functional data are intriguing, a more proximal measure of therapeutic value is likely to be behavioral pharmacological activity in an animal model of the indication of interest. Therefore, we decided to further test these compounds for their antidepressant profile in vivo.

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 it spends passively floating in the water (immobility) is recorded (Figure 4). Most traditional antidepressants decrease the amount of time the mouse spends immobile. Mice were administered compounds **43** (1 and 5 mg/kg ip) or the selective serotonin reuptake inhibitor (SSRI) antidepressant, sertraline, as a positive control (10 mg/kg). Drug administration produced a reduction in immobility. Fisher's post hoc tests showed that compound **43** reduced immobility at both doses (1 and 5 mg/kg), suggestive of a
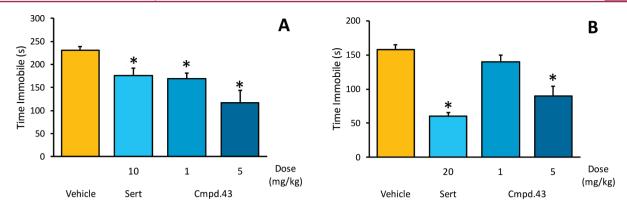
Figure 4. Compound **43** reduced immobility in the mouse forced swim test at low (1 and 5 mg/kg) doses. The SSRI, sertraline, produced the expected decrease in immobility. (ANOVAs: Fs > 6.845, ps < 0.0010. *Fisher's PLSD posthoc test: ps < 0.05 vs vehicle). All drugs were administered intraperitoneally (A) or orally (B); n = 9−10/group). Compound **43** reduced immobility in the forced swim test in mice at 5 mg/kg, but was inactive at 1 mg/kg when administered orally.

potent antidepressant-like effect. Moreover, **43** was also active at an orally administered dose of 5 mg/kg.

Broad Screening at Other Neurotransmitter Receptors and Transporters. A broad-range screening study was carried out for compound **43** to further determine its effects at 10 μM on about 45 other CNS neurotransmitter receptors and transporters, including serotonin receptors, dopamine receptors, GABA receptors, biogenic amine transporters, adrenergic receptors, muscarinic receptors, opioid receptors, sigma receptors, and histamine receptors (NIMH-PDSP, University of North Carolina, Chapel Hill). No inhibition caused by 10 μM of compound **43** was greater than 50% in the preliminary binding screen, indicating that **43** has no significant activity at these targets (see Supporting Information).

Preliminary in Vitro ADME-Tox Profile. Compound **43** showed good properties in assays for cytochrome P450 (CYP) inhibition, metabolic stability, and plasma protein binding (SRI, Stanford Research Institute), as well as hERG (human ether-a-go-go-related gene) inhibition (NIMH-PDSP) (see the Supporting Information).

CYP Inhibition. The inhibitory effect of **43** on in vitro CYP activity in human liver microsomes was screened using a high-throughput multiple CYP assay for CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, and CYP3A4. In the presence of 1 μM and 10 μM of **43**, none of these CYP isoforms' activity was reduced to less than 70% of the control, suggesting that **43** will not alter the metabolism of other xenobiotics or endogenous compounds that are substrates for the CYP isoforms tested.

Metabolic Stability. The metabolic stability of **43** was studied using human and mouse liver microsomes. The test article was incubated at two concentrations (1 μM and 10 μM), and aliquots (100 μL) were removed at various time points (0, 15, 30, and 60 min) for analysis by LC-MS/MS. This study showed that liver microsomes from both species caused a time-dependent decrease in parent drug at both concentrations. Human liver microsomes metabolized **43** to a greater extent in 60 min than mouse liver microsomes; 53.4% and 67.2% of the parent drug remained unchanged at 1 μM and 10 μM, respectively. Incubation with mouse liver microsomes resulted in 67.5% and 73.7% of **43** remaining unchanged after 60 min incubation at 1 μM and 10 μM, respectively.

PPB (Plasma Protein Binding). The binding of **43** to proteins in human and mouse plasma was determined using equilibrium dialysis. Binding of **43** was evaluated at

concentrations of 0, 0.1, 1, and 10 μM. The mean percentage of binding of this compound to human plasma ranged from 8.2% to 17.7%. Its mean percentage of binding to mouse plasma ranged from 12.9% to 19.3%.

hERG Inhibition. HEK293 cells stably expressing recombinant human hERG were used in a fluorescence-based membrane potential assay. The observed hERG inhibition of **43** at 10 μM was similar to that of the negative control, giving 0% hERG blockade.

■ CONCLUSIONS

In the present study, a new series of isoxazole ether nAChR ligands have been identified, and their preliminary SAR has been explored. In the PDSP binding study, most of the 5-alkoxyisoxazole ligands were found to bind to the rat $\alpha 4\beta 2$-nAChR with a significantly higher affinity than to the $\alpha 3\beta 4^*$-nAChR. Compound **43** was identified as the lead compound from this series as it displayed favorable in vitro nAChR binding affinities. The functional potency of isoxazole **43** at the $\alpha 4\beta 2$-nAChR is similar to that of compound **1**, but it has higher efficacy, helping to distinguish it from compound **1**. When tested in vivo, compound **43** demonstrated a potent antidepressant-like activity at both 5 mg/kg and 1 mg/kg ip, and was orally active at 5 mg/kg in the mouse forced swim test. Preliminary in vitro ADME-Tox characterization further suggested promising drug-like properties of **43**. The PDSP broad range screening indicated that compound **43** was highly selective for nAChRs and did not have significant binding affinity to the other 45 neurotransmitter receptors and transporters tested.

It remains unclear whether activity at the $\alpha 3\beta 4^*$-nAChR subtype is an attribute or a detriment for treatment of depression. Further studies are required to fully understand the importance of $\alpha 3\beta 4^*$-nAChR involvement in depression. Our previously reported nicotinic ligands such as sazetidine-A and 3-alkoxy-5-aminopyridine derivatives, that contain appropriate substituents in the 5-position of their pyridine rings, exhibited improved selectivity for $\beta 2$- over $\beta 4$-containing nAChRs.[6,52−55] Considering that the present isoxazole ligands are easily accessible through substitution reactions performed upon a preassembled isoxazole building block, the 5-alkoxyisoxazole scaffold should be a useful starting point for a broader optimization campaign to discover more $\alpha 4\beta 2$-selective nAChR ligands, perhaps importantly with higher efficacy than

compound **1**, that may in due course lead to a novel treatment option for depression.

■ EXPERIMENTAL SECTION

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 unless stated otherwise. ^{1}H NMR spectra were recorded at a spectrometer frequency of 300 or 400 MHz, ^{13}C NMR spectra at 75 or 100 MHz. ^{1}H chemical shifts are reported in δ (ppm) using the δ 7.26 signal of CDCl$_3$, the δ 4.80 signal of D$_2$O, the δ 3.31 signal of CD$_3$OD, or the δ 2.50 signal of DMSO-d_6 as internal standards. ^{13}C chemical shifts are reported in δ (ppm) using the δ 77.23 signal of CDCl$_3$, the δ 49.15 signal of CD$_3$OD, or the δ 39.51 signal of DMSO-d_6 as internal standards. ^{13}C NMR spectra in D$_2$O were not adjusted. Purities of 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 220 or 254 nm on a variable wavelength detector G1314A; flow rate = 1.4 mL/min; gradient of 0 to 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 × 20 mm; flow, 17 mL/min; all solvents containing 0.05 vol% TFA; UV detection at 254 and 220 nm; Gradient I: 25−100% MeOH in water in 30 min, 100% for 5 min, return to 25% in 4 min, and equilibration at 25% 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; Gradient III: 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.

General Procedure for the Deprotection of N-Boc-Amines to Afford TFA Salts (Method A). 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), 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 Mitsunobu Reaction of 1-(tert-Butoxycarbonyl)-2(S)-azetidinylmethanol (15) or 1-(tert-Butoxycarbonyl)-2(S)-pyrrolidinylmethanol (25) with Hydroxyisoxazoles to Afford Alkoxyisoxazoles (Method B). To a stirred solution of a hydroxyisoxazole (1 mmol), alcohol **15** or **25** (1.2 mmol), and PPh$_3$ (1.5 mmol) in anhydrous THF (20 mL) was added diethyl azodicarboxylate or diisopropyl azodicarboxylate (1.5 mmol) dropwise. After stirring overnight at rt, the solvent was evaporated, and the residue was dissolved in EtOAc. The solution was washed with water (20 mL) and brine (15 mL), dried over Na$_2$SO$_4$, filtered, and concentrated under a vacuum. The residue was purified by CC on SiO$_2$ to give the alkoxyisoxazole.

General Procedure for the Reduction of Isoxazolecarboxylic Acid Esters to Alcohols (Method C). To a solution of an isoxazolecarboxylic acid ester (1 mmol) in anhydrous THF (20 mL) was added LiBH$_4$ (4 mmol) with ice cooling under Ar. After stirring overnight at rt, saturated aqueous NH$_4$Cl solution was added with ice cooling. Extraction with EtOAc, drying over Na$_2$SO$_4$, and CC on SiO$_2$ gave the alcohol.

General Procedure for the Preparation of Iodides from Alcohols (Method D). To a stirred solution of an isoxazolylmethanol (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 Ar. 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 Phenyl Ethers from Iodides (Method E). To a stirred solution of an (iodomethyl)-isoxazole (1 mmol) and phenol (2 mmol) in anhydrous DMF (4 mL) was added K$_2$CO$_3$ (6 mmol) under Ar. After stirring overnight at rt, saturated aqueous NH$_4$Cl solution was added. The mixture was extracted with EtOAc (2 × 15 mL), and the combined organic phases were washed with water (3 × 10 mL), dried over Na$_2$SO$_4$, and evaporated. The residue was purified by CC on SiO$_2$ to give the product.

General Procedure for the Preparation of Alkyl Ethers from Alcohols (Method F). To a stirred solution of an isoxazolylmethanol (1 mmol) in anhydrous DMF (2 mL) was added NaH (60% dispersion in oil, 1.9 mmol) with ice cooling under Ar. After stirring for 30 min at rt, alkyl halide (1 mmol) was added. Stirring was continued for 2 h at rt, then the reaction was quenched with saturated aqueous NH$_4$Cl solution with ice cooling. The mixture was extracted with EtOAc (2 × 15 mL), and the combined organic phases were washed with water (3 × 10 mL), dried over Na$_2$SO$_4$, and evaporated. The residue was purified by CC on SiO$_2$ to give the product.

General Procedure for the Preparation of Amines from Iodides (Method G). To a solution of an (iodomethyl)isoxazole (1 mmol) in anhydrous CH$_3$CN (10 mL) were added at rt K$_2$CO$_3$ (6 mmol) and amine (4 mmol). After stirring overnight, the reaction mixture was concentrated. The residue was purified by CC on SiO$_2$ (CH$_2$Cl$_2$/MeOH) to obtain the product.

General Procedure for the Preparation of Carbamates from Alcohol 29 (Method H). A solution of **29** (1 mmol), isocyanate (2 mmol), and 4-(dimethylamino)pyridine (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 SiO$_2$ (acetone/hexane) to obtain the product.

3-[[1-(tert-Butoxycarbonyl)-2(S)-azetidinyl]methoxy]-5-iso-xazolylmethanol (17). This compound was obtained from 1-(tert-butoxycarbonyl)-2(S)-azetidinylmethanol and 3-hydroxyisoxazole-5-carboxylic acid methyl ester in two steps as a colorless oil in 84% yield employing Method B and Method C. ^{1}H NMR (400 MHz, CDCl$_3$) δ 6.57 (s, 1H), 4.58−4.54 (m, 1H), 4.48 (m, 1H), 4.35 (d, 2H, J = 10.8 Hz), 3.92 (s, 1H), 3.84 (t, 2H, J = 7.6 Hz), 2.33−2.28 (m, 1H), 2.22−2.18 (m, 1H), 1.39 (s, 9H).

3-[(2(S)-Azetidinyl)methoxy]-5-isoxazolylmethanol (18). This compound was obtained from **17** employing Method A and Gradient III. Colorless oil; yield 51%; purity 99.8%. $[\alpha]_D^{20}$ 3.1 (c = 0.42, MeOH); ^{1}H NMR (400 MHz, D$_2$O) δ 6.20 (s, 1H), 4.95 (m, 1H), 4.66 (s, 2H), 4.60−4.52 (m, 2H), 4.15 (dd, 1H, J = 9.2, 18.8 Hz), 4.04 (dd, 1H, J = 9.2, 18.4 Hz), 2.67 (q, 2H, J = 8.8 Hz); ^{13}C NMR (100 MHz, D$_2$O) δ 172.8, 171.0, 162.3 (TFA), 115.5 (TFA), 93.0, 67.6, 58.5, 54.7, 43.1, 19.8. Anal. Calcd for C$_8$H$_{12}$N$_2$O$_3$·1.0TFA·0.25H$_2$O (FW 305): C, 39.68; H, 4.49; N, 9.25; F, 18.98. Found: C, 39.94; H, 4.25; N, 8.92; F, 18.98.

3-[(2(S)-Azetidinyl)methoxy]-5-(phenoxymethyl)isoxazole (19). This compound was obtained from **17** and phenol employing Methods D, E, and A and Gradient I. Colorless solid; yields of the individual steps 93%, 89%, and 81%; purity ~ 100%. $[\alpha]_D^{20}$ 1.2 (c = 0.68, MeOH); ^{1}H NMR (400 MHz, D$_2$O) δ 7.02−7.00 (m, 2H), 6.70 (m, 3H), 6.01 (s, 1H), 4.75−4.70 (m, 3H), 4.34 (s, 2H), 4.02 (m, 1H), 3.90 (m, 1H), 2.52−2.43 (m, 2H); ^{13}C NMR (100 MHz, D$_2$O) δ 170.7, 168.8, 161.9 (TFA), 156.8, 129.1, 121.3, 115.9 (TFA), 114.2, 94.7, 67.5, 60.3, 58.1, 43.0, 19.8. Anal. Calcd for C$_{14}$H$_{16}$N$_2$O$_3$·1.1TFA·0.55H$_2$O (FW 398): C, 49.18; H, 4.64; N, 7.08; F, 15.85. Found: C, 48.89; H, 4.27; N, 7.06; F, 15.90.

3-[(2(S)-Azetidinyl)methoxy]-5-[(hexyloxy)methyl]isoxazole (20). This compound was obtained from **17** and MeI employing Methods F and A and Gradient I. Colorless oil; yields of the individual steps 84% and 76%; purity ~ 100%. $[\alpha]_D^{20}$ 2.8 (c = 0.14, MeOH); ^{1}H NMR (400 MHz, D$_2$O) δ 6.10 (s, 1H), 4.90−4.86 (m, 1H), 4.47−4.43

(m, 2H), 4.41 (s, 2H), 4.09 (m, 1H), 4.02−3.95 (m, 1H), 3.45 (t, 2H, J = 6.8 Hz), 2.64−2.52 (m, 2H), 1.53−1.48 (m, 2H), 1.28−1.23 (m, 6H), 0.83−0.80 (m, 3H); ^{13}C NMR (100 MHz, D_2O) δ 170.8, 170.4, 161.8 (TFA), 120.2, 115.8 (TFA), 94.1, 70.7, 67.5, 62.8, 58.2, 43.1, 30.9, 28.6, 24.9, 21.8, 19.9, 13.0. Anal. Calcd for $C_{14}H_{24}N_2O_3$·1.0TFA·0.05H_2O (FW 385): C, 50.14; H, 6.60; N, 7.31; F, 14.87. Found: C, 49.93; H, 6.26; N, 7.18; F, 14.56.

3-[(2(S)-Azetidinyl)methoxy]-5-[(cyclopropylmethoxy)-methyl]isoxazole (21). This compound was obtained from **17** and (bromomethyl)cyclopropane employing Methods F and A and Gradient II. Colorless oil; yields of the individual steps 77% and 59%; purity 99.8%. $[\alpha]_D^{20}$ −0.1 (c = 2.8, MeOH); ^{1}H NMR (400 MHz, D_2O) δ 6.25 (s, 1H), 4.93 (m, 1H), 4.60 (s, 2H), 4.57−4.51 (m, 2H), 4.12 (m, 1H), 4.05 (m, 1H), 3.40 (d, 2H, J = 7.2 Hz), 2.69−2.62 (m, 2H), 1.04 (m, 1H), 0.56−0.52 (m, 2H), 0.22−0.18 (m, 2H); ^{13}C NMR (100 MHz, D_2O) δ 170.9, 170.2, 162.2 (TFA), 115.9 (TFA), 94.6, 75.7, 67.6, 62.2, 58.4, 43.2, 19.9, 9.2, 2.1. Anal. Calcd for $C_{12}H_{18}N_2O_3$·1.1TFA·0.65H_2O (FW 378): C, 45.43; H, 5.48; N, 7.46; F, 16.70. Found: C, 45.20; H, 5.19; N, 7.31; F, 16.84.

3-[[1-(tert-Butoxycarbonyl)-2(S)-azetidinyl]methoxy]-5-methylisoxazole (23). This compound was obtained from 1-(tert-butoxycarbonyl)-2(S)-azetidinylmethanol and 3-hydroxy-5-methylisoxazole as a pale-yellow solid in 67% yield employing Method B. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.63 (s, 1H), 4.48−4.42 (m, 2H), 4.29−4.26 (m, 2H), 3.83 (t, 2H, J = 9.4 Hz), 2.31−2.16 (m, 5H), 1.39 (s, 9H); ^{13}C NMR (100 MHz, D_2O) δ 172.3, 170.6, 156.3, 93.1, 79.8, 69.9, 60.2, 47.0, 28.5, 21.9, 19.0, 13.0.

3-[(2(S)-Azetidinyl)methoxy]-5-methylisoxazole (24). This compound was obtained employing Method A and Gradient III. Colorless oil; yield 58%; purity 99.7%. $[\alpha]_D^{20}$ −0.2 (c = 2.4, MeOH); ^{1}H NMR (400 MHz, D_2O) δ 5.90 (s, 1H), 4.98 (m, 1H), 4.46 (m, 1H), 4.12 (m, 1H), 4.00 (m, 1H), 2.66−2.58 (m, 2H), 2.30 (s, 3H); ^{13}C NMR (100 MHz, D_2O) δ 172.3, 171.2, 162.1 (TFA), 115.9 (TFA), 92.4, 67.3, 58.5, 43.2, 19.8, 11.6. Anal. Calcd for $C_8H_{12}N_2O_2$·1.15TFA·0.55H_2O (FW 312): C, 40.01; H, 4.64; N, 9.06; F, 21.20. Found: C, 39.88; H, 4.43; N, 9.11; F, 21.41.

5-[(1-(tert-Butoxycarbonyl)-2(S)-azetidinyl)methoxy]-isoxazole-3-carboxylic Acid Ethyl Ester (27). This compound was obtained from 1-(tert-butoxycarbonyl)-2(S)-azetidinylmethanol and 5-hydroxyisoxazole-3-carboxylic acid ethyl ester (Princeton Bio) as a pale-yellow solid in 55% yield employing Method B. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.71 (s, 1H), 4.52 (m, 2H), 4.41 (q, 2H, J = 7.2 Hz), 4.31 (dd, 1H, J = 2.4, 10.0 Hz), 3.89−3.84 (m, 2H), 1.41 (s, 9H), 1.25 (t, 3H, J = 7.2 Hz).

5-[(1-(tert-Butoxycarbonyl)-2(S)-pyrrolidinyl)methoxy]-isoxazole-3-carboxylic Acid Ethyl Ester (28). This compound was obtained from 1-(tert-butoxycarbonyl)-2(S)-pyrrolidinylmethanol and 5-hydroxyisoxazole-3-carboxylic acid ethyl ester as a pale-yellow solid in 65% yield employing Method B. ^{1}H NMR (400 MHz, $CDCl_3$; two rotamers about the N-Boc bond in an approximate ratio of 1:1) δ 5.66 (br, 1H), 4.35 (q, 2H, J = 7.2 Hz), 4.28 (br, 1.5H), 4.07 (br, 1.5H), 3.29 (br, 2H), 2.01−1.83 (m, 4H), 1.41 (s, 9H), 1.34 (t, 3H, J = 7.2 Hz).

5-[(1-(tert-Butoxycarbonyl)-2(S)-pyrrolidinyl)methoxy]-3-isoxazolylmethanol (30). This compound was obtained from 5-[[1-(tert-butoxycarbonyl)-2(S)-pyrrolidinyl]methoxy]isoxazole-3-carboxylic acid ethyl ester as a pale-yellow solid in 94% yield employing Method C. ^{1}H NMR (400 MHz, $CDCl_3$; two rotamers about the N-Boc bond in an approximate ratio of 1:1) δ 5.28 (br, 0.5H), 5.22 (br, 0.5H), 4.43 (d, 2H, J = 5.6 Hz), 4.25−3.97 (m, 4H), 3.22 (br, 2H), 1.90−1.75 (m, 4H), 1.32 (s, 9H).

5-[(2(S)-Azetidinyl)methoxy]-3-isoxazolylmethanol (31). 5-[[1-(tert-Butoxycarbonyl)-2(S)-azetidinyl]methoxy]-3-isoxazolylmethanol (29) was obtained from 27 as a pale-yellow solid in quantitative yield employing Method C. The title compound 31 was obtained from 29 employing Method A and Gradient III. Colorless oil; yield 67%; purity 100%. $[\alpha]_D^{20}$ −3.0 (c = 1.2, MeOH); ^{1}H NMR (400 MHz, D_2O) δ 5.52 (s, 1H), 4.85 (m, 1H), 4.53−4.45 (m, 4H), 4.06 (m, 1H), 3.95 (m, 1H), 2.60−2.53 (m, 2H); ^{13}C NMR (100 MHz, D_2O) δ 172.3, 166.1, 162.3 (TFA), 115.9 (TFA), 77.4, 69.8, 58.1, 55.5, 43.2,

19.8. Anal. Calcd for $C_8H_{12}N_2O_3$·1.4TFA (FW 344): C, 37.73; H, 3.93; N, 8.15; F, 23.21. Found: C, 37.82; H, 4.10; N, 8.47; F, 23.26.

5-[(2(S)-Azetidinyl)methoxy]-3-(phenoxymethyl)isoxazole (32). The title compound was obtained from **29** employing Method A and Gradient II. Colorless solid; yield 38%; purity 98.9%. $[\alpha]_D^{20}$ −1.0 (c = 1.1, MeOH); ^{1}H NMR (400 MHz, CD_3OD) δ 7.30−7.26 (m, 2H), 7.00−6.94 (m, 3H), 5.69 (s, 2H), 4.90 (m, 1H), 4.59 (m, 1H), 4.52 (m, 1H), 4.09 (m, 1H), 4.00 (m, 1H), 2.62 (m, 1H); ^{13}C NMR (100 MHz, CD_3OD) δ 172.6, 163.0, 160.1 (TFA), 157.7, 128.8, 120.8, 115.9 (TFA), 114.1, 77.4, 69.9, 61.3, 58.0, 42.8, 19.9. Anal. Calcd for $C_{14}H_{16}N_2O_3$·1.55TFA·0.45H_2O (FW 445): C, 46.14; H, 4.18; N, 6.29; F, 19.85. Found: C, 45.98; H, 4.04; N, 6.39; F, 20.03.

3-(Phenoxymethyl)-5-[(2(S)-pyrrolidinyl)methoxy]isoxazole (33). This compound was obtained employing Method A and Gradient II. Colorless solid; yield 75%; purity 100%. $[\alpha]_D^{20}$ −1.7 (c = 1.5, MeOH); ^{1}H NMR (400 MHz, CD_3OD) δ 7.30−7.26 (m, 2H), 7.00−6.94 (m, 3H), 5.69 (s, 2H), 5.04 (s, 2H), 4.62 (m, 1H), 4.40 (m, 1H), 4.06 (m, 1H), 3.36 (t, 2H, J = 7.2 Hz), 2.26 (m, 1H), 2.13−2.05 (m, 2H), 1.87 (m, 1H); ^{13}C NMR (100 MHz, CD_3OD) δ 172.3, 162.8, 160.0 (TFA), 157.5, 128.6, 120.6, 115.7 (TFA), 113.8, 77.1, 69.8, 61.1, 57.7, 45.0, 25.1, 22.7. Anal. Calcd for $C_{15}H_{18}N_2O_3$·1.2TFA·0.35H_2O (FW 417): C, 50.06; H, 4.80; N, 6.71; F, 16.38. Found: C, 50.22; H, 4.73; N, 6.67; F, 16.14.

3-(Anilinomethyl)-5-[(2(S)-azetidinyl)methoxy]isoxazole (34). The title compound was obtained from **29** employing Methods D, G, and A and Gradient III. Pale-yellow oil; yields of the individual steps 76%, quantitative and 35%; purity 99.9%. $[\alpha]_D^{20}$ −2.7 (c = 0.79, MeOH); ^{1}H NMR (400 MHz, D_2O) δ 7.52 (t, 2H, J = 7.6 Hz), 7.44 (t, 1H, J = 7.4 Hz), 7.35 (d, 2H), 5.61 (s, 1H), 4.94−4.92 (m, 1H), 4.63 (s, 1H), 4.58−4.53 (m, 2H), 4.17−4.10 (m, 1H), 4.06−3.99 (m, 1H), 2.70−2.60 (m, 2H); ^{13}C NMR (100 MHz, D_2O) δ 172.8, 162.5 (TFA), 159.0, 135.7, 129.8, 127.8, 120.7, 115.9 (TFA), 78.8, 69.9, 58.0, 44.6, 43.2, 19.7. Anal. Calcd for $C_{14}H_{17}N_3O_2$·1.8TFA·1.25 H_2O (FW 487): C, 43.40; H, 4.41; N, 8.63; F, 21.06. Found: C, 43.34; H, 4.27; N, 8.76; F, 21.09.

5-[(2(S)-Azetidinyl)methoxy]-3-[(4-fluoroanilino)methyl]-isoxazole (35). This compound was obtained from **29** employing Methods D, G, and A and Gradient III. Pale-yellow oil; yields of the individual steps 76%, quantitative, and 74%; purity 99.5%. $[\alpha]_D^{20}$ −2.0 (c = 1.4, MeOH); ^{1}H NMR (400 MHz, D_2O) δ 7.13−7.08 (m, 4H), 5.58 (s, 1H), 4.92 (m, 1H), 4.63 (s, 1H), 4.58−4.54 (m, 2H), 4.44 (s, 2H), 4.13 (m, 1H), 4.02 (m, 1H), 2.68−2.59 (m, 2H); ^{13}C NMR (100 MHz, D_2O) δ 172.5, 162.2, 162.2 (TFA), 158.7 (d, J_{C-F} = 20.3 Hz), 137.5, 119.1 (d, J_{C-F} = 8.3 Hz), 115.9 (TFA), 115.9 (d, J_{C-F} = 238.4 Hz), 78.3, 69.8, 58.0, 43.2, 42.5, 19.7. Anal. Calcd for $C_{14}H_{16}FN_3O_2$·1.4TFA·0.55H_2O (FW 447): C, 45.16; H, 4.17; N, 9.40; F, 22.11. Found: C, 45.17; H, 4.13; N, 9.35; F, 22.09.

5-[(2(S)-Azetidinyl)methoxy]-3-[(N-phenylcarbamoyloxy)-methyl]isoxazole (36). This compound was obtained from **29** and phenyl isocyanate employing Methods H and A and Gradient II. Colorless oil; yields of the individual steps 93% and 40%; purity 99.8%. $[\alpha]_D^{20}$ −3.7 (c = 3.4, MeOH); ^{1}H NMR (400 MHz, D_2O) δ 7.22 (d, 2H, J = 8.0 Hz), 7.09 (t, 2H, J = 8.0 Hz), 6.87 (t, 2H, J = 7.4 Hz), 5.39 (s, 1H), 4.93 (s, 2H), 4.74 (m, 1H), 4.31−4.25 (m, 2H), 4.01 (m, 1H), 3.90−3.85 (m, 1H), 2.55−2.36 (m, 2H); ^{13}C NMR (100 MHz, D_2O) δ 172.2, 162.2, 162.0 (TFA), 153.8, 137.0, 128.5, 123.4, 118.8, 115.9 (TFA), 77.7, 69.7, 58.0, 57.8, 43.1, 20.0. Anal. Calcd for $C_{15}H_{17}N_3O_4$·1.55TFA·2.35 H_2O (FW 552): C, 41.62; H, 4.49; N, 8.04; F, 16.91. Found: C, 41.51; H, 4.46; N, 8.19; F, 16.94.

5-[(2(S)-Azetidinyl)methoxy]-3-[(N-ethylcarbamoyloxy)-methyl]isoxazole (37). This compound was obtained from **29** and ethyl isocyanate employing Methods H and A and Gradient III. Colorless oil; yields of the individual steps 99% and 50%; purity 99.6%. $[\alpha]_D^{20}$ −3.0 (c = 2.8, MeOH); ^{1}H NMR (400 MHz, D_2O) δ 5.62 (s, 1H), 5.04 (s, 2H), 4.96 (m, 1H), 4.63−4.55 (m, 2H), 4.15 (m, 1H), 4.04 (m, 1H), 3.13 (q, J = 7.2 Hz, 2H), 2.72−2.62 (m, 2H), 1.08 (t, J = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, D_2O) δ 172.4, 163.1, 162.2 (TFA), 157.0, 115.9 (TFA), 77.7, 69.9, 58.1, 58.0, 43.2, 35.2, 19.8, 13.6. Anal. Calcd for $C_{11}H_{17}N_3O_4$·1.4TFA·0.95H_2O (FW 432): C, 38.37; H, 4.74; N, 9.73; F, 18.47. Found: C, 38.13; H, 4.86; N, 9.97; F, 18.63.

5 - [(2 (S) - A z e t i d i n y l) m e t h o x y] - 3 - [(N - cyclopentylcarbamoyloxy)methyl]isoxazole (38). This compound was obtained from **29** and cyclopentyl isocyanate employing Methods H and A and Gradient III. Colorless oil; yields of the individual steps 94% and 40%; purity 99.6%. $[\alpha]_D^{20}$ −2.2 (c = 1.7, MeOH); ^{1}H NMR (400 MHz, D_2O) δ 5.63 (s, 1H), 5.07 (s, 2H), 4.96 (m, 1H), 4.65−4.57 (m, 2H), 4.13 (m, 1H), 4.06 (m, 1H), 3.87 (m 1H), 2.70−2.64 (m, 2H), 1.91−1.85 (m, 2H), 1.66−1.57 (m, 4H), 1.49−1.43 (m, 2H); ^{13}C NMR (100 MHz, D_2O) δ 172.4, 163.1, 162.4 (TFA), 156.7, 115.7 (TFA), 77.7, 69.8, 58.0, 57.9, 52.3, 43.2, 31.8, 22.7, 19.7. Anal. Calcd for $C_{14}H_{21}N_3O_4$·1.7TFA·1.65H_2O (FW 522): C, 40.28; H, 5.05; N, 8.10; F, 18.67. Found: C, 40.09; H, 4.81; N, 8.28; F, 18.81.

5-[(2(S)-Azetidinyl)methoxy]-3-(fluoromethyl)isoxazole Trifluoroacetate (39). To a solution of Et_2NSF_3 (0.22 mL, 1.7 mmol) in anhydrous CH_2Cl_2 (10 mL) was added at −78 °C 5-[[1-(*tert*-butoxycarbonyl)-2(S)-azetidinyl]methoxy]-3-isoxazolylmethanol (425 mg, 1.5 mmol) in CH_2Cl_2 (5 mL) under Ar. The solution was stirred for 2 h at −78 °C, then allowed to warm to rt. After the reaction was quenched with saturated aqueous $NaHCO_3$ solution, the phases were separated. The aqueous layer was extracted with CH_2Cl_2 (2 × 10 mL), and the combined organic phases were washed with water (5 mL), dried over Na_2SO_4, and concentrated. The residue was subjected to CC on SiO_2 to obtain 5-[(1-(*tert*-butoxycarbonyl)-2(S)-azetidinyl)methoxy]-3-(fluoromethyl)isoxazole (70 mg, 16%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.39 (s, 1H), 5.32 (s, 1H), 5.21 (s, 1H), 4.48−4.44 (m, 2H), 4.26 (m, 1H), 3.87−3.78 (m, 2H), 2.35−2.22 (m, 2H), 1.39 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.2, 161.9 (d, J_{C-F} = 22.4 Hz), 156.1, 80.1, 77.2, 76.6 (d, J_{C-F} = 165.9 Hz), 72.6, 59.1, 47.3, 28.4, 18.7.

This title compound was obtained employing Method A and Gradient III. Colorless oil; yield 46%; purity 99.4%. $[\alpha]_D^{20}$ −2.8 (c = 1.9, MeOH); ^{1}H NMR (400 MHz, D_2O) δ 5.74 (s, 1H), 5.48 (s, 1H), 5.36 (s, 1H), 4.97 (m, 1H), 4.66−4.58 (m, 2H), 4.15 (m, 1H), 4.06 (m, 1H), 2.72−2.64 (m, 2H); ^{13}C NMR (100 MHz, D_2O) δ 172.6, 162.3 (TFA), 162.3 (d, J_{C-F} = 20.3 Hz), 115.9 (TFA), 77.8, 76.2 (d, J_{C-F} = 162.3 Hz), 70.0, 58.1, 43.2, 19.8; ^{19}F NMR (376 MHz, D_2O) δ −76, −222. Anal. Calcd for $C_8H_{11}FN_2O_2$·1.05TFA·0.85 H_2O (FW 323): C, 37.77; H, 4.31; N, 8.72; F, 24.54. Found: C, 37.50; H, 4.03; N, 8.65; F, 24.32.

5-[[1-(*tert*-Butoxycarbonyl)-2(S)-azetidinyl]methoxy]-3-methylisoxazole (41). This compound was obtained from **15** and 3-methylisoxazol-5(4H)-one morpholine salt (**40**; Fluka) as a pale-yellow solid in 67% crude yield employing Method B. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.07 (s, 1H), 4.45−4.39 (m, 2H), 4.18 (dd, 2H, J = 10.0, 1.5 Hz), 3.78 (t, 2H, J = 8.4 Hz), 2.30−2.21 (m, 2H), 2.13 (s, 3H), 1.36 (s, 9H).

5-[(2(S)-Azetidinyl)methoxy]-3-methylisoxazole (43). This compound was obtained from **41** employing Method A and Gradient III. Colorless oil; yield 38%; purity 100%. $[\alpha]_D^{20}$ −1.9 (c = 0.47, MeOH); ^{1}H NMR (400 MHz, D_2O) δ 5.46 (s, 1H), 4.56 (m, 1H), 4.39 (m, 1H), 4.10 (m, 1H), 3.40 (t, 2H, J = 7.2 Hz), 2.27 (m, 1H), 2.20 (s, 3H), 2.16−2.06 (m, 2H), 1.90 (m, 1H); ^{13}C NMR (100 MHz, D_2O) δ 171.9, 164.0, 162.3 (TFA), 115.9 (TFA), 79.2, 69.6, 58.1, 43.1, 19.8, 10.9. Anal. Calcd for $C_8H_{12}N_2O_2$·1.1TFA·0.25H_2O (FW 300): C, 41.09; H, 4.60; N, 9.40; F, 21.03. Found: C, 40.94; H, 4.40; N, 9.23; F, 21.02.

3-Methyl-5-[(2(S)-pyrrolidinyl)methoxy]isoxazole (44). The title compound was obtained from **25** and **40** employing Method B and A and Gradient III. Colorless oil; yields of the individual steps 58% and 38%; purity 99.5%. $[\alpha]_D^{20}$ 6.7 (c = 2.6, MeOH); ^{1}H NMR (400 MHz, D_2O) δ 5.46 (s, 1H), 4.56 (m, 1H), 4.39 (m, 1H), 4.10 (m, 1H), 3.40 (t, 2H, J = 7.2 Hz), 2.27 (m, 1H), 2.20 (s, 3H), 2.16−2.06 (m, 2H), 1.90 (m, 1H); ^{13}C NMR (100 MHz, D_2O) δ 171.9, 164.0, 162.3 (TFA), 115.9 (TFA), 79.2, 69.7, 57.9, 45.5, 25.2, 22.9, 10.9. Anal. Calcd for $C_9H_{14}N_2O_2$·1.1TFA·0.8H_2O (FW 324): C, 41.77; H, 5.23; N, 8.70; F, 19.47. Found: C, 41.57, H, 4.85; N, 8.58; F, 19.13.

In Vitro Binding Studies. [^{3}H]Epibatidine competition studies and broad-range screening were carried out by the National Institute of Mental Health's Psychoactive Drug Screening Program, Contract #

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 $\alpha1$*-nAChRs, containing $\alpha1$, $\beta1$, γ, and δ subunits, with function detectable using [86]Rb^{+} efflux assays.[56] The human neuroblastoma cell line SH-SY5Y naturally expresses autonomic $\alpha3\beta4$*-nAChRs, containing $\alpha3$, $\beta4$, probably $\alpha5$, and sometimes $\beta2$ subunits, and also displays function detectable using [86]Rb^{+} efflux assays.[57] SH-SY5Y cells also express homopentameric $\alpha7$-nAChRs; however, their function is not detected in the [86]Rb^{+} efflux assay under the conditions used. SH-EP1 human epithelial cells stably transfected with human $\alpha4$ and $\beta2$ subunits (SH-EP1-h$\alpha4\beta2$ cells) have been established and characterized with both ion flux and radioligand binding assays.[58]

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.[56] 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.

[86]Rb^{+} Efflux Assays. Function of nAChR subtypes was investigated using an established [86]Rb^{+} efflux assay protocol.[56] 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 μL 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 μL per well of complete medium supplemented with ~350000 cpm of [86]Rb^{+} (NEN; counted at 40% efficiency using Cerenkov counting and the Packard TriCarb 1900 Liquid Scintillation Analyzer). After at least 4 h and typically overnight, [86]Rb^{+} efflux was measured using the "flip-plate" technique. Briefly, after aspiration of the bulk of [86]Rb^{+} loading medium from each well of the "cell plate," each well containing cells was rinsed with 2 mL of fresh [86]Rb^{+} efflux buffer (130 mM NaCl, 5.4 mM KCl, 2 mM $CaCl_2$, 5 mM glucose, and 50 mM HEPES; pH 7.4) to remove extracellular [86]Rb^{+}. 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 minutes 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 ~EC_{90} concentration of the full agonist carbamylcholine for 5 min (~EC_{90} concentrations were 200 μM for SH-EP1-h$\alpha4\beta2$ 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 [86]Rb^{+} in cell plates and efflux/drug plates was defined to confirm material balance (i.e., that the sum of [86]Rb^{+} released into the efflux/drug plates and [86]Rb^{+} remaining in the cell plate were the same for each well). Similarly, the sum of [86]Rb^{+} in cell plates and efflux/drug plates also determined the efficiency of [86]Rb^{+} loading (the percentage of applied [86]Rb^{+} actually loaded into cells). Furthermore, the sum of [86]Rb^{+} in cell

plates and the second efflux/drug plates defined the amount of intracellular $^{86}Rb^+$ 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 $^{86}Rb^+$ efflux was assessed in the presence of a fully efficacious concentration of carbamylcholine alone (1 mM for SH-EP1-hα4β2 and TE671/RD cells, or 3 mM for SH-SY5Y cells). Nonspecific $^{86}Rb^+$ efflux in another set of control samples was measured either in the presence of the fully efficacious concentration of carbamylcholine plus 100 μM 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 $^{86}Rb^+$-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 ~EC$_{90}$ 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 $^{86}Rb^+$ 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 $^{86}Rb^+$. Even in samples previously giving an efflux response during the initial 10 min exposure to a partial or full agonist, residual intracellular $^{86}Rb^+$ 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 $^{86}Rb^+$ available at the time of the assay, as absolute levels of total, nonspecific, or specific efflux varied in cells partially depleted of intracellular $^{86}Rb^+$ 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 $^{86}Rb^+$ typically were corrected for any variation in the electrochemical gradient of $^{86}Rb^+$ created by intracellular ion depletion after the first (agonism/pretreatment) drug treatment.

Ion flux assays ($n \geq 3$ 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 EC$_{50}$ ($n > 0$ for agonists) or IC$_{50}$ ($n < 0$ 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.

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 **43** were synthesized as described above, and sertraline was purchased from Toronto Research Chemicals (Ontario, Canada). All compounds were dissolved in injectable water and administered by intraperitoneal (IP) injection or oral gavage (PO) 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 ×10 cm wide, 1 L beakers) containing 23 ± 1 °C water 12 cm deep (approximately 800 mL). Mice were administered vehicle, the SSRI sertraline (10 or 20 mg/kg) as a positive control, or compound **43** (1 or 5 mg/kg). Thirty minutes following IP or PO 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 **43** (1 and 5 mg/kg)) 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 test.

ASSOCIATED CONTENT

Ⓢ Supporting Information

Broad screening data and detailed preliminary in vitro ADME-Tox profile. This material is available free of charge via the Internet at http://pubs.acs.org.

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ACKNOWLEDGMENTS

ABBREVIATIONS

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); SAR, structure−activity relationship; ADME-Tox, absorption, distribution, metabolism, excretion, and toxicity; SSRI, selective serotonin reuptake inhibitor; CYP, cytochrome P450; PPB, plasma protein binding; hERG, human ether-a-go-go-related gene; CC, column chromatography; rt, room temperature; TFA, trifluoroacetic acid

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