Design and Synthesis of Specific Probes for Human 5-HT$_4$ Receptor Dimerization Studies

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Recently, human 5-HT$_4$ receptors have been demonstrated to form constitutive dimers in living cells. To evaluate the role of dimerization on the 5-HT$_4$ receptor function, we investigated the conception and the synthesis of bivalent molecules able to influence the dimerization process. Their conception is based on a model of the 5-HT$_4$ receptor dimer derived from protein/protein docking experiments. These bivalent ligands are constituted by two ML10302 units, a specific 5-HT$_4$ ligand, linked through a spacer of different sizes and natures. These synthesized bivalent ligands were evaluated in binding assays and cyclic AMP production on the 5-HT$_{4(e/g)}$ receptor isoform stably transfected in C6 glial cells. Our data showed that bivalent ligands conserved a similar affinity compared to the basal ML10302 unit. Nevertheless, according to the nature and the size of the spacer, the pharmacological profile of ML10302 is more or less conserved. In view of the interest of bivalent ligands for investigating the GPCR dimerization process, these 5-HT$_4$ specific bivalent ligands constitute valuable pharmacological tools for the study of 5-HT$_4$ receptor dimerization.

Introduction

The concept that most G protein coupled receptors (GPCRs) can exist as dimeric entities is now largely accepted. However the reason for this dimerization remains elusive. Although dimerization or oligomerization of GPCR has been proved to participate in receptor function, including agonist affinity, potency and efficacy and G protein specificity, it is difficult to conclude whether dimerization or oligomerization is functionally essential for all GPCRs.[1−5]

Elucidating the role of dimerization in the activation process of GPCR became a new challenge which might lead to develop novel pharmaceutical agents able to promote activation or inhibition of GPCR signaling. Recently, constitutive human 5-HT$_4$ receptor dimers were observed in living cells and membrane preparations of CHO and HEK293 cells as detected by co-immunoprecipitation and BRET experiments. 5-HT$_4$ receptor dimerization was not influenced by the binding of classical 5-HT$_4$ ligands: agonists such as serotonin, prucalopride, ML10302 or antagonists such as GR113808.[6] Considering the ubiquitous involvement of the 5-HT$_4$ receptor in major diseases such as memory disorders, gastrointestinal disorders, hypertension, atrial arrhythmia and dysfunction of the urinary tract, the research of new 5-HT$_4$-related drugs is fundamental.[7] Moreover, recent data have underlined a role of the 5-HT$_4$ receptor in the amyloid precursor protein (APP) metabolism, a key gene involved in Alzheimer's disease (AD).[8] Together with its role in learning and memory, these results suggest that the 5-HT$_4$ receptor may constitute a new pharmacological target for the treatment of AD.[9]

Although many 5-HT$_4$ ligands were synthesized during this past decade, only two drugs indicated for the irritable bowel syndrome became commercially available.[7] It now appears evident that a better knowledge of GPCR signaling pathways as well as the characterization of the physiological role of GPCR dimerization process could open new avenues for the design of efficient drugs.

In this context, bivalent ligands, which were proved to modulate the dimerization process by binding dimeric receptors, may represent valuable pharmacological tools. In a recent study, Bushan et al. demonstrated the importance of bivalent ligands approach in the study of $\delta-\kappa$ opioid receptor heterodimers. Their heterobivalent ligands selective for heterodimeric opioid receptors constituted probes for targeting different tissues.[10,11]

In this paper, we describe the design and the synthesis of specific 5-HT$_4$ bivalent ligands, based on molecular modeling studies and more particularly on receptor−receptor docking experiments. Those bivalent ligands are constituted by two 5-HT$_4$ pharmacophoric parts linked with a spacer. Many spacers were used varying in nature and in size. The pharmacological properties of the synthesized bivalent ligands were evaluated.

Identification of Dimer Interfaces. To understand the functional role of dimerization, and the structural mechanism for cross-talk between receptors in a dimeric complex, we planed to design 5-HT$_4$ bivalent ligands as

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chemical tools. For that we focused first on the identification of the dimer interfaces.

Molecular modeling studies were performed and more particularly protein−protein docking experiments for investigating likely dimer arrangements. Docking was realized from the three-dimensional model of 5-HT$_4$ receptor recently published, based on the crystallized bovine rhodopsin structure,[12] and the specialized software GRAMM (global range molecular matching), which considers the proteins as rigid entities, was used.[13] The procedure implements an exhaustive grid search for the receptor−receptor structure matches. For quantifying possible interactions between two receptors, GRAMM places each monomer in a three-dimensional grid. Then, an exhaustive research in the three-dimensional spaces is realized using translations and rotations. Finally, this software evaluates the surface complementarities, penalizes covering and orders the obtained results.

In this study we have applied the low-resolution docking with a 6.8 Å grid step, using the special mode for helix in privileging the hydrophobic interactions. One of the two monomers was rotated with 10°-angle intervals. For each complex, the 100 lowest-energy matches were sorted from low to high energy and then analyzed visually. The values of the different parameters were determined as quasi optimal after many experiments of validation and based to the results of Vasker et al.[14,15]

From the 100 structures generated by GRAMM with the optimized parameters, two main structures of dimers emerged with 40% of II−IV/II−IV complexes including helices II and IV in their dimer interfaces and 40% of IV−VI/IV−VI complexes including helices IV and VI (Figure 1). So, the two most favorable complexes obtained by this docking procedure both involve helix IV. This result agrees well with results of literature which established that in GPCR A family, helix IV is likely to be involved in receptor−receptor contacts. Different biological experiments have identified this transmembrane domain as potential interfaces for dopamine D$_2$ receptors dimerization,[16] γ-opioid receptors or C5a receptors.[17] The most convincing argument derives from the application of atomic force microscopy to the organization of rhodopsin in its native membrane.[18] Models based on these pictures were inferred to indicate that receptor−receptor links might be provided by contacts between helices IV and V. Moreover, there is some evidence in the literature that similar dimerization motifs in transmembrane domains may be involved in dimerization interfaces. Thus in several mammalian GPCRs, (AGSTP)XXX(GAS) sequence types in transmembrane domains are known to play a key role in receptor dimerization and can be used as a consensus motif to identify potential dimerization sequences.[19] In the 5-HT$_4$ receptor, analysis of the receptor sequence revealed the presence of a A4.43XXXG4.47 motif in helix IV and visualization of our molecular model indicated a correct orientation of this motif to allow receptor−receptor interactions.

Design of Bivalent Ligands. Bivalent ligands should be constituted by two 5-HT$_4$ ligands linked through a spacer. ML10302,[20] a 5-HT$_4$ receptor specific ligand synthesized in our laboratory was selected (Scheme 1). This molecule allows different anchor points for the spacer. For example, a spacer could be introduced on

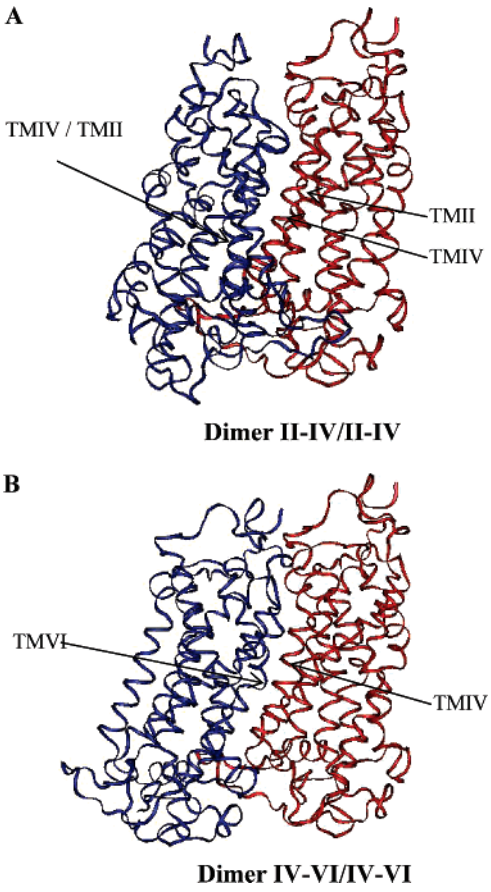
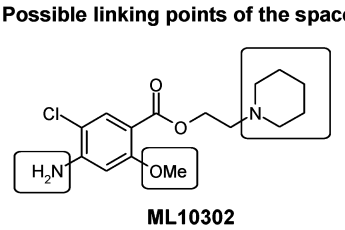


Figure 1. 5-HT$_4$ receptor dimer models obtained by protein−protein docking experiments using the GRAMM software. A: dimer where dimer interfaces included helices II and IV. B: dimer where dimer interfaces included helices IV and VI.

Scheme 1

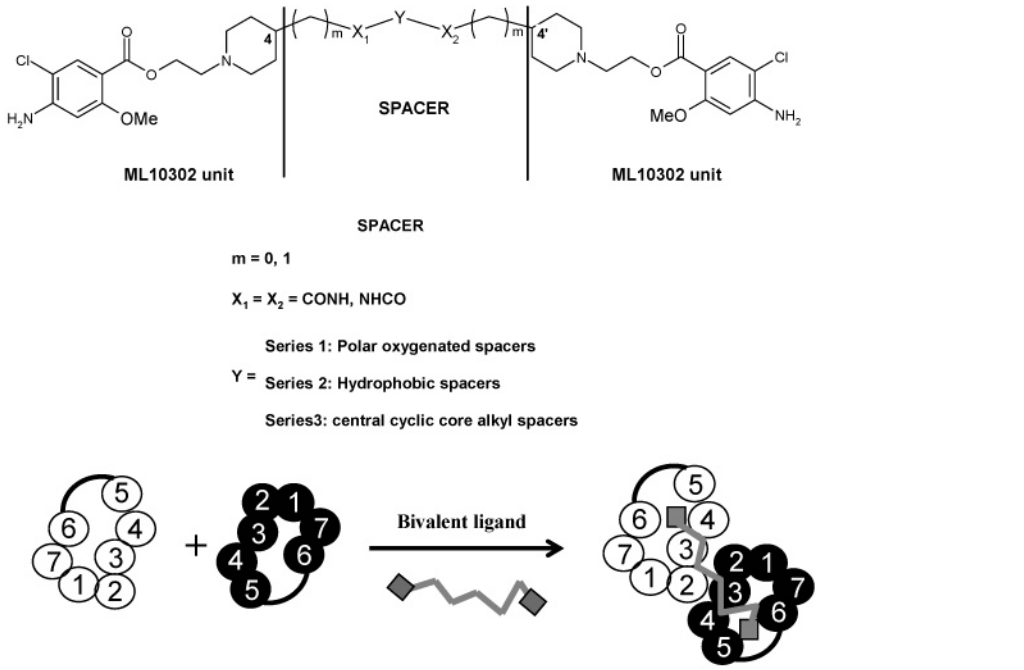


the methoxy group affording an ether bivalent ligand, or on the amino function giving rise to bivalent ligands linked through the anilic position of ML10302. Finally, a link from the 4 position of the piperidine ring of ML10302 can also be afforded.

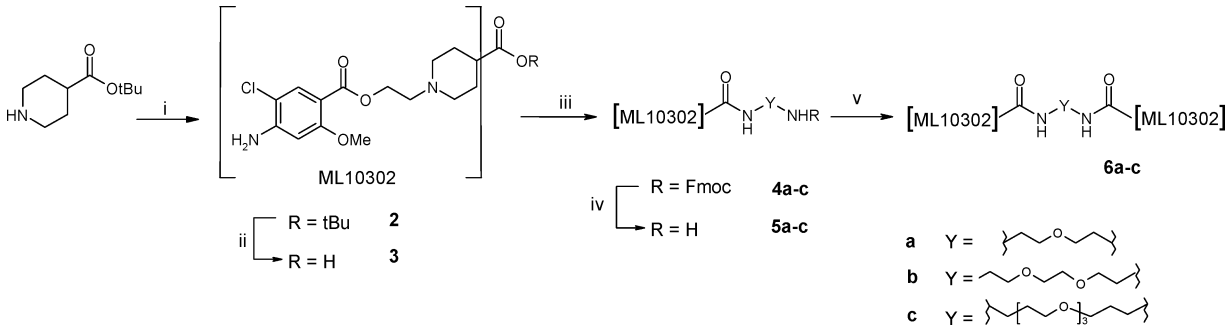
The two complexes suggested by the GRAMM docking procedure can be used to orient the design of bivalent ligands. For example, in the hypothesis of receptor dimers interfaced by helices II and IV, a convenient positioning of bivalent ligands suggests to link ML10302 parts through the 4 position of the piperidine ring of ML10302 (Scheme 2). In contrary, the hypothesis of complexes IV−VI/IV−VI should require bivalent ligands linked through the methoxy or the amino function of the aromatic ring of ML10302.

We first focused on II−IV/II−IV complexes and on bivalent ligands linked through the 4 position of the piperidine ring of ML10302. Previous studies on ML10302 molecules had underlined the possibility of substitution of the basic amino part with a voluminous

Scheme 2



Scheme 3[a]



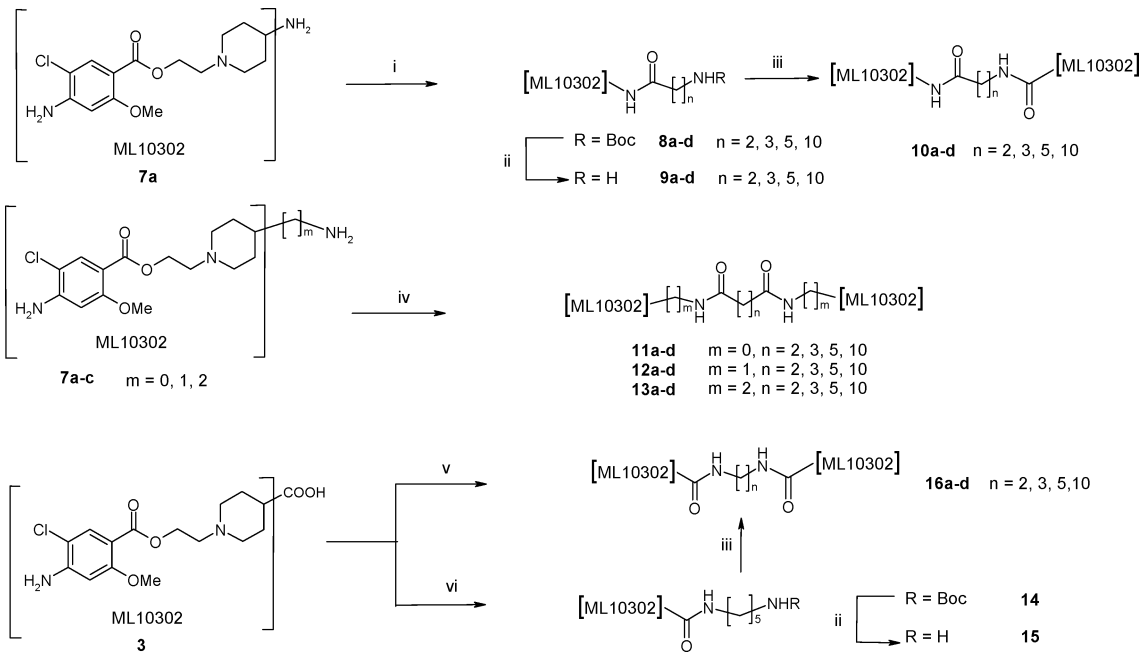
[a] Reagents and conditions: (i) 4-amino-5-chloro-2-methoxybenzoic acid 2-bromoethyl ester,[20] DIEA, DMF, 40−50 °C, 24 h; (ii) CF_3COOH, CH_2Cl_2, rt, 24 h; (iii) EDC, HOBt, NEt_3, CH_2Cl_2, Fmoc-2-(2-aminoethoxy)ethylamine hydrochloride, or Fmoc-1-amino-3,6-dioxa-8-octanamine hydrochloride, or Fmoc-1-amino-4,7,10-trioxa-13-tridecamine hydrochloride, 0 °C to room temperature, 24 h; (iv) 20% piperidine, DMF, 10−15 min, 4 N MeOH/HCl; (v) **3**, EDC, HOBt, NEt_3, CH_2Cl_2, 0 °C to room temperature, 24 h.

group such as fluorescent probes without penalizing the affinity of molecules.[21] These results were supported by the site-directed mutagenesis and molecular modeling results which showed a large pocket between helices III and VI extended to helix VII.[12] Altogether, these observations indicated that the substitution of the spacer on this part of ML10302 should not affect the affinity of bivalent ligands (Scheme 2).

The theoretical II−IV/II−IV complexes obtained were used as templates in order to evaluate the optimal length of the spacer. To identify compounds specifically interacting with receptor dimers, we synthesized bivalent ligands possessing different spacer lengths: short length spacers, insufficient for allowing the two pharmacophoric parts to bind to receptor dimers, longer spacers, allowing this binding via the lipid bilayer and excessively long spacer to permit bridging of receptor dimers. The influence of the chemical nature of the spacers was also examined with polar oxygenated chains, hydrophobic alkyl chains and alkyl chains including a central cyclic core. The spacers were connected through an amidic function to the 4 position of the piperidine ring of ML10302. To introduce some flexibility at this level, the amidic function was first introduced directly on the piperidine ring and then through one and two methylene groups. Taken as a whole, three different series of bivalent ligands were synthesized according to the nature of the spacer: (i) a polar oxygenated spacer series (compounds **6a**−**c**); (ii) a hydrophobic spacer series (compounds **10a**−**d**, **11a**−**d**, **12a**−**d**, **13a**−**d**, **16a**−**d** and **18a**−**b**); (iii) a central cyclic core alkyl spacer series (compounds **23**, **24**, **27** and **32**). In hydrophobic spacer series, two ligands possessing only one pharmacophore head linked to capped spacers were also synthesized, for comparison with bivalent ligands.

Synthesis of Bivalent Ligands. Molecules **6a**−**c** were prepared as described in Scheme 3. In the first step, condensation of piperidine-4-carboxylic acid *tert*-butyl ester with 4-amino-5-chloro-2-methoxy-benzoic acid 2-bromoethyl ester[20] in the presence of diisopropylethylamine at 40−50 °C in dry DMF afforded **2**. The acidic hydrolysis of **2** with excess of CF_3COOH in dry CH_2Cl_2 gave the acid **3** with a good yield. Coupling of **3** with Fmoc-2-(2-aminoethoxy)-ethylamine hydrochloride or Fmoc-1-amino-3,6-dioxa-8-octanamine or Fmoc-1-

Scheme 4[a]



[a] Reagents and conditions: (i) HOOC(CH$_2$)$_n$NHBoc, EDC, HOBt, NEt$_3$, CH$_2$Cl$_2$, 0 °C to room temperature, 24 h; (ii) 4 N MeOH/HCl, rt, 3−5 h; (iii) **3**, EDC, HOBt, NEt$_3$, CH$_2$Cl$_2$, 0 °C to room temperature, 24 h; (iv) HOOC(CH$_2$)$_n$COOH, EDC, HOBt, NEt$_3$, CH$_2$Cl$_2$, 0 °C to room temperature, 24 h; (v) NH$_2$(CH$_2$)$_n$NH$_2$, EDC, HOBt, NEt$_3$, CH$_2$Cl$_2$, 0 °C to room temperature, 24 h; (vi) NH$_2$(CH$_2$)$_5$NHBoc, EDC, HOBt, NEt$_3$, CH$_2$Cl$_2$, 0 °C to room temperature, 24 h.

amino-4,7,10-trioxa-13-tridecamine hydrochloride using a general procedure (EDC, HOBt, and NEt$_3$ in dry CH$_2$Cl$_2$) provided respectively **4a**−**c** with moderate yields. Removal of the Fmoc protecting group using piperidine 20% in DMF and subsequent treatment with MeOH/HCl gave compounds **5a**−**c.** Compounds **5a**−**c** were then coupled to **3** by the general coupling reaction as previously described to yield dimers **6a**−**c**.

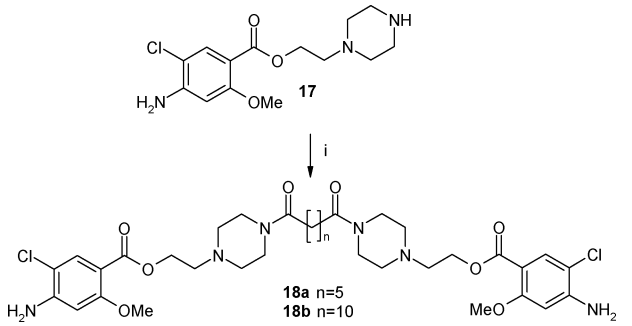
Synthesis of molecules of hydrophobic spacer series **10a**−**d**, **11a**−**d**, **12a**−**d**, **13a**−**d** and **16a**−**d**, is described in Scheme 4. The synthesized molecules differ in the length of the alkyl spacer *n*, in the direction of the amidic function (CONH or NHCO) and in the distance between the ML10302 part and the amidic function (*m*), constituting five groups of hydrophobic bivalent ligands.

For the group which possesses the NHCO- - - -NHCO motif, directly fixed on the ML10302 pharmacophoric part, the bivalent molecules **10a**−**d** were synthesized from compound **7a**[21] by a first coupling reaction with appropriate N-Boc protected amino acids activated with EDC, HOBt as previously described, and, after deprotection of the obtained compounds **8a**−**d**, a second coupling reaction between the resulted compounds **9a**−**d** and molecule **3**.

For the group which possesses the NHCO- - - -OCNH motif fixed on the ML10302 pharmacophoric part directly or through a chain of one and two carbons respectively, bivalent molecules **11a**−**d**, **12a**−**d**, **13a**−**d** were yielded by direct coupling of **7a**−**c**[21] with suitable acids.

Bivalent ligands **16a**−**b, d**, which possess the CONH- - - -HNCO motif directly fixed on ML10302 pharmacophore, were obtained by direct coupling of **3** according to the general procedure with the suitable diamine. This procedure applied to the synthesis of compound **16c** gave no product, for this reason this compound was obtained after two steps. In a first step

Scheme 5[a]



[a] Reagents and conditions: (i) HOOC(CH)$_n$COOH, EDC, HOBt, NEt$_3$,CH$_2$Cl$_2$, 0 °C to room temperature, 24 h.

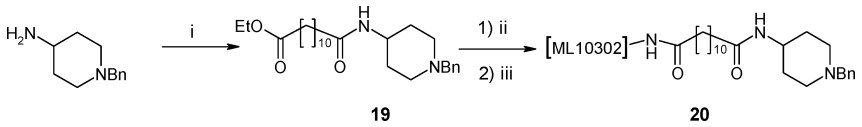
coupling of **3** with *tert*-butyl *N*-(6-aminohexyl)carbamate using the general procedure afforded compound **14**, followed by a classical deprotection with MeOH/HCl and coupling of the resulted compound **15** with **3** to afford the bivalent ligand **16c**.

The two bivalent ligands **18a**,**b**, possessing a piperazine ring instead of a piperidine ring were synthesized according to Scheme 5, using appropriate diacides by the same way.

For comparison of activities of the bivalents ligands, compound **20**, an analogue of compound **11d**, swapping one ML10302 unit for a benzylpiperidine group was synthesized (Scheme 6). Coupling of the commercially available 1-benzyl-piperidine-4-ylamine with dodecanedioic acid monoethyl ester[22] using the general coupling conditions gave **19** in a moderate yield. The ester **19** was saponified with LiOH in H$_2$O/dioxane and used without purification in a second coupling step with **7a**[21] using the general coupling protocol except that CH$_2$Cl$_2$ was replaced by anhydrous DMF as solvent.

In the third series, four molecules were synthesized. The bivalent ligand **23** containing a benzyl core in the

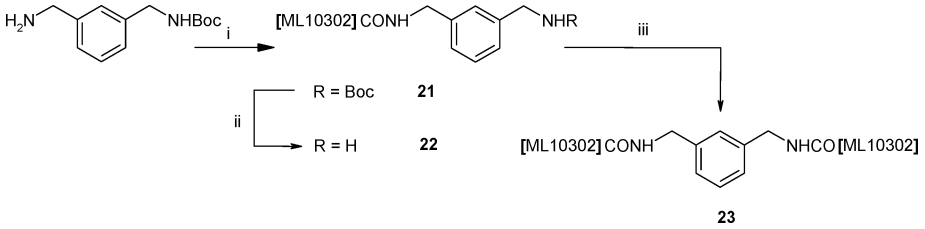
Scheme 6[a]



19 **20**

[a] Reagents and conditions: (i) $HOOC(CH_2)_{10}COOEt$,[22] EDC, HOBt, NEt_3, CH_2Cl_2, rt, 5 h; (ii) LiOH, H_2O/dioxane 20:80, rt, 7 h; (iii) **7a**,[21] EDC, HOBt, NEt_3, DMF, rt, 8 h.

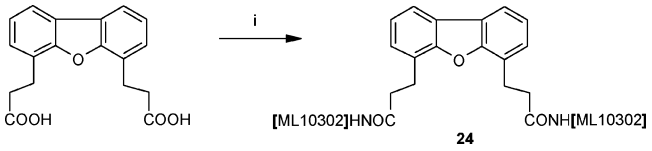
Scheme 7[a]



21 **22** **23**

[a] Reagents and conditions: (i) **3**, EDC, HOBt, NEt3, CH_2Cl_2, 0 °C to room temperature, 24 h; (ii) 4 N MeOH/HCl,2 h; (iii) **3**, EDC, HOBt, NEt_3, CH_2Cl_2/DMF, 0 °C to room temperature, 24 h.

Scheme 8[a]



24

[a] Reagents and conditions: (i) **7a**,[21] EDC, HOBt, NEt_3, CH_2Cl_2/DMF, 0 °C to room temperature, 24 h.

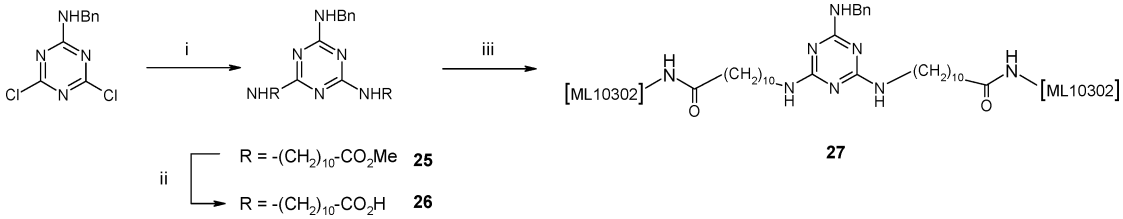
middle of the spacer was synthesized from the 3-(Boc-aminomethyl)-benzylamine hydrochloride as described in Scheme 7. A first coupling step with **3** using the general procedure afforded compound **21**. Classical removal of the Boc protecting group with HCl/MeOH gave the hydrochloride amine salt **22** which was further coupled with **3** according to the general procedure.

The preparation of the bivalent ligand **24**, outlined in Scheme 8, resulted from the coupling reaction (general procedure) of 3-[6-(2-carboxy-ethyl)-dibenzofuran-4-yl]-propionic acid[23] with **7a**.[21]

Compound **27** was obtained via the route depicted in Scheme 9. Reaction of benzyl-(4,6-dichloro-[1,3,5]triazin-2-yl)-amine[24] with a slight excess of 11-amino-undecanoic acid methyl ester[25] in refluxing DMF in the presence of DIEA afforded diester **25** with a good yield. Saponification of the resulting compound with NaOH in H_2O/dioxane and acidification with concentrated HCl gave the desired diacid **26**. Coupling of compound **26** with **7a** using the general procedure provided the bivalent ligand **27** with a good yield.

Finally the bivalent ligand **32** was synthesized as shown in Scheme 10 from the 1-Boc-4-aminomethyl-piperidine hydrochloride. The general coupling reaction with Z-Gly-OH yielded compound **28** which was further hydrogenated affording **29**. The alkylation by methyl 11-bromo-undecanoic acid methyl ester in the presence of Cs_2CO_3 in DMF gave **30**, with a moderate yield. The methyl ester was then hydrolyzed with lithium hydroxide, and the resulting acid was coupled with **7a**[21] according to the general procedure to yield the bivalent

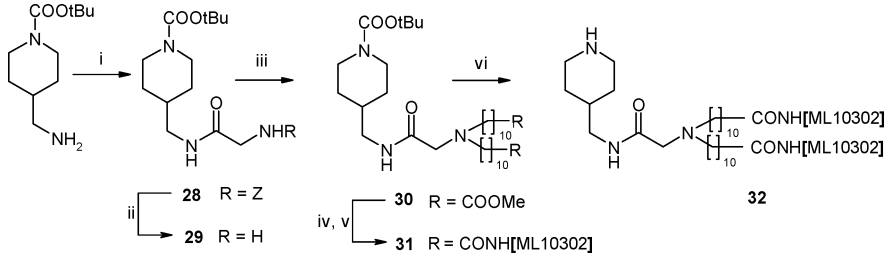
Scheme 9[a]



25 **26** **27**

[a] Reagents and conditions: (i) $NH_2(CH_2)_{10}COOMe$,[25] DIEA, CH_3CN, reflux, 24 h; (ii) NaOH, H_2O/dioxane 8/2, rt, 3 h, then reflux 1 h; (iii) **7a**,[21] EDC, HOBt, NEt_3, DMF, 0 °C to room temperature, 24 h.

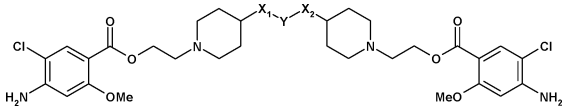
Scheme 10[a]



28 **29** **30** **31** **32**

[a] Reagents and conditions: (i) Z-Gly-OH, EDC, HOBt, NEt_3, CH_2Cl_2, 0 °C to room temperature, 24 h; (ii) Pd/C, H_2, MeOH, rt, 3 h; (iii) Br-$(CH_2)_{10}$-COOMe, Cs_2CO_3, DMF, rt, 48 h; (iv) LiOH, THF/H_2O, rt, 7 h; (v) EDC, HOBt, NEt_3, **7a**,[21] DMF, 0 °C to room temperature, 24 h; (vi) HCl/MeOH 4N, rt, 3 h.

Table 1



no.	X_1	Y	X_2	spacer size (atom number)	K_i (nM)	cAMP (%)
ML10302					5 ± 2.5	45
6a	CONH	$(CH_2)_2O(CH_2)_2$	NHCO	9	19 ± 9.4	0
6b	CONH	$(CH_2)_2O(CH_2)_2O(CH_2)_2$	NHCO	12	3 ± 2.6	3
6c	CONH	$CH_2(CH_2CH_2O)_3CH_2$	NHCO	15	6 ± 1.8	0
10a	NHCO	$(CH_2)_2$	NHCO	6	24 ± 11.6	10
10b	NHCO	$(CH_2)_3$	NHCO	7	31 ± 6.9	42
10c	NHCO	$(CH_2)_5$	NHCO	9	7 ± 3.2	46
10d	NHCO	$(CH_2)_{10}$	NHCO	14	20 ± 12.7	5
11a	NHCO	$(CH_2)_2$	CONH	6	21 ± 1,4	47
11b	NHCO	$(CH_2)_3$	CONH	7	5 ± 0,8	32
11c	NHCO	$(CH_2)_5$	CONH	9	9 ± 3,1	26
11d	NHCO	$(CH_2)_{10}$	CONH	14	16 ± 0,5	20
16a	CONH	$(CH_2)_2$	NHCO	6	37 ± 12.6	3
16b	CONH	$(CH_2)_3$	NHCO	7	13 ± 4.1	13
16c	CONH	$(CH_2)_5$	NHCO	9	5 ± 3.5	5
16d	CONH	$(CH_2)_{10}$	NHCO	14	8 ± 1.7	8

ligand **31** which was classically deprotected to afford the bivalent ligand **32**.

Biological Results and Discussion

All compounds were evaluated on C6 glial cells stably transfected with the human 5-HT$_{4(e/g)}$ receptor isoform. For some ligands, the pharmacological evaluation was completed by studies on CHO cell lines transfected with the same 5-HT$_{4(e/g)}$ receptor isoform. The affinities on the 5-HT$_4$ bivalent ligands were determined in binding studies using the 5-HT$_4$ receptor antagonist [^{3}H]-GR113808, and their functional properties were investigated by measuring their ability to regulate cAMP production.

The interest of mono-oxygenated spacer (compound **6a**), or poly-oxygenated spacer (compounds **6b**,**c**) was to introduce a hydrophilic character at the spacer chain level. These compounds showed good affinities (Table 1), more particularly compounds **6b**,**c** with nanomolar K_i close to that of ML10302 (K_i = 5 nM). However in contrast to ML10302, compounds **6b**−**c** shared a clear antagonist profile. Compared to analogous more hydrophobic compounds **16c**,**d** which possess equivalent spacer length chains (Table 1), no difference appeared on both the affinity and the activity of bivalent ligands. Compounds **10a**−**d**, **11a**−**d** and **16a**−**d** have their spacer chain attached to the pharmacophoric ML10302 part, directly through NHCO or CONH functions. In this set of compounds the spacer was a polyalkyl chain of variable size from 2 to 10 methylene units. All these compounds have good nanomolar affinities (Table 1), with a slight superiority for a spacer size of 9 atoms. However we observed differences in their activity profiles. Thus, the partial agonist tendency of ML10302 is more or less conserved when the two functional groups through which the 4 position of piperidine ring is attached to the spacer are NHCO (compounds **11a**−**d**), since an antagonist profile is favored when these two functional groups are CONH (compounds **16a**−**d**). A mixed situation occurred when the attachment groups are NHCO and CONH (compounds **10a**−**d**). In this case compounds **10b** and **10c** behave as partial agonists since **10d** is an antagonist with K_b = 7 nM (Figures 2 and 3).

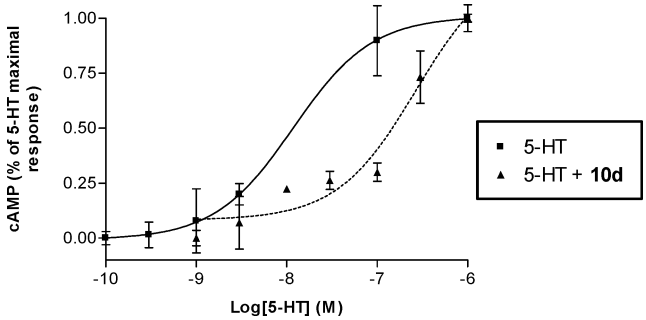


Figure 2. Concentration-effect curves for 5-HT on adenylyl cyclase activity in C6 glial cells stably transfected with the 5-HT$_{4(e/g)}$ receptor isoform: ■, 5-HT alone; ▲, 5-HT + **10d** (200 nM).

This phenomenon is confirmed by the observation that compounds **9d** (K_i = 13 nM, cAMP = 50%) and **20** (K_i = 14 nM, and cAMP = 35%) which both contain only one pharmacophoric part with the 4 position of the piperidine ring substituted with a chain through a NHCO function, conserve the partial agonist profile of ML10302 (Figure 3A). Taken into account that a partial agonist can behave as an antagonist in a different cellular system, the pharmacological profiles were also evaluated on CHO cells and similar results were obtained (Figure 3B). It was also interesting to examine if the flexibility at the attachment point level between the spacer chain and the piperidine ring plays a sensitive role on the biological properties of the bivalent ligands. For that, we introduced one (compounds **12a**−**d**) or two (compounds **13a**−**d**) methylene units between the attachment function NHCO and the piperidine ring. All those compounds showed nanomolar affinities, and we did not find any differences in their biological activity (Table 2). These results indicate that for up to 18 atoms the size of a polyalkyl spacer could not clearly differentiate the biological properties of bivalent ligands. Previous results have shown that replacement of piperidine ring with piperazine led to 5-HT$_4$ ligands with antagonist profile.[26] Piperazine ring is interesting since it allows easy derivatization of the ring in the 4 position. However, in contrast to the piperidine-based ligands, the synthesized bivalents compounds **18a**,**b** showed

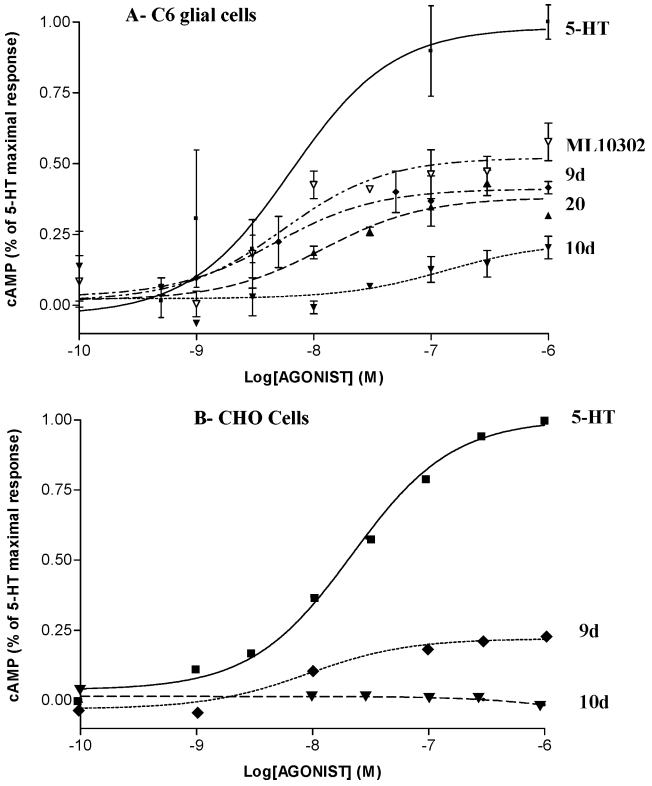
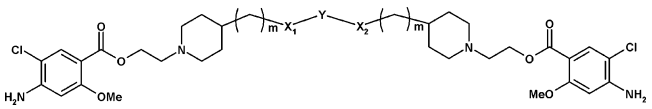


Figure 3. Concentration-effect curves for ligands on adenylyl cyclase activity. A: In C6 glial cells stably transfected with the h5-$HT_{4(e/g)}$ receptor isoform: ■, 5-HT; ▽, ML10302; ◆, **9d**; ▲, **20**; ▼, **10d**. B: In CHO cells stably transfected with the h5-$HT_{4(e/g)}$ receptor isoform: ■, 5-HT; ◆, **9d**; ▼, **10d**.

Table 2



no.	*m*	X_1	Y	X_2	spacer size (atom number)	K_i (nM)	cAMP (%)
11a	0	NHCO	$(CH_2)_2$	CONH	6	21 ± 1.4	47
11b	0	NHCO	$(CH_2)_3$	CONH	7	5 ± 2.8	32
11c	0	NHCO	$(CH_2)_5$	CONH	9	9 ± 3.1	26
11d	0	NHCO	$(CH_2)_{10}$	CONH	14	16 ± 9.3	20
12a	1	NHCO	$(CH_2)_2$	CONH	8	7 ± 2.3	16
12b	1	NHCO	$(CH_2)_3$	CONH	9	13 ± 6.5	20
12c	1	NHCO	$(CH_2)_5$	CONH	11	26 ± 15.7	20
12d	1	NHCO	$(CH_2)_{10}$	CONH	16	22 ± 13.1	21
13a	2	NHCO	$(CH_2)_2$	CONH	10	5 ± 2.4	15
13b	2	NHCO	$(CH_2)_3$	CONH	11	3 ± 3.2	24
13c	2	NHCO	$(CH_2)_5$	CONH	13	11 ± 3.4	31
13d	2	NHCO	$(CH_2)_{10}$	CONH	18	9 ± 4.0	6

weak affinities (Table 3). Finally, to introduce more important modulations of the spacer size up to 29 atoms, molecules **23**, **24**, **27** and **32** were designed and synthesized. Independently of the chemical structure of the central core of these molecules, results of Table 3 indicate that increasing spacer size from 9 atoms (compound **23**) to 29 atoms (compound **27**) is detrimental to affinity, since no correlation of their activities can be established. Taken together, these data show that, compared to ML10302, the bivalent piperidine-based ligands have generally good nanomolar affinities for the 5-HT_4 receptor except for those with large spacer sizes (15 or more atoms). However, these compounds have different activity profiles. So, compounds **11a**−**d** having a polyalkyl chain attached symmetrically by a NHCO function to the pharmacophoric parts behave like ML10302 as partial agonists, since their analogues attached symmetrically by a CONH function (compounds **16a**−**d**), and the oxygenated spacer-containing compounds **6a**−**c** behave clearly as antagonists.

In conclusion, since none of the bivalent ligands displayed a higher affinity than ML10302,[10] these results do not provide clear information about the possibility for any bivalent ligand to bind its two pharmacophoric parts to the 5-HT_4 receptor in its dimeric form. It would now be very interesting to evaluate these ligands on more pertinent biological models including notably BRET experiments. Taken into account that classical monovalent ligands of the 5-HT_4 receptor do not influence the receptor dimerization process,[6] these bivalent ligands could provide new useful information concerning the structure of 5-HT_4 receptor dimers. Co-immunoprecipitation and BRET experiments have revealed the presence of constitutive dimers but the monomer−dimer proportion is not clear.[6] The binding of bivalent ligands to dimeric receptors could enhance the dimer proportion which would be revealed by BRET signal modification.

Directed mutagenesis studies are also in progress in order to identity the dimer interfaces. The results of these studies and of BRET evaluation of our ligands will orient the conception and the synthesis of other bivalent ligands for example by linking the ML10302 parts through its methoxy or amino group. Moreover, the study of heterodimers has revealed the possible dimerization process between adrenergic receptors and 5-HT_4 receptors. In this context, the synthesis of bivalent ligands including adrenergic and 5-HT_4 pharmacophoric parts is also envisaged.

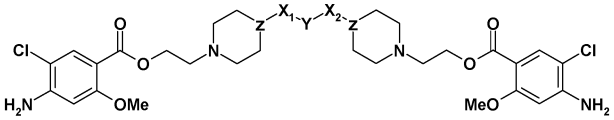
Conclusion

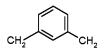
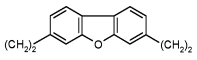
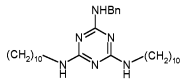
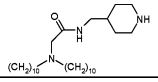
To understand the functional importance of the 5-HT_4 receptor dimerization process, we synthesized a family of bivalent ligands possessing two 5-HT_4 pharmacophoric parts linked through a spacer to the amino basic function. Their conception was based on the molecular modeling of the 5-HT_4 receptor dimer from our published model of the monomer receptor using protein/protein docking experiments. In this context, the basal monomer was ML10302, and several spacers differing in their nature and in their size were chosen and linked by an amidic function to the fourth position of the piperidine ring of ML10302. Three series of bivalent ligands were then defined, synthesized and pharmacologically evaluated. However, no synthesized bivalent ligand showed biological properties which could reflect a specific interaction with a receptor dimer, which would mean binding of each ML10302 part in a distinct monomer of receptor dimers. The evaluation of the synthesized bivalent ligands in BRET experiments is now envisaged.

Experimental Section

Chemistry. Melting points were determined on a Kofler melting point apparatus. NMR spectra were performed on a Bruker AMX 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), doublet (d), triplet (t), multiplet (m), quintet (q), broad doublet

Table 3



N°	Z	X_1	Y	X_2	Spacer Size (Atom number)	Ki (nM)	cAMP (%)
18a	N	CO	$(CH_2)_5$	CO	7	488 ± 245	nd
18b	N	CO	$(CH_2)_{10}$	CO	12	200 ± 60	nd
23	C	CONH		NHCO	9	5 ± 1.2	16
24	C	NHCO		CONH	15	47 ± 17.5	23
27	C	NHCO		CONH	31	113 ± 85	12
32	C	NHCO		CONH	25	50 ± 32	10

(bd), broad multiplet (bm), broad triplet (bt) and broad singlet (bs). 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 otherwise stated. Mass spectra were obtained using a Bruker Esquire electrospray ionization apparatus.

Materials. DMF distilled from BaO, CH_2Cl_2 distilled from calcium hydride, and usual solvents were purchased from SDS (Paris, 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. Boc-*β*-Ala-OH, Boc-4-aminobutyric acid, Boc-6-aminocaproic acid, Boc-11-aminoundecanoic acid, 3-(Boc-aminomethyl)-benzylamine hydrochloride, 1-Boc-4-aminomethylpiperidine hydrochloride, succinic acid, glutaric acid, pimelic acid, dodecanedioïc acid, Fmoc-2-(2-aminoethoxy)-ethylamine hydrochloride, Fmoc-1-amino-3,6-dioxa-3-octanamine, Fmoc-1-amino-4,7,10-trioxa-13-tridecamine hydrochloride, ethylenediamine, 1,3-diaminopropane, *tert*-butyl *N*-(6-aminohexyl)-carbamate, 4-amino-1-benzylpiperidine dihydrochloride and 1,10-diaminodecane and methyl 11-bromodecane were purchased from commercial sources. *tert*-Butyl 4-piperidine carboxylate,[27] dodecanedioic acid monoethyl ester,[22] *N*-benzyl-4,6-dichloro-1,3,5-triazin-2-amine,[24] methyl 11-aminododecanoate[25] and 3-[6- (3-hydroxy-3-oxopropyl)dibenzo[*b*,*d*]furan-4-yl]propanoic acid[23] were prepared according to methods reported in the literature. 4-Amino-5-chloro-2-methoxybenzoic acid 2-bromoethyl ester[20] and compounds **7a**−**c**[21] and **17**[26] were synthesized as previously described.

Representative Procedure for the Synthesis of Bivalent Compounds. Preparation of 2-{4-[11-(9*H*-Fluoren-9-yl)-9-oxo-5,10-dioxa-2,8-diazaundec-1-anoyl]ethylpiperidino-4-amino-5-chloro-2-methoxybenzoate (4a). To a solution of the trifluoroacetate salt of **3** (0.4 g, 0.85 mmol, 1 equiv) in dry CH_2Cl_2 (60−70 mL) and cooled at 0 °C were added HOBt (0.22 g, 1.66 mmol, 1.95 equiv), EDC (0.21 g, 85 mmol, 1.3 equiv) and NEt_3 (0.30 g, 2.97 mmol, 3.5 equiv). The reaction mixture was stirred at this temperature for 5−10 min, and then Fmoc-2-(2-aminoethoxy)-ethylamine hydrochloride (0.31 g, 0.11 mmol, 1 equiv) was added. After maintaining the mixture for 24 h at room temperature, the organic layer was washed with 10% aqueous $KHSO_4$ (25 mL), saturated $NaHCO_3$ (25 mL) and brine (25 mL) and dried ($MgSO_4$). A gum which separated from the CH_2Cl_2 layer when washing with $KHSO_4$ was immediately taken up in MeOH, and the methanolic solution was dried ($MgSO_4$). This methanolic solution was added to the dried CH_2Cl_2 organic phase. After concentration of the combined organic layers, the residue was purified by chromatography (CH_2Cl_2/MeOH 90:10) to afford 0.31 g (55%) of **4a** as a resinous amber solid. R_f (CH_2Cl_2/MeOH 90:10) 0.30; ^{1}H NMR (200 MHz): δ 7.80 (s, 1H), 7.75 (m, 2H), 7.58 (m, 2H), 7.40 (m, 2H), 7.32 (m, 2H), 6.25 (s, 1H), 5.88 (bs, 1H), 5.11 (bs, 1H), 4.41 (m, 4H), 4.33 (t, J = 5.9 Hz, 2H), 4.22 (t, J = 6.07 Hz, 1H), 3.82 (s, 3H), 3.60−3.25 (m, 8H), 3.00 (m, 2H), 2.70 (t, J = 5.9 Hz, 2H), 2.19−1.99 (m, 3H), 1.88−1.58 (m, 4H). Anal. ($C_{35}H_{41}ClN_4O_7 \cdot 0.75H_2O$), C, H, N.

Biology. Membrane Preparation and Radioligand Binding Assays. Briefly, C6 glial cells stably transfected with h5-HT$_{4(e)}$ receptors, grown to confluence, were incubated with serum-free medium for 4 h, washed twice with phosphate-buffered-saline (PBS) and centrifuged at 300*g* for 5 min. The pellet was 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 40 000*g* for 20 min at 4°C. The resulting pellet was resuspended in 15 volumes of HEPES buffer (50 mM, pH 7.4). The protein concentration was determined by the method of Bradford using bovine serum albumin as the standard.

Radioligand binding studies were performed in 500 μL of HEPES buffer (50 mM, pH 7.4), 20 μL of the studied ligand (7 concentrations), 20 μL of [^{3}H]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. Filters were subsequently 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 LS 6500C liquid scintillation counter. Binding data (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 determinations in triplicate.

Measurement of cAMP. C6 glial cells stably transfected with human 5-HT$_{4(e)}$ receptors 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 were added and incubated for an additional 15 min at 37 °C in 5% CO_2. The reaction was stopped by aspiration of the medium and addition of 50 μL of ice-cold perchloric acid (20%). After a 30 min period, neutralization buffer was added (HEPES 25 mM, KOH 2 N), supernatant was extracted after centrifugation at 2000*g* for 5 min and cAMP was quantified using radioimmu-

noassay kit (cAMP competitive radioimmunoassay, Beckman, France). The 5-HT concentration-effect curve was calculated using seven concentrations (10^{-10}−10^{-5}) alone or in the presence of compounds. The ligand concentration-effect curves were calculated using seven concentrations (10^{-10}−10^{-5}).

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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