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Synthesis and Behavioral Studies of Chiral Cyclopropanes as Selective α4β2-Nicotinic Acetylcholine Receptor Partial Agonists Exhibiting an Antidepressant Profile. Part III.

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KEYWORDS: Nicotinic acetylcholine receptors, selective α4β2 partial agonists, N-methylpyrrolidine, cyclopropane.

Abstract

We report the synthesis and biological characterization of novel derivatives of 3-[(1-methyl-2(*S*)-pyrrolidinyl)methoxy]-5-cyclopropylpyridine (**4a**-**f** and **5**) as potent and highly selective α4β2-nicotinic acetylcholine receptor (nAChR) full or partial agonists. A systematic structure-activity study was carried out on the previously described compound **3b**, particularly concerning its (2-methoxyethyl)cyclopropyl side-chain, in an effort to improve its metabolic stability while maintaining receptor selectivity. Compound **4d** exhibited very similar subnanomolar binding affinity for α4β2- and α4β2*-nAChRs compared to **3b**, and it showed excellent potency in activating high-sensitivity (HS) α4β2-nAChRs with an EC_{50} value of 8.2 nM. Testing of **4d** in the SmartCube® assay revealed that the compound has a combined antidepressant plus antipsychotic signature. In the forced swim test at a dose of 30 mg/kg given intraperitoneally, **4d** was found to be as efficacious as sertraline, thus providing evidence of the potential use of the compound as an antidepressant. Additional promise for use of **4d** in humans comes from pharmacokinetic studies in mice indicating brain penetration, and additional assays show compound stability in the presence of human microsomes and hepatocytes. Thus, **4d** has a very favorable preclinical drug profile.

INTRODUCTION

Neuronal nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels that are widely expressed throughout the peripheral and central nervous systems. nAChRs are pentameric assemblies made of various combinations of protein subunits (α1−α10, β1−β4, γ, δ, and ε). The predominant subtypes found in the mammalian central nervous system (CNS) are the homomeric α7-nAChR and the heteromeric α4β2*-nAChR (the asterisk indicates that the receptor complex may, or is known to, contain additional subunits). nAChRs subtypes have been targeted over the years with the aim of identifying drugs that could potentially treat various central nervous system disorders, such as schizophrenia, Alzheimer's disease, nicotine addiction, and depression.[1-4] The pharmacological and physiological properties of nAChRs subtypes are primarily dependent on their subunit makeup. For instance, α4β2*-nAChR subtypes have a higher affinity for acetylcholine (ACh) than most subtypes and have been implicated to play a vital role in depression, cognition, anxiety, and nicotine addiction.[4-6]

Numerous pre-clinical studies using murine models have reported the anti-depressant-like effects observed after the administration of α4β2*-nAChR partial agonists or antagonists.[4, 6] The smoking cessation drug varenicline (**1**), a partial agonist at α4β2-nAChRs and full agonist at α7- and α3β4-nAChRs, has been reported to display antidepressant-like properties in animal models.[7, 8] However, the use of varenicline has been associated with side effects such as nausea, sleep disturbance, and constipation, which may be related to its insufficient selectivity over α3β4*-nAChRs.[9-11] Mecamylamine, a nonselective and noncompetitive nAChR antagonist, also possess antidepressant-like effects in wild-type mice, which are absent in nAChR β2 or α7 subunit knockout mice, suggesting that these nAChR subunits play an important role in mood regulation.[12] However, mecamylamine could not be further developed due to its failure to show efficacy in human clinical trials,[13] again, perhaps because of its lack of selectivity toward α4β2*-nAChRs. Identifying novel ligands that offer high selectivity for a specific nAChR subtype over the plethora of existing nAChRs could facilitate the development of therapeutic agents for various neurological disorders, but that would lack side-effects commonly related to poorly selective ligands.

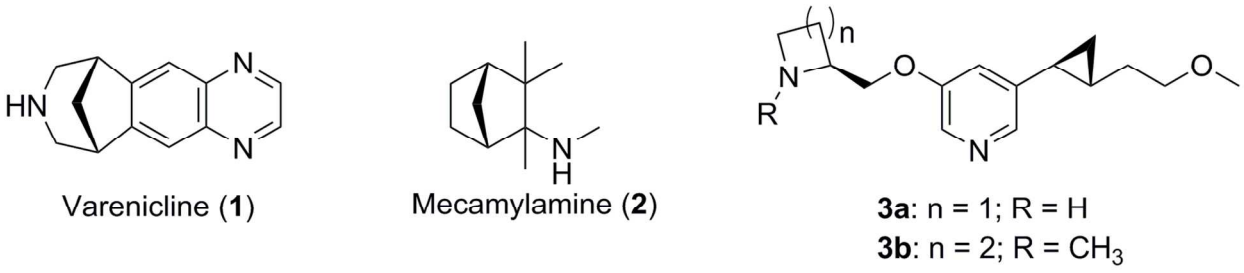


Figure 1. Examples of nicotinic receptor ligands (**1**-**3**).

Our group recently reported the discovery of compound **3a**, an antidepressant agent, with high selectivity for α4β2*-nAChRs, where it acts as a partial agonist. Compound **3a** showed remarkable binding affinity to α4β2*-nAChRs (K_i = 0.1 nM) with little or no affinity observed at α3β4*- and α7-nAChRs. This compound also showed a favorable behavioral profile in the SmartCube® assay and proved to be active in the mouse forced swim test (FST).[14] Unfortunately, the protonated form of compound **3a** (hydrochloride or trifluoroacetate salts) was found to partially decompose forming a dimer (unpublished).[15]

In an effort to address this problem, compound **3b** was designed to eliminate the labile azetidine ring by replacing it with a pyrrolidine ring (Figure 1).[16] Compound **3b** showed promising binding affinity and selectivity at nAChR subtypes containing β2 subunits (α4β2-nAChR, K_i = 0.3 nM; α4β2*-nAChR, K_i = 0.5 nM; inactive at α3β4-nAChR), as well as excellent antidepressant-like properties, confirmed by the forced swim test (FST).[16] As expected, compound **3b** proved to be stable and not prone to dimerization. However, upon ADMET (absorption, distribution, metabolism, excretion and toxicity) screening, compound **3b** proved metabolically unstable in assays with both mouse and rat hepatocytes, thus rendering it less attractive as a potential pharmacotherapeutic.

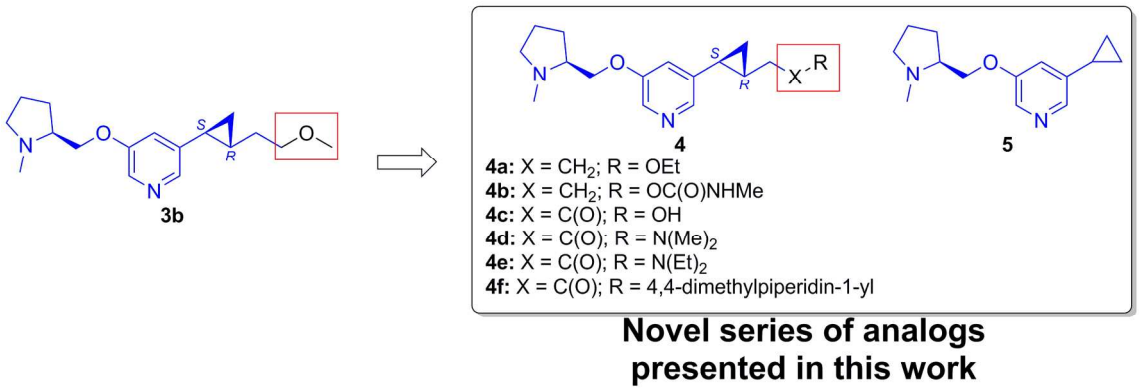


Figure 2. Design of novel cyclopropane derivatives presented in this work as selective α4β2*-nAChR ligands.

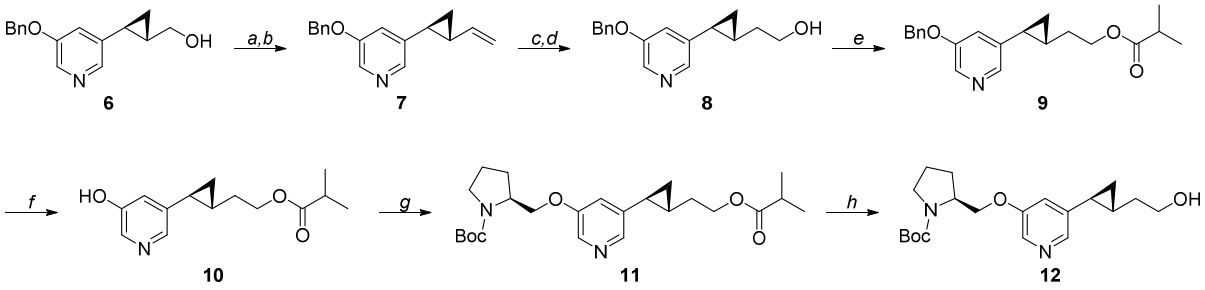
Based on the structure of our lead compound, **3b**, we embarked on a small, structure-activity relationship campaign with the main goal of improving its ADMET properties while maintaining high and selective binding affinity toward α4β2*-nAChRs, as well as excellent functional activity and behavioral properties. Given the fact that the *N*-methylpyrrolidine substituent on the position C3 of the pyridine core structure proved to be the best option so far, we focused on modifications to the (2-methoxyethyl)cyclopropyl side-chain attached to position 5 of the pyridine ring, while maintaining the cyclopropane linker in the preferred 1*S*,2*R* configuration (Figure 2).

We first chose to elongate the terminal ether functional group by one carbon atom, leading to the ethoxy-substituted compound **4a**. Next, we replaced the ether group by a moiety capable of additional receptor interactions, namely by a carbamate group as found in compound **4b**. The other analogs prepared (**4c**-**4f**) are all carboxylic acid derivatives in which the methoxy group of compound **3b** is replaced by a carboxyl group, afford the free carboxylic acid **4c**, dimethylamide **4d**, diethylamide **4e,** and the bulkier 4,4-dimethylpiperidin-1-yl derivative **4f**, respectively. Finally, compound **5**, lacking the side-chain attached to the cyclopropane ring, and thus not bearing any stereogenic centers on the C5 side of the pyridine core, was chosen to further explore the importance of the side-chain to receptor affinity. We believed that this side-chain, constrained to the proper orientation by the rigidity of the cyclopropane structure, is essential to achieving the selectivity profile of these analogs across the nAChR subtypes and thereby avoiding potential side effects associated with α3β4*-nAChR activity.

CHEMISTRY

The synthesis of the key intermediate **12**, that was used to prepare analogs **4a**-**f**, is depicted in Scheme 1. The optically pure starting material (1*S*,2*S*)-2-[5-(benzyloxy)-3-pyridyl]-cyclopropylmethanol **6** was prepared according to the previously reported procedure.[14] Standard Swern oxidation and Wittig reaction converted compound **6** into alkene **7**. Compound **7** was subjected to a hydroboration-oxidation sequence to yield alcohol **8**, which was then protected by acylation using isobutyric anhydride to furnish ester **9**. After removal of the benzyl group by hydrogenolysis, the Boc-protected pyrrolidine group was appended using the Mitsunobu reaction to give intermediate **11**. Subsequent removal of the isobutyryl group gave compound **12**.

Scheme 1. Synthesis of intermediate **12**.[a]

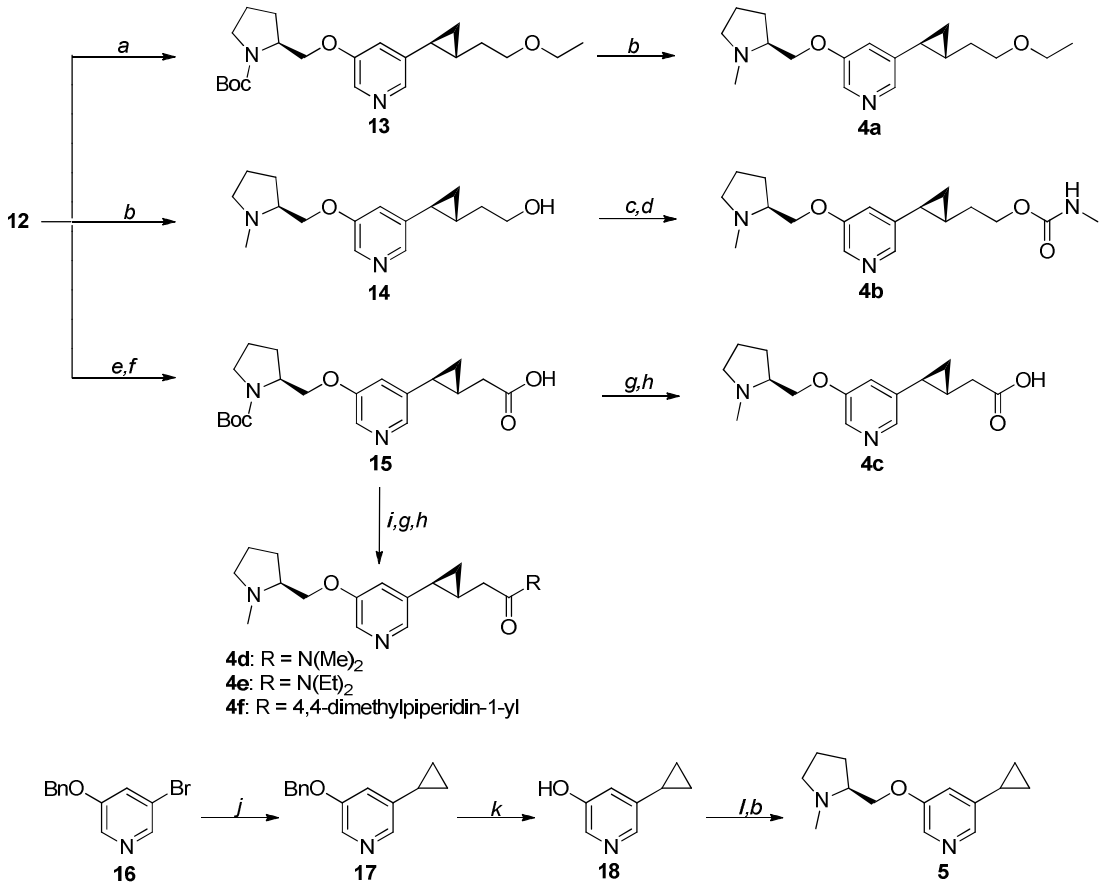


[a]Reagents and conditions: *a*) $(COCl)_2$, DMSO, TEA, DCM, -78 °C, 2 h; *b*) methyltriphenylphosphonium bromide, LiHMDS, THF, -78 °C to rt, 2.5 h; *c*) cyclohexene, BH_3·THF, THF, 0 °C to rt, overnight; *d*) MeOH, 3 N aqueous NaOH, 35% aqueous H_2O_2, 55 °C, 1 h; *e*) DMAP, TEA, isobutyric anhydride, DCM, 0 °C, 2.5 h; *f*) 10% Pd/C, H_2, EtOAc/MeOH, rt, 1.5 h; *g*) 1,1'-azodicarbonyldipiperidine (ADDP), P^{n}Bu$_3$, *N*-Boc-L-prolinol, toluene/THF, 0 °C to rt, overnight; *h*) NaOMe, MeOH, 40 °C, 1.5 h.

Alkylation of **12** with iodoethane in the presence of NaH followed by $LiAlH_4$-mediated reduction of the Boc group afforded compound **4a** (Scheme 2). Application of the same reduction procedure to intermediate **12** gave **14**, which was then converted into the carbamate **4b** by reaction with 1,1'-carbonyldiimidazole (CDI) followed by reaction with methylamine. The carboxylic acid derivatives **4c**-**f** were synthesized from **12** by first carrying out a Swern oxidation followed by Pinnick oxidation of the intermediate aldehyde, to afford the Boc-protected compound **15**. Deprotection of **15** with triflouroacetic acid (TFA) followed by reductive

amination with formaldehyde (HCHO) afforded compound **4c** as its trifluoroacetate salt. By employing standard amide coupling techniques, compound **15** was reacted with the appropriate dialkylamines and then deprotected and *N*-methylated to yield the desired compounds **4d-f**. Compound **5** was synthesized starting from 3-(benzyloxy)-5-bromopyridine (**16**)[17] via Suzuki coupling with cyclopropylboronic acid to afford **17**, followed by hydrogenative debenzylation (**18**), Mitsunobu reaction, and reduction of the Boc group.

Scheme 2. Synthesis of compounds **4a**-**f** and **5**.[a]



[a]Reagents and conditions: *a*) NaH, CH_3CH_2I, DMF, 2 h, rt; *b*) $LiAlH_4$, THF, reflux, 3.5 h; *c*) CDI, toluene, rt, 2 h; *d*) $MeNH_2$, THF, rt, overnight; *e*) $(COCl)_2$, DMSO, TEA, DCM, -78 °C, 2 h; *f*) KH_2PO_4, 2-methyl-2-butene, $NaClO_2$, $^tBuOH/H_2O$, 0 °C to rt, overnight; *g*) TFA, DCM, rt, 1 h; *h*) HCHO, $Na(OAc)_3BH$, CH_3CN, rt, overnight; *i*) EDCI, HOBt, TEA, dialkylamine, DCM, rt, overnight; *j*) cyclopropylboronic acid, K_3PO_4, tricyclohexylphosphine, $Pd(OAc)_2$, toluene/H_2O, 100 °C, 3 h; *k*) 10% Pd/C, H_2, EtOAc/MeOH, rt, 1.5 h; *l*) ADDP, P^nBu_3, *N*-Boc-L-prolinol, toluene/THF, 0 °C to rt, overnight.

DISCUSSION

Binding affinity and subtype selectivity

All of the synthesized compounds were assayed for their radioligand binding competition potencies toward [^{3}H]epibatidine at eight rat nAChR subtypes (Table 1). Generally, these compounds displayed sub-nanomolar to low nanomolar affinities and high selectivities for nAChR subtypes containing β2 subunits (α2β2-, α3β2-, α4β2-, and α4β2*-nAChRs) over those containing β4 subunits (α2β4-, α3β4-, and α4β4-nAChRs), confirming the validity of the 3-[(1-methyl-2(*S*)-pyrrolidinyl)methoxy]-5-cyclopropylpyridine scaffold as a building block for the design of potent and selective α4β2* ligands.

The one-carbon elongation of the alkyl substituent attached to the side-chain of compound **3b** led to **4a**, which displayed binding affinity for α4β2- and α4β2*-nAChR subtypes comparable to that for the parent compound (**3b**), but with a significant decrease in selectivity over α3β4-nAChRs (> 33000-fold *vs* 9900-fold). The replacement of the alkoxy group of **4a** with a carbamate unit (**4b**) resulted in a ~30-fold increased K_i, associated with a further 6-fold loss in selectivity compared to **4a**. The binding affinity of ligand **4c**, bearing the carboxylic acid group, at most β2*-nAChRs decreased by a factor of 4 relative to affinities of **4a**, and selectivity decreased ~2.5-fold. On the other hand, the amide derivatives **4d**-**f** showed greater selectivity than compounds **4a-c** for β2*-nAChRs, comparable to, or even higher than that of compound **3b** acting at α4β2- or α4β2*-nAChRs. Moreover, compound **4d** exhibited a sub-nanomolar K_i for both α4β2- and α4β2*-nAChRs (0.2 and 0.6 nM, respectively). Finally, compound **5**, lacking the side-chain, maintains reasonable affinity for β2*-nAChR subtypes, but shows the greatest loss of selectivity over β4*-nAChRs compared to the other ligands in this series. This profile, along with data from previous work performed by our group,[14, 16-18] confirms the need of an appropriately oriented side-chain in the search for selective α4β2/α4β2*-nAChR ligands. It is noteworthy that these compounds were found to be inactive at the highest concentration used (10 μM) when tested at the α7-nAChR, with the exception of **4a** and **5**, which showed binding affinities of 9.3 and 8.1 μM, respectively.

Table 1. Binding affinities of ligands at eight nAChR subtypes, defined by competition for [^{3}H]epibatidine binding. Varenicline (1), compound **3b**, and nicotine were used as reference compounds.

Compound	K_i (nM)[a]								α3β4/α4β2
	α2β2	α2β4	α3β2	α3β4	α4β2	α4β2*[b]	α4β4	α7	K_i
4a	0.1	346	1.5	990	0.1	0.4	83.6	9330	9900
4b	3.0	1930	26	4710	2.9	12.7	6123	> 10000	1620
4c	0.2	146	6.4	1570	0.4	1.7	72.1	NA[c]	3930
4d	0.2	3050	1.8	6507	0.2	0.6	628	NA[c]	32800
4e	0.2	4270	3	> 10000	0.2	1.2	760	NA[c]	> 50000
4f	0.4	7710	2.9	> 10000	0.6	1.4	1160	NA[c]	> 16700
5	0.4	323	4.5	591	0.7	2.7	172	8090	844
1[d]				86	0.4		110		215
3b[e]				> 10000	0.3	0.5			> 33300
Nicotine[f]	5.5	70	29	260	4.9	9.8	23		53

[a]See Supporting Information. [b]α4β2*, endogenous receptors prepared from rat forebrain. Besides α4 and β2, other unidentified subunits may also be present. [c]NA: not active, defined as < 50% inhibition of binding in the primary assay at 10 μM. [d]K_i values for varenicline (**1**) are from the literature.[19] [e]K_i values for **3b** are from the literature.[16] [f]K_i values for nicotine were taken from the PDSP Assay Protocol Book (http://pdsp.med.unc.edu/).

In vitro functional characterization

After assessing binding affinity and selectivity, all of the new ligands were characterized for their functional activity at human α4β2-, α3β4*-, and α1β1γδ-nAChRs using SH-EP1-hα4β2, SH-SY5Y, and TE671/RD cells, respectively, and the $^{86}Rb^+$ efflux assay (Table 2). Note that α4β2-nAChRs naturally exist as a mixture of isoforms having two or three copies of α4 subunits and functionally exhibiting either just high sensitivity (HS) or a combination of HS and low sensitivity (LS), respectively, in their responses to ACh and nicotine.[20, 21] High sensitivity (HS) responses occur due to agonist binding at the α4:β2 subunit interfaces expressed by both α4β2-nAChR isoforms, but low sensitivity (LS) interactions are due to agonist binding at the α4:α4

subunit interface unique to $(\alpha4)_3(\beta2)_2$-nAChR "LS" isoform. Due to the nature of nAChRs, both agonist activity (marked as "Agonism" that refers to the maximal response of the ligand to stimulate ion flux, normalized to that of the cholinomimetic drug carbamylcholine) and desensitizing activity (marked as "Inactivation" that refers to the ability of the ligand to inactivate the receptor after incubation for 10 minutes prior to the application of 200 μM carbamylcholine) were recorded, and these data are expressed as EC_{50} and IC_{50} values, respectively.

As is apparent from Table 2, all ligands exhibit high potencies in activating HS responses of α4β2-nAChRs[20, 21] with EC_{50} values < 180 nM. None had substantial activity as agonists activating LS responses. Compounds **4e** and **5** act as full agonists toward α4β2-nAChR HS responses, while the efficacies of the remaining compounds toward those responses ranged between 49% and 77%. None of the tested ligands activated the ganglionic α3β4*- or muscle-type α1β1γδ-nAChRs at the highest concentration tested of 10 μM (data not shown). Notably, compound **4c** (carboxylic acid analog) had α4β2-nAChR agonist potency for HS responses equal to that of **3b** (EC_{50} = 14 nM), while **4d** (EC_{50} = 8.2 nM) was ~2-fold more potent than **3b**. Functional α4β2-nAChR inactivation potencies were similar to those for agonism, with the minor exception of compound **4b**, having an IC_{50} nearly 5-fold lower than its EC_{50}.

Table 2. Functional potencies and efficacies of ligands: agonism and inactivation of human α4β2-nAChRs.[a] **3b** and nicotine were used as reference compounds.

	Agonism				Inactivation		
Compound	**EC_{50} (nM)**	**pEC_{50}**	**HS-α4β2 efficacy (%)**	**LS-α4β2 efficacy (%)**	**IC_{50} (nM)**	**pIC_{50}**	**efficacy (%)**
4a	54	7.3 ± 0.07	69 ± 4.0	-3.4 ± 3.3	55	7.3 ± 0.02	81 ± 1.2
4b	170	6.8 ± 0.26	65 ± 5.6	2.0 ± 3.4	35	7.5 ± 0.08	73 ± 1.5
4c	14	7.8 ± 0.06	74 ± 12	2.1 ± 5.2	12	7.9 ± 0.09	79 ± 2.8
4d	8.2	8.1 ± 0.07	77 ± 11	-5.2 ± 4.6	8.9	8.1 ± 0.10	82 ± 2.9
4e	45	7.3 ± 0.06	97 ± 5.0	-13 ± 4.1	41	7.4 ± 0.02	79 ± 1.0
4f	27	7.6 ± 0.12	70 ± 8.1	0.92 ± 3.8	19	7.7 ± 0.12	83 ± 2.7

5	52	7.3 ± 0.03	110 ± 5.5	-4.4 ± 4.5	51	7.3 ± 0.02	76 ± 1.1
3b	14	7.8 ± 0.13	49 ± 5.2	6.1 ± 2.0	20	7.7 ± 0.09	88 ± 2.7
Nicotine[c]	295	6.5 ± 0.05	124 ± 8.5	70 ± 5.9	427	6.4 ± 0.06	92 ± 2.1

[a]See Experimental Section for details. The term "inactivation" is used because compounds may be acting to desensitize receptors or act as competitive or non-competitive antagonists, and further work is needed to make such distinctions. Potencies (EC_{50} or IC_{50} values) and efficacies were measured for actions at a mixture of high-sensitivity (HS) and low-sensitivity (LS) α4β2-nAChRs. Reported errors are the standard error of the mean (SEM) for all values. [c]Results for nicotine were taken from reference.[19]

In vivo behavioral studies

Preliminary *in vivo* evaluation of selected nicotinic ligands **4b-e** for behavioral effects was carried out using the SmartCube® assay, an automated system that analyzes the behavioral responses of compound-treated mice captured on digital video with the aid of computer algorithms.[22] Mice were treated with two doses of test compounds (5 mg/kg and 10 mg/kg) and subjected to sequential challenges. The behavioral signatures of test compound, that is the collection of endpoint behavioral measures obtained in this test system, were then compared with a database of signatures obtained from a large set of diverse reference compounds/drugs, to evaluate the potential use of these chemicals to treat neurological disorders. The probability that the compound belongs to one or more classes (see Fig. 3 legend) is then based on the similarity of the compound's own signature to the class signatures, as assessed with machine learning classifiers.

In particular, **4b** showed a promising antidepressant signature at 10 mg/kg. At 5 mg/kg, **4d** also showed antidepressant-like properties with the addition of an antipsychotic signature.

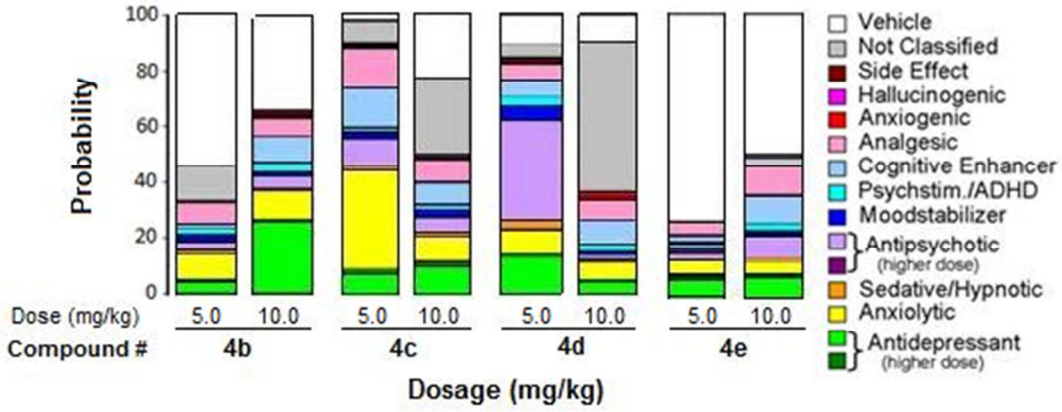


Figure 3. SmartCube® signature of compounds **4b** – **4e**.

Based on the SmartCube® data, compounds **4b** and **4d** were selected for further evaluation in the classical mouse FST,[23] to further probe their possible antidepressant-like activity. In the FST, a mouse is placed into a beaker of water, and the time spent passively floating in the liquid (immobility) is recorded. Most traditional antidepressants decrease the amount of time the mouse spends immobile. The mice were treated with compounds **4b**, **4d**, or the selective serotonin reuptake inhibitor sertraline (20 mg/kg) as positive control (Figure 4). Compounds **4b** (10 and 20 mg/kg salt; intraperitoneally (IP) administered) and **4d** (10 and 30 mg/kg of free base; orally (PO) administered) displayed an antidepressant-like effect and induced a weak to large decrease in the time spent immobile compared to saline. Although the antidepressant effect of compound **4b** increased somewhat when tested at 20 mg/kg, the overall effect was still weaker than the 20 mg/kg dose of the reference drug, sertraline (Figure 4a, left panel). On the other hand, at a dose of 30 mg/kg (free base), **4d** was at least as efficacious as sertraline (Figure 4b).

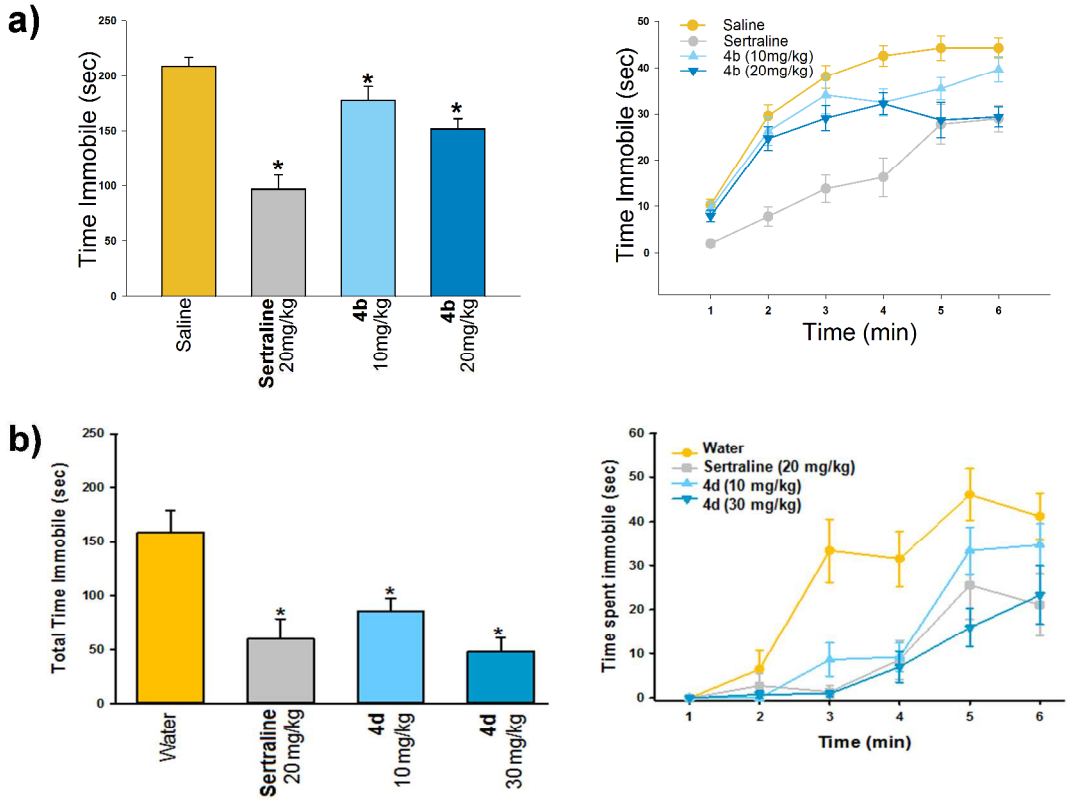


Figure 4. Results of the FST for compounds **4b** and **4d.** Each of the compounds was administered at the two doses shown.

PK and ADMET Studies.

Next, based on the above data, compound **4d** was selected for mouse *in vivo* pharmacokinetic (PK) and ADMET studies to further assess its drug-like properties. In the PK study, compound **4d** was administered intraperitoneally (10 mg/kg) to male CD-1 mice, and the plasma and brain concentrations were measured at 30 and 120 minutes after injection. Brain concentrations of **4d** were 250 and 231 ng/g at 30 and 120 min, respectively, while concentrations in plasma were 1420 and 218 ng/mL at the same time points (Table 3). The brain to plasma ratio for compound **4d** was found to be 0.18 at 30 min and 1.06 at 120 min, suggesting acceptable CNS penetration, which is consistent with the effects observed in the SmartCube® and FST studies.

Table 3. Pharmacokinetic parameters of **4d** in mouse plasma and brain following IP (10 mg/kg) administration.

Dose of 4d (mg/kg)	Plasma concentration (ng/mL)		Brain concentration (ng/g)		Brain/plasma ratio	
	30 min	120 min	30 min	120 min	30 min	120 min
10	1420	218	250	231	0.18	1.06

We also conducted some additional ADMET studies on compound **4d** to further assess its drug-like properties (Table 4). When incubated with human or male mouse liver microsomes, 95% or 86% of compound **4d** remained unchanged after 60 minutes incubation at a starting concentration of 2 μM. Incubation with human and rat hepatocytes showed 92 and 69%, respectively, of compound **4d** remaining unchanged after 2 h incubation at 1 μM. Compound **4d** thus proved to be more stable in the rat hepatocyte study than **3b**, for which only 0.35% remained under the same conditions, while 88% of **3b** remained using human hepatocytes. At a concentration of 10 μM of compound **4d**, none of the human CYP isoforms tested (CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4) showed more than 10% inhibition, suggesting minimal adverse drug-drug interactions. The low (2.6% at 30 μM) inhibition of the hERG channel observed for compound **4d** indicates that it should not cause QT prolongation. Plasma protein binding (PPB) assays were conducted using both human and mouse (CD-1) plasma at a final concentration of 1 μM of compound **4d**. Compound **4d** showed 4.9% binding to mouse plasma while no bound fraction was observed in human plasma.

Table 4. ADMET Properties of Compound **4d**.

Assay	Results found for compound 4d
Liver microsome stability (human / mouse, 2 μM) (% remaining after 60 mins)	95 / 86
Hepatocyte stability (human / rat, 1 μM) (% remaining after 2 hrs)	92 / 69
CYP inhibition (10 μM) (%)	

CYP 1A2	9.1
CYP2C9	0.17
CYP2C19	-6.9
CYP2D6	-5.5
CYP3A4	6.2
hERG inhibition (30 μM) (%)	2.6
Plasma protein binding (mouse / human, 1 μM) (%)	4.9 / 0

CONCLUSION

In summary, we describe the synthesis, structure-activity relationships, pharmacological evaluation, and behavioral characterization of some 3-[(1-methyl-2(*S*)-pyrrolidinyl)methoxy]-5-cyclopropylpyridine derivatives as nAChR ligands. Modifications to the side-chain attached to position 5 of the pyridine ring in the lead compound **3b** have been explored to create compounds with improved viabilities as therapeutic agents. Compound **4d** was identified as a potent and highly selective α4β2-nAChR partial agonist activating HS responses that exhibits comparable or improved pharmacological parameters in relation to compound **3b** in both [^{3}H]epibatidine binding studies and functional ($^{86}Rb^{+}$ ion flux) assays. Its high selectivity for α4β2-nAChRs over ganglionic α3β4- and muscle-type α1β1δ*-nAChRs suggests that it will have little side effect liability. However, additional off-target evaluation and toxicity studies are needed to further validate the viability of compound **4d**. Unlike mecamylamine, **4d** has strong selectivity for α4β2-nAChRs. Its agonist activity toward HS responses of α4β2-nAChR due to productive binding at the α4:β2 subunit interface is in contrast to its inactivity toward LS responses f α4β2-nAChR function mediated by ligand binding at the α4:α4 subunit interface. This distinguishes compound **4d** and other sazetidine-A analogues from ligands that act as agonists at both sites, such as the natural neurotransmitter, ACh, or the bioactive tobacco product compound, nicotine, and from varenicline, which acts as a partial agonist toward both HS and LS responses of α4β2-nAChRs with nearly equal efficacy and with slow functional kinetics not typical of other ligands. Even though varenicline and ligands such as **4d** are both "partial agonists" at α4β2-nAChRs, they

differ in mechanisms of action. This also means that compounds such as **4d** could have narrower effects on nAChR function than nicotine or ACh and be very unlikely to share the same side effect liabilities seen for varenicline. Compound **4d** displayed robust antidepressant-like efficacy in the mouse forced swim test, consistent with PK studies showing favorable brain penetration in the mouse. Compound **4d** also possesses a favorable metabolic profile, being stable in the presence of human hepatocytes and liver microsomes. Of particular note is the finding that **4d** is as efficacious as sertraline in the FST when tested at a dose of 30 mg/kg. These results support the further development of compound **4d** for potential treatment of human mood disorders.

Experimental details

General. All chemicals were purchased from Sigma-Aldrich, and solvents were purchased from Fisher Scientific or Sigma-Aldrich and used without further purification. All moisture- and air-sensitive reactions were carried out under an argon atmosphere. Anhydrous tetrahydrofuran (THF) and methylene chloride (DCM) were obtained by distillation over sodium wire and calcium hydride, respectively. Reactions were monitored by TLC on SiO_2; spots were visualized by UV light or by dipping into an I_2/SiO_2 mixture. In most cases, purification of intermediates was achieved by column chromatography on 230-400 mesh SiO_2. NMR spectra were recorded on a Bruker AVANCE III instrument at a ^1H frequency of 400 MHz and a ^{13}C frequency of 100.6 MHz. All chemical shifts (δ) are quoted in parts per million (ppm), and coupling constants (*J*) are recorded in Hertz. Splitting pattern abbreviations are as follows: *s* = singlet, *d* = doublet, *t* = triplet, *dd* = doublet of doublets, *m* = multiplet, *q* = quartet, *br* = broad. Chemical shifts are referenced to the solvent peak using the δ 7.26 signal of $CHCl_3$ (^1H NMR), the δ 4.80 signal of HDO (^1H NMR), and the δ 77.23 signal of $CDCl_3$ (^{13}C NMR) as internal standards at room temperature. Optical rotation was measured on an Autopol IV automatic polarimeter. Final compounds were purified by preparative HPLC on an ACE 5 AQ column (150 x 20 mm), with detection at 254 and 280 nm on a Shimadzu SPD-10A VP detector; flow rate = 17.0 mL/min; gradient of 0 to 50% methanol in water (both containing 0.05 vol% of TFA) in 20 min, to 100% methanol in 5 min, then return to 25% methanol in 1 min. Purity of > 98% was established for all final compounds by analytical HPLC carried out on an Agilent 1100 HPLC system with a Synergi 4 µm Hydro-RP 80A column, with detection at 254 or 280 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.

Synthesis of 3-(Benzyloxy)-5-((1*S*,2*R*)-2-vinylcyclopropyl)pyridine (7)

A solution of oxalyl chloride [$(COCl)_2$, 5.71 mL, 66.6 mmol] in anhydrous DCM (35 mL) was cooled to -78 °C in a dry ice/acetone bath. A solution of dimethyl sulfoxide (DMSO, 5.56 mL, 78.3 mmol) in anhydrous DCM (10 mL) was added dropwise with stirring, over a period of 15 minutes. The reaction mixture was stirred at -78 °C under an argon atmosphere for additional 15 minutes. Then a solution of [(1*S*,2*S*)-2-(5-(benzyloxy)pyridin-3-yl)cyclopropyl]methanol (**6**, 10.0 g, 39.2 mmol) in anhydrous DCM (35 mL) was added dropwise with stirring over a period of 30 minutes. Stirring was continued at -78 °C for an additional 20 minutes, after which anhydrous triethylamine (TEA, 32.8 mL, 235 mmol) was added dropwise over a period of 10 minutes. The resulting reaction mixture was stirred at -78 °C for 10 minutes, then allowed to slowly warm to room temperature and stirred for additional 20 minutes. TLC after this time (hexanes/EtOAc, 1/1) showed complete consumption of the starting material. Water was added to the reaction mixture, and it was extracted with DCM. The organic phase was washed with water and brine, dried over anhydrous Na_2SO_4, and filtered. Evaporation of the solvent under reduced pressure afforded 11.1 g (44.0 mmol) of the crude aldehyde product as a brown oil, which was immediately used in the following step without further purification or characterization.

A 1M solution of lithium bis(trimethylsilyl)amide in anhydrous THF (LiHMDS, 74.5 mL, 74.5 mmol) was added dropwise at -78 °C to a stirred suspension of methyltriphenylphosphonium bromide (23.5 g, 65.7 mmol) in anhydrous THF (25 mL) under an argon atmosphere. The reaction mixture was stirred at -78 °C for 20 minutes, then a solution of the aldehyde (11.1 g, 43.8 mmol) in anhydrous THF (20 mL) was added dropwise. The resulting reaction mixture was stirred at -78 °C for 30 minutes, then warmed to room temperature and stirred for additional 1.5 hours. TLC after this time (hexanes/EtOAc, 1/1) showed complete consumption of the starting material. Saturated aqueous NH_4Cl was added to the reaction mixture, and it was extracted with EtOAc. The organic phase was washed with brine, dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. Purification by flash chromatography on SiO_2, eluting with a hexanes/EtOAc gradient from 9/1 to 6/4, afforded 6.85 g (27.0 mmol, 69% yield over 2 steps) of pure target product as a yellow oil. ^{1}H-NMR ($CDCl_3$): δ 8.20 (*d*, *J* = 2.8 Hz, 1 H), 8.07 (*d*, *J* =

1.2 Hz, 1 H), 7.32-7.43 (*m*, 5 H), 6.88 (*d*, *J* = 2.0 Hz, 1 H), 5.52 (*m*, 1 H), 5.12 (*d*, *J* = 17.2 Hz, 1 H), 5.06 (*s*, 2 H), 4.99 (*d*, *J* = 10.0 Hz, 1 H), 1.88-1.93 (*m*, 1 H), 1.71 (*m*, 1 H), 1.13-1.21 (*m*, 2 H); ^{13}C-NMR ($CDCl_3$): δ 154.9, 140.9, 139.8, 138.7, 136.2, 135.1, 128.7 (2 C), 128.2, 127.5 (2 C), 118.5, 113.3, 70.3, 27.3, 22.5, 16.6.

Synthesis of 2-[(1*R*,2*S*)-2-[5-(Benzyloxy)pyridin-3-yl]cyclopropyl]ethanol (8)

Cyclohexene (16.4 mL, 162 mmol) was added dropwise over a period of 5 minutes to a stirred 1M solution of borane tetrahydrofuran complex in THF (BH_3·THF, 81 mL, 81 mmol) at 0 °C. A white precipitate formed almost immediately, and the resulting mixture was allowed to warm to room temperature and stirred for 30 minutes under an argon atmosphere, then it was cooled to 0 °C. A solution of compound **7** (5.80 g, 23.1 mmol) in anhydrous THF (25 mL) was added dropwise over a period of 15 minutes. The resulting reaction mixture was subsequently warmed to room temperature and stirred overnight.

Methanol (1.15 mL) was subsequently added dropwise, followed by 3 N aqueous NaOH (69.2 mL, 208 mmol) and, slowly, 35% aqueous H_2O_2 (40.4 mL, 415 mmol). The resulting biphasic reaction mixture was heated to 55 °C and vigorously stirred at this temperature for 1 hour. TLC after this time (DCM/MeOH, 9/1) showed complete consumption of the starting material. After cooling to room temperature, the mixture was extracted with EtOAc. The organic phase was washed with brine, dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. Purification by flash chromatography on SiO_2, eluting with DCM, then with a DCM/MeOH gradient from 99/1 to 97/3, afforded 6.22 g (23.1 mmol, quantitative yield) of pure target product as a colorless oil. ^{1}H-NMR ($CDCl_3$): δ 8.14 (*d*, *J* = 2.4 Hz, 1 H), 8.03 (*s*, 1 H), 7.32-7.44 (*m*, 5 H), 6.86-6.87 (*t*, *J* = 2.0 Hz, 1 H), 5.07 (*s*, 2 H), 3.76-3.79 (*t*, *J* = 6.2 Hz, 2 H), 2.30 (*br s*, 1 H), 1.63-1.72 (*m*, 3 H), 1.15 (*m*, 1 H), 0.87-0.97 (*m*, 2 H). ^{13}C-NMR ($CDCl_3$): δ 154.9, 140.8, 140.0, 136.2, 134.7, 128.7 (2 C), 128.2, 127.5 (2 C), 118.6, 70.3, 62.4, 37.2, 20.6, 20.2, 15.7.

Synthesis of 2-[(1*R*,2*S*)-2-[5-(Benzyloxy)pyridin-3-yl]cyclopropyl]ethyl Isobutyrate (9)

4-(*N*,*N*-Dimethylamino)pyridine (DMAP, 281 mg, 2.30 mmol) and TEA (12.8 mL, 92.1 mmol) were added to a solution of compound **8** (6.20 g, 23.0 mmol) in anhydrous DCM (23 mL). The mixture was cooled to 0 °C and stirred, then isobutyric anhydride (7.63 mL, 46.0 mmol) was

added dropwise. The resulting reaction mixture was stirred at this temperature for 2.5 hours. TLC after this time (DCM/MeOH, 95/5) showed almost complete consumption of the starting material. The reaction mixture was washed with water and brine, dried over Na_2SO_4, and evaporated to dryness, then the residue was purified by flash chromatography on SiO_2, eluting with DCM, then DCM/MeOH, 99/1, to afford 7.34 g (21.6 mmol, 94% yield) of pure target product as a colorless oil. ^{1}H-NMR ($CDCl_3$): δ 8.19-8.20 (*d*, *J* = 2.4 Hz, 1 H), 8.06 (*s*, 1 H), 7.34-7.44 (*m*, 5 H), 6.90 (*t*, *J* = 2.0 Hz, 1 H), 5.10 (*s*, 2 H), 4.14-4.24 (*m*, 2 H), 2.55 (*m*, 1 H), 1.68-1.80 (*m*, 3 H), 1.18 (*d*, *J* = 6.8 Hz, 6 H), 1.11 (*m*, 1 H), 0.87-0.98 (*m*, 2 H); ^{13}C-NMR ($CDCl_3$): δ 177.1, 155.0, 140.4, 139.9, 136.1, 134.2, 128.7 (2 C), 128.3, 127.5 (2 C), 119.3, 70.4, 63.8, 34.0, 33.2, 20.3, 20.2, 19.1, 19.0, 15.6,

Synthesis of 2-[(1*R*,2*S*)-2-(5-Hydroxypyridin-3-yl)cyclopropyl]ethyl isobutyrate (10)

10% Palladium on charcoal (1.08 g) was added to a solution of compound **9** (7.30 g, 21.5 mmol) in EtOAc/MeOH, 1/1 (108 mL) under an argon atmosphere. The flask was evacuated and purged with argon three times, then a hydrogen-filled balloon was connected, and the reaction mixture was stirred at room temperature for 1.5 hours. TLC after this time (DCM/MeOH, 95/5) showed complete consumption of the starting material. The reaction mixture was filtered over a pad of Celite®, then the solvents were evaporated under reduced pressure. Purification by flash chromatography on SiO_2, eluting with DCM, then a DCM/MeOH gradient from 99/1 to 95/5, afforded 4.34 g (17.4 mmol, 81% yield) of pure target product as a colorless oil. ^{1}H-NMR ($CDCl_3$): δ 8.08 (*s*, 1 H), 7.92 (*s*, 1 H), 6.89 (*s*, 1 H), 4.16-4.25 (*m*, 2 H), 2.55 (*m*, 1 H), 1.78 (*dd*, J_1 = 13.2 Hz, J_2 = 6.4 Hz, 2 H), 1.69 (*m*, 1 H), 1.18 (*d*, *J* = 6.8 Hz, 6 H), 1.09 (*m*, 1 H), 0.95 (*m*, 1 H), 0.89 (*m*, 1 H); ^{13}C-NMR ($CDCl_3$): δ 177.0, 154.0, 140.5, 138.1, 133.6, 120.7, 63.5, 33.6, 32.8, 20.0, 19.8, 18.6, 18.6, 15.2.

Synthesis of *tert*-Butyl (*S*)-2-[[[5-[(1*S*,2*R*)-2-[2-(Isobutyryloxy)ethyl]cyclopropyl]pyridin-3-yl]oxy]methyl]pyrrolidine-1-carboxylate (11)

An oven-dried, three-necked 250 mL round-bottomed flask, equipped with a magnetic stir bar and a dropping funnel, was charged with a solution of 1,1'-azodicarbonyldipiperidine (ADDP, 6.53 g, 25.9 mmol) in anhydrous toluene (43 mL). The orange-colored solution was cooled to 0 °C, then tri-*n*-butylphosphine (6.38 mL, 25.9 mmol) was added dropwise with stirring under an argon atmosphere. The resulting reaction mixture was stirred at 0 °C for 30 minutes (the color

disappeared during this time), then a solution of compound **10** (4.30 g, 17.3 mmol) and *N*-Boc-L-prolinol (2.43 g, 12.1 mmol) in anhydrous THF (43 mL) was added dropwise over a period of 15 minutes at 0 °C. The resulting reaction mixture was subsequently allowed to warm to room temperature and stirred overnight. TLC after this time (DCM/MeOH, 95/5) showed complete consumption of the starting material. The solvents were evaporated under reduced pressure, then the residue was purified by flash chromatography on SiO_2, eluting with a hexanes/diethyl ether gradient from 1/1 to 4/6, to afford 5.52 g (12.3 mmol, 74% yield) of pure target product as a yellow oil. ^{1}H-NMR ($CDCl_3$): δ 8.09 (*s*, 1 H), 8.01 (*s*, 1 H), 6.83 (*br s*, 1 H), 4.13-4.21 (*m*, 4 H), 3.83-3.93 (*br s*, 1 H), 3.39 (*m*, 2 H), 2.57 (*m*, 1 H), 2.01 (*m*, 3 H), 1.88 (*m*, 1 H), 1.65-1.79 (*m*, 3 H), 1.47 (*s*, 9 H), 1.18 (*m*, 1 H), 1.16 (*d*, *J* = 6.8 Hz, 6 H), 0.96 (*m*, 1 H), 0.86 (*m*, 1 H); ^{13}C-NMR ($CDCl_3$): δ 177.1, 155.0, 154.7, 140.6, 139.5, 135.4, 117.7, 79.4, 77.2, 68.3, 63.8, 55.9, 46.9, 34.0, 33.2, 28.5 (3 C), 23.8, 22.9, 20.2, 19.0, 18.9, 15.5.

Synthesis of *tert*-Butyl (*S*)-2-[[[5-[(1*S*,2*R*)-2-(2-Hydroxyethyl)cyclopropyl]pyridin-3-yl]oxy]methyl]pyrrolidine-1-carboxylate (12)

A 25% (wt/vol) solution of sodium methoxide in MeOH (NaOMe, 12.7 mL) was added to a solution of compound **11** (5.50 g, 12.7 mmol) in anhydrous MeOH (32 mL). The reaction mixture was heated to 40 °C and stirred at this temperature for 1.5 hours. TLC after this time (DCM/MeOH, 95/5) showed almost complete consumption of the starting material. The reaction mixture was concentrated under reduced pressure, re-dissolved in DCM (30 mL) and then washed with water and brine, dried over anhydrous Na_2SO_4, and evaporated to dryness. Purification by flash chromatography on SiO_2, eluting with DCM, then with a DCM/MeOH gradient from 99/1 to 95/5, afforded 3.27 g (9.03 mmol, 71% yield) of pure target product as a colorless oil. ^{1}H-NMR ($CDCl_3$): δ 8.07 (*s*, 1 H), 8.00 (*s*, 1 H), 6.95 (*s*, 1 H), 4.11-4.16 (*m*, 2 H), 3.90 (*m*, 1 H), 3.79 (*t*, *J* = 6.2 Hz, 2 H), 3.37-3.39 (*m*, 2 H), 2.51 (*br s*, 1 H), 2.00 (*m*, 3 H), 1.89 (*m*, 1 H), 1.62-1.69 (*m*, 3 H), 1.46 (*s*, 9 H), 1.17 (*m*, 1 H), 0.93-0.97 (*m*, 1 H), 0.88 (*m*, 1 H); ^{13}C-NMR ($CDCl_3$): δ 155.0, 140.7, 135.4, 120.1, 117.8, 102.6, 79.6, 68.1, 62.3, 55.8, 46.9, 37.1, 32.7, 28.5 (3 C), 28.0, 23.7, 20.2, 15.5.

Synthesis of 3-[[1-(*tert*-Butoxycarbonyl)-2(*S*)-pyrrolidinyl]methoxy]-5-[(1*S*,2*R*)-2-(2-ethoxyethyl)cyclopropyl]pyridine (13)

Compound **12** (0.20 g, 0.55 mmol) was dissolved in anhydrous DMF (5 mL). The solution was cooled to 4 °C with an ice bath, and NaH (20 mg, 0.83 mmol) was added. After stirring for 10 min, iodoethane (66 μL, 0.83 mmol) was added slowly. The reaction was further stirred at 4 °C for 20 min and allowed to warm to rt with continuous stirring overnight. The reaction was quenched with aqueous NH_4Cl and extracted with EtOAc (2 x 15 mL), and the combined organic extracts were washed successively with water (10 mL) and brine (10 mL). The organic solution was dried over anhydrous Na_2SO_4, concentrated under reduced pressure, and purified by column chromatography on silica gel with DCM/MeOH, 9/1 as eluent to obtain the target product **13** as a colorless oil (0.14 g, 66% yield). ^{1}H NMR ($CDCl_3$) δ 8.04-7.97 (*m*, 2 H), 6.85 (*m*, 1 H), 4.10-3.79 (*m*, 3 H), 3.50-3.31 (*m*, 6 H), 1.97-1.84 (*m*, 4 H), 1.64-1.60 (*m*, 3 H), 1.43 (*s*, 9 H), 1.18-1.10 (*m*, 4 H), 0.91-0.82 (*m*, 2 H); ^{13}C NMR ($CDCl_3$) δ 155.3, 140.7, 140.3, 135.1, 134.1, 118.1, 117.8, 79.6, 70.2, 68.5, 66.4, 56.1, 55.6, 47.1, 34.5, 28.6, 20.9, 20.5, 15.8, 15.4.

Synthesis of 3-[(1-Methyl-2(*S*)-pyrrolidinyl)methoxy]-5-[(1*S*,2*R*)-2-(2-ethoxyethyl)cyclopropyl]pyridine Trifluoroacetate (4a)

Lithium aluminum hydride solution (0.9 mL, 2.0 M in THF, 1.8 mmol) was slowly added to a solution of the product (0.14 g, 0.36 mmol) obtained from the previous step in anhydrous THF (5 mL) at 0 °C under Ar. The reaction mixture was heated to 70 °C and stirred for 3 h. After cooling to rt, saturated aqueous Na_2SO_4 was added dropwise to quench the reaction. The resulting suspension was filtered, and the filtrate was evaporated to dryness. After a pre-purification by flash chromatography on SiO_2, eluting with DCM, then with a DCM/MeOH gradient from 99/1 to 95/5, the obtained product was further purified by preparative HPLC to afford compound **4a** as a colorless oil. Purity: 99.8%; $[\alpha]_D^{20}$ +39.0 (*c* 0.42, MeOH). ^{1}H NMR (D_2O) δ 8.34 (*d*, *J* = 2.4 Hz, 1 H), 8.26 (*s*, 1 H), 7.84 (*s*, 1 H), 4.66 (*dd*, *J* = 2.8, 3.2 Hz, 1 H), 4.50 (*dd*, *J* = 6.0 Hz, 1 H), 4.00 (*m*, 1 H), 3.78 (*m*, 1 H), 3.65 (*dd*, *J* = 6.8, 6.4 Hz, 2 H), 3.60 (*q*, *J* = 7.2 Hz, 2 H), 3.28 (*m*, 1 H), 3.07 (*s*, 3 H), 2.45 (*m*, 1 H), 2.25 (*m*, 1 H), 2.14-2.09 (*m*, 2 H), 2.02 (*m*, 1 H), 1.81-1.68 (*m*, 2 H), 1.37-1.33 (*m*, 1 H), 1.20-1.16 (*m*, 5 H); ^{13}C NMR (D_2O) δ 162.6 (TFA), 156.1, 146.7, 132.6, 128.3, 125.6, 117.8 (TFA), 69.7, 67.4, 66.4, 66.2, 57.2, 40.6, 32.8, 25.9, 22.5, 22.1, 21.0, 19.9, 16.6, 14.1. Anal. Calcd for $C_{18}H_{28}N_2O_2 \cdot 2.4C_2HF_3O_2 \cdot 0.15H_2O$: C, 47.15; H, 5.13; F, 23.55; N, 4.82. Found: C, 47.05; H, 5.19; F, 23.43; N, 4.82.

Synthesis of 2-[(1*R*,2*S*)-2-[5-[((*S*)-1-Methylpyrrolidin-2-yl)methoxy]pyridin-3-yl]cyclopropyl]ethanol (14)

Lithium aluminum hydride ($LiAlH_4$, 60 mg, 1.57 mmol) was added in one portion to a solution of compound **12** (190 mg, 0.52 mmol) in anhydrous THF (2.5 mL) under Ar. The reaction mixture was heated to reflux and stirred at this temperature for 3.5 hours. TLC after this time ($CHCl_3$/MeOH, 9/1) showed complete consumption of the starting material. After cooling to room temperature, the reaction mixture was cooled to 0 °C, and saturated aqueous $NaHCO_3$ was carefully added. The resulting mixture was extracted with EtOAc. The organic phase was washed with brine, dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. Purification by preparative HPLC, followed by treatment of the collected fractions with saturated aqueous $NaHCO_3$, extraction with chloroform, and evaporation of the solvent, afforded 50 mg of pure compound **14** as a colorless oil (35% yield). ^{1}H-NMR ($CDCl_3$): δ 8.07 (*s*, 1 H), 8.00 (*s*, 1 H), 6.80 (*s*, 1 H), 3.98 (*m*, 1 H), 3.89 (*m*, 1 H), 3.74-3.77 (*t*, *J* = 6.4 Hz, 2 H), 3.10 (*t*, *J* = 7.4 Hz, 1 H), 2.52-2.64 (*m*, 2 H), 2.47 (*s*, 3 H), 2.30 (*dd*, J_1 = 16.8 Hz, J_2 = 9.2 Hz, 1 H), 2.04 (*m*, 1 H), 1.25-1.86 (*m*, 6 H), 1.14 (*m*, 1 H), 0.93 (*m*, 1 H), 0.88 (*m*, 1 H); ^{13}C-NMR ($CDCl_3$): δ 155.2, 140.6, 139.9, 134.5, 118.0, 71.0, 64.2, 62.3, 57.7, 41.7, 37.2, 28.6, 23.0, 20.5, 20.2, 15.6.

Synthesis of 2-[(1*R*,2*S*)-2-[5-[[(*S*)-1-Methylpyrrolidin-2-yl)methoxy]pyridin-3-yl]cyclopropyl]ethyl Methylcarbamate (4b)

1,1'-Carbonyldiimidazole (CDI, 32 mg, 0.20 mmol) was added to a solution of compound **14** (45 mg, 0.16 mmol) in anhydrous toluene (1.3 mL). The reaction mixture was stirred at room temperature for 2 hours under Ar. Anhydrous THF (0.65 mL) was added, and stirring was continued for an additional hour. A 2.0M solution of methylamine in THF (163 μL, 0.33 mmol) was then added, and the resulting reaction mixture was stirred at room temperature overnight. TLC after this time, ($CHCl_3$/MeOH, 9/1) showed complete consumption of the starting material. The solvents were removed by evaporation under reduced pressure, and the residue was purified by preparative HPLC affording, after evaporation of the solvents and lyophilization, 84 mg (0.15 mmol, 92% yield) of pure target product (TFA salt) as a colorless viscous oil. Purity: 98.6%; $[\alpha]_D^{20}$ +30.5 (*c* 0.4, MeOH). ^{1}H-NMR ($CDCl_3$): δ 15.97 (*br s*, 1 H), 12.78 (*br s*, 1 H), 8.31 (*s*, 1 H), 8.25 (*s*, 1 H), 7.51 (*s*, 1 H), 5.30 (*br s*, 1 H), 4.70 (*m*, 1 H), 4.46 (*d*, *J* = 9.2 Hz, 1 H), 4.13-4.18 (*m*, 2 H), 3.90 (*m*, 1 H), 3.73 (*m*, 1 H), 3.02 (*s*, 4 H), 2.73 (*s*, 3 H), 2.39 (*m*, 1 H), 2.11-2.33

(*m*, 3 H), 1.80 (*m*, 2 H), 1.62 (*m*, 1 H), 1.24 (*m*, 1 H), 1.04-1.10 (*m*, 2 H); ^{13}C-NMR (DMSO-d_6): δ 158.4, 157.2, 154.8, 141.6, 139.7, 133.5, 119.8, 67.2, 67.0, 63.8, 57.1, 41.4, 33.5, 27.3, 26.7, 22.7, 21.5, 20.0, 16.1. Anal. Calcd. for $C_{18}H_{27}N_3O_3 \cdot 1.65C_2HF_3O_2 \cdot 1.6H_2O$: C, 46.48; H, 5.83; F, 17.09; N, 7.63. Found: C, 45.83; H, 5.18; F, 16.61; N, 7.24.

Synthesis of 2-[(1*S*,2*R*)-2-[5-[[1-(*tert*-Butoxycarbonyl)-2(*S*)-pyrrolidinyl]methoxy]-3-pyridyl]cyclopropyl]acetic acid (15)

A 100 mL three-necked flask with magnetic stirrer, two septa, and an Ar balloon was charged with anhydrous DCM (15 mL) and oxalyl chloride (0.51 mL, 6.0 mmol). The flask was immersed in an acetone/CO_2 bath, and the solution was stirred. A solution of anhydrous DMSO (0.47 mL, 6.6 mmol) in anhydrous DCM (2 mL) was added dropwise in 5 min. The Swern reagent solution was stirred at approx. -70 °C for another 15 min. A solution of compound **12** (1.20 g, 3.3 mmol) in anhydrous DCM (10 mL) was added in 25 min. Stirring at approx. -70 °C was continued for another 30 min. Anhydrous triethylamine (2.76 mL, 1.8 mmol) was added dropwise in 10 min. The reaction mixture was stirred for another 10 min at approx. -70 °C and then allowed to warm to rt. The mixture was subsequently stirred at rt for 25 min, then washed with two 15 mL portions of water. The aqueous phases were back-extracted with DCM. The combined organic phases were dried over Na_2SO_4 and evaporated to furnish the intermediate aldehyde as a yellowish syrup which was directly used for the next step without purification.

To a mixture of the aldehyde (1.20 g, 3.3 mmol), monobasic potassium phosphate (0.55 g, 4.0 mmol), and 2-methyl-2-butene (0.52 mL, 5.0 mmol) in *tert*-butanol (15 mL) and H_2O (5 mL) at 0 °C, was added sodium chlorite (0.36 g, 4.0 mmol, technical grade, 80%) in several portions over 5 min. The mixture was warmed to rt slowly and stirred overnight. The solution was concentrated, and the residue was taken up in EtOAc and washed successively with water and brine. The organic layer was dried over anhydrous Na_2SO_4 and concentrated to obtain 0.82 g (66% yield) of the target product **15** as a yellowish solid, which was used in the next step without further purification). ^{1}H NMR (DMSO-d_6): δ 12.14 (*br s*, 1 H), 8.07 (*s*, 1 H), 8.01 (*s*, 1 H), 7.05 (*s*, 1 H), 4.07-3.94 (*m*, 3 H), 3.27 (*m*, 2 H), 2.35-2.32 (*m*, 2 H), 1.94-1.76 (*m*, 5 H), 1.39 (*s*, 10 H), 1.04 (*m*, 1 H), 0.89 (*m*, 1 H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 173.5, 154.8, 153.8, 140.3, 134.8, 117.8, 78.7, 68.6, 55.5, 46.5, 38.1, 31.3, 28.1, 23.2, 22.3, 19.6, 19.0, 15.2.

General Procedure for Amide Coupling Reactions, Deprotection, and Reductive Methylation.

To a solution of the appropriate carboxylic acid (**15**) (1 equiv) in anhydrous DCM (4 mL per mmol) at room temperature were added anhydrous hydroxybenzotriazole (HOBt, 1 equiv) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC·HCl, 1 equiv) under Ar. After stirring for 10 min, the appropriate amine (1 equiv) and triethylamine (1.5 equiv) were added, and the reaction mixture was stirred at room temperature until disappearance of the starting material (usually 12 to 16 h). After this time, water (2 mL) was added, and the mixture was extracted with EtOAc (3 × 10 mL). The organic layers were separated, washed with brine, dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography on SiO_2 with DCM/MeOH to obtain the desired product.

To a stirred solution of the amide/carboxylic acid (0.7 mmol) in DCM (5 mL) was added TFA (1 mL), and the reaction was monitored by TLC until completion. The solution was concentrated. The resulting TFA salt was treated with PL-HCO_3 MP-resin to afford the free base, which was then dissolved in CH_3CN (8 mL) and treated with formaldehyde (25-30% in H_2O, 7 mL) with stirring for 30 min followed by the addition of $Na(OAc)_3BH$ (1.4 mmol). The reaction mixture was stirred overnight at rt and concentrated, and the residue was purified by flash chromatography on SiO_2 eluting with $CHCl_3$/MeOH/NH_3 (aq), 88:10:2 to obtain the products in yields ranging from 65 to 85%. These crude products were further purified by preparative HPLC to recover the desired products in high purity and good yield.

Synthesis of 2-[(1*S*,2*R*)-2-[5-[[(*S*)-1-Methylpyrrolidin-2-yl]methoxy]pyridin-3-yl]cyclopropyl]acetic acid Trifluoroacetate (4c).

This compound was obtained following the procedure reported above. Colorless oil; 77% yield; purity: 99.7%; $[\alpha]_D^{20}$ +29.3 (*c* 0.4, MeOH). ^{1}H NMR (D_2O) δ 8.35 (*s*, 1 H), 8.30 (*s*, 1 H), 7.90 (*s*, 1 H), 4.66 (*m*, 1 H), 4.49 (*m*, 1 H), 3.97 (*s*, 1 H), 3.78 (*m*, 1 H), 3.27 (*m*, 1 H), 3.06 (*s*, 3 H), 2.70-2.64 (*m*, 1 H), 2.53-2.39 (*m*, 2 H), 2.24 (*m*, 1 H), 2.15-2.08 (*m*, 3 H), 1.54 (*m*, 1 H), 1.30-1.19 (*m*, 2 H). ^{13}C NMR (D_2O) δ 177.2, 162.6 (TFA), 156.1, 145.6, 132.9, 128.7, 126.0, 117.7 (TFA), 67.3, 66.4, 57.2, 40.5, 37.5, 25.8, 22.1, 21.0, 19.9, 19.7, 15.7. Anal. Calcd for

$C_{16}H_{22}N_2O_3 \cdot 2C_2HF_3O_2 \cdot 0.05H_2O$: C, 46.26; H, 4.68; F, 21.95; N, 5.39. Found: C, 46.26; H, 4.72; F, 21.95; N, 5.45.

Synthesis of *N,N*-Dimethyl-2-[(1*S*,2*R*)-2-[5-[[(*S*)-1-methylpyrrolidin-2-yl]methoxy]pyridin-3-yl]cyclopropyl]acetamide Trifluoroacetate (4d).

This compound was obtained following the procedure reported above. Colorless oil; 74% yield; purity: 99.8%; $[\alpha]_D^{20}$ +43.0 (*c* 0.2, CH_3OH). ^{1}H NMR (D_2O) δ 8.34 (*s*, 1 H), 8.26 (*s*, 1 H), 7.87 (*s*, 1 H), 4.64 (*m*, 1 H), 4.48 (*m*, 1 H), 3.97 (*s*, 1 H), 3.76 (*m*, 1 H), 3.25 (*m*, 1 H), 3.05 (*s*, 6 H), 2.92 (*m*, 3 H), 2.67 (*m*, 1 H), 2.56 (*m*, 1 H), 2.42 (*m*, 1 H), 2.22-2.04 (*m*, 4 H), 1.51 (*m*, 1 H), 1.26-1.19 (*m*, 2 H). ^{13}C NMR (D_2O) δ 174.5, 156.1, 145.8, 132.7, 128.7, 125.9, 117.7 (TFA), 67.3, 66.4, 57.2, 40.6, 37.3, 36.6, 35.3, 25.8, 22.1, 20.5, 19.8, 15.9. Anal. Calcd for $C_{18}H_{27}N_3O_2 \cdot 2.05C_2HF_3O_2 \cdot 0.75H_2O$: C, 47.01; H, 5.45; F, 20.69; N, 7.44. Found: C, 47.05; H, 5.35; F, 20.56; N, 7.57.

Synthesis of *N,N*-Diethyl-2-[(1*S*,2*R*)-2-[5-[[(*S*)-1-methylpyrrolidin-2-yl]methoxy]pyridin-3-yl]cyclopropyl]acetamide Trifluoroacetate (4e).

This compound was obtained following the procedure reported above. Colorless oil; 71% yield; purity: 99.8%; $[\alpha]_D^{20}$ +46.5 (*c* 0.19, MeOH). ^{1}H NMR (D_2O) δ 8.35 (*s*, 1 H), 8.29 (*s*, 1 H), 7.89 (*s*, 1 H), 4.67 (*m*, 1 H), 4.49 (*m*, 1 H), 3.99 (*m*, 1 H), 3.78 (*m*, 1 H), 3.44-3.35 (*m*, 4 H), 3.30 (*m*, 1 H), 3.06 (*s*, 3 H), 2.73 (*m*, 1 H), 2.57 (*m*, 1 H), 2.42 (*m*, 1 H), 2.24 (*m*, 1 H), 2.13-2.07 (*m*, 3 H), 1.54 (*m*, 1 H), 1.28-1.09 (*m*, 8 H). ^{13}C NMR (D_2O) δ 173.7, 156.1, 145.8, 132.8, 128.7, 126.0, 67.3, 66.4, 57.2, 42.7, 40.7, 40.5, 36.4, 25.8, 22.1, 20.9, 19.8, 15.9, 13.1, 12.1. Anal. Calcd for $C_{20}H_{31}N_3O_2 \cdot 2C_2HF_3O_2 \cdot 0.8H_2O$: C, 49.03; H, 5.93; F, 19.39; N, 7.15. Found: C, 48.70; H, 5.57; F, 19.29; N, 6.90.

Synthesis of 1-(4,4-Dimethylpiperidin-1-yl)-2-[(1*S*,2*R*)-2-[5-[[(*S*)-1-methylpyrrolidin-2-yl]methoxy]pyridin-3-yl]cyclopropyl]ethanone Trifluoroacetate (4f).

This compound was obtained following the procedure reported above. Colorless oil; 68% yield; purity: 99.5%; $[\alpha]_D^{20}$ +45.5 (*c* 0.28, MeOH). ^{1}H NMR (D_2O) δ 8.34 (*d*, *J* = 2.4 Hz, 1 H), 8.24 (*s*, 1 H), 7.85 (*t*, *J* = 2.0 Hz, 1 H), 4.62 (*dd*, *J* = 2.8 Hz, 1 H), 4.46 (*dd*, *J* = 5.6, 6.0 Hz, 1 H), 3.95 (*m*, 1 H), 3.74 (*m*, 1 H), 3.48 (*m*, 4 H), 3.25 (*m*, 1 H), 3.02 (*s*, 3 H), 2.72 (*dd*, *J* = 6.0, 6.4 Hz, 1 H), 2.55 (*dd*, *J* = 7.6, 7.2 Hz, 1 H), 2.40 (*m*, 1 H), 2.19 (*m*, 1 H), 2.08-2.01 (*m*, 3 H), 1.49 (*m*, 1

H), 1.35-1.17 (*m*, 6 H), 0.93 (*s*, 3 H), 0.91 (*s*, 3 H); ^{13}C NMR (D_2O) δ 172.4, 162.5 (TFA), 156.1, 145.8, 132.7, 128.7, 126.0, 117.7 (TFA), 67.3, 66.4, 57.2, 43.1, 40.5, 39.2, 38.4, 37.7, 36.6, 28.2, 26.9, 26.7, 25.8, 22.1, 21.0, 19.8, 16.0. Anal. Calcd for $C_{23}H_{35}N_3O_2 \cdot 2.35C_2HF_3O_2 \cdot 0.75H_2O$: C, 49.88; H, 5.87; F, 20.08; N, 6.30. Found: C, 49.80; H, 5.52; F, 19.74; N, 6.37.

Synthesis of 3-(Benzyloxy)-5-cyclopropylpyridine (17).

To a solution of 3-(benzyloxyl)-5-bromopyridine (**16**)[17] (0.50 g, 1.9 mol), cyclopropylboronic acid (0.195 g, 2.27 mol, 1.2 equiv), K_3PO_4 (1.40 g, 6.62 mmol, 3.5 equiv), and tricyclohexylphosphine (53 mg, 0.19 mmol, 0.1 equiv) in toluene (8 mL) and water (500 μL) under Ar was added $Pd(OAc)_2$ (21 mg, 0.095 mmol, 0.05 equiv). The mixture was heated to 100 °C for 3 h and cooled to rt. Water (10 mL) was added, and the mixture was extracted with EtOAc (3 x 15 mL). The combined organic phases were washed with brine, dried over Na_2SO_4, and concentrated. The residue was purified by flash chromatography on SiO_2 with hexane/EtOAc, 3/1 to obtain 0.42 g (97% yield) of the target product. ^{1}H NMR (DMSO-d_6) δ 8.13 (*d*, *J* = 2.8 Hz, 1 H), 8.03 (*d*, *J* = 1.6 Hz, 1 H), 7.47-7.32 (*m*, 5 H), 7.05 (*m*, 1 H), 5.15 (*s*, 2 H), 1.92 (*m*, 1 H), 1.00 (*m*, 2 H), 0.76-0.72 (*m*, 2 H); ^{13}C NMR (DMSO-d_6) δ 154.6, 140.3, 140.1, 136.6, 134.9, 128.4, 127.9, 127.8, 117.5, 69.4, 12.5, 9.4.

Synthesis of 5-Cyclopropylpyridin-3-ol (18).

In a 100 mL round-bottom flask, compound **17** (1.66 g, 7.4 mmol) was dissolved in a mixture of EtOAc (4 mL) and methanol (16 mL), and 10% Pd/C (500 mg) was added. A H_2 balloon was connected after the atmosphere was exchanged three times with Ar. The reaction was allowed to proceed at rt and monitored via TLC. After completion, the catalyst was filtered off over Celite®. The filtrate was evaporated, and the residue was purified by flash chromatography on SiO_2 with DCM/MeOH, 9/1 to furnish the hydroxypyridine (0.88 g, 88%) as a colorless oil. ^{1}H NMR (DMSO-d_6): δ 9.75 (*s*, 1 H), 7.91 (*d*, *J* = 2.4 Hz, 1 H), 7.87 (*d*, *J* = 2.0 Hz, 1 H), 6.74 (*s*, 1 H), 1.86 (*m*, 1 H), 0.97-0.92 (*m*, 2 H), 0.68-0.65 (*m*, 2 H); ^{13}C NMR (DMSO-d_6): δ 153.6, 140.1, 138.6, 135.1, 118.0, 12.4, 9.2.

Synthesis of (S)-3-Cyclopropyl-5-[(1-methylpyrrolidin-2-yl)methoxy]pyridine Trifluoroacetate (5).

In a 250 mL side-arm flask with stir bar and Ar balloon, tri-*n*-butylphosphine (1.2 mL, 4.7 mmol) was added dropwise in 5 min to a solution of *N*,*N*'-azodicarbonyldipiperidine (1.2 g, 4.7 mmol) in anhydrous toluene (30 mL). Stirring was continued at rt for 45 min to complete the formation of the Mitsunobu reagent. A solution of the hydroxypyridine intermediate **18** (0.40 g, 3.0 mmol) and 1-(*tert*-butoxycarbonyl)-(2*S*)-pyrrolidinylmethanol (0.95 g, 4.7 mmol) in anhydrous toluene (30 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 flash chromatography on SiO_2 eluting with hexanes/EtOAc, 2/1 to 1/1 to furnish impure target product (1.1 g) as a colorless oil, which was used in the next step without further purification.

Lithium aluminum hydride solution (3.5 mL, 2.0 M in THF, 7.0 mmol) was slowly added to a solution of the intermediate (0.44 g, 1.4 mmol) obtained from the previous step in anhydrous THF (10 mL) at 0 °C under Ar. The reaction mixture was heated to 70 °C and stirred for 3 h. After cooling to rt, saturated aqueous Na_2SO_4 solution was added dropwise to quench the reaction. The resulting suspension was filtered, and the filtrate was evaporated to dryness. The residue (0.25 g, 78%) was further purified by preparative HPLC to afford compound **5** as a colorless oil. Purity: 99.8%; ^{1}H NMR (D_2O) δ 8.35 (*d*, *J* = 2.8 Hz, 1 H), 8.29 (*s*, 1 H), 7.87 (*s*, 1 H), 4.67 (*dd*, *J* = 3.2, 2.8 Hz, 1 H), 4.52 (*dd*, *J* = 6.0, 5.6 Hz, 1 H), 4.01 (*m*, 1 H), 3.80 (*m*, 1 H), 3.31 (*m*, 1 H), 3.07 (*s*, 3 H), 2.44 (*m*, 1 H), 2.26-2.12 (*m*, 4 H), 1.30-1.25 (*m*, 2 H), 0.96-0.93 (*m*, 2 H). ^{13}C NMR (D_2O) δ 162.7 (TFA), 156.1, 147.2, 132.8, 128.4, 125.8, 117.8 (TFA), 67.4, 66.4, 57.2, 40.6, 25.9, 22.1, 12.8, 10.3. Anal. Calcd for $C_{14}H_{20}N_2O \cdot 2.25C_2HF_3O_2 \cdot 0H_2O$: C, 45.45; H, 4.59; F, 26.23; N, 5.73. Found: C, 45.18; H, 4.29; F, 26.09; N, 5.68.

General Procedures for Binding and Functional Studies

***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 α1*-nAChRs, containing α1, β1, γ, and δ subunits, with function detectable using $^{86}Rb^+$ efflux assays.[24] The human neuroblastoma cell line SH-SY5Y naturally expresses autonomic α3β4*-nAChRs, containing α3, β4, probably α5, and sometimes β2 subunits, and also displays function detectable using $^{86}Rb^+$ efflux assays.[25] SH-SY5Y cells also express homopentameric α7-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 α4 and β2 subunits (SH-EP1-hα4β2 cells) have been established and the human α4β2-nAChRs that they stably and heterologously express have been characterized with both ion flux and radioligand binding assays.[26] They are known to express a mixture of "HS" $(\alpha4\beta2)_2\beta2$-nAChR [$(\alpha4)_2(\beta2)_3$-nAChR] and "LS" $(\alpha4\beta2)_2\alpha4$-nAChR [$(\alpha4)_3(\beta2)_2$-nAChR] isoforms having the indicated subunit stoichiometries.

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.[24] 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.[24] 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 (Life Technologies, Inc., USA; for TE671/RD or transfected SH-EP1 cells) were then suspended in complete medium and evenly seeded into 24-well plates. After cells had adhered and grown to confluence, the medium was removed and replaced with 250 μL per well of complete medium supplemented with ~350000 cpm of $^{86}Rb^+$ (Perkin-Elmer; 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. 10 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 ~EC_{90} concentration of the full agonist carbamylcholine for 5 min (~EC_{90} concentrations were 200 μM for SH-EP1-hα4β2 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 measured 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 tested drugs was ascertained during the first 9.5 min of the initial 10 min exposure period using samples containing only test drug at different concentrations and was normalized, after subtraction of nonspecific efflux, to specific efflux in carbamylcholine 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 5 (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.

To determine the efficacies of tested ligands in activation of at both HS and LS responses of α4β2-nAChR, an unusual property of satetidine A was exploited. Sazetidine A is a full agonist at "HS" $(\alpha4\beta2)_2\beta2$-nAChR isoform and of the HS responses of the $(\alpha4\beta2)_2\alpha4$-nAChR isoform but has nearly zero efficacy in activation of the predominant LS responses for the "LS" $(\alpha4\beta2)_2\alpha4$-nAChR isoform, whereas carbamylcholine is fully efficacious at both α4β2-nAChR isoforms (i.e., for both HS and LS responses). The SH-EP1-hα4β2 cell line used expresses a mixture of "HS" and "LS" α4β2-nAChR isoforms. The ratio of isoforms can vary, as then does the ratio of HS and LS responses. The proportion of the "HS" $(\alpha4)_2(\beta2)_3$-nAChR can usually be increased by incubation of cells at 29 degrees Centigrade. This allows us to manipulate these ratios to some degree and to use assessments of efficacy of sazetidine A to define the proportion of function due

to HS responses activated by it or LS responses insensitive to sazaetidijne A These results are then used to define efficacy of test ligands at both α4β2-nAChR isoforms. Plotting the efficacy of test ligands relative to carbamylcholine (ordinate) versus the efficacy of sazetidine A relative to carbamylcholine (abscissa) reveals a linear relationship. Extrapolation of the plot to 0% or 100% sazetidine A efficacy allows determination of test ligand efficacies toward purely LS or purely HS responses of α4β2-nAChR, respectively.

General Procedures for Behavioral Studies

Animals

Balb/cJ male mice (8-9 weeks of age at the start of the study) were obtained from Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed 5 to a cage in a colony room maintained at a 12 h light–dark cycle and a temperature of 22±3 °C with a relative humidity between 30% and 70%. All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council 2010) and the Harvard Medical School Animal Care and Use Committee (for compound **4b**), and the PsychoGenics Animal Care and Use Committee (for compound **4d**).

Drugs

Sertraline hydrochloride (Matrix Scientific, Columbia, SC) and compound **4b** were dissolved in saline and IP administered in a volume of 10 mL/kg.

Sertaline (Toronto Research Chemicals, Ontario, Canada) and compound **4d** were dissolved in sterile water and IP or PO administered in a volume of 10 mL/kg.

Procedure

Procedures were based on methods initially described[23] and in use in our laboratory[27] to test acute antidepressant responsiveness in mice. Mice were given a 30 min pretreatment (IP or PO administration) with either saline (n=10), water (n = 10), sertraline (20 mg/kg) (n=10) as a positive control, **4b** (10 mg/kg salt) (n=10), **4b** (20 mg/kg salt) (n=10), **4d** (10 mg/kg free-base) (n = 10) or **4d** (30 mg/kg free-base) (n = 10) then placed individually into a glass cylinder (i.e., 15 cm tall x 10 cm wide, 1 L beakers) containing 23±1°C water 12 cm deep (approximately 800

mL). The time the animal spent immobile was recorded over a 6-min trial. Immobility was described as the postural position of floating in the water.

Statistical Analyses

All data were analyzed with analysis of variance (ANOVA). Total time immobile was used as the dependent variable and treatment [saline, water, sertraline, **4b** (10 mg/kg salt), **4b** (20 mg/kg salt), **4d** (10 mg/kg free-base) or 4d (30 mg/kg free-base)] as the independent variable. Main effects were compared between groups and analyzed for significance with the post hoc Fisher's PLSD test.

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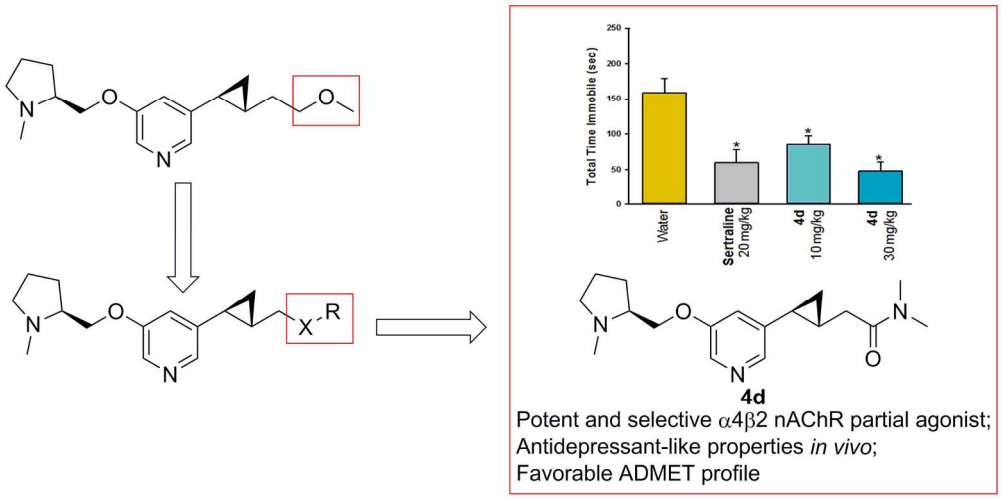

TOC Graphic
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