Design, Synthesis, and Biological Evaluation of New 5-HT$_4$ Receptor Agonists: Application as Amyloid Cascade Modulators and Potential Therapeutic Utility in Alzheimer's Disease

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Serotonin 5-HT$_4$ receptor (5-HT$_4$R) agonists are of particular interest for the treatment of Alzheimer's disease because of their ability to ameliorate cognitive deficits and to modulate production of amyloid β-protein (Aβ). However, despite the range of 5-HT$_4$R agonists synthesized to date, potent and selective 5-HT$_4$R agonists are still lacking. In the present study, two libraries of molecules based on the scaffold of ML10302, a highly specific and partial 5-HT$_4$R agonist, were efficiently prepared by parallel supported synthesis and their binding affinities and agonist activities evaluated. Furthermore, we showed that, in vivo, the two best candidates exhibited neuroprotective activity by increasing the level of the soluble form of the amyloid precursor protein (sAPPα) in the cortex and hippocampus of mice. Interestingly, one of these compounds could also inhibit Aβ fibril formation in vitro.

Introduction

The serotonin 5-HT$_4$[a] receptor (5-HT$_4$R) is one of the seven subtypes of serotonin receptors that constitute the largest single family of G-protein coupled receptors (GPCR). Their widespread activity is mediated by several splice variants that differ in their intracellular C-terminal ends and are largely distributed throughout central and peripheral systems.[1,2]

Besides important effects on the gastrointestinal and urinary tracts,[3] 5-HT$_4$R has gained a lot of attention for its physiological role in the brain. To date, 5-HT$_4$R has been described as modulating mood,[4] feeding,[5] memory, and cognition,[2] and these features could be exploited for therapeutic purposes. Alzheimer's disease (AD), for instance, could benefit from the positive effects of 5-HT$_4$R on learning and memory performances.[6] Moreover, 5-HT$_4$R regulates also the production of the neurotoxic amyloid β-peptide (Aβ), which represents one of the major AD pathogenic pathways.[7] Indeed, 5-HT$_4$R agonists can stimulate the nonamyloidogenic pathway, leading to the release of the soluble form of the amyloid precursor protein (sAPPα), which, in contrast to Aβ, has putative neurotrophic and neuroprotective properties.[6,8,9] The role played by 5-HT$_4$R in amyloid precursor protein (APP) processing as well as in cognitive processes clearly elucidates the great hope that 5-HT$_4$R agonists represent for the treatment of AD.

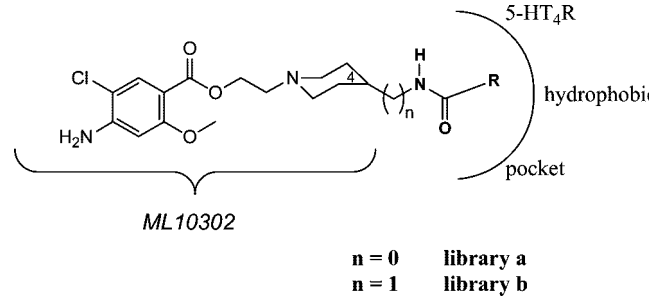

Figure 1. Design of 5-HT$_4$ agonists.

Despite the number of described 5-HT$_4$R agonists belonging to the benzamide, indole, or benzimidazoline families,[1] only two partial agonists SL 65.0155 (Sanofi-Aventis)[10] and PRX-03140 (Epix Pharmaceuticals)[11] have reached phase II clinical trials for the treatment of AD. Therefore, more work needs to be done to design and synthesize more effective 5-HT$_4$R agonists.

In a recent study, we demonstrated that ML10302,[12] a 5-HT$_4$R partial agonist with a benzoate-based structure, could selectively enhance sAPPα levels in the hippocampus and cortex of mice.[13] We therefore decided to design new, potential 5-HT$_4$R agonists by tuning the basal structure of ML10302 to favor possible interactions with Asp3.32, a highly conserved amino acid in the GPCR family, which is involved both in ligand recognition and activation of 5-HT$_4$R.[14,15] Two libraries of molecules differing by the absence (library **a**) or the presence (library **b**) of a methylene unit linking a substituted amide group to the ML10302 piperidine ring were thus prepared by parallel supported synthesis (Figure 1). One original additional molecule constituted by two ML10302 pharmacophores linked by a spacer (compound **14**)[16] and its monovalent counterpart (molecule **19**) were also prepared in order to highlight the potential benefit of

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[a] Abbreviations: 5-HT$_4$R, serotonin 5-HT$_4$ receptor; cAMP, cyclic adenosine monophosphate; Aβ, amyloid β-protein; AD, Alzheimer's disease; (s)APPa, (soluble form of) amyloid precursor protein; BAL, backbone amide linker; GPCR, G-protein coupled receptor; ML10302, 4-amino-5-chloro-2-methoxy-benzoic acid 2-piperidin-1-yl-ethyl ester; GR113808, 1-methyl-1*H*-indole-3-carboxylic acid 1-(2-methanesulfonylamino-ethyl)-piperidin-4-ylmethyl ester.

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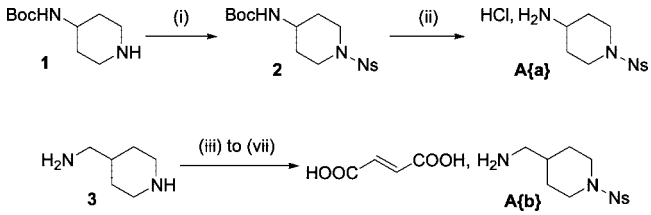
Scheme 1. Synthesis of Building Blocks A[a]



[a] Legend: (i) NsCl, NEt_3, CH_2Cl_2, 0 °C to RT; (ii) HCl/MeOH 4 N, RT; (iii) PhCHO, toluene, reflux, Dean−Stark trap, 1.5 h; (iv) NsCl, NEt_3, 0 °C to RT; (v) KHSO4 aq 1 M; (vi) NaOH, extraction; (vii) fumaric acid, EtOH, RT.

the "bivalent-ligand" approach on agonist activity.[17−19] Because molecules **14** and **19** do not have a methylene unit linking the ML10302 piperidine ring to the amide group, these molecules were classified as belonging to library **a**. Binding affinity and functional activity of all synthesized molecules were then evaluated in C6 glial cells that stably express 5-HT$_4$R. In addition, the possible neuroprotective effects of the best candidates were investigated in vivo (i.e., APP processing in the hippocampus and cortex of C57BL/6j mice) and in vitro (i.e., inhibition of Aβ peptide polymerization).

Results

Design and Chemistry. Design. With the aim of synthesizing new, more active 5-HT$_4$R agonists, we decided to design molecules structurally based on the scaffold of ML10302, a specific 5-HT$_4$R partial agonist.[12] The observation that mutations in Asp3.32 (Asp3.32Ala[14,20] or Asp3.32Asn[21]) had no impact on ML10302 affinity for the receptor suggests that ML10302 interacts very weakly with Asp3.32. Therefore, to develop other interactions with Asp3.32, we decided to introduce in position four of ML10302 piperidine ring a substituted amide group. Preliminary molecular modeling studies (data not shown) indicated that Asp3.32 could be equidistant from the basic piperidine amino group and from the amide function of ML10302, thus allowing the formation of an ionic interaction with the first and a hydrogen bond with the second. Furthermore, the substituent could be located in a large hydrophobic pocket of the 5-HT$_4$R (also called secondary binding site)[22] and develop supplementary interactions potentially favorable to the activation of the receptor (Figure 1).

Parallel Solid-Phase Synthesis. Two libraries of compounds that used ML10302 as a scaffold were thus prepared by a parallel solid-phase synthesis using a BAL resin. Following our synthetic strategy, first, we synthesized two piperidine moieties (i.e., **A**{**a**} and **A**{**b**}) that possessed a protected cyclic nitrogen and a free exocyclic amine (Scheme 1). Aminopiperidine **A**{**a**} was easily synthesized from the commercially available 4-*N*-*tert*-butoxycarbonylaminopiperidine **1** by introducing a *nosyl* group in dichloromethane and triethylamine.[23] The Boc group of the fully protected compound **2** was then selectively removed with HCl in MeOH to obtain the monoprotected molecule **A**{**a**} in the form of hydrochloride salt.

The protected aminomethylpiperidine **A**{**b**} was prepared from piperidin-4-ylmethyl-amine **3** (Scheme 1) following a modified version of an existing protocol for selective protection of secondary amines in the presence of a primary amine.[24] The primary amine was temporarily neutralized with benzaldehyde, followed by protection of the intracyclic amine with nosyl chloride. After hydrolysis of the imine and extraction, the free primary amine was converted into its hemifumarate salt **A**{**b**}.

We then produced the two amide libraries by solid-phase synthesis (Scheme 2). The chosen protocol allowed us to introduce three points of diversity: the aminopiperidines **A**{**a**} and **A**{**b**}, the carboxylic acids **B**{**a**−**x**} and a halide **C**, which are presented in Figure 2. Specifically, the primary amines **A**{**a**} (hydrochloride salts) and **A**{**b**} (hemifumarate salts) were loaded onto the BAL resin **4** by reductive amination using 3 equiv of amine salts and 5 equiv of $NaBH(OAc)_3$ in DMF (Scheme 2). Using this procedure, the loading of resins **5**{**a**} and **5**{**b**} was quantitative for both aminopiperidines as determined by picric acid assay.[25]

The acylation step was optimized in parallel. The best results were obtained with 6 equiv of carboxylic acid (130 mM, final concentration), 6 equiv of HBTU (130 mM, final concentration), and 7 equiv of DIEA in a 7/3 mixture of DMF and dichloromethane at room temperature for 18 h. This procedure led to compounds **6**{**a**; **a**−**x**} and **6**{**b**; **a**−**x**}. Deprotection of the nosyl amine (**7**{**a**; **a**−**x**} and **7**{**b**; **a**−**x**}) was realized by treatment with a DMF solution containing 0.5 M of thiophenol and 1 M of potassium carbonate in 2 cycles of 30 min/each. The *N*-alkylation step to prepare compounds **8**{**a**; **a**−**x**} and **8**{**b**; **a**−**x**} was difficult to optimize. Finally, the best conditions obtained were the use of 5 equiv of bromoester **7** (150 mM, final concentration) and 7 equiv of DIEA in DMF in 2 cycles of 20 h/each at 40 °C. Products **9**{**a**; **a**−**x**} and **9**{**b**; **a**−**x**} were cleaved from the BAL resin with a 1/4 mixture of TFA and dichloromethane and air-dried. Purification of the crude products was done by ion-exchange chromatography on a sulfonic resin (DSC-SCX). All compounds (48 molecules in total, distributed between the two libraries) were then analyzed by LC-MS, and a selection of compounds (i.e., half of the library) was analyzed by ^{1}H NMR. Overall yields, HPLC purities, and mass data are presented in Table 1.

Compounds **9**{**a**;**b**}, **9**{**a**;**d**}, **9**{**a**;**n**}, **9**{**a**;**t**}, **9**{**a**;**u**}, **9**{**a**;**x**}, **9**{**b**;**m**}, and **9**{**b**;**n**} were not further tested due to their low purity.

Synthesis in Solution. On the basis of the pharmacological results, compounds **9**{**a**;**q**} and **9**{**b**;**w**} were also synthesized in solution using the same protocol, purified, and fully analyzed (NMR, LC-MS, elementary analysis).

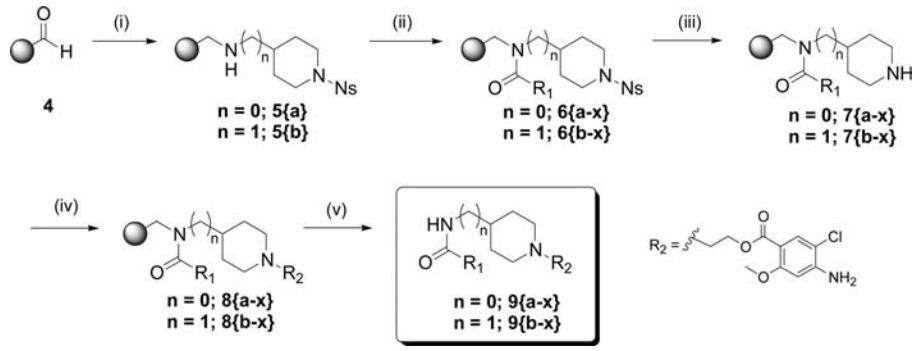
Conversely, molecules **9**{**a**;**y**}, **14**, and **19** were prepared only in solution as they were difficult to obtain by solid-phase synthesis.

To prepare molecule **9**{**a**;**y**} (Scheme 3), amine **10**[26] was coupled with 4-nitro-1,8-naphthalimido-*N*-caproic acid[27] using a general procedure (EDC, HOBt, and NEt_3 in dry DMF) to obtain the nitro compound **11** with very moderate yield. The nitro group of **11** was then reduced by catalytic hydrogenation with hydrogen under atmospheric pressure and Raney Ni. Compound **9**{**a**;**y**} was thus easily obtained, avoiding the sensitive reducible chlorine group of the aryl moiety.

Preparation of the bivalent molecule **14** started from the previously described compound **12**.[28] Boc protecting groups were removed by HCl in MeOH, and the resulting, very hygroscopic hydrochloride salt **13** was converted to the more stable tetrafumarate salt **14** (Scheme 4).

To synthesize ligand **19** (Scheme 5), a Sonogashira−Linstrumelle coupling reaction was performed at room temperature between the propargylic acid **15**[28] and iodobenzene, leading to the aryl propargyl compound **16**. After classical hydrogenation on Pd/C (molecule **17**), a condensation with amine **10** using EDC, HOBt, and ET_3N allowed preparation of molecule **18**. A Boc protecting

Scheme 2. Solid-Phase Synthesis of Amides **9**[a]



[a] Legend: (i) **A**{**a**−**x**} (3 equiv), $NaBH(OAc)_3$ (5 equiv), DMF, RT, 46 h; (ii) barboxylic acids **B**{**a**−**x**} (6 equiv), HBTU (6 equiv), DIEA (7 equiv), DMF/CH_2Cl_2 (7/3), RT, 18 h; (iii) PhSH 0.5 M, K_2CO_3 1 M, DMF, RT, 2 × 30 min; (iv) halide **C** (5 equiv), DIEA (7 equiv), DMF, 40 °C, 2 × 20 h; (v) TFA/CH_2Cl_2 (1/4), RT, 2 h.

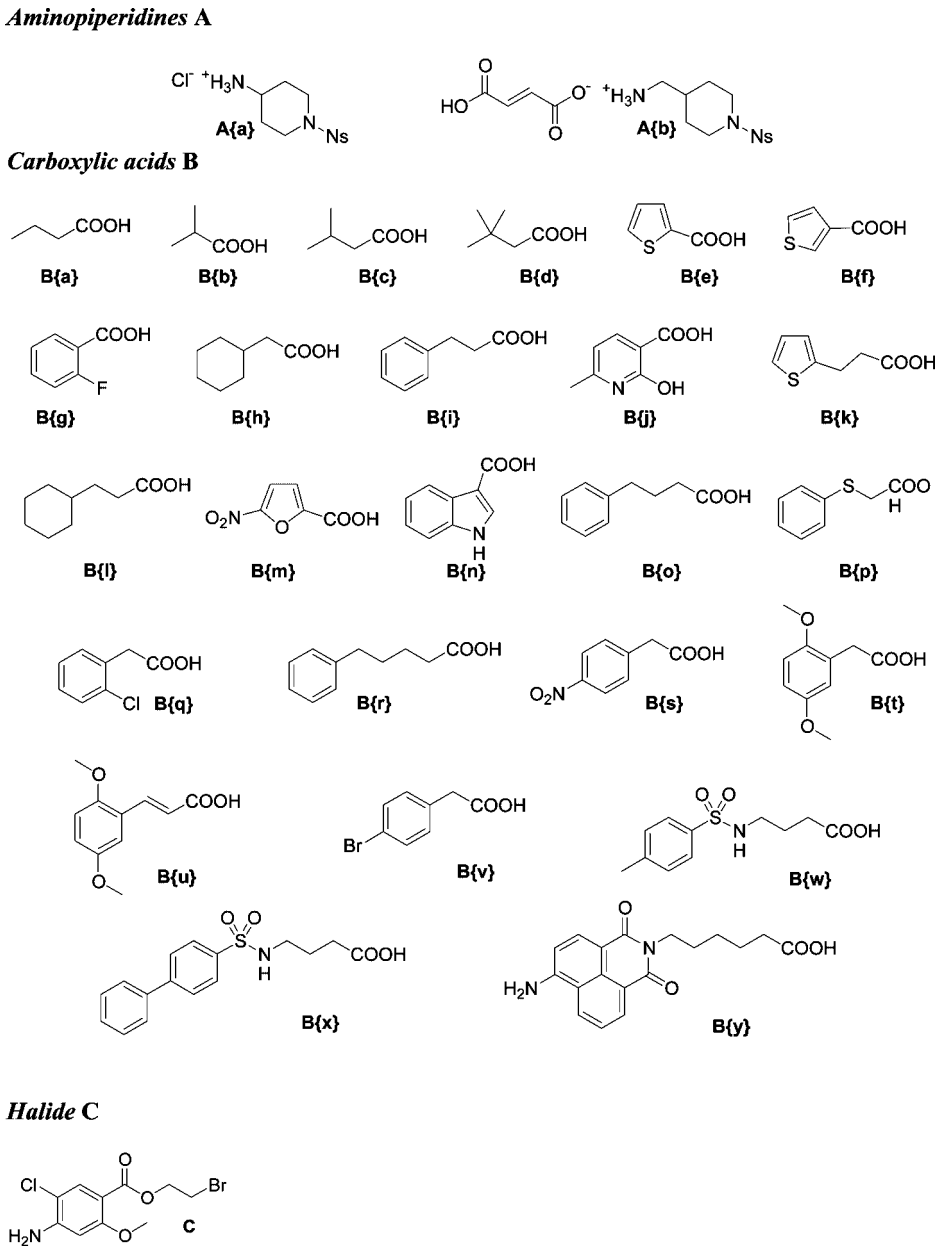


Figure 2. Building blocks used for the solid-phase synthesis of amides **9**.

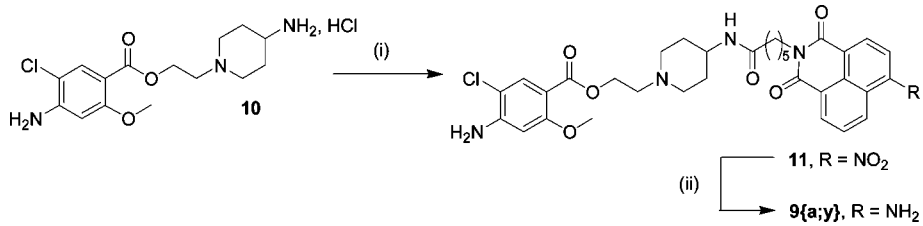
group was then removed by HCL in MeOH, and as for the bivalent molecule **14**, the obtained hydrochloride salt was converted to the more stable fumarate salt **19**.

Pharmacological and Functional Properties of the Synthesized Molecules. Binding affinities of the synthesized ligands (10 nM/each) were analyzed in C6 glial cells that stably

Table 1. Overall Yields, Physicochemical Data, and Purity of Amides **9** Synthesized on Solid-Phase

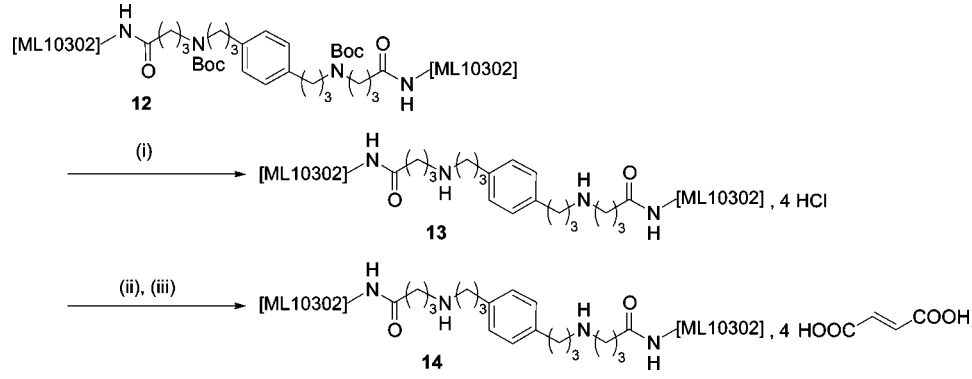
compd	overall yield, %	t_R (min)	LC purity, %	molecular weight	mass $[M + H]^+$	compd	overall yield, %	t_R (min)	LC purity, %	molecular weight	mass $[M + H]^+$
9{**a**;**a**}	98	12.26	76	397.18	398	**9**{**b**;**a**}	35	12.64	82	411.19	412
9{**a**;**b**}	41	13.52	28	397.18	398	**9**{**b**;**b**}	36	12.52	83	411.19	412
9{**a**;**c**}	42	14.28	90	411.19	412	**9**{**b**;**c**}	36	14.20	84	425.21	426
9{**a**;**d**}	24	16.15	40	425.21	426	**9**{**b**;**d**}	32	16.02	83	439.22	440
9{**a**;**e**}	38	14.13	81	437.12	438	**9**{**b**;**e**}	37	14.20	68	451.13	452
9{**a**;**f**}	59	13.98	78	437.12	438	**9**{**b**;**f**}	41	14.23	65	451.13	452
9{**a**;**g**}	64	14.26	73	449.15	450	**9**{**b**;**g**}	43	14.53	65	463.17	464
9{**a**;**h**}	27	18.18	93	451.22	452	**9**{**b**;**h**}	36	18.12	77	465.24	466
9{**a**;**i**}	55	16.48	82	459.19	460	**9**{**b**;**i**}	42	15.98	68	473.21	474
9{**a**;**j**}	35	12.78	57	462.17	463	**9**{**b**;**j**}	20	13.28	60	476.18	477
9{**a**;**k**}	28	15.88	79	465.15	466	**9**{**b**;**k**}	21	15.52	58	479.16	480
9{**a**;**l**}	22	18.48	84	465.24	466	**9**{**b**;**l**}	34	20.09	66	479.26	480
9{**a**;**m**}	21	13.82	48	466.13	467	**9**{**b**;**m**}	36	13.79	28	480.14	544
9{**a**;**n**}	30	11.83	35	470.17	471	**9**{**b**;**n**}	19	15.73	18	484.19	485
9{**a**;**o**}	53	17.94	82	473.21	474	**9**{**b**;**o**}	44	18.14	68	487.22	488
9{**a**;**p**}	9	16.63	77	477.15	478	**9**{**b**;**p**}	39	16.17	61	491.16	492
9{**a**;**q**}	66	16.38	75	479.14	480	**9**{**b**;**q**}	44	16.64	57	493.15	494
9{**a**;**r**}	93	19.00	78	487.22	488	**9**{**b**;**r**}	44	19.23	66	501.24	502
9{**a**;**s**}	63	15.09	60	490.16	491	**9**{**b**;**s**}	37	15.46	65	504.18	505
9{**a**;**t**}	24	16.03	25	505.20	506	**9**{**b**;**t**}	35	16.14	58	519.21	520
9{**a**;**u**}	24	18.32	41	517.20	518	**9**{**b**;**u**}	27	18.14	51	531.21	532
9{**a**;**v**}	41	17.93	76	523.09	524	**9**{**b**;**v**}	37	18.05	60	537.10	538
9{**a**;**w**}	18	16.75	58	566.20	567	**9**{**b**;**w**}	11	19.80	55	580.21	581
9{**a**;**x**}	20	18.00	41	628.21	629	**9**{**b**;**x**}	30	22.89	64	642.23	643

Scheme 3. Synthesis of Amide **9**{**a**;**y**}[a]



[a] Legend: (i) 4-nitro-1,8-naphthalimido-*N*-caproic acid, EDC, HOBt, NEt_3, CH_2Cl_2, 0 °C to RT, 20 h; (ii) Raney Ni, H_2, MeOH, atmospheric pressure, RT, 14 h.

Scheme 4. Synthesis of Bivalent Ligand **14**[a]



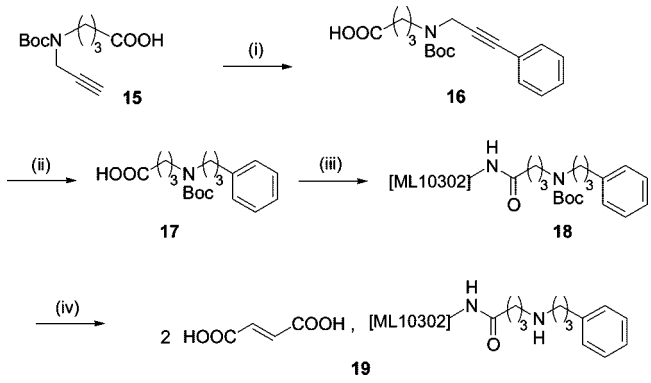
[a] Legend: (i) HCl/MeOH 4 N, RT, 30 min; (ii) aq sat. Na_2CO_3, extraction; (iii) fumaric acid, EtOH, RT.

express the human 5-HT$_{4(e/g)}$R isoform[29] (Table 2) using [^{3}H]-GR113808, a 5-HT$_4$R specific antagonist, as radioligand for the binding assays. Displacement results revealed that compounds **9**{**a**;**a**−**y**} from library **a**, in which the amide function is directly attached to the piperidine ring, had weaker binding affinities than ML10302. At 10 nM, compounds with the highest displacements (**9**{**a**;**e**}, **9**{**a**;**i**}, **9**{**a**;**o**}, **9**{**a**;**p**}, and **9**{**a**;**s**}) did not exceed 30% of the bound radioligand compared to 40−50% of displacement for ML10302 (Table 2). Conversely, agonists **9**{**b**;**a**−**x**} of library **b**, where the amide function is linked to the piperidine ring through a methylene unit, showed binding affinities that were globally better than those of library **a**, with **9**{**b**;**w**} and **9**{**b**;**q**} displacing more than 50% of the bound radioligand (Table 2).

Then, their functional properties were assessed by measuring their ability to induce cAMP production at a concentration of 10 nM using a previously reported radioimmunoassay technique[29] (Table 2). In library **a**, compounds **9**{**a**;**q**}, **9**{**a**,**y**}, and **14** induced higher cAMP production than ML10302 (more than 50%). In library **b**, four molecules (**9**{**b**;**c**}, **9**{**b**;**l**}, **9**{**b**;**s**}, and **9**{**b**;**w**}) also were found to stimulate cAMP production better than ML10302.

A particular attention was devoted to identify the influence of the different chemical substituents used on the pharmacologi-

Scheme 5. Synthesis of Molecule **19**[a]



[a] Legend: (i) iodobenzene, $PdCl_2(PPh_3)_2$ 5 mol%, CuI 10 mol%, NEt_3, DMF, 80 °C. (ii) H_2 (1 bar), Pd/C 10%, MeOH, RT. (iii) **10**, HBTU, NEt_3, DMF, RT. (iv) (a) HCl/MeOH 4 N, RT, 30 min; (b) aq sat. Na_2CO_3, extraction; (c) fumaric acid, EtOH, RT.

cal properties of the agonists we synthesized. Specifically, among the agonist with aliphatic substituents, the best activities were observed with molecules **9**{**b**;**c**} and **9**{**b**;**l**} (library **b**), which are lipophilic and conformationally flexible. When aromatic substituents were used, the presence of an aromatic ring not directly bound to the amide functional group could be advantageous for agonist activity independently from the absence/presence of a methylene unit and the size of the aryl ring (five or six) (see Table 2, agonists **9**{**a**;**o**}, **9**{**a**;**q**}, **9**{**a**;**s**}, **9**{**a**;**v**}, and **9**{**b**;**i**}, **9**{**b**;**k**}, **9**{**b**;**q**}, and **9**{**b**;**s**}). Among the agonists with phenyl substituents, **9**{**b**;**s**} (library **b**), which has an electron-withdrawing group, was a better agonist compared to **9**{**b**;**q**}, **9**{**b**;**t**}, and **9**{**b**;**v**} whose aryl rings were substituted by electron-donating groups. Conversely, in library **a**, the presence of electron-donating groups on the aryl ring, as in **9**{**a**;**q**}, led to a better agonist activity. Furthermore, in library **b**, a polar group, such as a sulfonamide, gave the best agonist compound (**9**{**b**;**w**}) of this series. However, a bulkier substituent on the sulfonamide group (**9**{**b**;**x**}) prevented both binding and activity.

On the basis of these general results, we selected the four best agonist molecules (i.e., **9**{**a**;**q**}, **9**{**a**,**y**}, **14**, and **9**{**b**;**w**}) to determine their K_i, EC_{50}, and E_{max} values on purified samples (Table 3). [^{3}H]-GR113808 competition curves of pure **9**{**a**;**q**} and **9**{**b**;**w**} were monophasic, and their affinity values (5 ± 2 nM and 2 ± 1 nM, respectively) were in the same range as that of ML10302. The dose−response cAMP production measurements revealed EC_{50} of 18 ± 5 nM for **9**{**a**;**q**} and 68 ± 12 nM for **9**{**b**;**w**} and E_{max} respectively of 29% of 5-HT and 65% of 5-HT. Although **9**{**a**,**y**} and **14** displayed lower affinities than **9**{**a**;**q**} and **9**{**b**;**w**} (K_i values respectively of 111 ± 16 nM for **9**{**a**,**y**} and 14 ± 7 nM for **14**), their EC_{50} (4 ± 3 nM for **9**{**a**;**y**} and 9 ± 4 nM for **14**) and E_{max} values (63% for **9**{**a**;**y**} and 70% for **14**) were very similar to those of 5-HT (EC_{50} of 12 ± 2 nM and E_{max} of 100%).[29] Interestingly, the bivalent molecule **14**, despite a similar 5-HT_4R affinity, presented a better E_{max} than its monovalent counterpart **19**.

According to these data, compound **9**{**a**,**y**} and the bivalent molecule **14** were considered the best candidates for in vivo and in vitro biological experiments to test their neuroprotective effects.

Effects of 5 HT_4 Receptor Agonists on sAPPα Levels in Mice. To measure in vivo the neuroprotective effects of agonists **9**{**a**,**y**} and **14**, mice were subcutaneously injected with a single dose of these molecules (5 mg/kg) or vehicle alone (5 mL/kg) and sacrificed 90 min after the injection. The level of sAPPα was then determined by Western blot analysis in extracts from hippocampus and cortex. Both agonists (5 mg/kg) increased sAPPα levels in hippocampus (Figure 3A,B) and cortex (Figure 3D,E) as compared to vehicle alone (0 mg/kg). When we quantified our results in hippocampus and cortex (Figure 3C,F), we observed that compound **9**{**a**,**y**} had a stronger effect than agonist **14** in both. Moreover, the effect of **9**{**a**,**y**} and **14**, at 5 mg/kg, on sAPPα concentration were in the same range or higher than that previously reported of ML10302, but at 20 mg/kg,[13] confirming the real potential of the proposed molecules.

Effects of 5-HT_4 Receptor Agonists on Aβ Fibril Formation. Because compounds **9**{**a**,**y**} and **14** present structural similarities with the antiamyloidogenic molecules curcumin and polyphenols, which possess two phenolic rings linked by a spacer, we decided to test whether the two 5-HT_4R agonists (at the concentration of 10 μM) could inhibit Aβ fibril formation by using an in vitro assay based on UV−visible measurements.[30] Specifically, this assay examines the effects of candidate molecules on the β amyloid peptide 25−35 ($A\beta_{25-35}$) that presents neurotoxic and aggregation properties comparable to those of Aβ.[31,32] Curcumin, which has been shown to possess in vitro[33] and in vivo[34] antiamyloidogenic activities and to inhibit fibril formation using $A\beta_{1-40}$[33] or $A\beta_{25-35}$[30] fragments, was used as reference.

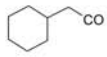
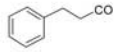
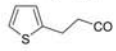
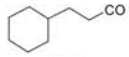
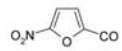
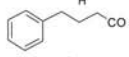
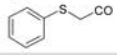
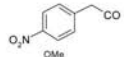
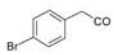
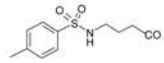
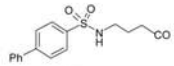
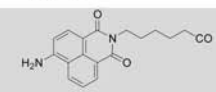
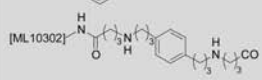
Compound **9**{**a**,**y**} did not show any important inhibitory activity compared to curcumin, whereas the bivalent compound **14** inhibited Aβ fibril formation (EC_{50} value of 9 μM) as well as curcumin (Table 4).

Discussion

During the last decades, many 5-HT_4R agonists have been synthesized, but so far, only two (SL65.0155 and PRX-03140) manifest a promising future for the treatment of AD. The aim of this study was to discover new 5-HT_4R agonists to be used as drug candidates for brain disorders. We therefore prepared two libraries in which ML10302,[12] was used as a scaffold. Compounds were designed to improve the agonist properties of ML10302 by introducing a substituted amide group on the piperidine moiety (position four) of the molecule to facilitate interactions both with Asp3.32 and with residues of the 5-HT_4R hydrophobic pocket. In addition, one original 5-HT_4R bivalent ligand (**14**) and its monovalent counterpart (**19**) were added to the series of potential agonists to be assessed.

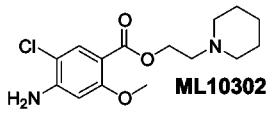
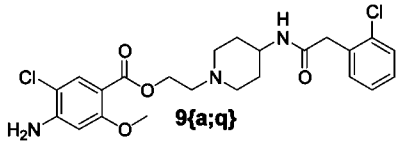
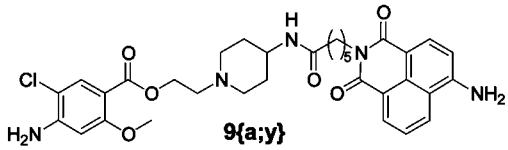
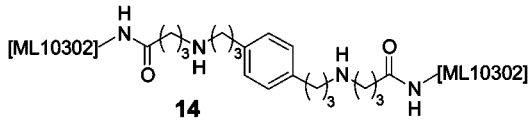
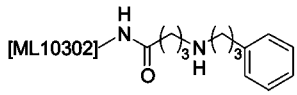
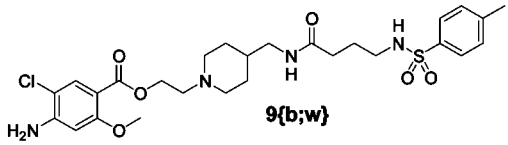
The introduction of a substituted amide function onto the piperidine ring of ML10302 allowed us to obtain four molecules with comparable or higher agonist activity than ML10302: **9**{**a**,**y**}, **14**, **9**{**b**,**w**}, and to a lesser extent **9**{**a**,**q**} (Figure 4). The presence of the amide group has been shown to be essential for the molecules to exhibit agonist properties. For instance, an analogue of molecule **9**{**a**,**y**} that lacks the amide group displayed an antagonist profile.[26] Nevertheless, it is worth noting that for several molecules, the presence of the amide group is necessary but not sufficient to activate 5-HT_4R. Therefore, the development of supplementary interactions with residues of the hydrophobic pocket of the 5-HT_4R binding site could improve their agonist activity. This idea is supported by the observation that three of the best candidates possess a polar group that is located four or five atoms away from their amido piperidine ring and can interact with the hydrophobic pocket: a naphtalimide group for **9**{**a**,**y**}, a basic amino group for the bivalent molecule **14**, and a sulfonamide functional group for **9**{**b**,**w**} (Figure 4). Interestingly, when this amino group is Boc-protected, it looses its agonist properties.[28] This further high-

Table 2. Binding Affinities to 5-HT$_4$R and Effect on cAMP Production of Amides **9**[d]

Carbonyl group	Compound	Bound % [a,b]	cAMP prod % [a,c]	Compound	Affinity % [a,b]	cAMP prod % [a,c]
-	**ML10302**	40-50	30-40	-	-	-
	**9{a;a}**	0	n.d.	**9{b;a}**	20-30	<10
	**9{a;b}**	n.d.	n.d.	**9{b;b}**	20-30	10-20
	**9{a;c}**	<10	10-20	**9{b;c}**	20-30	40-50
	**9{a;d}**	n.d.	n.d.	**9{b;d}**	10-20	10-20
	**9{a;e}**	20-30	<10	**9{b;e}**	20-30	<10
	**9{a;f}**	10-20	20-30	**9{b;f}**	10-20	0
	**9{a;g}**	10-20	10-20	**9{b;g}**	20-30	0
	**9{a;h}**	<10	n.d.	**9{b;h}**	20-30	<-10
	**9{a;i}**	20-30	<10	**9{b;i}**	20-30	20-30
	**9{a;j}**	<10	n.d.	**9{b;j}**	10-20	10-20
	**9{a;k}**	10-20	10-20	**9{b;k}**	30-40	30-40
	**9{a;l}**	0	n.d.	**9{b;l}**	10-20	>50
	**9{a;m}**	<10	n.d.	**9{b;m}**	n.d.	n.d.
	**9{a;n}**	n.d.	n.d.	**9{b;n}**	n.d.	n.d.
	**9{a;o}**	20-30	20-30	**9{b;o}**	20-30	0
	**9{a;p}**	20-30	<10	**9{b;p}**	30-40	<10
	**9{a;q}**	10-20	>50	**9{b;q}**	>50	20-30
	**9{a;r}**	<10	n.d.	**9{b;r}**	10-20	10-20
	**9{a;s}**	20-30	20-30	**9{b;s}**	40-50	>50
	**9{a;t}**	n.d.	n.d.	**9{b;t}**	40-50	10-20
	**9{a;u}**	n.d.	n.d.	**9{b;u}**	20-30	n.d.
	**9{a;v}**	10-20	10-20	**9{b;v}**	40-50	0
	**9{a;w}**	10-20	10-20	**9{b;w}**	>50	>70
	**9{a;x}**	n.d.	n.d.	**9{b;x}**	10-20	<10
	**9{a;y}**	<10	>50	-	-	-
	**14**	10-20	>50	-	-	-

[a] Mean of at least two independent determinations made in triplicate. [b] Percentage of displacement of [^{3}H]GR113808 by agonists at 10 nM. [c] cAMP production induced by compounds at a 10 nM concentration. Results were normalized to the maximal response induced by 5-HT. All experiments were performed in C6 glial cells. [d] n.d.: not determined.

Table 3. K_i, EC_{50}, and E_{max} of Agonists **9**{**a**;**q**}, **9**{**a**;**y**}, **14**, **19**, and **9**{**b**;**w**}

Compounds	Ki (nM) [a,b]	EC_{50} (nM) [a,c]	E_{max} [a,d] (% of 5-HT)
5-HT[29]	1049 ± 300	12 ± 2	100%
 **ML10302**	5 ± 2	51 ± 11	45%
 **9{a;q}**	5 ± 2	18 ± 5	29%
 **9{a;y}**	111 ± 16	4 ± 3	63%
 **14**	14 ± 7	9 ± 4	70%
 **19**	12 ± 4	-	50%
 **9{b;w}**	2 ± 1	68 ± 12	65%

[a] Mean ± SEM of at least two or three independent determinations made in triplicate. [b] K_i values were calculated using the Cheng−Prussof equation from the IC_{50} values with the PRISM program. [c] EC_{50} values correspond to the concentration of agonist required to obtain half-maximal stimulation of adenylyl cyclase. [d] The maximum response produced by each molecule was normalized to the maximum response induced by 5-HT (E_{max}).

lights the crucial role not only of the amide group but also of the nature of the amide substituent for 5-HT_4R activation. Finally, the presence of flexibility around the amide group seems secondary for the agonist activity.

Concerning bivalent molecule **14**, it has been previously reported that bimultivalent ligands could show enhanced binding and better activity because of entropic considerations.[20−22] This is indeed the case for molecule **14** that presented the best agonist profile of all synthesized molecules. This result was corroborated by the fact that its monovalent counterpart **19**, constituted by one ML10302 unit and a truncated spacer, displayed lower agonist properties.

As previously reported for ML10302 and prucalopride, which selectively enhanced sAPPα level in vivo in adult mice hippocampus and cortex, the best candidates **14** and **9**{**a**;**y**} seem to cross the blood−-brain barrier and act on APP processing. Interestingly, the neuroprotective effects were not always linked to best biological activities. For instance, the bivalent molecule **14** showed better K_i and E_{max} than **9**{**a**,**y**}, but the latter had a stronger effect on APP processing.

Nevertheless, during in vitro inhibition assays of fibril formation, bivalent ligand **14** only exhibited an inhibition level higher than the reference curcumin. The naphtalimide group of **9**{**a**,**y**}, which we think is important to explain its strong agonist activity, could be the cause of its weak inhibition of Aβ fibril formation through steric hindrance and rigidity of the naphtalimide extremity. On the other hand, the long size of the spacer of the bivalent compound **14**, compared to curcumin and

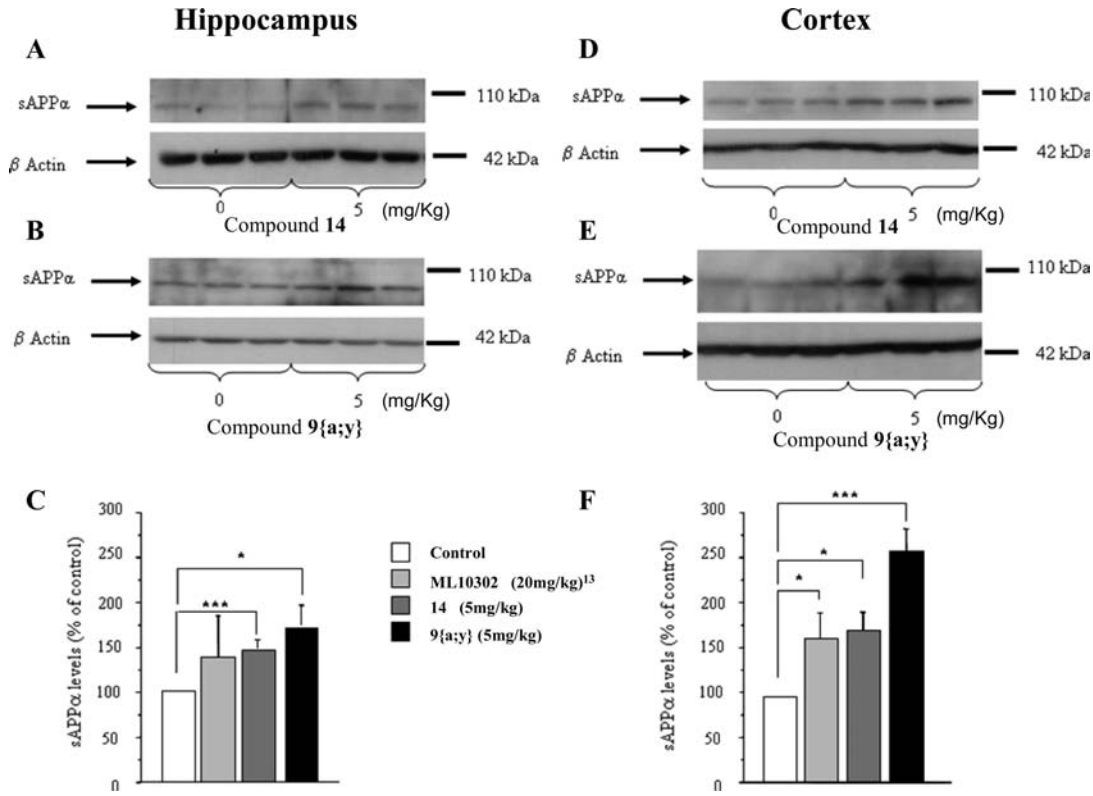
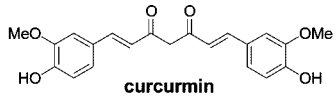
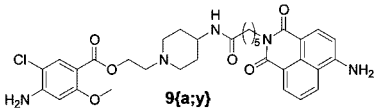
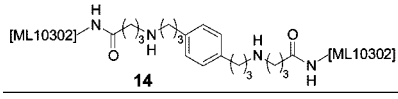


Figure 3. Study of the effect of compounds **9**{**a**;**y**} and **14** on sAPPα level in the hippocampus (A, B, and C) and cortex (D, E, and F) in 8 week old male C57BL/6j mice. The mice (7−9 mice per group) were treated with a single dose of ligand (5 mg/kg for compounds **9**{**a**;**y**} and **14**) or saline (5 mL/kg). sAPPα and β-actin levels (used as internal control) were measured in brain homogenates 90 min after the injection. A, B, D, and E, representative immunoblots illustrating the effects of 5-HT$_4$ agonists (5 mg/kg) or saline (0 mg/kg) on sAPPα levels. C and F, the mean densitometric values (±SEM) of sAPPα, relatively to β-actin (used as internal control), are expressed as percentages of the values observed in untreated control mice. Values for ML10302 were retrieved from a previous work[13] in which the agonist was injected subcutaneously at a dose of 20 mg/kg, mice were sacrificed after 90 min and hippocampus and cortex processed in the same way as for the present study. Data show results of a typical experiment. One-way ANOVA, $F(1,13) = 19.53$; ***$p < 0.001$ in the hippocampus and $F(1,13) = 10.25$; *p0.05 in the cortex for compound **14** as compared to saline treated animals (white bar). One-way ANOVA, $F(1,15) = 6.25$; *p0.05 in the hippocampus and $F(1,17) = 26.37$; ***$p <$ 0.001 in the cortex for **9**{**a**;**y**} as compared to saline treated animals (white bar).

Table 4. Inhibition of Aβ Fibril Formation

Compounds	Inhibition %	[a] IC_{50} (μM)
 curcumin	45 ± 6	10
 9{a;y}	14 ± 2	-
 14	49 ± 5	9

[a] For agonists exhibiting inhibitory activity at least equal to that of curcumin, IC_{50} was calculated by using a least-squares fitting technique to match the experimental data with a sigmoidal curve. IC_{50} was the effective concentration of agonist inhibiting the formation of Aβ fibrils to 50% of the control value. Three independent measurements were made for all cases.

polyphenols,[30,35] did not prevent inhibition of Aβ fibril formation. The strong inhibitory effect of this molecule more likely resides in the nature of the aromatic ring (methoxy and ester substituents), which presents structural similarities with polyphenols and certainly orients the molecule in the same manner to interact with the Aβ_{25-35} fragment.

Conclusions

We have prepared new 5-HT$_4$R agonists mainly synthesized by a rapid and efficient parallel solid-phase strategy. ML10302, a specific 5-HT$_4$R partial agonist, was used as a scaffold, and a substituted amide group was introduced on the piperidine moiety of the molecule to allow interaction with Asp3.32 and with residues of the 5-HT$_4$R hydrophobic pocket. After preliminary pharmacological screenings to test the binding and activity properties of the synthesized agonists, two (**9**{**a**,**y**} and the bivalent molecule **14**) were selected for in vivo and in vitro biological tests (APP processing and Aβ fibril formation). They both increased sAPPα concentration in the hippocampus and cortex of mice, suggesting that they could be useful for the treatment of AD. Moreover, molecule **14** displayed an inhibitory effect on Aβ fibril formation. To our knowledge, this is the first report showing that a 5-HT$_4$ ligand can interfere in the process of Aβ fibril formation. Further experiments are needed to test the potential neuroprotective properties of these new 5-HT$_4$ agonists in animal models of AD.

Experimental Section

Chemistry. Melting points were determined on a Kofler melting point apparatus. NMR spectra were performed on a Bruker AMX

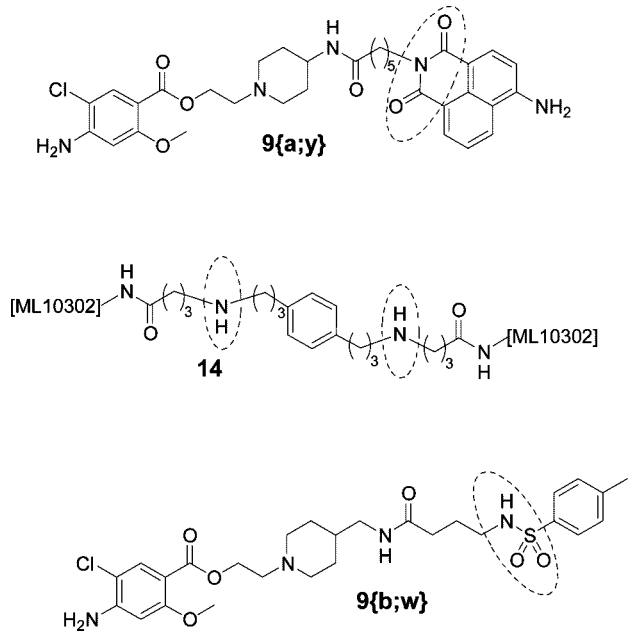


Figure 4. Best 5-HT_4 synthesized agonists.

200 (^{1}H, 200 MHz; ^{13}C, 50 MHz) or Bruker AVANCE 400 (^{1}H, 400 MHz; ^{13}C, 100 MHz). Unless otherwise stated, $CDCl_3$ was used as solvent. Chemical shifts δ are in ppm, and the following abbreviations are used: singlet (s), broad singlet (bs), doublet (d), triplet (t), and multiplet (m). Elemental analyses (C, H, N) were performed at the Microanalyses Service of the Faculty of Pharmacy at Châtenay-Malabry (France) and were within 0.4% of the theorical values unless otherwise stated. Mass spectra were obtained using a Bruker Esquire electrospray ionization apparatus.

Materials. DMF distilled from $CaSO_4$, CH_2Cl_2 distilled from calcium hydride, and standard solvents were purchased from VWR International (Fontenay-sous-Bois, France). Liquid chromatography was performed on Merck silica gel 60 (70/30 mesh), and TLC was performed on silica gel, 60F-254 (0.26 mm thickness) plates. Visualization was achieved with UV light and Dragendorff reagent unless otherwise stated.

Synthesis of *N-tert*-Butoxycarbonyl-1-(2-nitrophenylsulfonyl)piperidin-4-amine 2. To a solution of *N-tert*-butoxycarbonylpiperidin-4-amine **1** (1.83 g, 9.13 mmol, 1 equiv) and NEt_3 (1.46 mL, 10.50 mmol, 1.15 equiv) in CH_2Cl_2 (35 mL) at 0 °C was added dropwise 2-nitrobenzenesulfonyl chloride (2.13 g, 9.59 mmol, 1.05 equiv) in CH_2Cl_2 (8.5 mL). The resulting solution was stirred and allowed to reach room temperature. After 2.5 h, the solution was diluted with H_2O (25 mL), the organic phase was separated, successively washed with citric acid 10% (25 mL), $NaHCO_3$ 1 M (25 mL), and brine (25 mL) and dried (Na_2SO_4) to give 3.46 g (98%) of **2** as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.03−7.87 (m, 1H), 7.74−7.66 (m, 2H), 7.63−7.58 (m, 1H), 4.50 (bs, 1H), 3.77 (d, J = 12.9 Hz, 2H), 3.53 (bs, 1H), 3.01−2.76 (m, 2H), 1.99 (dd, J = 12.9 and 2.9 Hz, 2H), 1.48 (m, 11H). ^{13}H NMR (75 MHz, $CDCl_3$) δ 155.0, 148.2, 133.7, 131.7, 131.6, 130.8, 124.1, 79.6, 47.0, 44.9, 32.0, 28.3; mp 150−152 °C. Anal. ($C_{16}H_{23}N_3O_6S$), C, H, N.

Synthesis of 1-(2-Nitrophenylsulfonyl)piperidin-4-amine Hydrochloride A{a}. To a solution of **2** (3.41 g, 8.84 mmol) in MeOH (50 mL) was added HCl/MeOH 4 N (50 mL). After stirring at room temperature for 3 h, the solvent was removed to give 2.84 g (100%) of **A{a}** as a white solid. ^{1}H NMR (300 MHz, CD_3OD) δ 8.12−7.97 (m, 4H), 3.96 (d, J = 11.2 Hz, 2H), 3.30 (m, 1H), 2.92 (dt, J = 11.2 and 2.2 Hz, 2H), 2.07 (m, 2H), 1.65 (dd, J = 12.1 and 4.0 Hz, 2H). ^{13}C NMR (75 MHz, CD_3OD) δ 149.4, 135.3, 132.8, 131.9, 131.5, 125.1, 48.6, 45.1, 30.6; mp 246−248 °C. Anal. ($C_{11}H_{15}N_3O_4S\cdot HCl$), C, H, N.

(1-(2-Nitrophenylsulfonyl)piperidin-4-yl)methylamine Fumarate A{b}. To a solution of piperidin-4-ylmethylamine **3** (2.00 g, 17.51 mmol, 1.00 equiv) in toluene (30 mL) was added all at once benzaldehyde (1.78 mL, 17.51 mmol, 1.00 equiv), and the mixture was refluxed under argon with a Dean−Stark trap for 1.5 h. After cooling to 0 °C, NEt_3 (2.81 mL, 20.14 mmol, 1.15 equiv) was added, followed by slow addition of nitrobenzenesulfonyl chloride (4.08 g, 18.39 mmol, 1.05 equiv). The solution was allowed to reach room temperature and stirred overnight. After removal of the solvent, the residue was stirred vigorously with aqueous $KHSO_4$ 1 M (25 mL) for 1.5 h and diluted with brine (25 mL). This aqueous solution was extracted with Et_2O (1 × 50 and 2 × 25 mL) and made strongly basic with NaOH 2 N, followed by extraction with CH_2Cl_2 (1 × 50 and 2 × 25 mL). The combined organic layers were dried (Na_2SO_4), filtered, and concentrated. The residue was dissolved in EtOH (25 mL), treated with a solution of fumaric acid (2.03 g, 17.51 mmol, 1.00 equiv) in EtOH (50 mL), and the resulting mixture was refluxed for 15 min. After cooling, the obtained precipitate was filtered, washed with cold EtOH, and dried. Recrystallization from MeOH gave 6.99 g (96%) of **A{b}** as a white solid. ^{1}H NMR (300 MHz, CD_3OD/DMSO-d_6) δ 7.29 (m, 1H), 7.13 (m, 3H), 5.94 (s, 2H), 3.16 (d, J = 10.3 Hz, 2H), 2.08 (m, 4H), 1.17 (m, 3H), 0.63 (m, 2H). ^{13}H NMR (75 MHz, CD_3OD/DMSO-d_6) δ 170.6, 149.4, 136.0, 135.3, 132.9, 131.4, 131.3, 125.1, 46.4, 44.7, 34.4, 29.8; mp 197−199 °C. Anal. ($C_{12}H_{17}N_3O_4S\cdot C_4H_4O_4$), C, H, N.

Resin 5{a}. BAL resin **4** (3.65 g, 0.79 mmol/g, 1 equiv) was suspended in DMF (3 × 65 mL for 5 min). Following addition of DMF (39 mL), the amine **A{a}** (2.78 g, 8.65 mmol, 3 equiv) and $NaBH(OAc)_3$ (3.06 g, 14.42 mmol, 5 equiv) were added. The reactor was purged with argon, sealed, and agitated on an orbital shaker at room temperature for 46 h. After a negative DNPH test and a positive Kaiser test, the mixture was diluted with MeOH (30 mL), agitated for 5 min, and filtered. The resin was successively washed with MeOH (2 × 65 mL for 5 min), DMF (3 × 65 mL for 5 min), MeOH (3 × 65 mL for 5 min), CH_2Cl_2 (3 × 65 mL for 5 min), and dried overnight to give 4.45 g of resin **5{a}**. The loading level was determined to 0.67 mmol/g (100%) by picric acid assay.

Resin 5{b}. Resin **5{b}** was prepared by following the same procedure described for **5{a}**, using BAL resin **4** (4.00 g, 0.79 mmol/g, 1 equiv), amine **A{b}** (3.94 g, 9.48 mmol, 3 equiv), and $NaBH(OAc)_3$ (3.35 g, 15.80 mmol, 5 equiv) to obtain 4.96 g of resin **5{b}**. The loading level was determined to 0.66 mmol/g (100%) by picric acid assay.

Amide Library 9. Acylation Step (6{a−x} and 6{b−x}). Resin **5{a}** (approximately 100 mg, 0.067 mmol, 1 equiv) or resin **5{b}** (approximately 100 mg, 0.066 mmol, 1 equiv) was distributed into Teflon tubes, swelled in CH_2Cl_2 (3 × 3 mL for 3 min), followed by a 9/1 mixture of CH_2Cl_2/DIEA (3 × 3 mL for 3 min) and a 7/3 mixture of DMF/CH_2Cl_2 (3 × 3 mL for 3 min). A solution of each acid **B{a−x}** (0.402 mmol for resin **5{a}**, 0.396 mmol for resin **5{b}**, 6 equiv) in a 7/3 mixture of DMF/CH_2Cl_2 (1.50 mL) was added to the resin, followed by 1.50 mL of a stock solution containing HBTU (4.03 g, 10.61 mmol) and DIEA (2.06 mL, 12.38 mmol) in a 7/3 mixture of DMF/CH_2Cl_2 (40 mL), and the resulting mixtures were agitated at 25 °C for 18 h (the final concentration of the acid and HBTU is 130 mM). The resins were filtered, washed alternatively with DMF, MeOH, CH_2Cl_2 (5 × (3 mL of DMF for 3 min followed by 3 mL of MeOH for 3 min, followed by 3 mL of CH_2Cl_2 for 3 min)), and CH_2Cl_2 (2 × 3 mL) and air-dried for 10 min.

Deprotection Step (7{a−x} and 7{b−x}). Resins **6{a−x}** and **6{b−x}** were swelled in DMF (2 × 3 mL for 3 min) and 3 mL of a stock solution containing thiophenol 0.5 M, and K_2CO_3 1 M in DMF was added to each resin. The resulting mixtures were agitated at 25 °C for 30 min. Each reaction was diluted with a 1/1 mixture of THF/H_2O (2 mL), agitated for 1 min, and then the resins were filtered, washed with a 1/1 mixture of THF/H_2O (2 × 3 mL for 3 min) and DMF (2 × 3 mL for 3 min). Resins were replaced in 3 mL of a stock solution containing thiophenol 0.5 M and K_2CO_3 1 M in DMF, and the resulting mixtures were agitated at 25 °C for

30 min. Each reaction was diluted with a 1/1 mixture of THF/H_2O (2 mL), agitated for 1 min, and then the resins were filtered, washed successively with a 1/1 mixture of THF/H_2O (2 × 3 mL for 3 min), DMF (4 × 3 mL for 3 min), and CH_2Cl_2 (2 × 3 mL for 3 min) and air-dried for 10 min.

***N*-Alkylation Step (8{a−x} and 8{b−x}).** Resins **7**{**a**−**x**} and **7**{**b**−**x**} were swelled in a 9/1 mixture of DMF/DIEA (3 mL for 3 min), DMF (2 × 3 mL for 3 min), and 2.33 mL of a stock solution containing **C** (2.73 g, 8.84 mmol) and DIEA (2.16 mL, 12.38 mmol) in 57 mL of DMF were added to each resin (the final concentration of the bromoester **C** is 150 mM). The mixtures were agitated at 40 °C for 20 h, cooled to room temperature, filtered, and washed three times sequentially with DMF, MeOH, and CH_2Cl_2 (3 mL of DMF for 2 min, followed by 3 mL of MeOH for 2 min, followed by 3 mL of CH_2Cl_2 for 3 min) and then with DMF (3 × 3 mL for 2 min). Then, 2.33 mL of the **C**/DIEA stock solution were added to each resin. Mixtures were agitated at 40 °C for 20 h, cooled to room temperature, filtered, and washed three times sequentially with DMF, MeOH, and CH_2Cl_2 (3 mL of DMF for 2 min, followed by 3 mL of MeOH for 2 min, followed by 3 mL of CH_2Cl_2 for 3 min), three times sequentially with CH_2Cl_2 and MeOH (3 mL of CH_2Cl_2 for 3 min, followed 3 mL of MeOH for 3 min) and finally with CH_2Cl_2 (3 × 3 mL for 2 min).

Cleavage (9{a−x} and 9{b−x}). Resins **8**{**a**−**x**} and **8**{**b**−**x**} were swelled in CH_2Cl_2 (2 × 3 mL for 3 min) and cleaved with a 1/4 mixture of TFA/CH_2Cl_2 (2 mL) for 2 h at 25 °C. Resins were filtered, and filtrates were concentrated.

Purification by Solid-Phase Extraction (SPE). SPE cartridges (Supelco, DSC-SCX, 1 g/6 mL) were conditioned in MeOH (2 × 3 mL) and charged with the crude products dissolved in MeOH (1 mL). Columns were washed with MeOH (4 × 4 mL), and the final products were eluted with a solution of NH_4OH 2 M in MeOH (2 × 3 mL). Filtrates were concentrated to give amides **9**.

Analytical Control of Library 9. Amides **9** were dissolved in MeOH (1.50 mL, HPLC grade), and 100 μL of this solution were diluted with 900 μL of H_2O milliQ + 0.025% TFA. The solutions were analyzed by HPLC using the standard conditions.

A selection of compounds (**9**{**a**;**a**}, **9**{**a**;**c**}, **9**{**a**;**e**}, **9**{**a**;**h**}, **9**{**a**;**i**}, **9**{**a**;**o**}, **9**{**a**;**q**}, **9**{**a**;**r**}, **9**{**a**;**s**}, **9**{**a**;**v**}, **9**{**b**;**a**}, **9**{**b**;**c**}, **9**{**b**;**e**}, **9**{**b**;**g**}, **9**{**b**;**i**}, **9**{**b**;**j**}, **9**{**b**;**l**}, **9**{**b**;**p**}, **9**{**b**;**q**}, **9**{**b**;**r**}, and **9**{**b**;**w**}) was analyzed by ^{1}H NMR (and ^{13}C NMR for **9**{**a**;**g**} and **9**{**b**;**w**}). Elemental analyses were carried out on pure samples of **9**{**a**;**q**} and **9**{**b**;**w**}. See Supporting Information for full details.

2-[4-({6-[6-Nitro-1,3-dioxo-1*H*-benzo[*de*]isoquinolin-2(3*H*)-yl]hexanoyl}amino)piperidino]ethyl 4-Amino-5-chloro-2-methoxybenzoate 11. To a solution of 4-nitro-1,8-naphthalimido-*N*-caproic acid[27] (500 mg, 1.4 mmol, 1 equiv) in dry DMF (40 mL) and cooled at 0 °C were added 1-hydroxbenzotriazole hydrate (HOBT; 586 mg, 4.3 mmol, 3.1 equiv), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC; 0.665 g, 3.4 mmol, 2.5 equiv), and triethylamine (641 mg, 6.3 mmol, 4.5 equiv). The reaction mixture was stirred at this temperature for 5−10 min, and then 2-(4-aminopiperidin-1-yl)ethyl 4 amino-5-chloro-2-methoxybenzoate hydrochloride **10**[26] (562 mg, 1.4 mol, 1 equiv) was added. The reaction was then stirred at room temperature for 20 h before it was evaporated to dryness. The residue was purified twice by chromatography on silica. Elution first with (CH_2Cl_2/MeOH 90:10) and then with (CH_2Cl_2/*i*PrOH 60:40) gave 80 mg (8.5%) of **11** ($C_{33}H_{36}ClN_5O_8$) as an ochre foam. R_f (CH_2Cl_2/*i*PrOH 60:40) 0.36. ^{1}H NMR (200 MHz) δ 8.69 (m,1H), 8.63 (m,1H), 8.50 (m, 1H), 8.35 (m, 1H), 7.94 (s, 1H), 7.76 (m, 1H), 6.23 (s, 1H), 5.94 (bd, 1H), 4.46 (m, 4H), 4.16 (m, 2H), 3.89 (m, 1H), 3.80 (s, 3H), 3.29 (m, 2H), 3.02 (t, J = 5.4 Hz, 2H), 2.55 (m, 2H), 2.21 (t, J = 7.3 Hz, 2H), 1.95 (m, 2H), 1.76 (m, 6H), 1.43 (m, 2H).

2-[4-({6-[6-Amino-1,3-dioxo-1*H*-benzo[*de*]isoquinolin-2(3*H*)-yl]hexanoyl}amino)piperidino]ethyl 4-Amino-5-chloro-2-methoxybenzoate 9{a;y}. To a solution of **11** (75 mg, 0.113 mmol) in methanol (6 mL) was added Raney nickel. The reaction mixture was stirred under atmospheric pressure at room temperature for 14 h and filtered through celite. The solvent was removed under reduced pressure, and the residue was purified twice by chromatography on silica. Eluting first with (CH_2Cl_2/MeOH 80:20) and second with (CH_2Cl_2/toluene/MeOH 50:30:20) gave 65 mg (91%) of **9**{**a**;**y**} as an orange foam. R_f (CH_2Cl_2/toluene/MeOH 50:30:20) 0.57. ^{1}H NMR (400 MHz, CD_3OD) δ 8.55 (dd, 1H, J = 8.3 Hz and J = 1.2 Hz), 8.46 (dd, 1H, J = 7.3 Hz and J = 1.2 Hz), 8.33 (d, 1H, J = 8.3 Hz), 7.73 (s, 1H), 7.63 (dd, 1H, J = 8.3 Hz and J = 7.4 Hz), 6.98 (d, 1H, J = 8.3 Hz), 6.43 (s, 1H), 4.37 (t, 2H, J = 5.4), 4.10 (t, 2H, J = 7.3 Hz), 3.78 (s, 3H), 3.67 (m, 1H), 3.06 (m, 2H), 2.88 (t, 2H, J = 5.4 Hz), 2.39 (m, 2H), 2.18, (t, 2H, J = 7.2 Hz), 1.83 (m, 2H), 1.65 (m, 4H), 1.54 (m, 2H), 1.39 (m, 2H). ^{13}C NMR (100 MHz, CD_3OD) δ 175.5, 166.4, 166.2, 165.6, 164.2, 162.0, 151.6, 135.3, 134.2, 132.6, 131.3, 130.8, 126.0, 124.7, 123.1, 113.0, 111.3, 110.3, 108.2, 98.7, 62.2, 57.6, 56.2, 53.6, 49.4, 49.2, 49.0, 48.8, 48.6, 48.4, 47.1, 40.8, 36.8, 31.9, 28.8, 27.5, 26.7. ESI m/z 637 (M + H^+), 659 (M + Na); mp 150 °C; Anal. ($C_{33}H_{38}ClN_5O_5 \cdot 3H_2O$), C, H, N.

Bivalent Ligand 13. A solution of **12**[28] (301 mg, 0.25 mmol) in HCl/MeOH 4 N (15 mL) was stirred at room temperature for 30 min, and the solvent was removed to give 285 mg (99%) of **11** as a beige hygroscopic foam. ^{1}H NMR (200 MHz, CD_3OD) δ 7.71 (s, 2H), 7.06 (s, 4H), 6.52 (s, 2H), 4.51 (bs, 4H), 3.76−3.64 (m, 8H), 3.59−3.32 (m, 8H) 2.92 (m, 8H), 2.57 (m, 4H), 2.28 (m, 4H), 2.15−1.65 (m, 20H). ^{13}C NMR (50 MHz, CD_3OD) δ 174.1, 165.7, 161.7, 149.5, 139.7, 134.4, 129.6, 111.8, 109.4, 98.4, 59.9, 56.9, 56.7, 53.6, 45.6, 33.8, 33.1, 30.1, 29.0, 27.6, 23.1. m/z = 984 [M + H]$^+$. Anal. ($C_{50}H_{72}Cl_2N_8O_8 \cdot 4HCl + 9.5H_2O$), C, H, N.

Bivalent Ligand 14. Compound **13** (200 mg, 0.18 mmol, 1 equiv) was treated with a saturated aqueous solution of Na_2CO_3 (5 mL), and the aqueous phase was extracted twice with CH_2Cl_2 (1 × 15 mL and 2 × 10 mL). The two organic phases were pooled, dried (Na_2SO_4), filtered, and concentrated. The resulting foam was dissolved in absolute EtOH (10 mL) and treated with a solution of fumaric acid (82 mg, 0.71 mmol, 4 equiv) in absolute EtOH (10 mL). The solution was stirred 10 min at room temperature, and the solvent was removed. The residue was solubilized in hot EtOH, and a resin was formed upon cooling. The supernatant was removed, and the procedure was repeated. The resin was finally taken in hot EtOH, concentrated, and dried to give 187 mg (73%) of **14** a white foam. ^{1}H NMR (200 MHz, CD_3OD) δ 7.80 (s, 2H), 7.18 (s, 4H), 6.73 (s, 8H), 6.49 (s, 2H), 4.61 (bs, 4H), 4.08−3.77 (m, 8H), 3.66 (m, 4H), 3.51 (m, 4H), 3.30−2.92 (m, 8H), 2.71 (m, 4H), 2.40 (m, 4H), 2.25−1.77 (m, 20H). ^{13}C NMR (50 MHz, CD_3OD) δ 174.2, 170.8, 165.9, 162.0, 151.9, 139.7, 136.2, 134.4, 129.6, 110.5, 107.6, 98.7, 59.8, 56.6, 56.4, 52.8, 45.4, 33.8, 33.1, 29.7, 29.0, 27.2, 23.5. Anal. ($C_{50}H_{72}Cl_2N_8O_8 \cdot 4C_4H_4O_4 + 4.5\ H_2O$), C, H, N.

4-(*N*-*tert*-Butoxycarbonyl(3-phenylprop-2-ynyl)amino)butanoic Acid 16. To a stirred solution of iodobenzene (612 mg, 3.00 mmol, 1.00 equiv), $PdCl_2(PPh_3)_2$ (106 mg, 0.15 mmol, 0.05 equiv), and CuI (56 mg, 0.30 mmol, 0.10 equiv) in a 1/1 mixture of freshly distilled NEt_3 and anhydrous DMF (20 mL) at room temperature was added dropwise a solution of **15**[28] (763 mg, 3.15 mmol, 1.05 equiv) in anhydrous DMF (10 mL). The solution was stirred at 80 °C for 3.5 h and cooled to room temperature. After removal of the solvents, the crude mixture was purified by chromatography on silica gel, using AcOEt/cHex (2/8) + 0.5% AcOH followed by AcOEt/cHex (3/7) + 0.5% AcOH, to give 780 mg (82%) ($C_{18}H_{23}NO_4$) of **16** as a yellow oil. ^{1}H NMR (300 MHz) δ 7.41 (dd, J = 6.7 and 3.0 Hz, 2H), 7.35−7.27 (m, 3H), 4.26 (s, 2H), 3.46 (t, J = 7.2 Hz, 2H), 2.42 (t, J = 7.2 Hz, 2H), 1.97 (m, J = 7.2 Hz, 2H), 1.49 (s, 9H). ^{13}C NMR (75 MHz) δ 176.3, 155.5, 131.8, 128.4, 123.0, 85.1, 80.2, 45.9, 31.3, 28.5, 27.1, 23.4.

4-(*N*-*tert*-Butoxycarbonyl(3-phenylpropyl)amino)butanoic Acid 17. To a stirred solution of **16** (500 mg, 1.58 mmol) in MeOH (15 mL) at room temperature was added Pd/C 10% (47 mg). The mixture was hydrogenated at atmospheric pressure overnight and filtered through a pad of celite. After evaporation of the solvent, a purification by chromatography on silica gel, using CH_2Cl_2 + 0.5% AcOH followed by CH_2Cl_2/MeOH (95/5) + 0.5% AcOH, gave 478 mg (94%) of **17** ($C_{18}H_{27}NO_4$) as a colorless oil. ^{1}H NMR (300 MHz) δ 10.40 (bs, 1H), 7.52 (m, 5H), 3.26 (bs, 4H), 2.62 (t, J = 7.7 Hz, 2H), 2.37 (t, J = 7.2 Hz, 2H), 1.88 (m, 4H), 1.46 (s, 9H). ^{13}C

NMR (75 MHz) δ 177.2, 155.6, 141.8, 128.5, 128.4, 126.0, 79.9, 47.1, 46.3, 33.4, 31.4, 30.2, 28.5, 23.7.

2-(4-(4-(*N*-*tert*-Butoxycarbonyl(3-phenylpropyl)amino)butylamido)piperidin-1-yl)ethyl 4-Amino-5-chloro-2-methoxybenzoate 18. To solution of **17** (506 mg, 1.24 mmol, 1.0 equiv) in dry DMF (10 mL) at room temperature was added **10**[26] (403 mg, 1.24 mmol, 1.0 equiv), HBTU (520 mg, 1.37 mmol, 1.1 equiv), and NEt_3 (694 μL, 4.98 mmol, 4.0 equiv), and the mixture was stirred overnight at room temperature. After solvent removal, the crude product was dissolved in AcOEt (30 mL), washed with saturated aqueous Na_2CO_3 (20 mL), and brine (20 mL). The organic phase was dried (Na_2SO_4) and concentrated. Purification by chromatography on silica gel, using AcOEt/MeOH (90/10) followed by AcOEt/MeOH/NH4OHaq 20% (87/10/3), afforded 540 mg (68%) of **18** as white foam. 1H NMR (300 MHz) δ 7.90 (s, 1H), 7.34 (m, 5H), 6.83 (bs, 1H), 6.39 (s, 1H, H3), 4.61 (bs, 2H), 4.47 (t, J = 5.8 Hz, 2H), 3.93 (m, 4H, 12), 3.33 (m, 4H), 3.07 (m, 2H), 2.89 (t, J = 5.8 Hz, 2H), 2.69 (t, J = 7.7 Hz, 2H), 2.44 (m, 2H), 2.23 (t, J = 6.8 Hz, 2H), 2.10−1.84 (m, 6H), 1.65 (m, 2H), 1.54 (s, 9H). 13C NMR (75 MHz) δ 172.2, 164.6, 160.4, 156.5, 148.0, 141.7, 133.5, 128.5, 128.4, 126.4, 110.1, 109.7, 98.4, 79.8, 62.2, 56.8, 56.2, 52.8, 47.0, 46.4, 45.8, 33.7, 33.4, 32.0, 30.2, 28.6, 24.7. m/z = 632 [M + H]+. Anal. (C33H47N4O6 + 2H2O), C, H, N.

2-(4-(4-(3-Phenylpropylamino)butylamido)piperidin-1-yl)ethyl 4-Amino-5-chloro-2-methoxybenzoate, Fumarate 19. A solution of **18** (495 mg, 0.78 mmol) in MeOH (10 mL) and HCl/MeOH 4N (10 mL) was stirred at room temperature for 30 min, and the solvent was removed to give 467 mg (99%) of a white foam. This foam was treated with a saturated aqueous solution of Na2CO3 (5 mL), and the aqueous phase was extracted with AcOEt (10 mL). The organic phase was washed with brine (5 mL), dried (Na2SO4), filtered, and concentrated. The resulting foam was dissolved in absolute EtOH (10 mL) and treated with a solution of fumaric acid (165 mg, 1.42 mmol, 2 equiv) in absolute EtOH (10 mL). The solution was stirred 10 min at room temperature, and the solvent was removed. The residue was solubilized in hot *i*PrOH, and a resin was formed upon cooling. The supernatant was removed, and the processus was repeated. The resin was finally taken in hot *i*PrOH, concentrated, and dried to give 331 mg (89%) of **19** as a white foam. 1H NMR (300 MHz, CD3OD) δ 7.80 (s, 1H), 7.24 (m, 5H), 6.70 (s, 4H), 6.48 (s, 1H), 4.53 (t, J = 5.0 Hz, 2H), 3.89 (m, 1H), 3.82 (s, 3H), 3.52 (m, 2H), 3.34 (m, 2H), 3.10−2.92 (m, 6H), 2.73 (t, J = 7.6 Hz, 2H), 2.37 (t, J = 6.9 Hz, 2H), 2.15−1.90 (m, 6H), 1.82 (m, 2H). 13C NMR (75 MHz, CD3OD) δ 174.1, 171.4, 166.1, 162.1, 151.9, 141.6, 136.2, 134.4, 129.6, 129.4, 127.4, 110.5, 107.7, 98.6, 60.4, 56.8, 56.3, 53.1, 45.9, 48.3, 33.6, 33.5, 30.3, 29.1, 23.1. m/z = 531 [M + H]+. Anal. (C28H39ClN4O4·2C4H4O4 + H2O), C, H, N.

Biology. Membrane Preparation and Radioligand Binding Assays. C6 glial cells, which were stably transfected with h5-HT4(e) receptor, were grown to confluence and incubated with serum-free medium for 4 h, washed twice with phosphate-buffered-saline (PBS), and centrifuged at 300*g* for 5 min. Pellets were used immediately or stored at −80 °C. The pellet was resuspended in 10 volumes of ice-cold HEPES buffer (50 mM, pH 7.4) and centrifuged at 40000*g* for 20 min at 4 °C. The pellet was then suspended in 15 volumes of HEPES (50 mM, pH 7.4), and protein concentration was determined by the method of Bradford using bovine serum albumin as the standard. Radioligand binding studies were performed in 250 μL of HEPES buffer (50 mM, pH 7.4), 20 μL of the studied ligand (at 10 nM for the screening of the libraries or 7 concentrations for *K*i determination), and 20 μL of [3H]-GR113808 at a concentration of 0.2 nM and 50 μL of membranes preparation (100−200 μg of protein). Nonspecific binding was determined with 10 μM GR113808. Tubes were incubated at 25 °C for 30 min, and the reaction was terminated by filtration through Whatman GF/B Filter paper using the Brandel 48R cell harvester. Filters were presoaked in a 0.1% solution of polyethylenimine, washed with ice-cold buffer (50 mM Tris-HCl, pH 7.4), and placed overnight in 4 mL of ready-safe scintillation cocktail. Radioactivity was measured using a Beckman model LS6500C liquid scintillation counter. Binding data are expressed as a percentage of displacement for the screening of the library compounds. For selected compounds, *K*i were analyzed by computer-assisted nonlinear regression analysis (Prism, Graphpad Software, San Diego, CA). The data are the results of two or three independent determinations performed in triplicate.

cAMP Accumulation. C6 glial cells stably transfected with the h5-HT4(e)R were grown to confluence and incubated with serum-free medium for 4 h before the beginning of the assay. Then, the cells were preincubated for 15 min with serum-free medium supplemented with 5 mM theophylline and 10 μM pargyline. 5-HT (1 μM) and/or compounds (10 nM) were added and incubated for an additional 15 min at 37 °C in 5% CO2. The reaction was stopped by aspiration of the medium and addition of 50 μL of ice-cold perchloric acid (20%). After 30 min, neutralization buffer was added (HEPES 25 mM, KOH 2 N), supernatant was eliminated by 5 min centrifugation at 2000*g* and cAMP was quantified using a radioimmunoassay kit (cAMP competitive radioimmunoassay, Beckman, France). The ligand concentration−effect curves were calculated using seven concentrations (10−10−10−5). EC50 values correspond to the concentration of agonists required to obtain half-maximal stimulation of adenylyl cyclase. The maximum response produced by each molecule was normalized to the 5-HT induced maximum response (*E*max). Values are the means ± SEM of two or three experiments performed in triplicate.

Effects of Agonists on sAPPα Levels in Mice. Adult male C57BL/6j wild-type mice (8 week/old, weighing 23−27 g) from Janvier (Le Genest-Saint-Isle, France) were used in this study. Mice were housed in a room with a 12 h light−dark cycle with food and water ad libitum. They received a single subcutaneous injection of 0.9% NaCl (control), or **12** or **9**{**a**;**y**} at 5 mg/kg dissolved in 0.9% NaCl. Mice were sacrificed 90 min after the injection by cervical dislocation, and their brains were then treated as previously described.[13] Hippocampus and cortex were homogenized by sonication in 150 and 300 μL of 50 mM Tris, pH 7.4, 150 mM NaCl, and proteinase inhibitors (Sigma, France), respectively, and cleared by centrifugation (100000*g*, 45 min, 4 °C). Only the supernatant was used for Western blot experiments (polyclonal antiserum R1736, 1:4000 dilution (a gift from Dr. Selkoe), goat antirabbit immunoglobulin antibody, 1:15000, Amersham Pharmacia Biotech, (Orsay, France). Immunoreactive bands were then visualized with the ECL plus detection kit (Amersham Pharmacia Biotech, Orsay, France) on Kodak ML lights films. The membrane was stripped and blocked again before being probed with an anti-β-actin antibody (monoclonal anti-β-actin, 1:5000 dilution; Abcam, Cambridge, UK), used as internal control. After 1 h incubation with a horseradish peroxidase-linked goat antimouse immunoglobulin antibody (Cell Signaling, Ozyme, Saint-Quentin-en-Yvelines, France) at 1:2000 dilution, immunoreactive bands were visualized with the ECL detection kit (Amersham Pharmacia Biotech, Orsay, France) on Kodak ML lights films. After digitization, densitometric values were quantified by using an image analysis. Statistical analyses were performed using the computer software StatView 4.02 (Abacus Concepts Inc., Berkeley, CA). Data are means ± SEM of sAPPα levels expressed as percentage of control values (mice which received a saline injection). Values were compared between the different groups (7−9 mice/group) by using a one-way analysis of variance (ANOVA) followed by protected least significant difference tests (PLSD). The significance level was set at $p < 0.05$.

Measurement of Inhibitory Activity on Aβ25−35 Polymerization. Initial screening for inhibition was carried out using a reaction mixture containing 80 μL of phosphate buffer (10 mM final concentration), 10 μL of MeOH containing 10 μM final concentration of agonist and 10 μL of Aβ25−35 (100 μM final concentration), pH 7.2. All steps were carried out at 4 °C to prevent Aβ25−35 polymerization. UV−visible spectroscopy was performed on a Cary 300 bio UV−visible spectrophotometer. First, the UV−visible spectra of agonist alone were recorded by using the spectrometer in the range of 190−600 nm at 15 °C. Optimal measurements of Aβ25−35 were then recorded by using the spectrometer in the range of 190−600 nm at 15 °C to control for

any artifacts due to agonist aggregation. Finally, polymerization kinetics were monitored at 200 nm, this wavelength corresponding to the absorption of peptide bonds. Data were collected 6 h after incubation. For each inhibitory experiment, one sample containing Aβ_{25-35} alone and another containing the agonist alone were used in parallel as control in the same experimental conditions. Moreover, to rule out any influence due to agonist absorbance, their UV−visible spectra were subtracted from the Aβ_{25-35} absorption spectra. At least three independent measurements were recorded for all cases. All results are presented with means and standard deviation.

For agonists exhibiting inhibitory activity at least equal to that of curcumin, IC_{50} was calculated by using a least-squares fitting technique to match the experimental data with a sigmoidal curve. IC_{50} was the effective concentration of agonist inhibiting the formation of Aβ fibrils to 50% of the control value.

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Supporting Information Available: Chemistry experimental, spectroscopic data, and results from elemental analyses of all the listed compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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