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Synthesis, Structure−Affinity Relationships, and Radiolabeling of Selective High-Affinity 5-HT_4 Receptor Ligands as Prospective Imaging Probes for Positron Emission Tomography

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In a search for high-affinity receptor ligands that might serve for development as radioligands for the imaging of brain 5-HT_4 receptors in vivo with positron emission tomography (PET), structural modifications were made to the high-affinity 5-HT_4 antagonist (1-butylpiperidin-4-yl)methyl 8-amino-7-iodo-2,3-dihydrobenzo[*b*][1,4]dioxine-5-carboxylate (**1**, SB 207710). These modifications were made mainly on the aryl side of the ester bond to permit possible rapid labeling of the carboxylic acid component with a positron emitter, either carbon-11 ($t_{1/2}$ = 20.4 min) or fluorine-18 ($t_{1/2}$ = 109.7 min), and included (i) replacement of the iodine atom with a small substituent such as nitrile, methyl, or fluoro, (ii) methylation of the 8-amino group, (iii) opening of the dioxan ring, and (iv) alteration of the length of the *N*-alkyl goup. High-affinity ligands were discovered for recombinant human 5-HT_4 receptors with amenability to labeling with a positron emitter and potential for development as imaging probes. The ring-opened radioligand, (([methoxy-^{11}C]1-butylpiperidin-4-yl)methyl 4-amino-3-methoxybenzoate; [^{11}C]**13**), showed an especially favorable array of properties for future evaluation as a PET radioligand for brain 5-HT_4 receptors.

Introduction

Suitably effective radioligands, when applied with molecular imaging techniques, such as positron emission tomography (PET[a]) or single photon emission computed tomography (SPECT), provide a unique means for measuring brain neurotransmitter receptor concentrations in living subjects, and therefore, they constitute important clinical research tools.[1] Such radioligands may also be used with imaging techniques to assess the receptor binding of unlabeled ligands in vivo, whether exogenous (e.g., a developmental or therapeutic drug or a substance of abuse) or endogenous (the neurotransmitter), and hence, they are also useful for drug discovery and development.[2]

Serotonin (5-hydroxytryptamine, 5-HT) is an important neurotransmitter that is known to act on at least 14 receptors in seven major subclasses. Many of these receptors in brain have been implicated in neuropsychiatric disorders, and hence, they have become targets for deep biomedical investigation and also for drug discovery and development programs. Although many effective radioligands exist for the in vivo imaging of some of the 5-HT receptors, especially the 5-HT_{1A}, 5-HT_{2A}, and 5-HT_{1B} subtypes, radioligands for some other subtypes are not yet well explored (e.g., 5-HT_4).

The 5-HT_4 receptor is a well-characterized G-protein-coupled receptor that exists abundantly in brain, especially in limbic and striatonigral regions.[3] This receptor population is implicated in dopamine, serotonin, and acetylcholine release and possibly plays a significant role in normal cognition, learning, and memory. 5-HT_4 receptors have also been implicated in neuropsychiatric disorders, such as Alzheimer's disease, anxiety, and depression.[4−6]

A reason for the relatively slow development of 5-HT_4 PET radioligands is the wide array of properties that must ideally be found in any candidate. These include high target receptor affinity, high selectivity, generally moderate lipophilicity,[7] appropriate intrinsic activity, ability to cross the blood−brain barrier,[8] absence of troublesome radiometabolites,[8] and amenability to labeling with a suitable radioisotope.[9,10] Suitable radioisotopes are generally short-lived carbon-11 ($t_{1/2}$ = 20.4 min) or fluorine-18 ($t_{1/2}$ = 109.7 min) for PET and iodine-123 ($t_{1/2}$ = 13.2 h) for SPECT.

(1-Butylpiperidin-4-yl)methyl 8-amino-7-iodo-2,3-dihydrobenzo[*b*][1,4]dioxine-5-carboxylate (**1**, SB 207710; Chart 1) is an exceptionally high-affinity 5-HT_4 receptor antagonist.[11,12] The use of [^{123}I]**1** with SPECT provided the first demonstration of 5-HT_4 receptor imaging in primate brain in vivo.[13] Some analogues of **1**, labeled with either carbon-11 or fluorine-18 in the terminal *N*-alkyl group, have been prepared, and one of these, [^{11}C]**2** ([^{11}C]SB 207145, Chart 1) has shown promise for PET imaging in animal[14] and human subjects.[15,16] This radioligand is an ester and rapidly metabolized in vivo by hydrolysis. Here, we aimed to develop alternative radioligands for 5-HT_4 receptor imaging with PET, again based on **1**, in which

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[a] Abbreviations: BZP, peripheral benzodiazepine receptor; Cbz, benzyloxycarbonyl; CDI, *N*,*N*′-carbonyldiimidazole; D, dopamine; DAT, dopamine transporter; DOR, δ opiate receptor; DMAP, 4-(dimethylamino)-pyridine; DMF, dimethylformamide; EOS, end of synthesis; H, histamine; K 2.2.2, 4,7,13,18-tetraoxo-1,10-diazabicyclo[8,8,8]hexacosane; M, muscarinic; MOR, μ opiate receptor; NBS, *N*-bromosuccinimide; NCA, no-carrier-added; NET, noradrenalin transporter; NMP, *N*-methyl-2-pyrrolidinone; NXS, *N*-halosuccinimide (X = halo atom); PET, positron emission tomography; rt, room temperature; RCY, decay-corrected radiochemical yield; SERT, serotonin transporter; SPECT, single photon emission computed tomography; THF, tetrahydrofuran; TMS, trimethylsilyl; TPP, triphenylphosphine; 5-HT, serotonin.

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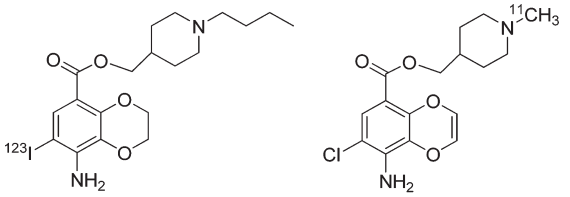
Chart 1. Current Analogues of **1** for 5-HT_4 Receptor Imaging in Vivo

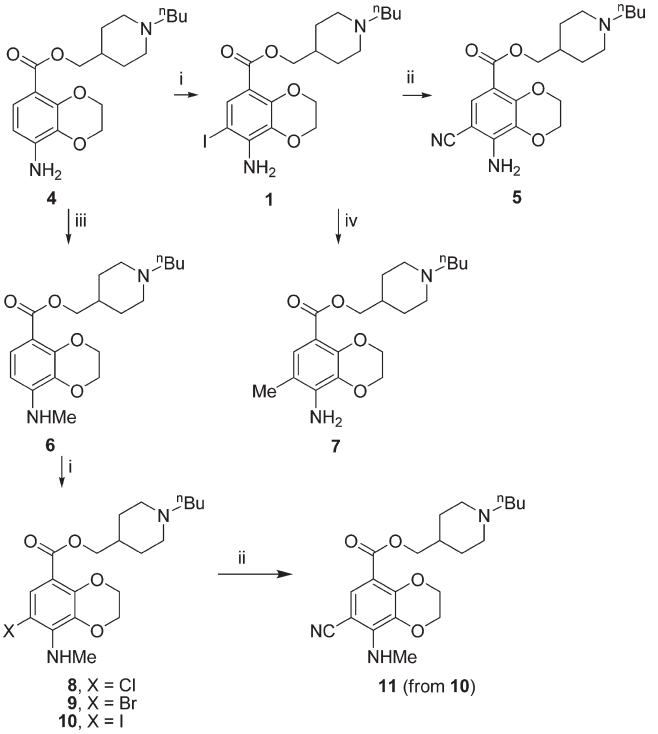


the radiolabel is located on the carbonyl side of the ester group to avoid potential issues from possibly brain-penetrant radiometabolites and to permit control of lipophilicity through adjustment of *N*-alkyl chain length. The ester hydrolysis of such radioligands outside brain would produce carboxylic acids as radiometabolites; these radiometabolites would be expected to have poor entry into brain and therefore would not be expected to interfere with the measurement of brain receptor density with the parent radioligand.[8] The 5-HT_4 radioligand [^{11}C]**2** has been shown to be amenable to quantification in human brain,[16] implying that ester hydrolysis to generate benzoates as radiometabolites within brain should not be a major concern for ligands related to **1**. Control of lipophilicity can be important for adjusting properties that may have an impact on the potential success of a radioligand, including its plasma free fraction, ability to penetrate the blood−brain barrier (permeability parameter),[17] and susceptibility to metabolism.[8−10] Our synthetic strategies included replacement of the aryl halo group and/or alkylation of the aryl amino group, opening of the dioxan ring, and manipulation of the *N*-alkyl chain length. As a result, several new high-affinity ligands were discovered and radiolabeled as prospective PET radioligands for 5-HT_4 receptors. The 3-methoxy compound, [^{11}C]**13**, was found to have an especially favorable array of properties for further evaluation as a new 5-HT_4 receptor PET radioligand.

Results

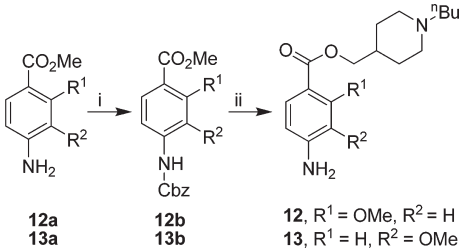
Chemistry. We considered several approaches for modifying the structure of **1** to allow carbon-11 to be introduced as a radiolabel into the structure on the carbonyl side of the ester bond, using readily accessible labeling agents such as [^{11}C]methyl iodide,[18] [^{11}C]methyl triflate,[19] or [^{11}C]cyanide ion.[20] These strategies included (i) replacing the iodo group in **1** with a group of similar or smaller size, namely, nitrile, as in **5** and **11**, or methyl as in **7** (Scheme 1), (ii) N-methylation, as in **6** and **8**−**11** (Scheme 1), and (iii) replacement of the dioxan ring by a single *O*-methyl group, as in **12** and **13** (Scheme 2). Ligand **5** was obtained in low but useful yield (19%) by treating **1** with potassium cyanide. The *N*-methyl compound **6** was obtained in good yield (60%) by treating the primary arylamine **4** with paraformaldehyde and then sodium borohydride. Treatment of **6** with *N*-halosuccinimides (NXS, X = Cl, Br, or I) gave the respective halo derivatives **8**−**10** in moderate yields (30−56%). Ligand **7** was obtained from **1** in moderate yield (44%) through Pd-catalyzed methylation with tetramethyltin. Ligand **11** was similarly obtained from ligand **10** but in much lower yield (7%). The ring *O*-methyl derivatives, **12** and **13**, were obtained in moderate (32%) and low (8%) overall yields, respectively, from the corresponding methyl esters **12a** and **13a** in two steps, namely, N-protection and trans-esterification (Scheme 2). Analogues of **13**, in which either the ester

Scheme 1. Synthesis of Ring Methyl, Halo, and Nitrile Ligands and *N*-Methyl Ligands **5**−**11**[a]



[a] Reagents and conditions: (i) NXS (X = Cl, Br, or I), AcOH; (ii) KCN, CuI, 1,10-phenanthroline, DMF, 42 h, 110 °C; (iii) (1) paraformaldehyde, EtOH, 20 h, 60 °C; (2) $NaBH_4$; (iv) Me_4Sn, CuI, $Pd_2(dba)_3$, TPP, NMP, 70 °C, 48 h.

Scheme 2. Synthesis of Ring Methoxy Ligands **12** and **13**[a]



[a] Reagents and conditions: (i) CbzCl, $NaHCO_3$, overnight; (ii) (1) lithium (l-butylpiperidin-4-yl)methanolate, THF, 0 °C, then rt overnight; (2) H_2,Pd/C.

group was replaced with an amido group, as in **14**, or in which the length of the *N*-alkyl group was altered to vary lipophilicity, as in **15** and **16**, were also prepared in two steps and moderate overall yields (30−69%) from 4-amino-3-methoxybenzoic acid (Scheme 3).

For the purpose of creating a radioligand that might be labeled with fluorine-18, we succeeded in preparing **17**, a 7-fluoro analogue of **1**, in six steps from 5-fluoro-2-hydroxybenzoic acid (Scheme 4). All steps proceeded in > 75% yield except the final two-stage esterification, involving Cbz protection (34%) and trans-esterification (59%). Also, two fluoroalkoxy analogues of **13**, the fluoromethoxy compound **18** and the 2-fluoroethoxy compound **19**, were prepared in two steps from methyl 4-amino-3-hydroxybenzoate in moderate overall yields of 37% and 20%, respectively (Scheme 5).

Potential precursors for radiolabeling were also synthesized, including the phenol precursor **20** by demethylation of

13 (42% yield) (Scheme 6), and the nitro compound **21** in three steps from 8-amino-7-nitro-2,3-dihydrobenzo[*b*]-[1,4]dioxine carboxylic acid methyl ester (**21a**) in 42% overall yield (Scheme 7).

Pharmacological Assays and Screen. Assay of compounds **1**, **5**, **8**, **13**, **15**, and **17**−**19** for binding to 5-HT_4 receptors in guinea pig striatal membranes showed all these compounds to have subnanomolar K_i values (Table 1). However, assay of the same ligand set against human recombinant 5-HT_4 receptors (h5-HT_4), expressed in HEK293T cells, revealed a greater variation in K_i, with only some of the compounds (**1**, **8**, **13**, and **17**) showing low nanomolar values. This assay was applied to the full range of new ligands and revealed interesting structure−activity information (Table 1).

Replacement of the iodine atom in **1** with a nitrile, methyl, or nitro group resulted in approximately 15-, 3-, and 3.5-fold decrease in binding affinity, respectively, whereas replacement with fluorine retained binding affinity and replacement with hydrogen slightly increased affinity. N-Methylation of **1** resulted in about a 4-fold reduction in binding affinity, while the N-methylation of the corresponding nitrile, **5**, resulted in only a marginal reduction in binding affinity. The *N*-methyl 8-chloro analogue **8** showed a binding affinity comparable to that of **1**.

Replacement of the dioxan ring in **1** with a 2-methoxy group gave ligand **12** with much reduced affinity, whereas replacement with a 3-methoxy group gave ligand **13** with affinity comparable to that of **1**. Shortening or lengthening of the *N*-alkyl chain length in **13** gave ligands of 5- and 7-fold lower affinity, respectively.

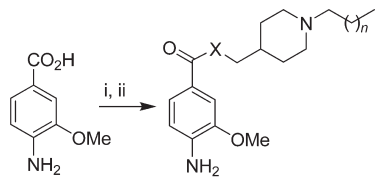
Replacement of the ester group in **13** with an amido group drastically reduced binding affinity.

Replacement of the methoxy group in **13** with fluoromethoxy or 2-fluoroethoxy led to 7- and 5-fold reduction in binding affinity, respectively.

A selection of the compounds was also assessed for intrinsic activity (Table 1). Most of these ligands were found to be quite potent partial or full inverse agonists with pEC_{50} values in the range 7.70−8.23. The 2-methoxy ligand **12** was found to be a potent full agonist with a pEC_{50} value of 9.75. In stark contrast, the high-affinity regional isomer **13** was found to be an antagonist. The arylmethyl analogue of **1** was also found to be an antagonist, as was the previously known ligand, **4**.

Ligand **13** showed greater than 2000-fold selectivity for h5-HT_4 receptors versus 12 other h5-HT receptors and binding sites (Table 2). Ligands **8** and **17** were not quite as selective among h5-HT receptors and binding sites.

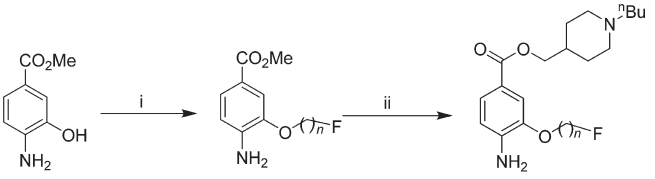
Scheme 3. Synthesis of Amide (**14**) and *N*-Alkyl Analogues (**15** and **16**) of **13**[a]



14, X = NH, *n* = 2
15, X = O, *n* = 1
16, X = O, *n* = 3

[a] Reagents and conditions: (i) CDI; (ii) (l-butylpiperidin-4-yl)methanamine or lithium (1-propylpiperidin-4-yl)methanolate or lithium (l-pentylpiperidin-4-yl)methanolate.

Scheme 5. Synthesis of Fluoroalkoxy Ligands **18** and **19**[a]

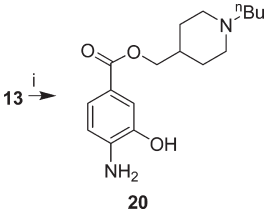


18a, *n* = 1
19a, *n* = 2

18, *n* = 1
19, *n* = 2

[a] Reagents and conditions: (i) $F(CH_2)_nCl$, Cs_2CO_3, DMF, rt; (ii) lithium (l-butylpiperidin-4-yl)methanolate, THF, 0 °C, then rt overnight.

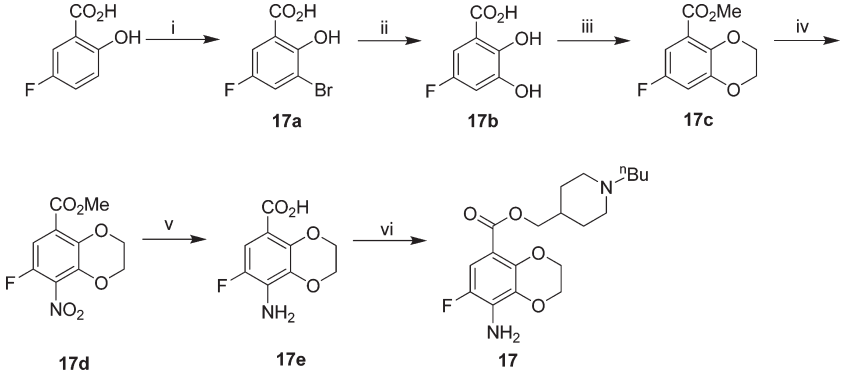
Scheme 6. Synthesis of Phenol Precursor **20**[a]



20

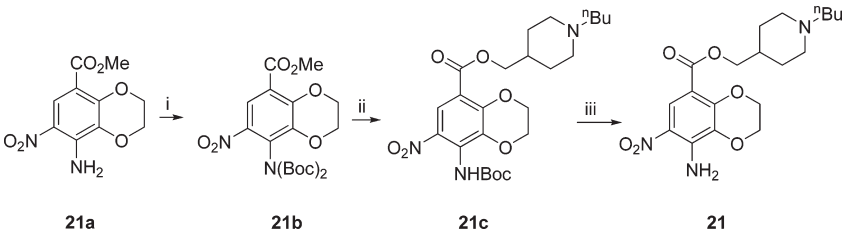
[a] Reagents and conditions: (i) $AlCl_3$, NaI, MeCN, reflux, overnight.

Scheme 4. Synthesis of Ring Fluoro Ligand **17**[a]



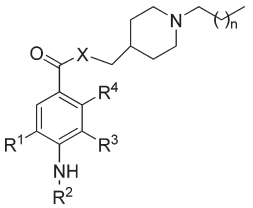
[a] Reagents and conditions: (i) NBS, AcOH; (ii) NaOH, $CuSO_4$; (iii) (1) $TMSCHN_2$; (2) Cs_2CO_3, 1,2-dibromoethane, DMF, 80 °C, 16 h; (iv) HNO_3, −50 °C, 16 min; (v) 10% Pd/C, HCOOK, MeOH, 80 °C, 2 h; (vi) (1) CbzCl, aqueous $NaHCO_3$; (2) CDI, MeCN, rt, 2 h; (3) lithium (l-butylpiperidin-4-yl)-methanolate, THF, 0 °C, then rt overnight; (4) H_2, Pd/C.

Scheme 7. Synthesis of Nitro Analogue **21**[a]



[a] Reagents and conditions: (i) $(Boc)_2O$, DMAP, 50 °C, 1 h; (ii) lithium (l-butylpiperidin-4-yl)methanolate, THF, 0 °C, then rt overnight; (iii) 4 M HC1 in dioxane, overnight.

Table 1. Binding Affinities ($1/K_i$),[a] Efficacies, and cLogD of New 5-HT_4 Ligands



compd	R^1	R^2	R^3	R^4	X	*n*	5-HT_4 K_i (nM)[a]	h5-HT_4 K_i (nM)[b]	efficacy[c,d]	pEC_{50}[d]	cLogD[e]
1	I	H	OCH_2	OCH_2	O	2	0.20	2.2 ± 0.3	inv ag	7.9	3.20
4	H	H	OCH_2	OCH_2	O	2		1.4 ± 0.2	antagonist		1.74
5	CN	H	OCH_2	OCH_2	O	2	0.45	33 ± 5	n.m.	n.m.	2.59
6	H	Me	OCH_2	OCH_2	O	2		9.1 ± 0.7	inv.ag	8.05	2.37
7	Me	H	OCH_2	OCH_2	O	2		6.3 ± 1	antagonist		2.20
8	Cl	Me	OCH_2	OCH_2	O	2	0.577	2.0 ± 0.2	inv ag	7.85	3.27
9	Br	Me	OCH_2	OCH_2	O	2		4.5 ± 0.4	inv ag	8.0	3.36
10	I	Me	OCH_2	OCH_2	O	2		7.4 ± 0.5	inv ag	7.7	3.41
11	CN	Me	OCH_2	OCH_2	O	2		37 ± 5	inv ag	7.8	2.52
12	H	H	H	OMe	O	2		50 ± 7	ag.	9.75	1.77
13	H	H	OMe	H	O	2	0.738	2.4 ± 0.3	antagonist		2.07
14	H	H	OMe	H	N	2		8664 ± 857	n.m.	n.m.	0.16
15	H	H	OMe	H	O	1	0.901	17 ± 1	n.m.	n.m.	1.54
16	H	H	OMe	H	O	3		12 ± 1	n.m.	n.m.	2.60
17	F	H	OCH_2	OCH_2	O	2	0.334	2.1 ± 0.3	inv ag	8.23	2.42
18	H	H	OCH_2F	H	O	2	0.334	17 ± 1	n.m.	n.m.	1.97
19	H	H	$O(CH_2)_2F$	H	O	2	0.215	11 ± 1	n.m.	n.m.	2.30
21	NO_2	H	OCH_2	OCH_2	O	2		7.8 ± 0.9	n.m.	n.m.	3.04

[a] For guinea pig striatal membrane 5-HT_4 receptors. Values are averages of triplicate measurements. [b] For h5-HT_4 receptors. Binding assay results are averages of triplicate measurements. [c] In agonist/inverse agonist assay: ag = agonist; inv ag = inverse agonist. [d] n.m. = not measured. [e] cLogD was calculated with ACD software. Estimated errors are approximately ±1.0.

Table 2. Binding Affinities of 5-HT_4 Ligands **8**, **13**, and **17** for Other 5-HT Receptors/Binding Sites

	binding affinity (K_i) (nM)		
binding site or receptor	ligand **8**	ligand **13**	ligand **17**
h5-HT_4	2.0	2.4	2.1
h5-HT_{1A}	300	> 10000	> 10000
h5-HT_{1B}	820	> 10000	> 10000
h5-HT_{1D}	1304	5686	2856
h5-HT_{1E}	> 10000	> 10000	> 10000
h5-HT_{2A}	1776	> 10000	> 10000
h5-HT_{2B}	66	2861	168
h5-HT_{2C}	685	> 10000	8363
h5-HT_3	1072	3511	> 10000
h5-HT_{5A}	8589	> 10000	> 10000
h5-HT_6	489	> 10000	> 10000
h5-HT_7	5702	> 10000	> 10000
hSERT	9006	> 10000	> 10000

The lowest selectivity for **8** was 33-fold versus h5-HT_{2B} receptors and for **17**, 80-fold versus the same receptors (Table 2). Ligand **13** also exhibited high selectivity for h5-HT_4 receptors versus a wide range of nonserotonergic receptors and binding sites (Table 3). For **13**, the lowest selectivities for h5-HT_4 receptors were versus σ_1 and σ_2 receptors, namely, 88- and 62-fold, respectively. Ligands **8** and **17** were also generally selective for h5-HT_4 receptors versus other receptors and binding sites except that they displayed much lower selectivities versus σ_1 and especially σ_2 receptors (Table 3). Ligand **8** also showed a quite low 22-fold selectivity versus D_4 receptors (Table 3).

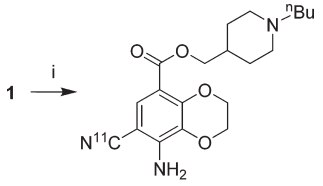
Computation of cLogD. The cLogD values of ligands ranged from 1.74 to 3.27 (Table 1).

Syntheses of Radioligands. Some ligands, namely, ligands **5**, **8**, **13**, **17**, and **18**, were selected to test their amenability for rapid labeling with a positron emitter, either carbon-11 or fluorine-18. Ligand [^{11}C]**5**, a [^{11}C]nitrile, was produced in an average of 26% decay-corrected radiochemical yield (RCY) by Pd-mediated exchange of the iodine atom with no-carrier-added (NCA) [^{11}C]cyanide ion in the presence of potassium

Table 3. Binding Affinities of 5-HT$_4$ Ligands **8**, **13**, and **17** for Non-Serotonergic Receptors and Binding Sites

	binding affinity (K_i) (nM)		
binding site or receptor	ligand **8**	ligand **13**	ligand **17**
hα_{1A}	522	> 10000	87.6
hα_{1B}	> 10000	4228	9076
hα_{1D}	2276	> 10000	> 10000
hα_{2A}	2239	> 10000	> 10000
hα_{2B}	671.2	> 10000	773
hα_{2C}	556	989	593
β_1 (rat)	> 10000	4407	> 10000
β_2 (rat)	> 10000	> 10000	> 10000
β_3 (rat)	> 10000	> 10000	> 10000
σ_1 (rat)	60	211	55
σ_2 (rat)	8	148	12.9
BZP (rat brain site)	> 10000	> 10000	> 10000
hDAT	2020	> 10000	> 10000
hDOR	> 10000	> 10000	> 10000
D_1 (rat)	2291	> 10000	> 10000
D_2 (rat)	220	> 10000	615
D_3 (rat)	336	6272	1011
D_4 (rat)	43	1272	316
hD_5	972	> 10000	> 10000
h$GABA_A$	> 10000	> 10000	> 10000
H_1 (guinea pig)	2727	> 10000	> 10000
H_2 (guinea pig)	> 10000	> 10000	> 10000
H_3 (guinea pig)	> 10000	1649	5038
H_4 (guinea pig)	> 10000	> 10000	> 10000
hKOR	> 10000	> 10000	> 10000
hMOR	> 10000	> 10000	> 10000
hM_1	> 10000	> 10000	> 10000
hM_2	433	> 10000	> 10000
hM_3	823	1095	> 10000
hM_4	760	2579	> 10000
hM_5	972	4058	> 10000
hNET	3226	> 10000	> 10000

Scheme 8. Radiosynthesis of [^{11}C]**5**[a]



[^{11}C]**5**

[a] Reagents, conditions and yield: (i) [^{11}C]HCN, Pd(PPh$_3$)$_4$, K_2CO_3, THF, K 2.2.2, 80 °C, 5 min, RCY = 26%.

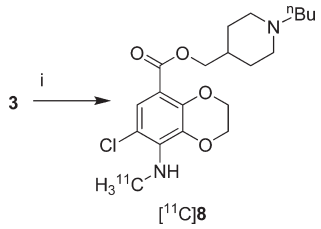
Table 4. RCYs for [^{11}C]**5** under Various Conditions

solvent	base	RCY [a](%)	*n*
THF	KH_2PO_4	2	1
DMSO	KH_2PO_4	10	2
THF	K_2CO_3, K 2.2.2	26	3

[a] From [^{11}C]HCN.

carbonate−K 2.2.2 in THF (Scheme 8). Use of potassium dihydrogen phosphate as base in THF or DMSO[21] gave much lower RCY (Table 4). The *N*-[^{11}C]methyl ligand, [^{11}C]**8**, was obtained in 11% RCY by ^{11}C-methylation of **1** with NCA [^{11}C]methyl iodide in the presence of the solid base, Li_3N, under the influence of ultrasound (Scheme 9). Use of Li_2O as solid base resulted in a lower RCY (7%) of [^{11}C]**8**. The *O*-[^{11}C]methyl ligand [^{11}C]**13** was obtained from the phenol **20** by methylation with either NCA [^{11}C]methyl

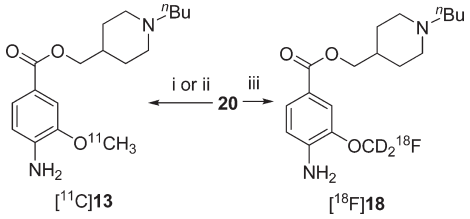
Scheme 9. Radiosynthesis of [^{11}C]**8**[a]



[^{11}C]**8**

[a] Reagents, conditions and yield: (i) [^{11}C]MeI, Li_3N, DMF, rt, ultrasound, 10 min. RCY = 11%.

Scheme 10. Radiosyntheses of [^{11}C]**13** and [^{18}F]**18**[a]



[^{11}C]**13** [^{18}F]**18**

[a] Reagents, conditions and yields: (i) [^{11}C]MeI, DMF, 1.0 M (*n*-Bu)$_4$NOH in MeOH, 80 °C, 5 min, RCY = 36%; (ii) [^{11}C]MeOTf, MeCN, 0.5 M NaOH, heat, 5 min, RCY = 27%; (iii) [^{18}F]FCD$_2$Br, MeCN, 0.5 M NaOH, 100 °C, 15 min. RCY = 13% from [^{18}F]fluoride ion.

Table 5. RCYs of [^{18}F]**18** under Various Conditions

solvent	base	*T* (°C)	time (min)	RCY [a](%)
MeCN	0.5 M NaOH	80	5	1
DMF	K_2CO_3, 18-crown-6	110	10	7
MeCN	0.5 M NaOH	100	15	13

[a] Overall starting from [^{11}C]fluoride ion.

iodide or [^{11}C]methyl triflate in either 36% or 27% RCY, respectively (Scheme 9). An experiment based on ^{13}C/^{11}C colabeling, followed by ^{13}C NMR,[22] confirmed the position of the radiolabel. This radioligand was obtained in moderately high specific radioactivity, namely, 2848 mCi/μmol from [^{11}C]methyl iodide and 2517 mCi/μmol from [^{11}C]-methyl triflate at the end of radiosynthesis.

All attempts to prepare [^{18}F]**17** by substitution of the nitro group in precursor **21** or the *N*-Boc-protected analogue **21c** with cyclotron-produced [^{18}F]fluoride ion were unsuccessful. However, the [^{18}F]fluorodideuteromethoxy ligand, NCA [^{18}F]**18** was obtained in 13% RCY from [^{18}F]fluoride ion by treating the phenol **21** with derived [^{18}F]fluorobromomethane-d_2 in acetonitrile with NaOH as base at 100 °C (Scheme 10). Other tested conditions gave inferior RCYs (Table 5).

Discussion

In this study, variation of the structure of **1** led to several new high-affinity ligands for guinea pig and h5-HT$_4$, receptors, some of which proved amenable to labeling with a positron emitter to provide candidate radioligands for imaging brain 5-HT$_4$ receptors with PET. Interestingly, all seven new ligands (**5**, **8**, **13**, **15**, **17**−**19**) that were tested for binding to guinea pig 5-HT$_4$ receptors showed subnanomolar affinity within a narrow range (K_i = 0.22−0.90 nM), while the same set of ligands assayed against human recombinant receptors (h5-HT$_4$) showed somewhat lower binding affinity across a wider range (K_i = 2.2−33 nM) (Table 1). This species

difference was unexpected, since 5-HT_4 receptor ligands from several other structural classes do not show such major differences in binding affinity between guinea pig 5-HT_4 and h5-HT_4 receptors.[23] Also, the affinities of **1**, the prototypic ligand for our series, were previously reported to be quite similar for human colon and guinea pig 5-HT_4 receptors.[12] Because any successfully developed PET radioligand would ultimately be used for imaging 5-HT_4 receptors in human brain, all new ligands from this study were assayed against h5-HT_4 receptors (Table 1).

Replacement of the iodine atom in **1** with a methyl or nitro substituent reduced h5-HT_4 receptor binding affinity by less than 1 order of magnitude, whereas replacement with a fluoro substituent caused negligible change, and replacement with hydrogen slightly improved affinity (Table 1). Replacement with a nitrile or nitro group caused a 15- or 4-fold reduction in affinity, respectively. Therefore, the h5-HT_4 receptor was quite tolerant of a small substituent ortho to the 8-amino group in **1**, irrespective of the electronic influence of the substituent.

Although a primary arylamino group is a frequent constituent of ligands reported for 5-HT_4 receptors,[24] the effect of N-alkylation of this group on binding affinity had not been examined previously. We found that the N-methylations of **1**, **4**, and **5** caused only small reductions of binding affinity of about 3-, 6.5-, and 1.1-fold, respectively. The N-methylated **4** recovered its affinity to 2.0 nM after chlorination in the ortho position as seen in **8**. Another prepared *N*-methyl ligand, **9**, also exhibited low nanomolar affinity. Thus, the h5-HT_4 receptor readily tolerates the secondary 8-*N*-methylamino substituent in this structural class of ligand.

SB 204070 (**3**) is the 7-chloro analogue of **1**. The non-dioxan 2-methoxy analogue of **3** has nanomolar affinity for guinea pig 5-HT_4 receptors.[25] Here, opening of the dioxan ring in the proto analogue of **1**, namely, ligand **4**, by removal of either OCH_2 group gave the two *O*-methyl compounds **12** and **13**. Binding affinity was reduced 36-fold in the 2-methoxy compound **12** but only 1.7-fold in the 3-methoxy compound **13**. Replacement of the 3-methoxy group in **13** with fluoromethoxy or 2-fluoroethoxy reduced affinity by 7- and 4.6-fold, respectively. Shortening or lengthening of the *N*-alkyl chain in **13** similarly reduced binding affinity by 7- and 5-fold, respectively.

We noted that the conversion of the ester function in the benzodioxan **3** into an amido function has negligible effect on binding affinity ($1/IC_{50}$)[24] and in the ring-opened 2-methoxy analogue causes only a 10-fold reduction in binding affinity.[25] We therefore considered replacing the ester function in the new radioligands with an amide function in order to confer greater resistance to hydrolysis in vivo. However, replacement of the ester group in the 3-methyl ether **13** with an amido group dramatically reduced affinity by several-thousand-fold.

The maximal receptor-specific signal to be expected from the use of a radioligand with PET is related to the binding potential (BP), expressed as B_{max}/K_D, where B_{max} is the local concentration of receptors and K_D is the equilibrium dissociation constant of radioligand from the receptor.[9,10] Therefore, high affinity ($1/K_D$ or as a surrogate measure, $1/K_i$) is a key parameter in determining whether a particular radioligand can be successful. Previously, it has been found that moderately sizable receptor-specific signals can be obtained in minipig,[14] monkey,[13] and human[16] subjects in vivo with 5-HT_4 receptor radioligands having K_D values in the low or subnanomolar range, such as [^{123}I]**1** and [^{11}C]**2**. Our new ligands **8**, **13**, and **17** showed K_i values less than or comparable with that of **1** for h5-HT_4 receptors, and they therefore met the high-affinity criterion for development as PET radioligands. Since target selectivity, intrinsic activity,[26] and lipophilicity also bear on the likely success of candidate PET radioligands,[9,10] these three ligands were also assessed for these parameters. For comparison, lipophilicity was also computed for the other synthesized ligands, and intrinsic activity was also assessed for **1**, **4**, **6**−**13**, and **17**.

Ligands **1**, **8**, and **17** appeared to be similarly potent inverse agonists in the GloSensor assay, whereas ligands **4** and **13** were antagonists (inactive) in the same assay (Table 1). Ligand **1** is widely considered to be an antagonist at 5-HT_4 receptors.[11,12] However, it is noted that ligands characterized as competitive antagonists often express inverse agonism in assays where constituitive receptor activity is present.[27] This fact likely explains why **1** appears to be an inverse agonist in the Glo-sensor assay. Ligand **7** was also inactive in the Glo-sensor assay, whereas ligands **6** and **9**−**11** were inverse agonists. Remarkably, ligand **12**, the close methoxy positional isomer of the antagonist **13**, was a potent agonist with quite high binding affinity.

Ligands **8**, **13**, and **17** showed generally high selectivity for binding to h5-HT_4 receptors versus binding to other h5-HT receptors and binding sites (Table 2). The lowest selectivity was 33-fold for ligand **8** versus h5-HT_{2B} receptors. Although there is strong evidence that 5-HT_{2B} receptors exist in brain, their distribution and density in human brain remain unknown and are suspected to be low.[28,29] Thus, the low-affinity binding of **8** to 5-HT_{2B} receptors in human brain in vivo would likely be insignificant compared to its high-affinity binding to relatively abundant 5-HT_4 receptors. Therefore, this off-target binding would probably not be a serious impediment to the development of **8** as a PET radioligand. Ligands **8**, **13**, and **17** showed only low affinity for a wide range of other receptors and binding sites except for σ_1 and σ_2 receptors in the cases of **8** and **17** and for D_4 receptors in the case of **8** (Table 3). Both σ receptors are present in brain,[30] and indeed σ_1 receptors may be imaged with PET in vivo with moderately high-affinity radioligands, such as [^{11}C]SA4503 (K_i = 17.4 nM).[30] Thus, the affinities of ligands **8** (K_i = 60 nM) and **17** (K_i = 55 nM) for σ_1 receptors may be insufficiently low for successful 5-HT_4 receptor PET imaging. Imaging data suggest that the density of σ_2 receptors in human brain is much lower than that of σ_1 receptors.[30] Hence, it is uncertain whether the high-affinities of **8** (K_i = 8 nM) and **17** (K_i = 13 nM) for σ_2 receptors may be problematic for their development as PET 5-HT_4 receptor radioligands. The moderate affinity of **8** for D_4 receptors is unlikely to be problematic, since D_4 receptors only exist in very low density in human brain.[31,32] As a candidate for development as a PET radioligand, **13** expressed excellent overall 5-HT_4 receptor selectivity, with greater than 60-fold selectivity against the full range of tested receptors and binding sites (Table 3).

Ligands **8**, **13**, and **17** had computed lipophilicities (cLogD values) between 2.0 and 3.3, and these are within the range considered desirable for achieving adequate brain entry from blood without incurring excessive nonspecific binding.[7−10]

Given the array of favorable properties expressed by ligands **8**, **13**, and **17**, it appeared especially attractive to attempt to label these ligands with a positron emitter, either carbon-11 or fluorine-18, to give radioligands that might be tested and evaluated in vivo with PET.

Various conditions were attempted for the labeling of **8** with carbon-11 in its *N*-methyl group, all based on ^{11}C-methylation

of the primary arylamine **3**. However, **3**, as is quite usual for primary arylamines, proved stubbornly reactive toward [^{11}C]methyl iodide or [^{11}C]methyl triflate under conventional conditions. Recently, we have developed methodology for labeling such precursors in the presence of a strong inorganic base under the influence of ultrasound agitation. This method, using DMF as solvent and Li_3N as solid base, gave [^{11}C]**8** in low but still useful RCY (11%) from [^{11}C]methyl iodide after HPLC separation (Scheme 9).

By contrast to **8**, the methoxy compound **13** was readily labeled by reaction of the phenol **20** with [^{11}C]methyl iodide or [^{11}C]methyl triflate under basic conditions. No protection of the anilino nitrogen was necessary because of its low reactivity toward these labeling agents. The selectivity of the ^{11}C-methylation reaction for the phenol oxygen versus the anilino nitrogen was confirmed through a ^{11}C/^{13}C colabeling experiment and subsequent ^{13}C NMR spectroscopy.[22] [^{11}C]**13** was readily separated by HPLC and was shown to be radiochemically pure by analytical HPLC.

The labeling of the fluoro compound **17** was first attempted through treatment of the *N*-Boc-protected **21** with NCA [^{18}F]fluoride ion because the presence of unprotected amino group and the electron-rich nature of the aryl ring were expected to oppose facile aromatic nucleophilic substitution.[33] The *N*-Boc group was found to be unstable when the temperature exceeded 120 °C. Nitro substitution occurred in deprotected **21** at high temperature (200 °C) but not without concomitant ester hydrolysis. The direct labeling of **21** also only gave [^{18}F]fluoride ion substituted benzoic acid at similarly high temperature. Another attempt based on halogen exchange in methyl 7-iodo-8-nitro-2,3-dihydrobenzo[*b*]-[1,4]dioxine-5-carboxylate precursor resulted in the replacement of the nitro group instead of the iodo group by the [^{18}F]fluoride ion. We considered other strategies for ^{18}F-labeling, including the production of a diaryliodonium salt precursor for radiofluorination.[34] However, we were unable to synthesize a suitable iodonium salt via a metalated-ring intermediate, since the aryl ring resisted clean stannylation, boronation, or mercuration by conventional reagents and methods. Curiously, the *n*-tributylstannyl analogue of **1** is known[13] but its synthesis, as far as we can ascertain, has never been published. Therefore, the radiosynthesis of [^{18}F]**17** remains a major challenge.

The radiolabeling of the relatively lower affinity ligands **5** and **18** with carbon-11 and fluorine-18, respectively, was shown to be feasible. Thus, [^{11}C]**5** was obtained by palladium-mediated ^{11}C-cyanation of **1** and [^{18}F]**18** by ^{18}F-fluoromethylation of the phenol **20** with [^{18}F]fluoromethyl-d_2 bromide. Deuterium was incorporated into [^{18}F]**18** to provide for greater resistance to defluorination in vivo.[35−37]

Conclusions

In this study, manipulations of the structure of **1** led to several new ligands with high affinity toward guinea pig 5-HT$_4$ receptors and a few (**8**, **13**, and **17**) with comparably high affinity toward h5-HT$_4$ receptors. Both **8** and **13** were amenable to labeling with carbon-11, whereas the labeling of **17** with NCA fluorine-18 remains a challenge. [^{11}C]**8** has affinity, selectivity, intrinsic activity, and computed lipophilicity comparable to those of [^{123}I]**1** (Table 1) and should prove to be similarly effective for imaging 5-HT$_4$ receptors in monkey in vivo. [^{11}C]**13** is an easily labeled, highly selective, high-affinity, and moderate lipophilicity antagonist for 5-HT$_4$ receptors and therefore merits evaluation as a PET radioligand for the study of 5-HT$_4$ receptors in vivo with PET. This radioligand is currently under evaluation in monkey, and findings will be published elsewhere.

Experimental Section

Materials. Methyl 3-methoxy-4-nitrobenzoate, 4-(aminomethyl)-1-(*n*-butyl)piperidine, and tetrakis(triphenylphosphine)-palladium(0) were purchased from Alfa Aesar (Ward Hill, MA). Chlorofluoromethane and 1-chloro-2-fluoroethane were purchased from SynQuest (Alachua, FL). Other chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI) and used as received. Compound **3** (SB 204070, 1-butylpiperidin-4-yl)-methyl 8-amino-7-chloro-2,3-dihydrobenzo[*b*][1,4]dioxane-5-carboxylate) and the des-chloro analogue **4** were synthesized from 2,3-dihydrobenzodioxin by modified literature procedures.[25,38,39]

General Methods. ^{1}H (400.13 MHz), ^{13}C (100.62 MHz), and ^{19}F (376.46 MHz) NMR spectra were recorded at room temperature on an Avance-400 spectrometer (Bruker, Billerica, MA). Chemical shifts are reported in δ units (ppm) downfield relative to the chemical shift for tetramethylsilane. Abbreviations s, d, t, dd, dt, and bs denote singlet, doublet, triplet, doublet of doublet, doublet of triplet, and broad singlet. Thin layer chromatography was performed with POLYGRAM SIL G/UV$_{254}$ layers (0.2 mm silica gel with fluorescent indicator; Grace Davison Discovery Sciences; Deerfield, IL); compounds were visualized under UV light (λ = 254 nm).

High resolution mass spectra were acquired from the Mass Spectrometry Laboratory, University of Illinois at Urbana—Champaign (Urbana, IL) under electron ionization conditions using a double-focusing high-resolution mass spectrometer (Autospec, Micromass Inc.) with samples introduced through a direct insertion probe.

LC−MS analyses of synthesized compounds were performed on an LCQ Deca model instrument (Thermo Fisher Scientific Corp.; Waltham, MA). A gradient or isocratic LC analysis of sample was carried out with binary solvents (A/B, 150 μL/min) composed of water−methanol−acetic acid (90:10:0.5 by volume) (A) and methanol−acetic acid (100:0.5, v/v) (B) on a Luna C18 column (3 μm, 50 mm × 2 mm; Phenomenex; Torrance, CA). Following electrospray ionization of the column effluent, ions m/z 150−750 were acquired.

Melting points were measured with a Mel-Temp manual apparatus (Electrothermal, Fisher Scientific) and were uncorrected.

γ-Radioactivity from ^{11}C and ^{18}F was measured using a calibrated dose calibrator (Atomlab 300, Biodex Medical Systems). Radioactivity measurements were corrected for physical decay. All radiochemistry was performed in lead-shielded hot cells for personnel protection from radiation.

Radioactive products were separated by HPLC on a Gemini or Gemini-NX C18 column (5 μm, 10 mm × 250 mm; Phenomenex) and eluted with 10 mM NH_4OH−MeCN or 100 mM $HCOONH_4$−MeCN at the stated composition and flow rate. Eluates were monitored for radioactivity (pin diode detector, Bioscan) and absorbance at 294 nm (System Gold 166 detector, Beckman).

The purity of each new nonradioactive compound was assessed by reverse phase HPLC under the conditions tabulated in Supporting Information. Each compound was shown to have a chemical purity of >98%. Radioactive compounds were analyzed with HPLC on a Gemini C18 column (5 μm, 4.6 mm × 150 mm) or Gemini-NX C18 column (5 μm, 4.6 mm × 250 mm; Phenomenex) and eluted with 10 mM NH_4OH−MeCN or 100 mM $HCOONH_4$−MeCN at the later stated composition and flow rate. Eluates were monitored for radioactivity (pin diode detector, Bioscan). Samples were injected alone and then co-injected with the reference nonradioactive compound to check for coelution. RCYs were calculated for labeled products isolated with HPLC.

Computation of cLogP and cLogD. cLogP and cLogD (at pH 7.4) values for ligands were computed with ACD software.

(1-Butylpiperidin-4-yl)methyl 8-amino-7-iodo-2,3-dihydrobenzo-[*b*][1,4]dioxine-5-carboxylate (1). *N*-Iodosuccinimide (498 mg, 2.21 mmol) was added portionwise to a solution of **4** (770 mg, 2.21 mmol) in acetic acid (5 mL) at 0 °C and stirred at rt for 2 h. The acetic acid was evaporated, and the residue was basified with $NaHCO_3$ solution and extracted twice with CH_2Cl_2. The combined organic layers were evaporated to dryness. Silica gel chromatography ($MeOH-CH_2Cl_2$, 1:20 v/v) of the residue gave **1** as a light yellow oil (410 mg, 40%). ^1H NMR ($CDCl_3$): δ 0.92 (3H, t, J = 7.2 Hz), 1.25−1.53 (6H, m), 1.74−1.79 (3H, m), 1.94 (2H, t, J = 11.2 Hz), 2.31−2.34 (2H, m), 2.96 (2H, d, J = 11.2 Hz), 4.10 (2H, d, J = 6.4 Hz), 4.30−4.38 (4H, m), 4.54 (2H, bs), 7.82 (1H, s). ^{13}C NMR ($CDCl_3$): δ 14.09, 20.92, 29.02, 29.17, 35.53, 53.44, 58.89, 63.97, 64.44, 68.86, 71.21, 110.80, 129.77, 133.29, 141.06, 144.75, 164.14.

(1-Butylpiperidin-4-yl)methyl 8-amino-7-cyano-2,3-dihydrobenzo-[*b*][1,4]dioxine-5-carboxylate (5).[40] A mixture of **1** (200 mg, 0.42 mmol), KCN (42 mg, 0.64 mmol), CuI (17 mg, 0.084 mmol), and 1,10-phenanthroline (30 mg, 0.17 mmol) in DMF (500 μL) was stirred at 110 °C in an oven-dried sealed tube under Ar for 42 h. Then the mixture was cooled to rt and filtered through Celite. The Celite pad was rinsed twice with CH_2Cl_2. The combined filtrates were evaporated to dryness and dissolved in MeOH. Separation by HPLC on an XTerra RP18 column (10 μm, 19 mm × 250 mm; Waters) eluted with MeOH−aqueous NH_4OH (0.025%) (9:1 v/v) at 10 mL/min) gave **5** as a pale yellow solid (t_R = 14 min; 30 mg, 19%). Mp: 108−110 °C. ^1H NMR ($CDCl_3$): δ 0.92 (3H, t, J = 7.2 Hz), 1.28−1.50 (6H, m), 1.81−1.84 (3H, m), 2.15 (2H, t, J = 11.4 Hz), 2.51 (2H, m), 3.16 (2H, d, J = 11.6 Hz), 4.12 (2H, d, J = 6.0 Hz), 4.34−4.44 (4H, m), 4.87 (2H, s), 7.67 (1H, s). ^{13}C NMR ($CDCl_3$): δ 14.09, 20.92, 29.06, 29.23, 35.53, 53.42, 58.89, 63.73, 64.85, 69.13, 88.00, 110.11, 116.81, 128.80, 129.90, 143.32, 147.47, 163.64. LC−MS m/z: $[M + H]^+$, 374.2. HRMS: calcd for $C_{20}H_{27}N_3O_4$ ($M^+ + H$), 374.2080; found, 374.2070.

(1-Butylpiperidin-4-yl)methyl 8-(methylamino)-2,3-dihydrobenzo-[*b*][1,4]dioxine-5-carboxylate (6).[41] A mixture of **4** (2.24 g, 6.44 mmol) and paraformaldehyde (580 mg) in ethanol (58 mL) was heated at 60 °C overnight, and then $NaBH_4$ (245 mg, 6.44 mmol) was added. The mixture was heated at 70 °C for 2 h and then evaporated to dryness. The residue was diluted with water and extracted thrice with CH_2Cl_2. The combined organic layers were dried on $MgSO_4$ and evaporated to dryness. Silica gel chromatography of the residue ($MeOH-CH_2Cl_2$, 1:20 v/v) gave **6** as a colorless oil (1.4 g, 60%). ^1H NMR ($CDCl_3$): δ 0.92 (3H, t, J = 7.2 Hz), 1.29−1.35 (2H, m), 1.52−1.53 (4H, m), 1.78−1.82 (3H, m), 1.99 (2H, t, J = 11.2 Hz), 2.39−2.35 (2H, m), 2.90 (2H, d, J = 5.2 Hz), 3.02 (2H, d, J = 11.2 Hz), 4.10 (2H, d, J = 6.0 Hz), 4.28−4.30 (2H, m), 4.34−4.36 (2H, m), 4.46−4.52 (1H, m), 6.18 (1H, d, J = 8.8 Hz), 7.51 (1H, d, J = 8.8 Hz). ^{13}C NMR ($CDCl_3$): δ 14.02, 20.85, 28.78, 28.88, 29.85, 35.47, 53.38, 58.75, 63.83, 64.50, 68.18, 100.99, 107.36, 125.60, 129.78, 143.15, 144.11, 165.40. LC−MS m/z: $[M + H]^+$, 363.3. HRMS: calcd for $C_{20}H_{30}N_2O_4$ ($M^+ + H$), 363.2284; found, 363.2281.

(1-Butylpiperidin-4-yl)methyl 8-amino-7-methyl-2,3-dihydrobenzo[*b*][1,4]dioxine-5-carboxylate (7).[42] **1** (210 mg, 0.44 mmol), *N*-methylpyrrolidone (560 μL), $Pd_2(dba)_3$ (14 mg, 0.015 mmol), and PPh_3 (28 mg, 0.11 mmol) were added to an oven-dried sealed tube. The mixture was heated at 50 °C for 10 min, and then CuI (6.0 mg, 0.031 mmol) was added. The mixture was stirred for another 10 min, and then Me_4Sn (91 μL, 0.65 mmol) was added. The mixture was heated at 70 °C for 48 h. The solvent was evaporated, and the residue was diluted with water and extracted thrice with CH_2Cl_2. The combined organic layers were dried over $MgSO_4$ and evaporated to dryness. Silica gel chromatography ($MeOH-CH_2Cl_2$, 1:20 v/v) of the residue gave **7** as a pale yellow oil (70 mg, 44%). ^1H NMR ($CDCl_3$): δ 0.92 (3H, t, J = 7.2 Hz), 1.25−1.58 (7H, m), 1.72−1.81 (3H, m), 1.78−1.82 (3H, m), 1.95 (2H, t, J = 11.2 Hz), 2.11 (3H, s), 2.31−2.35 (2H, m), 2.98 (2H, d, J = 11.6 Hz), 4.08 (2H, s), 4.10 (2H, d, J = 6.4 Hz), 4.30−4.36 (4H, m), 7.28 (1H, s). ^{13}C NMR ($CDCl_3$): δ 14.02, 16.48, 20.84, 28.69, 35.39, 53.34, 58.72, 63.87, 64.45, 68.27, 107.79, 113.41, 125.34, 130.17, 138.86, 143.34, 165.46. LC−MS: m/z $[M + H]^+$, 363.1. HRMS: calcd for $C_{20}H_{30}N_2O_4$ ($M^+ + H$), 363.2284; found, 363.2279.

(1-Butylpiperidin-4-yl)methyl 7-chloro-8-(methylamino)-2,3-dihydrobenzo[*b*][1,4]dioxine-5-carboxylate (8). *N*-Chlorosuccinimide (70 mg, 0.52 mmol) was added in portions to a stirred solution of **6** (190 mg, 0.52 mmol) in acetic acid (5 mL) at rt and left for 1 h. The acetic acid was evaporated, and the residue was basified with $NaHCO_3$ solution and extracted thrice with CH_2Cl_2. The combined organic layers were evaporated to dryness. Silica gel chromatography ($MeOH-CH_2Cl_2$, 1:20 v/v) of the residue gave **8** as a pale yellow oil (80 mg, 38%). ^1H NMR ($CDCl_3$): δ 0.92 (3H, t, J = 7.2 Hz), 1.29−1.35 (2H, m), 1.44−1.53 (4H, m), 1.75−1.80 (3H, m), 1.98 (2H, t, J = 11.2 Hz), 2.34−2.38 (2H, m), 3.00 (2H, d, J = 11.2 Hz), 3.12 (3H, d, J = 4.8 Hz), 4.10 (2H, d, J = 6.4 Hz), 4.28−4.36 (5H, m), 7.45 (1H, s). ^{13}C NMR ($CDCl_3$): δ 14.03, 20.84, 28.83, 28.97, 33.82, 35.42, 53.35, 58.76, 63.63, 64.26, 68.72, 109.54, 112.30, 124.79, 133.42, 140.14, 144.06, 164.27. LC−MS: m/z $[M + H]^+$, 397.6. HRMS: calcd for $C_{20}H_{29}ClN_2O_4$ ($M^+ + H$), 397.1894; found, 397.1886.

(1-Butylpiperidin-4-yl)methyl 7-bromo-8-(methylamino)-2,3-dihydrobenzo[*b*][1,4]dioxine-5-carboxylate (9). The procedure for the synthesis of **8** was used with a solution of **6** (140 mg, 0.39 mmol) in acetic acid (4 mL) and with *N*-bromosuccinimide (70 mg, 0.39 mmol) replacing *N*-chlorosuccinimide and gave **9** as a pale yellow oil (50 mg, 30%). ^1H NMR ($CDCl_3$): δ 0.92 (3H, t, J = 7.2 Hz), 1.29−1.52 (6H, m), 1.73−1.78 (3H, m), 1.92 (2H, dt, J = 2.4, 11.6 Hz), 2.29−2.33 (2H, m), 2.96 (2H, d, J = 11.2 Hz), 3.10 (3H, d, J = 5.2 Hz), 4.10 (2H, d, J = 6.4 Hz), 4.27−4.37 (5H, m), 7.62 (1H, s). ^{13}C NMR ($CDCl_3$): δ 14.09, 20.93, 29.11, 29.27, 34.11, 35.58, 53.48, 58.93, 63.60, 64.31, 68.96, 102.04, 110.64, 127.61, 133.67, 141.18, 144.76, 164.21. LC−MS: m/z $[M + H]^+$ 441.1. HRMS: calcd for $C_{20}H_{29}{}^{80}BrN_2O_4$ ($M^+ + H$), 441.1389; found, 441.1368.

(1-Butylpiperidin-4-yl)methyl 7-iodo-8-(methylamino)-2,3-dihydrobenzo[*b*][1,4]dioxine-5-carboxylate (10). The procedure for the synthesis of **8** was used with a solution of **6** (195 mg, 0.54 mmol) in acetic acid (4 mL) and with *N*-iodosuccinimide (122 mg, 0.54 mmol) replacing *N*-chlorosuccinimide to give **10** as a colorless oil (147 mg, 56%). ^1H NMR ($CDCl_3$): δ 0.92 (3H, t, J = 7.2 Hz), 1.19−1.54 (6H, m), 1.72−1.80 (3H, m), 1.96 (2H, t, J = 11.2 Hz), 2.33−2.37 (2H, m), 3.00 (2H, d, J = 11.2 Hz), 3.07 (3H, d, J = 3.2 Hz), 4.05 (1H, bs), 4.09−4.11 (3H, m), 4.28−4.38 (4H, m), 7.84 (1H, s). ^{13}C NMR ($CDCl_3$): δ 14.06, 20.89, 28.91, 29.06, 34.50, 35.47, 53.39, 58.83, 63.57, 64.35, 68.88, 112.25, 133.12, 133.80, 143.57, 145.67, 164.02. LC−MS: m/z $[M + H]^+$, 489.2. HRMS: calcd for $C_{20}H_{29}IN_2O_4$ ($M^+ + H$), 489.1250; found, 489.1258.

(1-Butylpiperidin-4-yl)methyl 7-cyano-8-(methylamino)-2,3-dihydrobenzo[*b*][1,4]dioxine-5-carboxylate (11).[40] A mixture of **10** (140 mg, 0.29 mmol), KCN (21 mg, 0.32 mmol), CuI (6.0 mg, 0.029 mmol), 1,10-phenanthroline (11 mg, 0.057 mmol) in DMF (300 μL) was stirred at 110 °C in an oven-dried sealed tube under Ar for 48 h. The mixture was cooled to rt and then filtered through Celite. The Celite pad was rinsed twice with CH_2Cl_2. The combined filtrates were evaporated to dryness and then dissolved in MeOH. Separation by HPLC on an XTerra RP18 column (10 μm, 19 mm × 250 mm; Waters) and elution with MeOH−aqueous NH_4OH (0.025%) (9:1 v/v) at 15 mL/min gave **11** (t_R = 10.31 min; 8 mg, 7%). ^1H NMR ($CDCl_3$): δ 0.92 (3H, t, J = 7.2 Hz), 1.25−1.52 (7H, m), 1.73−1.79 (3H, m), 1.90−1.96 (2H, t, J = 11.2 Hz), 2.30−2.34 (2H, m), 2.96 (2H, d, J = 11.2 Hz), 3.33 (3H, d, J = 5.2 Hz), 4.09 (2H, d, J = 6.0 Hz), 4.30−4.40 (4H, m), 5.04−5.06 (1H, m), 7.71 (1H, s). ^{13}C NMR ($CDCl_3$): δ 14.09, 20.92, 29.10, 29.26, 31.63, 35.53, 53.44, 58.91, 63.79, 64.61, 69.07, 85.21, 109.42, 119.52, 130.19, 131.81, 144.86, 145.98, 163.61. LC−MS: m/z $[M + H]^+$, 388.2. HRMS: calcd for $C_{21}H_{29}N_3O_4$ ($M^+ + H$), 388.2236; found, 388.2219.

Methyl 4-(Benzyloxycarbonylamino)-2-methoxybenzoate (12b).[43] Saturated $NaHCO_3$ solution (50 mL) and then CbzCl (1.70 mL, 12.1 mmol) were added to a solution of methyl 4-amino-2-methoxybenzoate (**12a**, 2.0 g, 11 mmol) in THF (50 mL). The mixture was stirred at rt for 4 h and then filtered through Celite. The filtrate was acidified to pH < 1 and extracted with EtOAc. After concentration of this solution, the residue was purified by recrystallization from EtOAc−hexane to give **12b** as a white solid (3.09 g, 89%). Mp: 132−134 °C. ^{1}H NMR ($CDCl_3$): δ 3.84 (3H, s), 3.87 (3H, s), 5.19 (2H, s), 6.77 (1H, dd, J = 1.6, 8.4 Hz), 6.66, 7.11 (1H, s), 7.35−7.39 (5H, m), 7.79 (1H, d, J = 8.4 Hz). ^{13}C NMR ($CDCl_3$): δ 51.84, 55.97, 67.30, 101.72, 109.43, 114.05, 128.32, 128.52, 128.68, 133.06, 135.67, 143.19, 152.96, 160.76, 166.07.

(1-Butylpiperidin-4-yl)methyl 4-Amino-2-methoxybenzoate (12).[44] A THF solution of *n*-BuLi (1.6 M, 612 μL, 0.98 mmol) was added dropwise to a solution of (4-butylpiperidin-1-yl)-methanol (190 mg, 1.11 mmol) in THF (3 mL) in an oven-dried flask under Ar at 0 °C. After the mixture was stirred for 10 min, a solution of **12b** (350 mg, 1.11 mmol) in THF (3 mL) was added dropwise. The mixture was stirrred for 2 h, poured into water, and extracted with CH_2Cl_2. The combined organic layers were dried on $MgSO_4$ and evaporated to dryness. Silica gel chromatography of the residue (MeOH−CH_2Cl_2, 1:15 v/v) gave a white solid (200 mg). MeOH (15 mL) was added to this solid (170 mg) plus Pd/C (10%, 25 mg). The mixture was degassed with H_2 for 30 min, stirred at rt overnight, filtered, and evaporated to dryness. Silica gel chromatography (MeOH−CH_2Cl_2, 1:20 v/v) of the residue gave **12** as a colorless oil (108 mg, 36%). ^{1}H NMR ($CDCl_3$): δ 0.91 (3H, t, J = 7.6 Hz), 1.28−1.53 (6H, m), 1.71−1.80 (3H, m), 1.95 (2H, t, J = 11.2 Hz), 2.31−2.35 (2H, m), 2.97 (2H, d, J = 11.6 Hz), 3.81 (3H, s), 4.09 (2H, d, J = 6.0 Hz), 4.22 (2H, br), 6.18−6.22 (2H, m), 7.72 (1H, d, J = 8.4 Hz). ^{13}C NMR ($CDCl_3$): δ 14.06, 20.88, 28.94, 29.07, 35.53, 53.45, 55.66, 58.84, 68.33, 97.58, 106.23, 108.78, 134.06, 152.29, 161.87, 165.63. LC−MS: m/z $[M + H]^+$, 321.1. HRMS: calcd for $C_{18}H_{28}N_2O_3$ ($M^+ + H$), 321.2178; found, 321.2181.

Methyl 4-Amino-3-methoxybenzoate (13a). Pd/C (10%, 1.8 g) and HCOOK (7.4 g, 88 mmol) were added to a solution of methyl 3-methoxy-4-nitrobenzoate (2.0 g, 9.5 mmol) in MeOH (50 mL). The mixture was refluxed at 80 °C for 1 h. The suspension was cooled and filtered through Celite. The filtrate was evaporated to dryness. Silica gel chromatography (30% EtOAc in hexane) of the residue gave **13a** as a white solid (1.72 g, 100%). Mp: 127−129 °C. Lit. mp: 127−128 °C.[45] ^{1}H NMR ($CDCl_3$): δ 3.86 (3H, s), 3.90 (3H, s), 4.22 (2H, br), 4.45 (1H, s), 6.66 (1H, d, J = 8.4 Hz), 7.55 (1H, d, J = 8.4 Hz). ^{13}C NMR ($CDCl_3$): δ 51.69, 55.58, 111.15, 113.09, 119.41, 124.09, 141.21, 146.10, 167.35.

Methyl 4-(Benzyloxycarbonylamino)-3-methoxybenzoate (13b). As described for **12b** from **12a**, **13b** was obtained from **13a** as a white solid (58%). Mp: 95−96 °C. ^{1}H NMR ($CDCl_3$): δ 3.89 (6H, 2s), 5.22 (2H, s), 7.34−7.52 (6H, m), 7.69 (1H, dd, J = 1.6, 8.4 Hz), 8.20 (1H, d, J = 8.4 Hz). ^{13}C NMR ($CDCl_3$): δ 52.05, 55.90, 67.27, 110.73, 116.92, 123.48, 124.25, 128.43, 128.49, 128.68, 132.06, 135.84, 146.96, 152.93, 166.81.

(1-Butylpiperidin-4-yl)methyl 4-Amino-3-methoxybenzoate (13). As described for **12** from **12b**, compound **13** was obtained from **13b** as a white solid in 13% yield. Mp: 162−164 °C. ^{1}H NMR ($CDCl_3$): δ 0.94 (3H, t, J = 7.2 Hz), 1.31−1.39 (2H, m), 1.72−1.80 (2H, m), 1.95−1.99 (5H, br), 2.55 (2H, br), 2.75−2.79 (2H, m), 3.37 (2H, d, J = 11.6 Hz), 3.90 (3H, s), 4.18 (2H, d, J = 4.0 Hz), 4.38 (2H, br), 6.69 (1H, d, J = 8.0 Hz), 7.43 (1H, d, J = 2 Hz), 7.52−7.53 (1H, dd, J = 1.6, 8 Hz). ^{13}C NMR: δ ($CDCl_3$) 14.09, 20.94, 29.10, 29.25, 35.71, 53.50, 55.63, 58.94, 68.84, 111.21, 113.05, 119.75, 124.02, 141.09, 146.16, 166.86. LC−MS: m/z $[M + H]^+$, 321.3. HRMS: calcd for $C_{18}H_{28}N_2O_3$ ($M^+ + H$), 321.2178; found, 321.2190.

4-Amino-*N*-((1-butylpiperidin-4-yl)methyl)-3-methoxybenzamide (14).[38] CDI (485 mg, 2.99 mmol) was added in portions to a suspension of 4-amino-3-methoxybenzoic acid (500 mg, 2.99 mmol) in MeCN (30 mL) in an oven-dried flask and then stirred for 2 h at rt. (1-Butylpiperidin-4-yl)methanamine (510 mg, 2.99 mmol) in MeCN (10 mL) was added dropwise. The above solution was stirred overnight and evaporated to dryness. Silica gel chromatography (from 9% to 33% MeOH in CH_2Cl_2) of the residue gave an oil which was then dissolved in MeCN and filtered through an Iso-Disc filter (PTFE 25-4, 25 mm × 0.45 μm). The filtrate was dried to give **14** as a light brown foamlike oil (660 mg, 69%). ^{1}H NMR ($CDCl_3$): δ 0.91 (3H, t, J = 7.2 Hz), 1.26−1.38 (4H, m), 1.42−1.48 (2H, m), 1.57−1.64 (1H, m), 1.88 (2H, dt, J = 2.0, 11.6 Hz), 2.27−2.31 (2H, m), 2.92 (2H, d, J = 11.6 Hz), 3.30 (2H, t, J = 6.4 Hz), 3.86 (3H, s), 4.14 (2H, bs), 6.34 (1H, t, J = 5.6 Hz), 6.63 (1H, d, J = 8.0 Hz), 7.14 (1H, dd, J = 2.0, 8.4 Hz), 7.37 (1H, d, J = 1.6 Hz). ^{13}C NMR ($CDCl_3$): δ 12.58, 19.41, 27.72, 28.58, 34.79, 43.98, 52.06, 54.08, 57.36, 108.34, 111.67, 118.05, 122.76, 138.15, 145.21, 166.04. LC−MS: m/z $[M + H]^+$, 320.2. HRMS: calcd for $C_{18}H_{30}N_3O_2$ ($M^+ + H$), 320.2338; found, 320.2328.

(1-Propylpiperidin-4-yl)methyl 4-Amino-3-methoxybenzoate (15). CDI (485 mg, 2.99 mmol) was added in portions to a suspension of 4-amino-3-methoxybenzoic acid (500 mg, 2.99 mmol) in MeCN (15 mL) in an oven-dried flask and stirred for 30 min at rt. Solvent was then evaporated and the residue dissolved in anhydrous THF (7 mL). In another oven-dried flask, (1-propylpiperidin-4-yl)methanol (319 mg, 2.03 mmol) was dissolved in anhydrous THF (7 mL) and cooled (ice bath). A THF solution of *n*-BuLi (1.6M, 1.27 mL, 2.03 mmol) was added dropwise to this solution and stirred for 10 min. The solution of CDI-activated acid was then added, stirred overnight, and evaporated to dryness. The residue was diluted with water and extracted thrice with CH_2Cl_2. The combined organic layers were dried on $MgSO_4$ and evaporated to dryess. Silica gel chromatography (MeOH−CH_2Cl_2, 1:30 v/v) of the residue gave **15** as a yellow oil (312 mg, 50%). ^{1}H NMR ($CDCl_3$): δ 0.89 (3H, t, J = 7.2 Hz), 1.37−1.56 (4H, m), 1.72−1.78 (2H, m), 1.94 (2H, dt, J = 2.0, 12.0 Hz), 2.25−2.29 (2H, m), 2.94 (2H, d, J = 11.6 Hz), 3.86 (3H, s), 4.13 (2H, d, J = 6.0 Hz), 4.36 (2H, bs), 6.63 (1H, d, J = 8.4 Hz), 7.44 (1H, d, J = 2.0 Hz), 7.54 (1H, dd, J = 1.6, 8.0 Hz). ^{13}C NMR ($CDCl_3$): δ 12.05, 20.15, 29.06, 35.67, 53.40, 55.50, 61.09, 68.76, 111.08, 112.90, 119.35, 124.03, 141.31, 146.03, 166.83. LC−MS: m/z $[M + H]^+$, 307.2. HRMS: calcd for $C_{17}H_{26}N_2O_3$ ($M^+ + H$), 307.2022; found, 307.2022.

(1-Pentylpiperidin-4-yl)methyl 4-Amino-3-methoxybenzoate (16). As described for **15**, compound **16** was obtained from 4-amino-3-methoxybenzoic acid and 1-pentylpiperidin-4-yl)-methanol as a pale yellow oil (200 mg, 30%). ^{1}H NMR ($CDCl_3$): δ 0.89 (3H, t, J = 7.2 Hz), 1.24−1.52 (8H, m), 1.77−1.79 (3H, m), 1.89−1.95 (2H, dt, J = 2.4, 12 Hz), 1.95−2.32 (2H, m), 2.96 (2H, d, J = 11.6 Hz), 3.89 (3H, s), 4.13 (2H, d, J = 6.4 Hz), 4.26 (2H, bs), 6.65 (1H, d, J = 8 Hz), 7.45 (1H, d, J = 1.6 Hz), 7.55 (1H, dd, J = 2, 8.4 Hz). ^{13}C NMR ($CDCl_3$): δ 14.06, 22.64, 26.78, 29.10, 29.94, 35.71, 53.49, 55.59, 59.23, 68.82, 111.16, 113.00, 119.61, 124.03, 141.17, 146.12, 166.86. LC−MS: m/z $[M + H]^+$, 335.2. HRMS: calcd for $C_{19}H_{30}N_2O_3$ ($M^+ + H$), 335.2335; found, 335.2325.

3-Bromo-5-fluoro-2-hydroxybenzoic Acid (17a). 5-Fluoro-2-hydroxybenzoic acid (20 g, 0.13 mol) and *N*-bromosuccinimide (23 g, 0.13 mol) were added to acetic acid (200 mL). The mixture was heated at 80 °C for 24 h. After evaporation of all acetic acid, the residue was recrystallized from EtOAc and hexane to give **17a** (27 g, 90%). Mp 233−235 °C. Lit. mp 233 °C.[46] ^{1}H NMR (MeOD): δ 7.48−7.50 (2H, br). ^{13}C NMR (MeOD): δ 111.91 (d, J = 10.0 Hz), 114.92 (d, J = 7.0 Hz), 116.22 (d, J = 24.0 Hz), 127.13 (d, J = 26.0 Hz), 155.70 (d, J = 239 Hz), 156.56, 172.24.

5-Fluoro-2,3-dihydroxybenzoic Acid (17b).[47] NaOH solution (2.5 M, 300 mL) was stirred under an aspirator for 2 h. Then $CuSO_4$ (250 mg, 1.57 mmol) was added and the solution further stirred for 1 h. To the filtrate of this solution was added **17a** (22 g, 0.094 mol), and the mixture was refluxed overnight. The reaction mixture was cooled, acidified with 37% HCl to pH < 2, and then evaporated to dryness. The residue was dissoved in

MeOH and filtered through Celite. The combined filtrates were evaporated to dryness. Silica gel chromatography (30% EtOAc in hexane with 1% HOAc) of the residue gave **17b** as a white solid (12 g, 75%). Mp 184−186 °C. ^{1}H NMR (MeOD): δ 6.58 (1H, dd, J = 3.2, 9.6 Hz), 7.01 (1H, dd, J = 3.0, 9.0 Hz). ^{13}C NMR (MeOD): δ 105.80 (d, J = 24.0 Hz), 109.44 (d, J = 26.0 Hz), 113.48 (d, J = 9.0 Hz), 148.36, 148.50 (d, J = 9.0 Hz), 156.24 (d, J = 234 Hz), 173.03. ^{19}F NMR ($CDCl_3$): δ −125.39. HRMS: calcd for $C_7H_5FO_4$ (M^+ + H), 172.017 19; found, 172.017 17.

Methyl 7-Fluoro-2,3-dihydrobenzo[*b*][1,4]dioxine-5-carboxylate (17c).[48] $TMSCHN_2$ (2.0 M, 45 mL, 90 mmol) was added in portions to a solution of **17b** (6.0 g, 35 mmol) in MeOH (150 mL) and Et_2O (150 mL). After 20 min the solvent was evaporated off. Silica gel chromatography (15% EtOAc in hexane) of the residue gave the methyl ester (5.6 g, 93%). A mixture of this ester (3.9 g, 20 mmol) and Cs_2CO_3 (16.2 g, 49.8 mmol) in DMF (40 mL) was stirred at rt for 0.5 h. Then 1,2-dibromoethane (5.07 g, 27.0 mmol) was added and the mixture stirred at 80 °C for 16 h. The mixture was cooled to rt and filtered through Celite, which was then rinsed twice with DMF. The combined DMF rinses were evaporated to dryness under high vacuum to give a dark red residue, which after silica gel chromatography (20% EtOAc in hexane with 1% HOAc) gave **17c** as a white solid (3.37 g, 77%). Mp 108−110 °C. ^{1}H NMR (MeOD): δ 3.89 (3H, s), 4.28−4.34 (4H, m), 6.77 (1H, dd, J = 3.2, 8.8 Hz), 7.11 (1H, dd, J = 3.2, 8.8 Hz). ^{13}C NMR (MeOD): δ 52.27, 64.10, 64.30, 108.71 (d, J = 26.0 Hz), 109.73 (d, J = 24.0 Hz), 120.07 (d, J = 9.0 Hz), 140.65, 144.69 (d, J = 12.0 Hz), 155.69 (d, J = 238 Hz), 164.99. ^{19}F NMR ($CDCl_3$): δ −121.14. HRMS: calcd for $C_{10}H_9FO_4$ (M^+ + H), 213.0563; found, 213.0561.

Methyl 7-Fluoro-8-nitro-2,3-dihydrobenzo[*b*][1,4]dioxine-5-carboxylate (17d). 17c (3.4 g, 15.9 mol) was added in portions to a flask cooled between −50 and −60 °C and containing HNO_3 (90%, 30 mL). After 16 min, the reaction mixture was warmed gradually and water was added. The precipitate was filtered off and washed with water to give **17d** as a yellow solid (3.58 g, 88%). **17d** was used in the next step without further purification. Mp 133−135 °C. ^{1}H NMR ($CDCl_3$): δ 3.92 (3H, s), 4.33−4.40 (4H, m), 6.83 (1H, d, J = 11.2 Hz). ^{13}C NMR ($CDCl_3$): δ 53.59, 64.22, 64.80, 107.12 (d, J = 25.0 Hz), 119.75 (d, J = 1.0 Hz), 137.57 (d, J = 3.0 Hz), 148.50 (d, J = 12.0 Hz), 150.10 (d, J = 257 Hz), 162.83 (d, J = 3.0 Hz). ^{19}F NMR ($CDCl_3$): δ −124.76. HRMS: calcd for $C_{10}H_8FNO_6$ (M^+ + Na), 280.0233; found, 280.0225.

8-Amino-7-fluoro-2,3-dihydrobenzo[*b*][1,4]dioxine-5-carboxylic Acid (17e). Pd/C (10%, 340 mg) and potassium formate (1.4 g, 17 mmol) were added to a solution of **17d** (500 mg, 1.94 mmol) in MeOH (10 mL). The mixture was refluxed at 80 °C for 2 h and then cooled and filtered through Celite. Then 37% HCl was added to the filtrate until no CO_2 was released. The white solid was filtered off and the filtrate evaporated to dryness. Silica gel chromatography (MeOH−CH_2Cl_2, 1:30 v/v) of the residue gave **17e** as light brown solid (350 mg, 85%). Mp 174−176 °C. ^{1}H NMR (MeOD): δ 4.08−4.10 (2H, m), 4.18−4.21 (2H, m), 6.67 (1H, d, J = 11.6 Hz). ^{13}C NMR (MeOD): δ 65.06, 66.29, 106.06 (d, J = 4.0 Hz), 109.21 (d, J = 23.0 Hz), 134.30 (d, J = 11.0 Hz), 134.44, 140.54 (d, J = 3.0 Hz), 147.01 (d, J = 232 Hz), 169.25. ^{19}F NMR (MeOD): δ −143.14. HRMS: calcd for $C_9H_8FNO_4$ (M^+ + H), 214.0516; found, 214.0510.

(1-Butylpiperidin-4-yl)methyl 8-Amino-7-fluoro-2,3-dihydrobenzo[*b*][1,4]dioxine-5-carboxylate (17). Saturated $NaHCO_3$ solution (5 mL) and THF (20 mL) followed by CbzCl (662 mg, 3.87 mmol) were added to a flask containing **17e** (750 mg, 3.52 mmol). After the mixture was stirred overnight, the THF was evaporated off. The solution was diluted with water, acidified with 37% HCl to pH < 1, and extracted thrice with EtOAc. The combined organic layers were dried on $MgSO_4$ and evaporated to dryness. Silica gel chromatography (30% EtOAc in hexane) of the residue gave Cbz-protected **17e** as a white solid (420 mg, 34%).

Cbz-protected **17e** (420 mg, 1.21 mmol), CDI (196 mg, 1.21 mmol), and MeCN (30 mL) were added to an oven-dried flask under Ar. The mixture was stirred for 2 h and evaporated to dryness. The residue was dissolved in THF (10 mL). In another oven-dried flask, a solution of (4-butylpiperidin-1-yl)methanol (207 mg, 1.21 mmol) in THF (10 mL) was added dropwise to a THF solution of *n*-BuLi (1.6 M, 760 μL, 1.21 mmol) under Ar at 0 °C and stirred at this temperature for 10 min. Then the activated acid in THF was added dropwise to the prepared lithium alkoxide solution. The mixture was warmed to rt and stirred overnight. The reaction mixture was evaporated to remove THF, diluted with water, and extracted with CH_2Cl_2. The residue was purified by silica gel chromatography (MeOH−CH_2Cl_2, 1:15 v/v) to give an oil. Pd/C (10%, 25 mg) was then added to a solution of the oil in MeOH (15 mL). The suspension was degassed for 30 min with H_2 and then stirred at rt overnight under H_2. The mixture was filtered through Celite and evaporated to dryness. Silica gel chromatography (MeOH−CH_2Cl_2, 1:15 v/v) of the residue gave **17** as a yellow solid (260 mg, 59%). Mp 160−162 °C. ^{1}H NMR ($CDCl_3$): δ 0.95 (3H, t, J = 7.4 Hz), 1.33−1.39 (2H, m), 1.70−1.95 (7H, m), 2.39−2.42 (2H, m), 2.67−2.71 (2H, m), 3.31 (2H, d, J = 11.6 Hz), 4.19−4.27 (6H, m), 4.88 (2H, s), 6.71 (1H, d, J = 11.6 Hz). ^{13}C NMR ($CDCl_3$): δ 13.74, 20.46, 26.97, 34.35, 52.56, 57.84, 63.84, 64.56, 68.14, 105.60 (d, J = 4.0 Hz), 108.19 (d, J = 23.0 Hz), 132.20 (d, J = 15.0 Hz), 133.43 (d, J = 11.0 Hz), 139.81 (d, J = 3.0 Hz), 145.24 (d, J = 232 Hz), 166.72 (d, J = 4 Hz). ^{19}F NMR ($CDCl_3$): δ −141.99. LC−MS: m/z [M + H]$^+$, 367.1. HRMS: calcd for $C_{19}H_{27}FN_2O_4$ (M^+ + H), 367.2033; found, 367.2033.

Methyl 4-Amino-3-(fluoromethoxy)benzoate (18a).[49] Chlorofluoromethane was bubbled into a tube (30 mL) containing anhydrous DMF (10 mL), methyl 4-amino-3-hydroxybenzoate (500 mg, 2.99 mmol), and Cs_2CO_3 (1.95 g, 5.98 mmol) for 14 min at −70 °C. The tube was then sealed and slowly warmed to rt. The mixture was stirred for 5 days and then filtered through Celite which was then rinsed thrice with EtOAc. The combined rinses were evaporated to dryness. Silica gel chromatography (20% EtOAc in hexane) of the residue gave **18a** as a pale yellow solid (368 mg, 62%). Mp: 69−70 °C. ^{1}H NMR ($CDCl_3$): δ 3.84 (3H, s), 4.46 (2H, br), 5.71 (2H, d, J = 54.4 Hz), 6.69 (1H, d, J = 8 Hz), 7.63 (1H, dd, J = 8.4, 1.6 Hz), 7.68 (1H, s). ^{13}C NMR ($CDCl_3$): δ 51.73, 100.22, 102.40, 114.25, 116.72, 119.23, 126.79, 142.44, 142.92, 142.95, 166.90. ^{19}F NMR ($CDCl_3$): δ −147.56. LC−MS: m/z [M + H]$^+$ 200.1. HRMS: calcd for $C_9H_{11}FNO_3$ (M^+ + H), 200.0723; found, 200.0723.

(1-Butylpiperidin-4-yl)methyl 4-Amino-3-(fluoromethoxy)-benzoate (18). *n*-BuLi (1.6 M, 1.6 mmol, 1 mL) was added dropwise to a solution of (4-butylpiperidin-1-yl)methanol (280 mg, 1.64 mmol) in THF (2 mL) contained in an oven-dried flask under Ar at 0 °C. The solution was stirred for 10 min, and **18a** (160 mg, 0.80 mmol) in THF (2 mL) was added dropwise. The mixture was stirred overnight, poured into water, and extracted with CH_2Cl_2. The combined organic layers were dried on $MgSO_4$ and evaporated to dryness. Silica gel chromatography (MeOH−CH_2Cl_2, 1:20 v/v) of the residue gave **18** as a pale yellow oil (160 mg, 59%). ^{1}H NMR ($CDCl_3$): δ 0.92 (3H, t, J = 7.6 Hz), 1.43−1.53 (6H, m), 1.75−1.80 (3H, m), 1.91−1.98 (2H, t, J = 12.0 Hz), 2.33 (2H, t, J = 8 Hz), 2.98 (2H, d, J = 11.6 Hz), 4.14 (2H, d, J = 6.0 Hz), 4.28 (2H, br), 5.76 (2H, d, J = 54.4 Hz), 6.71 (1H, d, J = 8.0 Hz), 7.67 (1H, dd, J = 2.0, 8.4 Hz), 7.70 (1H, s). ^{13}C NMR ($CDCl_3$): δ 12.81, 19.65, 27.73, 27.89, 34.37, 52.18, 57.63, 67.66, 100.17 (d, J = 219 Hz), 113.04, 115.81, 118.78, 125.58, 140.86, 141.85 (d, J = 2.0 Hz), 165.00. ^{19}F NMR ($CDCl_3$): δ −147.50. LC−MS m/z [M + H]$^+$, 214.1. HRMS: calcd for $C_{18}H_{28}FN_2O_3$ (M^+ + H), 339.2084; found, 339.2088.

Methyl 4-Amino-3-(2-fluoroethoxy)benzoate (19a). 1-Chloro-2-fluoroethane (760 mg, 8.97 mmol) was added to a tube (15 mL) containing anhydrous DMF (5 mL), methyl 4-amino-3-hydroxybenzoate (500 mg, 2.99 mmol), and Cs_2CO_3 (1.95 g, 5.98 mmol).

The tube was sealed, and the mixture was stirred for 88 h. The mixture was then filtered through Celite which was rinsed thrice with EtOAc. The combined filtrate and rinses were evaporated to dryness. Silica gel chromatography (20% EtOAc in hexane) of the residue gave **19a** as a white solid (270 mg, 42%). Mp 86−87 °C. ^{1}H NMR ($CDCl_3$): δ 3.84 (3H, s), 4.18−4.27 (2H, dt, J = 28.4, 4 Hz), 4.39 (2H, br), 4.65−4.79 (2H, dt, J = 47.2, 3.7 Hz), 6.66 (1H, d, J = 8 Hz), 7.43 (1H, d, J = 1.6 Hz), 7.55 (1H, dd, J = 8.4, 1.6 Hz). ^{19}F NMR ($CDCl_3$): δ −147.56. LC−MS: m/z [M + H]$^+$, 214.1. HRMS: calcd for $C_{10}H_{13}FNO_3$ (M^+ + H), 214.0879; found, 214.0876.

(1-Butylpiperidin-4-yl)methyl 4-Amino-3-(2-fluoroethoxy)-benzoate (19). As described for **18** from **18a**, compound **19** was obtained from **19a** as a white solid in 48% yield. Mp 72−74 °C. ^{1}H NMR ($CDCl_3$): δ 0.92 (3H, t, J = 7.6 Hz), 1.27−1.53 (6H, m), 1.74−1.79 (3H, m), 1.94 (2H, t, J = 12.0 Hz), 2.33 (2H, t, J = 7.6 Hz), 2.97 (2H, d, J = 11.2 Hz), 4.13 (2H, d, J = 6 Hz), 4.24−4.33 (2H, dt, J = 28.4, 4.0 Hz), 4.36 (2H, br), 4.69−4.83 (2H, dt, J = 47.2, 4.0 Hz), 6.67 (1H, d, J = 8.0 Hz), 7.45 (1H, d, J = 2.0 Hz), 7.57 (1H, dd, J = 1.6, 8.4 Hz). ^{13}C NMR ($CDCl_3$): δ 14.07, 20.89, 29.03, 29.17, 35.65, 53.45, 58.88, 67.92 (d, J = 20.0 Hz), 68.82, 81.77 (d, J = 170 Hz), 112.94, 113.39, 119.45, 124.79, 141.70, 144.70, 166.67. ^{19}F NMR ($CDCl_3$): δ −147.49. LC−MS m/z [M + H]$^+$, 353.2. HRMS: calcd for $C_{19}H_{30}FN_2O_3$ (M^+ + H), 353.2240; found, 353.2238.

(1-Butylpiperidin-4-yl)methyl 4-Amino-3-hydroxybenzoate (20).[50,51] $AlCl_3$ (311 mg, 2.34 mmol) and NaI (351 mg, 2.34 mmol) were added to a solution of **13** (500 mg, 1.56 mmol) in MeCN (10 mL). The mixture was refluxed overnight and extracted thrice with EtOAc. The combined organic layers were dried on $MgSO_4$ and evaporated to dryness. Silica gel chromatography ($MeOH/CH_2Cl_2$, 1:20 v/v) of the residue gave **20** as an orange-brown solid (200 mg, 42%). Mp: 210−212 °C. ^{1}H NMR ($CDCl_3$): δ 0.98 (3H, t, J = 7.6 Hz), 1.31−1.43 (2H, m), 1.51−1.63 (4H, m), 1.87−1.93 (3H, m), 2.28 (2H, t, J = 11.2 Hz), 2.54−2.58 (2H, m), 3.17 (2H, d, J = 12.0 Hz), 4.14 (2H, d, J = 5.6 Hz), 6.68 (1H, d, J = 8.0 Hz), 7.34 (1H, d, J = 2.0 Hz), 7.38−7.41 (1H, dd, J = 1.6, 8.0 Hz); ^{13}C NMR ($CDCl_3$): δ 13.96, 21.05, 27.46, 34.81, 53.45, 58.09, 68.05, 114.47, 116.05, 119.06, 124.34, 143.61, 144.84, 168.55. LC−MS: m/z [M + H]$^+$, 307.2. HRMS: calcd for $C_{17}H_{26}N_2O_3$ (M^+ + H), 307.2022; found, 307.2025.

(1-Butylpiperidin-4-yl)methyl 8-Amino-7-nitro-2,3-dihydrobenzo-[*b*][1,4]dioxine-5-carboxylate (21). A mixture of **21a**[52] (200 mg, 0.79 mmol), $(Boc)_2O$ (515 mg, 2.36 mmol), and DMAP (44 mg, 0.039 mmol) in CH_2Cl_2 (15 mL) was refluxed at 50 °C for 1 h, then quenched with water and extracted thrice with CH_2Cl_2. The combined organic layers were dried on $MgSO_4$ and then condensed to a crude product, which after silica gel chromatography (30% EtOAc in hexane) gave di-Boc-protected **21a** as a yellow solid (**21b**, 343 mg, 95%). ^{1}H NMR ($CDCl_3$): δ 1.41 (18H, s), 3.94 (3H, s), 4.37−4.49 (4H, m), 8.30 (1H, s). ^{13}C NMR ($CDCl_3$): δ 27.78, 52.63, 63.77, 64.80, 83.66, 118.16, 120.06, 126.55, 138.19, 140.71, 148.53, 149.49, 163.61.

A THF solution of *n*-BuLi (1.6 M, 0.95 mL, 1.52 mmol) was added dropwise to a solution of (4-butylpiperidin-1-yl)methanol (260 mg, 1.52 mmol) in THF (2 mL) in an oven-dried flask, under Ar at 0 °C. After this solution was stirred for 10 min, a solution of **21b** (343 mg, 0.76 mmol) in THF (4 mL) was added dropwise. The mixture was stirred overnight, poured into water, and extracted with CH_2Cl_2. The combined organic layers were dried on $MgSO_4$ and evaporated to dryness. Silica gel chromatography ($MeOH/CH_2Cl_2$, 1:15 v/v) of the residue gave mono-Boc-protected **21** as a white solid (**21c**, 186 mg, 50%). ^{1}H NMR ($CDCl_3$): δ 0.92 (3H, t, J = 7.2 Hz), 1.29−1.49 (15H, m), 1.78 (3H, t, J = 12.4 Hz), 1.93 (2H, t, J = 10.0 Hz), 2.31 (2H, t, J = 7.6 Hz), 2.96 (2H, d, J = 11.6 Hz), 4.16 (2H, d, J = 6.4 Hz), 4.39−4.48 (4H, m), 7.20 (1H, br), 8.15 (1H, s). ^{13}C NMR ($CDCl_3$) δ 13.06, 19.88, 27.05, 28.01, 28.24, 34.43, 52.34, 57.85, 62.79, 63.70, 68.82, 81.43, 119.75, 124.57, 134.69, 135.44, 146.92, 150.51, 162.21.

21c (100 mg, 0.20 mmol) was stirred overnight in a dioxane solution of HCl (4 M). The mixture was evaporated to dryness, diluted with water, and then neutralized to pH 7 with aqueous NH_4OH (1 M). The solution was extracted thrice with CH_2Cl_2. The organic layers were combined and dried on $MgSO_4$. Evaporation of solvent gave **21** as a yellow solid (71 mg, 89%). Mp 108−110 °C (n = 2). ^{1}H NMR ($CDCl_3$): δ 0.92 (3H, t, J = 7.2 Hz), 1.29−1.51 (7H, m), 1.79 (3H, d, J = 8.4 Hz), 1.92 (2H, t, J = 11.6 Hz), 2.33 (2H, t, J = 7.6 Hz), 2.98 (2H, d, J = 11.6 Hz), 4.14 (2H, d, J = 6.4 Hz), 4.38−4.46 (4H, m), 8.45 (1H, s). ^{13}C NMR ($CDCl_3$): δ 14.06, 20.89, 28.99, 29.16, 35.47, 53.38, 58.85, 63.82, 64.84, 69.28, 108.62, 123.12, 125.47, 130.17, 139.42, 147.16, 163.70. LC−MS: m/z [M + H]$^+$, 394.2. HRMS: calcd for $C_{19}H_{28}N_3O_6$ (M^+ + H), 394.1978; found, 394.1972.

Pharmacological Assay and Screen. Some ligands (**1**, **5**, **8**, **13**, **15**, **17**−**19**) were evaluated at Caliper Life Sciences (Hanover, MD) for binding to 5-HT_4 receptors in guinea pig striatal membranes at 37 °C with [^{3}H]GR 113808 as reference radioligand.[53]

All new ligands (**2**, **3**, and **5**−**21**) plus the already known ligands **1** and **4** were submitted to the National Institute of Mental Health Psychoactive Drug Screening Program (NIMH-PDSP) for assessment of binding affinity to human recombinant 5-HT_4 receptors (reference radioligand, [^{3}H]GR 113808) and a wide range of other receptors and binding sites (5-$HT_{1A-E,2A-C,3,5A,6,7}$, $\alpha_{1A,B,D,2A-C}$, BZP (rat brain site), β_{1-3}, $\sigma_{1,2}$, D_{1-5}, DAT, DOR, $GABA_A$, H_{1-4}, KOR, M_{1-5}, MOR, NET, and SERT). Selected ligands (**1**, **4**, **6**−**13**, and **17**) were also assessed for agonist/partial agonist activity in a GloSensor L9 assay for cAMP response and for antagonist activity. Detailed assay protocols are available at the NIMH-PDSP Web site (http://pdsp.cwru.edu).

Radiochemistry. Production of NCA [^{11}C]Carbon Dioxide. No-carrier-added (NCA) [^{11}C]carbon dioxide was produced with a PEtrace cyclotron (GE; Milwaukee, WI) according to the $^{14}N(p,\alpha)^{11}C$ reaction[20] by irradiation of nitrogen gas (300 psi) containing 1% oxygen with a proton beam (16.5 MeV, 45 μA) for either 20 or 40 min. A 40 min irradiation produced about 2.0 Ci of [^{11}C]carbon dioxide.

Production of NCA [^{11}C]Hydrogen Cyanide.[20] A PETrace radiotracer production system was used to produce this labeling agent as follows. Cyclotron-produced NCA [^{11}C]carbon dioxide was trapped on molecular sieves (13 Å, 80−100 mesh, 0.55 g) at 40 °C, while residual [^{13}N]nitrogen was directed to waste. The [^{11}C]carbon dioxide was then released with a stream of nitrogen (250 mL/min), mixed with a stream of hydrogen (30 mL/min), and passed through a heated (400 °C) glass tube (10 mm × 200 mm) containing nickel catalyst (Ni-3266 Engelhardt). The effluent containing the generated [^{11}C]methane was passed through an OXY-TRAP (part no. 4001R, Alltech), mixed with anhydrous ammonia (research grade, 20 mL/min), and then passed over a wad of platinum wire (d 0.127 mm, 2.6 g) at 920 °C. The generated [^{11}C]hydrogen cyanide was delivered to a hot cell for subsequent radiochemistry in a mixture of hydrogen, ammonia, and nitrogen carrier gas at 300 mL/min.

Production of NCA [^{11}C]Methyl Iodide. NCA [^{11}C]methyl iodide was produced from NCA [^{11}C]carbon dioxide (~2.0 Ci) via reduction to [^{11}C]methane and then vapor phase iodination,[18] either in a TRACERlab FX C Pro module (GE; Milwaukee, WI) for the synthesis of [^{11}C]**8** or a MeI MicroLab apparatus (GE; Milwaukee, WI) for the synthesis of [^{11}C]**13**.

Production of NCA [^{11}C]Methyl Triflate. A quartz column (2.6 mm i.d., 26 cm length) was packed around its center with a 6 cm length of AgOTf/Graphac (50:50 w/w) held in place at each end with glass wool. NCA [^{11}C]methyl triflate (~350 mCi) was produced by passing [^{11}C]methyl iodide in helium gas (17 mL/min) into the heated (180 °C) column.[19]

Production of NCA [^{18}F]Fluoromethyl-d_2 Bromide.[54] Cyclotron-produced [^{18}F]fluoride ion (~150 mCi) in [^{18}O]water was delivered into a glass vial containing K 2.2.2 (5.0 mg, 13.3 μmol) and potassium carbonate (0.50 mg, 3.6 μmol) in MeCN−H_2O

(0.1 mL, 9:1 v/v). This solution was transferred to a modified version of a TRACERlab FX_{F-N} module and diluted with MeCN (1 mL). The mixture was evaporated to dryness at 90 °C under reduced pressure with a nitrogen flow. MeCN (2 mL) was again added and then evaporated to dryness. The vessel was sealed, and then CD_2Br_2 (100 μL) in MeCN (1.0 mL) was added to the dry [^{18}F]fluoride ion−K 2.2.2−K^+ complex which was then heated at 95 °C for 15 min. The reaction vessel was then cooled to 35 °C. Nitrogen gas was used to transfer the volatile [^{18}F]fluoromethyl-d_2 bromide through a series of four silica gel cartridges (SepPak Plus) and then into a V-vial (1-mL) having a crimp-sealed silicon−Teflon septum cap. The RCY of [^{18}F]fluoromethyl-d_2 bromide was typically about 28%.[54]

Radiosynthesis of [^{11}C]5. NCA [^{11}C]hydrogen cyanide (~200 mCi) was trapped in a V-vial (5 mL) containing THF (500 μL), precursor (**1**, ~1.0 mg), $Pd(PPh_3)_4$ (1.5−2.0 mg), and base [K_2CO_3 (~2.0 mg) plus K 2.2.2 (~5.0 mg), or usually KH_2PO_4 (~2.0 mg) only]. The reaction mixture was heated at 80 °C for 5 min. HPLC mobile phase (3 mL) was added to the V-vial and [^{11}C]**5** isolated with radio-HPLC on a Gemini C18 column (5 μm, 10 mm × 250 mm; MeCN−aqueous NH_4OH (10 mM, 3:2 v/v, 6 mL/min, t_R = 7.2 min). The identity of [^{11}C]**5** was confirmed by analytical radio-HPLC on a Gemini C18 column (5 μm, 4.6 mm × 150 mm) eluted at 1 mL/min with MeCN−10 mM NH_4OH (16:9 v/v, t_R = 5.8 min) and also by LC−MS of the associated carrier. The radiochemical purity of [^{11}C]**5** was >99%. The decay-corrected radiochemical yield (RCY) from [^{11}C]hydrogen cyanide was 26% (n = 3).

Radiosynthesis of [^{11}C]8. [^{11}C]Methyl iodide (~15 mCi) was trapped in a capped fluoropolymer custom-made reaction vial (1.5-mL) containing anhydrous DMF (300 μL), precursor (**3**, ~1.0 mg) and base (Li_3N or Li_2O, ~5.0 mg). The mixture was sonicated in an ultrasound apparatus (UIS250L, Hielscher Ultrasonics, Germany) for 10 min and then filtered through an Iso-Disc filter (PFTE13-4, 13 mm × 0.45 μm) which was then rinsed twice with DMF. The combined filtrates were diluted with water, and then the [^{11}C]**8** was isolated with radio-HPLC (MeCN−$HCOONH_4$, 2:3 v/v, 3 mL/min, t_R = 13.1 min). The identity of [^{11}C]**8** was confirmed by analytical radio-HPLC on a Gemini-NX C18 column (5 μm, 4.6 mm × 250 mm) eluted with MeCN−100 mM $HCOONH_4$ (16:9 v/v) at 1 mL/min (t_R = 7.0 min) and also by LC−MS of the associated carrier. The RCYs from [^{11}C]methyl iodide were 7% (Li_2O) and 11% (Li_3N).

Radiosynthesis of [^{11}C]13 from [^{11}C]Methyl Triflate. [^{11}C]-Methyl triflate (30−20 mCi) was trapped in a tapered bottom vial (0.9 mL) containing **20** (0.15−0.8 mg), 0.5 M NaOH (3 equiv), and MeCN (300 μL). The mixture was heated at 80 °C for 5 min and diluted with HPLC mobile phase (500 μL), and then the [^{11}C]**13** (t_R = 8.7 min) was isolated with radio-HPLC (MeCN−$HCOONH_4$, 2:3 v/v, 4 mL/min). The identity of [^{11}C]**13** was confirmed by analytical radio-HPLC on a Gemini-NX C18 column (5 μm, 4.6 mm × 250 mm) eluted with MeCN−100 mM $HCOONH_4$ (2:3 v/v) at 1 mL/min (t_R = 4.4 min) and also by LC−MS of the associated carrier. The RCY was 27% (n = 12) from [^{11}C]methyl triflate and the radiochemical purity >99%. The absorbance detector response of the analytical HPLC system was calibrated for the mass of carrier ligand **13**. This allowed the mass of **13** in measured samples of [^{11}C]**13** to be determined and the specific radioactivity to be calculated. The specific radioactivity was 2517 mCi/μmol at the end of synthesis (EOS).

Radiosynthesis of [^{11}C]13 from [^{11}C]Methyl Iodide. [^{11}C]Methyl iodide (30−550 mCi) was trapped in a tapered bottom vial (0.9 mL) containing **20** (~1.0 mg, 3.2 μmol), 1.0 M (n-Bu)$_4$NOH (3 equiv, 10 μL, 3.2 μmol), and DMF (300 μL). The mixture was heated at 80 °C for 5 min and then diluted with water (500 μL). [^{11}C]**13** was separated and analyzed as described above. The RCY of [^{11}C]**13** from [^{11}C]iodomethane was 36% (n = 6) and the radiochemical purity >99%. The specific radioactivity was 2848 mCi/μmol at EOS.

The following experiment[22] was performed to confirm the position of radiolabel in [^{11}C]**13**. [^{11}C]Methyl iodide (~47 mCi) was trapped in a solution of **20** (~1.0 mg) in DMF (300 μL). Then [^{13}C]methyl iodide (10 μL from 47.7 mM stock solution in DMF) was added. [^{11}C]/[^{13}C]**13** was isolated by HPLC, as described above. A sample of the collected radioactive fraction was then analyzed by HPLC and LC−MS. The radioactive fraction was then evaporated to dryness, dissolved in $CDCl_3$, and analyzed by ^{13}C NMR {DEPT 135}.

Radiosynthesis of [^{18}F]18. Phenol **20** (~0.15 mg, 0.49 umol), MeCN (300 μL), and 0.5 M NaOH (1.5 μmol, 3 μL) were added to a V-vial (1 mL). [^{18}F]FCD_2Br (~20 mCi) was transferred to the solution under computer control from a TRACERlab FX_{F-N} module. Radioactivity transfer was monitored by two external radioactivity detectors (Bioscan) and was stopped when radioactivity was maximized. The mixture was heated at 100 °C for 15 min and then diluted with water (700 μL). [^{18}F]**18** was isolated with HPLC (MeCN−$HCOONH_4$, 2:3 v/v, 3 mL/min, t_R = 10.1 min). The identity of [^{18}F]**18** was confirmed by analytical HPLC on a Gemini-NX C18 column (5 μm, 4.6 mm × 250 mm) eluted with MeCN−100 mM $HCOONH_4$ (1:1 v/v) at 1 mL/min (t_R = 4.2 min) and also by LC−MS. The RCY of [^{18}F]**18** was 13% (n = 1) from [^{18}F]fluoride ion and the radiochemical purity >99%.

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Supporting Information Available: Chemical purities of compounds **1**−**21** and their HPLC methods of determination. This material is available free of charge via the Internet at http://pubs.acs.org.

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