# Synthesis of Specific Bivalent Probes That Functionally Interact with 5-HT$_4$ Receptor Dimers

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G-protein-coupled receptor dimerization directs the design of new drugs that specifically bind to receptor dimers. Here, we generated a targeted series of homobivalent ligands for serotonin 5-HT$_4$ receptor (5-HT$_4$R) dimers composed of two 5-HT$_4$R-specific ML10302 units linked by a spacer. The design of spacers was assisted by molecular modeling using our previously described 5-HT$_4$R dimer model. Their syntheses were based on Sonogashira−Linstrumelle coupling methods. All compounds retained high-affinity binding to 5-HT$_4$R but lost the agonistic character of the monomeric ML10302 compound. Direct evidence for the functional interaction of both pharmacophores of bivalent ligands with the 5-HT$_4$R was obtained using a bioluminescence resonance energy transfer (BRET) based assay that monitors conformational changes within 5-HT$_4$R dimers. Whereas the monovalent ML10302 was inactive in this assay, several bivalent derivatives dose-dependently increased the BRET signal, indicating that both pharmacophores functionally interact with the 5-HT$_4$R dimer. These bivalent ligands may serve as a new basis for the synthesis of potential drugs for 5-HT$_4$R-associated disorders.

## Introduction

In the past 2 decades, a large body of evidence has led to the reconsideration of the classical dogma of monomeric G-protein-coupled receptor (GPCR) entities. It is now well accepted that GPCRs can form homo- and/or heterodimers in cell membranes and that dimer formation may influence receptor function.[1−7] Several studies have shown the role of dimerization in the activation process of therapeutically relevant GPCRs, demonstrating the crucial importance of elucidating this phenomenon to develop novel pharmaceutical entities.[8] For this purpose, one of the obvious ways to create new tools is the design of specific bivalent ligands. This strategy has been successfully used for some GPCRs and has led to interesting results.[9−11] For instance, chimeric agonists comprising a somatostatin pharmacophore targeting somatostatin SST5 receptors and a dopamine pharmacophore targeting dopamine D2 receptors have been shown to enhance the potency of each pharmacophore separately. The physiological consequences are a suppression of growth hormone and prolactin secretion in human pituitary somatotroph adenoma cells.[12] Furthermore, bivalent ligands composed of an adenosine A1 and A3 receptor agonist linked by a spacer have been shown to activate both receptors. This coactivation induces a greater protection against myocardial ischemia than activation of each receptor individually.[13]

Serotonin 5-HT$_4$ receptors (5-HT$_4$R) belong to the GPCR superfamily and have been identified as a valuable target to treat gastrointestinal diseases.[14] Moreover, recent studies have outlined the role of 5-HT$_4$R activation in the amyloid precursor protein metabolism,[15,16] a key gene involved in Alzheimer's disease (AD). This observation and its well-known memory and learning-enhancing effects[17] indicate that 5-HT$_4$ receptors are promising pharmacological targets for the treatment of AD.[18] However, the plethora of 5-HT$_4$ ligands synthesized over the past decades has only led to the commercialization of two drugs used in the treatment of irritable bowel syndrome.[14] Thus, it clearly appears that additional studies are required to better understand the molecular determinants of 5-HT$_4$R activation. Recently, the 5-HT$_4$R has been shown to form constitutive homodimers,[19] opening a new avenue for the design of 5-HT$_4$ probes, which could serve as a basis for the pharmacotherapy of memory disorders such as AD. In a previous paper, we reported the design and the synthesis of 5-HT$_4$R-selective bivalent ligands[20] based on the structure of ML10302, a selective partial 5-HT$_4$ agonist.[21] Bivalent molecules composed of two ML10302 units and spacers ranging from 6 to 29 atoms were prepared (Figure 1). All bivalent ligands conserved high-affinity binding for 5-HT$_4$R, and those with the longest spacers (18, 25, and 29 atoms) appeared to be functionally different in the cAMP accumulation assay compared to the ML10302 reference compound.[20]

We now extend this work by determining the optimal spacer length for the ML10302-based bivalent ligands using molecular modeling on our recently published 5-HT$_4$R dimer model,[20] which has been validated by site-directed mutagenesis.[22] Modeling studies predicted an optimal spacer length of approximately 22 Å (20−24 atoms), which is in good agreement with recent studies on $\mu$-opioid receptor dimers and bivalent ligands.[9,23] Since our previous series of bivalent ligands did not cover spacer lengths between 18 and 25 atoms, we decided to synthesize two new series of 5-HT$_4$R-specific bivalent ligands containing flexible and constrained spacers of 20−24 atoms. Position 4 of the piperidin ring of ML10302 was conserved as the most suitable attachment point for spacers based on molecular modeling of 5-HT$_4$R dimers and structure−activity relationship studies.

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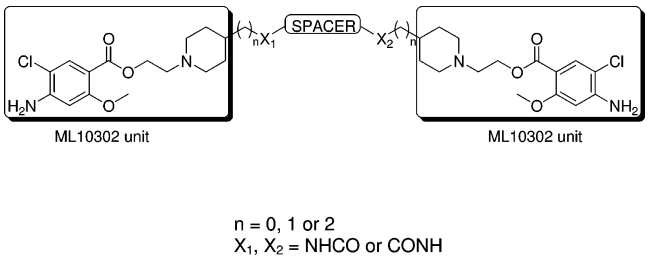



**Figure 1.** General structure of 5-$HT_4$ bivalent ligands.

We first determined the pharmacological and functional properties of these bivalent ligands and then addressed the important issue of whether both or only one pharmacophore of the bivalent compounds binds to 5-$HT_4$R dimers by using an innovative bioluminescence resonance energy transfer (BRET) assay. BRET determines the ability of the monovalent ML10302 molecule and its bivalent derivates to promote ligand-induced conformational changes within 5-$HT_4$R dimers.[19]

## Results

**Molecular Modeling Studies.** To estimate the optimal length and structure of the spacer of bivalent ligands, we performed molecular docking studies on our recently described 5-$HT_4$R dimer model. This model, which was initially generated by GRAMM software calculations[20] and subsequently confirmed by site-directed mutagenesis,[22] predicts the involvement of helices II, III, and IV in the dimer interface. Initial positioning of the two ML10302 pharmacophores into the 5-$HT_4$R binding site was based on experimental data and on a ML10302−5-$HT_4$R complex[24,25] that has been previously optimized by large-scale molecular dynamics in a virtual hydrated lipid bilayer.[26] On the basis of these docking studies, position 4 of the piperidin ring of ML10302 has been confirmed as the most suitable attachment point for spacers. The evaluation of the minimal length between these two spacer attachment points has to take into account the bypassing of the two helices III, revealing a minimal distance of approximately 22 Å. Modeling with short spacers of 14 atoms was possible but required an unfavorable highly extended conformation of the spacer. A more favorable and extended conformation was obtained for spacers of 20−24 atoms. The final structure obtained for each bivalent-ligand−5-$HT_4$R dimer complex was then refined using molecular dynamics and energy minimizations to allow a more favorable positioning of the spacer with respect to essential ML10302−5-$HT_4$R interactions (Figure 2, compound **9c** shown as a 22-atom spacer). Optimal docking of spacers depends not only on their lengths but also on their structures. Since the exact chemical composition of optimal spacers is difficult to predict, we decided to synthesize two series of bivalent ligands with either flexible or constrained spacers ranging from 20 to 24 atoms.

**Synthesis of Bivalent Ligands.** In order to modulate the spacer length by one-atom increments with homogeneous chemical compositions, we designed spacers, which are presented in Table 1. By variation of the number of atoms *n* and *m* and the positions of the substitutents on the central aromatic ring (1,3 or 1,4), the desired repertoire of spacers could be achieved. The synthesis of bivalent ligands was based on a Sonogashira−Linstrumelle coupling reaction and is described in Scheme 1. Introduction of a propargyl group on the nitrogen atom of Boc-glycine or Boc-$\gamma$-aminobutyric acid brought three supplementary carbons into the spacers. In this first step, Boc protected amines **1a** and **1b** were alkylated using NaH as a base in DMF to give the propargyl acids **2a** and **2b** in 58% and 77%

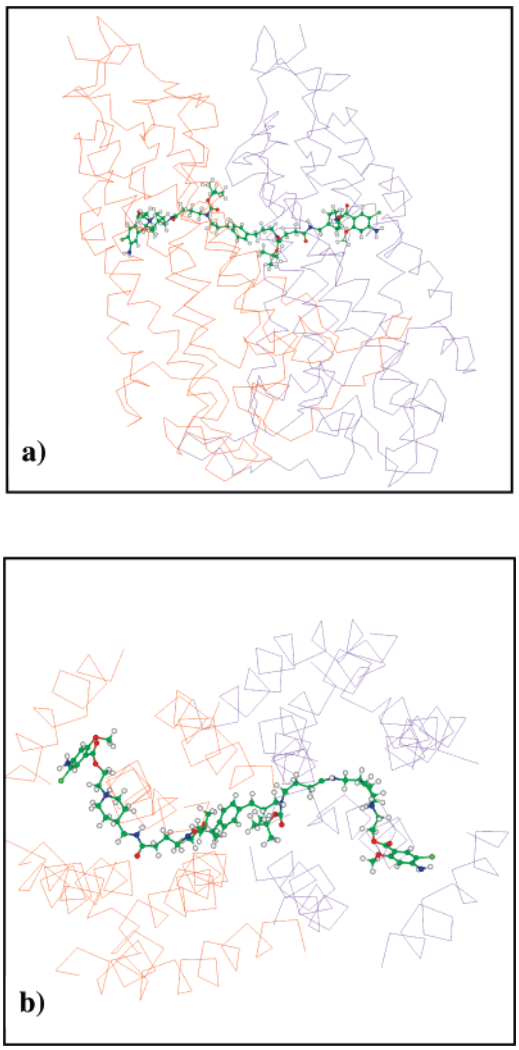


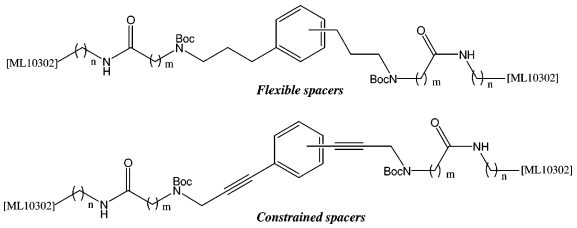

**Figure 2.** Molecular modeling studies: (a) side view of docked bivalent ligand **9c** in 5-$HT_4$R dimer model, where both protomers including helices and extra- and intracellular loops are represented in red and blue; (b) top view of docked of bivalent ligand **9c** in 5-$HT_4$R dimer model, where the loops are not displayed for a better visualization.

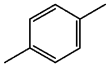
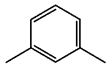
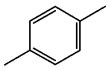
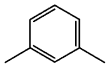
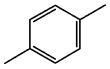
yields, respectively. Then a Sonogashira−Linstrumelle coupling with either 1,3 or 1,4-diiodobenzene allowed introduction of three or four atoms in the linker. This reaction was performed at 80 °C, allowing the synthesis of the constrained spacers **3a**, **3b**, and **4** in moderate yields (46−62%). Finally, conformational flexibility was introduced by reducing the triple bonds into single bonds by classical hydrogenation on Pd/C, producing molecules **5a**,**b** and **6**. The synthesis of the bivalent ligands was then performed by condensation between the already known amine **7a** or **7b**[20] derived from ML10302 and previously synthesized acids. A standard protocol using EDC, HOBt, and $NEt_3$ in anhydrous DMF was used to afford the constrained bivalent ligands **8a**−**e** (Scheme 2) or the flexible bivalent ligands **9a**−**e** (Scheme 3) in low to moderate yields (19−62%).

This straightforward synthesis is highly modular, avoiding the use of protecting groups, thus leading to spacers bearing terminal carboxylic functions ready for the coupling with amines derived from ML10302. This strategy allowed us to prepare both rigid and flexible bivalent molecules derived from ML10302 with spacer lengths ranging from 20 to 24 atoms.

Molecule **13**, which is not a bivalent ligand because one of the two ML10302 units of the bivalent ligands was replaced by a cyclohexyl ring, was also prepared as a control compound.

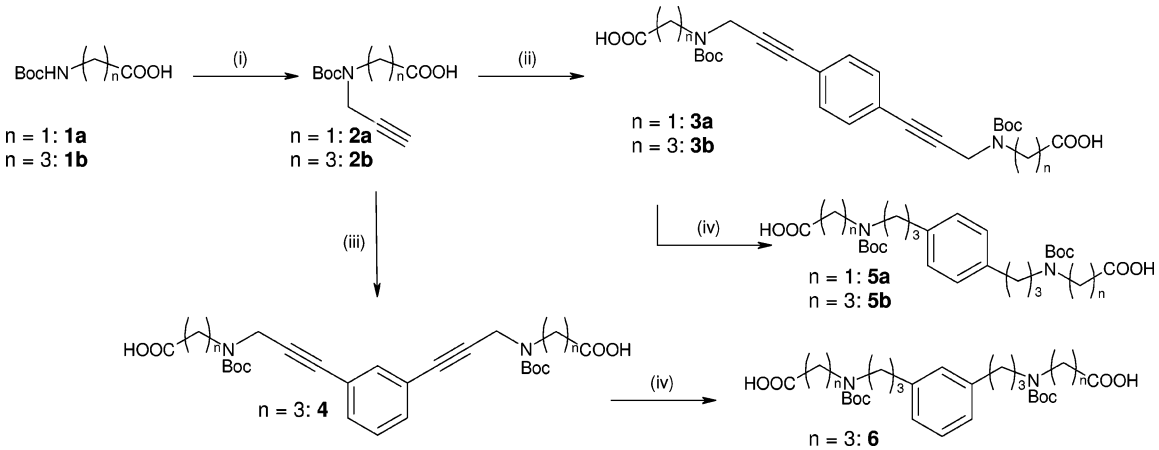
**Table 1.** General Structure of Flexible and Constrained 5-HT$_4$R Bivalent Ligands[a]




| n | m | Substitutions | spacer length |
|---|---|---|---|
| 1 | 1 |  | 20 |
| 0 | 3 |  | 21 |
| | |  | 22 |
| 1 | 3 |  | 23 |
| | |  | 24 |

[a] The meta or para substitution of the aromatic central ring mixed with adequat aliphatic chain combinations (*n* or *m*) allow a one by one atom incremented spacer length.

**Scheme 1.** Synthesis of Spacers[a]

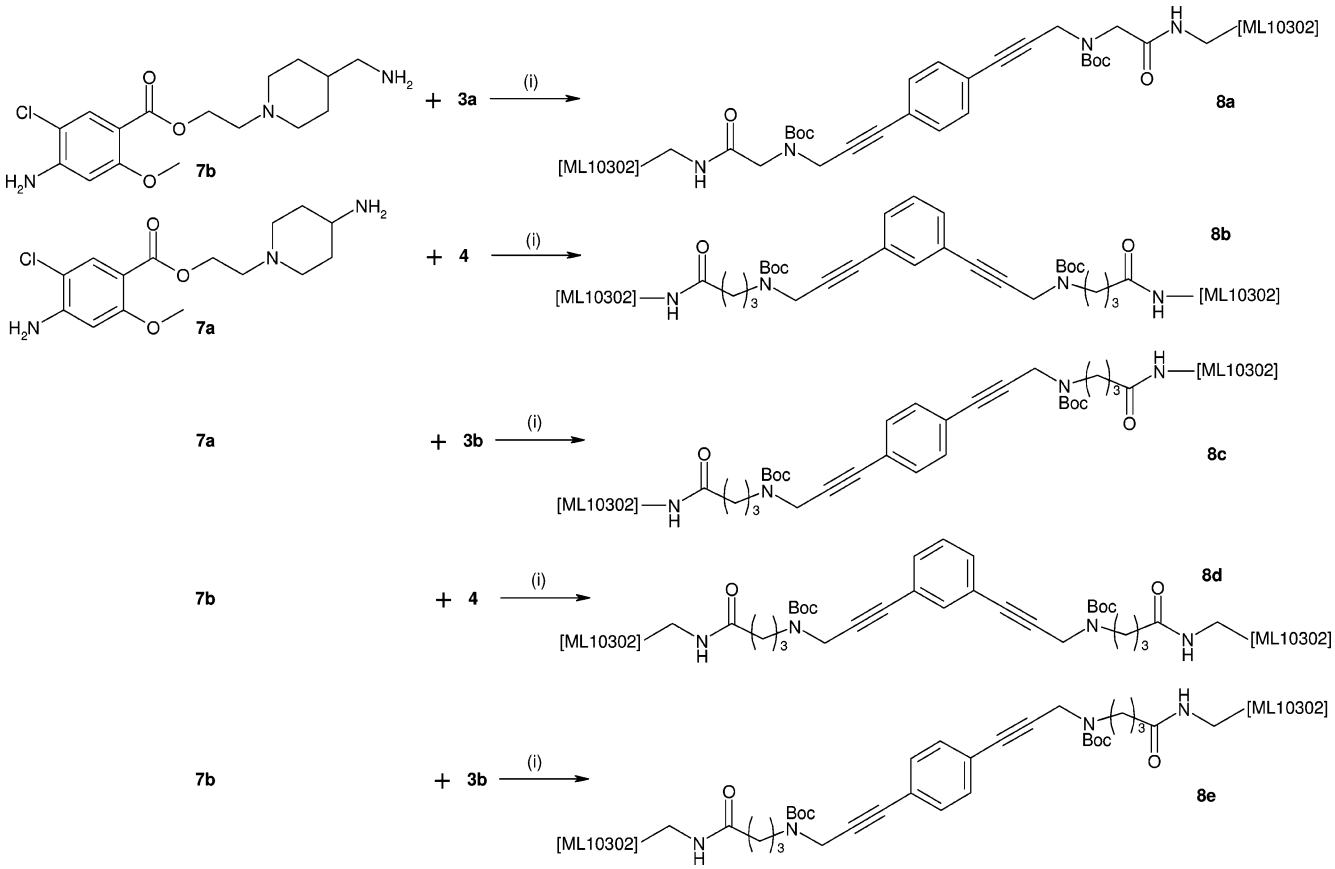



[a] Reagents and conditions: (i) (a) NaH, DMF, 0 °C, 1 h; (b) propargyl bromide 80% in toluene, room temp, overnight; (ii) 1,4-diiodobenzene, $PdCl_2(PPh_3)_2$ (5 mol %), CuI (10 mol %), $NEt_3$, DMF, 80 °C, (iii) 1,3-diiodobenzene, $PdCl_2(PPh_3)_2$ (5 mol %), CuI (10 mol %), $NEt_3$, DMF, 80 °C; (iv) $H_2$ (1 bar), Pd/C (10%), MeOH, room temp.

A similar reaction as that used to produce the propargyl acid **2b** was used again, except that dissymmetry was obtained using a large excess of 1,4-diiodobenzene in a Sonogashira−Linstrumelle coupling reaction at room temperature, to give **10** in 49% yield (Scheme 4). The cyclohexylamine part of **13** was obtained by coupling cyclohexylamine with **2b**, using EDC, HOBt, and $NEt_3$ in DMF to give **11** in 61% yield. A second Sonogashira−Linstrumelle coupling reaction between **10** and **11** at 80 °C yielded **12** in a moderate yield (53%). Compound **13** was then obtained with a good yield after hydrogenation of the triple bonds of **12** and coupling with **7a**, using HBTU and $NEt_3$ in anhydrous DMF. Bivalent molecules **14**−**18** (Figure 3), which possess other chemical composition spacers and which were previously synthesized,[20] were also used in this study.

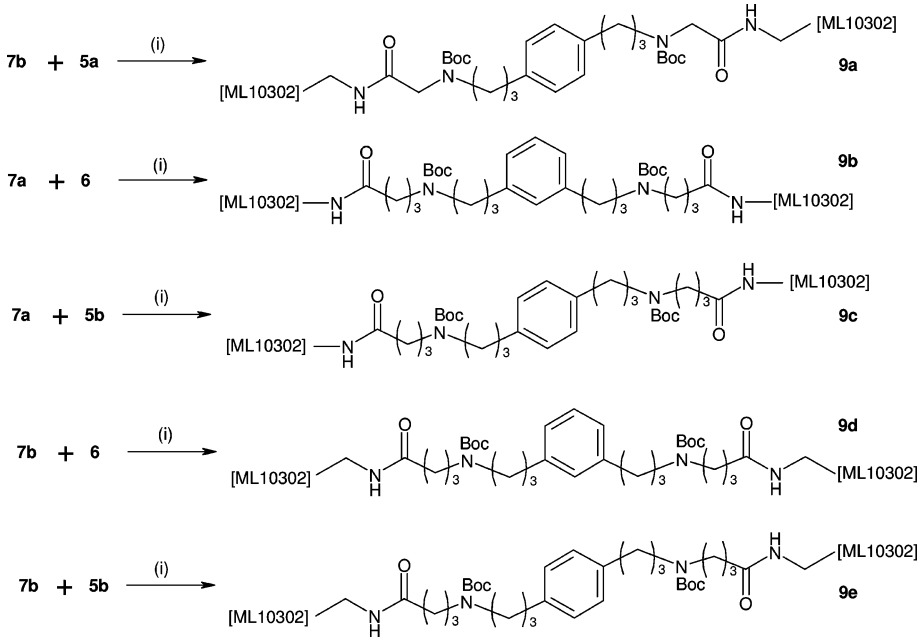
**Pharmacological and Functional Properties of 5-HT$_4$R-Specific Bivalent Molecules.** The pharmacological properties of the newly synthesized series of ligands were determined in C6 glial cells stably expressing the human 5-HT$_4$R.[27] [$^3$H]-GR113808 competition curves of bivalent ligands were monophasic (see Table 2 for $K_i$ values). All compounds retained high affinity for 5-HT$_4$R with $K_i$ values that were very similar to those obtained for recently published bivalent ligands and the monovalent ML10302 reference compound.[20] Similar results were obtained in Chinese hamster ovary (CHO) cells transiently expressing the 5-HT$_4$R (data not shown). Next, the ability of bivalent ligands to stimulate adenylyl cyclase activity was analyzed by measuring ligand-induced cAMP production. Compared to the monovalent ML10302, bivalent ligands either conserved a ML10302-like partial agonistic character (**8a**) or

**Scheme 2.** Synthesis of Constrained Bivalent Ligands[a]




[a] Reagents and conditions: (i) EDC, HOBt, $NEt_3$, anhydrous DMF, room temp.

**Scheme 3.** Synthesis of Flexible Bivalent Ligands[a]

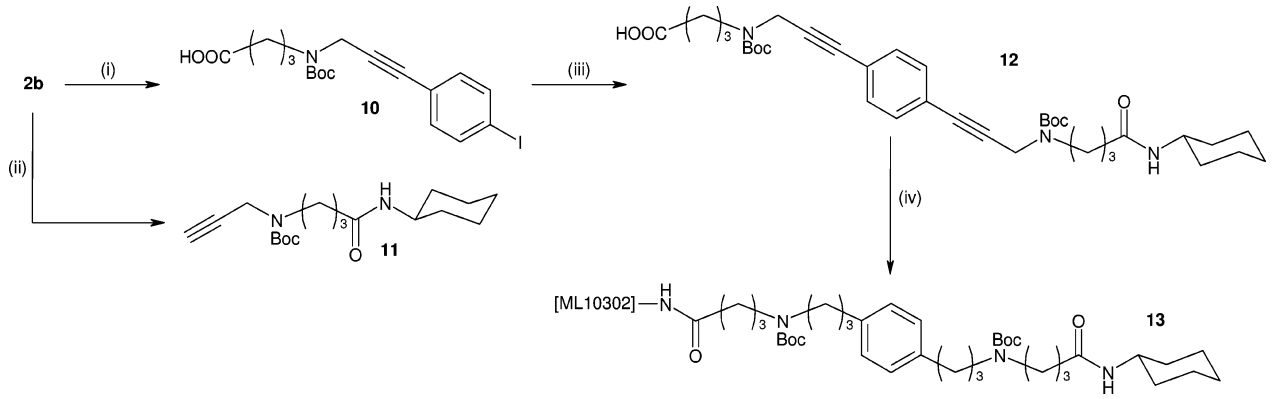



[a] Reagents and conditions: (i) EDC, HOBt, $NEt_3$, anhydrous DMF, room temp.

lost these agonistic properties (**8b**,**d**,**e**, **9b**−**e**) (Table 2). This confirms our previous observation that bivalent ligands with long spacers (20−29 atoms) are functionally different compared to ML10302.[20] To evaluate the respective impact of the spacer and/or the second pharmacophore of the bivalent ligands on these functional changes, we synthesized a ML10302 derivative that possessed a spacer of 22 atoms but only one pharmacophore (**13**). This compound retained high-affinity for 5-$HT_4$R and generated an intermediate cAMP production (17%), indicating that both the spacer and the second pharmacophore participate in the modification of the functional properties compared to the ML10302 reference compound.

**BRET Results.** The functional differences observed between ML10302 and the bivalent compounds suggest that ML10302

**Scheme 4.** Synthesis of a Nonbivalent Control Ligand[a]




[a] Reagents and conditions: (i) 1,4-diiodobenzene, $PdCl_2(PPh_3)_2$ (5 mol %), CuI (10 mol %), $NEt_3$, DMF, 0 °C to room temp, (ii) cyclohexylamine, EDC, HOBt, $NEt_3$, DMF/DCM (2/1), room temp; (iii) **11**, $PdCl_2(PPh_3)_2$ (5 mol %), CuI (10 mol %), $NEt_3$, DMF, 80 °C; (iv) (a) $H_2$ (1 bar), Pd/C (10%), MeOH, room temp; (b) **7a**, HBTU, $NEt_3$, DMF, room temp.

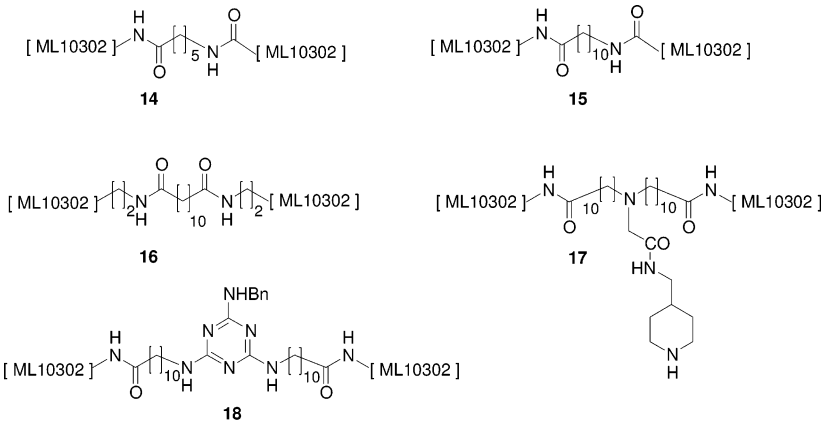



**Figure 3.** Previously synthesized 5-$HT_4$ bivalent ligands.[20]

**Table 2.** Pharmacological Evaluations of Synthesized 5-$HT_4$R Bivalent Ligands: Binding Affinities ($K_i$), cAMP Production (% of 5-HT), BRET Signal Measurements[a]

| compd | spacer size | $K_i$ (nM) | cAMP (% of 5-HT)[b] | BRET (% of control)[c] |
|---|---|---|---|---|
| GR113808 | | 0.5 ± 0.2 | 1 ± 1 | 99 ± 2 |
| ML10302 | | 5 ± 2.5 | 45 ± 1 | 101 ± 5 |
| **8a** | 20 | 44 ± 10 | 49 ± 26 | 107 ± 2 |
| **8b** | 21 | 8 ± 3 | 8 ± 8 | 109 ± 2 |
| **8c** | 22 | 18 ± 6 | 35 ± 10 | 106 ± 2 |
| **8d** | 23 | 40 ± 11 | 6 ± 9 | 108 ± 1 |
| **8e** | 24 | 25 ± 5 | 1 ± 2 | 115 ± 1* |
| **9a** | 20 | 19 ± 6 | 18 ± 1 | 114 ± 4* |
| **9b** | 21 | 15 ± 10 | 10 ± 8 | 111 ± 1 |
| **9c** | 22 | 12 ± 3 | 21 ± 3 | 118 ± 1* |
| **9d** | 23 | 23 ± 8 | 5 ± 6 | 108 ± 2 |
| **9e** | 24 | 15 ± 22 | 9 ± 1 | 115 ± 0* |
| **13** | | 34 ± 11 | 17 ± 3 | 105 ± 2 |
| **14** | 9 | 7 ± 3.2 | 46 ± 5 | 108 ± 1 |
| **15** | 14 | 20 ± 12 | 5 ± 1 | 110 ± 1 |
| **16** | 18 | 9 ± 4 | 6 ± 4 | 108 ± 4 |
| **17** | 25 | 50 ± 12 | 10 ± 2 | 114 ± 1* |
| **18** | 29 | 113 ± 32 | 12 ± 3 | 114 ± 2* |

[a] Very similar $K_i$ values of 5.7 ± 1 and 3.5 ± 2 (ML10302), 0.5 ± 0.1 and 0.7 ± 0.1 (GR113808), and 10 ± 2 (compound **9c**) were obtained for 5-$HT_4$R−RLuc and 5-$HT_4$R−YFP fusion proteins, respectively. 5-$HT_4$R−RLuc and 5-$HT_4$R−YFP fusion proteins behaved as the wild type receptor in the cAMP assay.[19] [b] Results are the mean ± of two independent experiments performed in triplicate. [c] Results are the mean ± of three independent experiments performed in duplicate (∗, $p < 0.001$).

interacts with 5-$HT_4$R differently than its bivalent derivatives. Recent studies indicate that functional differences (i.e., between partial and full agonists) can be explained by the stabilization of different conformational states of GPCRs.[28−30] To detect

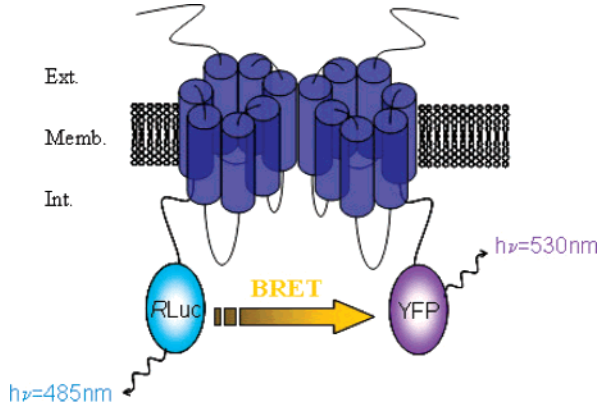



**Figure 4.** BRET technique.

ligand-induced conformational changes of ML10302 and its bivalent derivatives, we used a recently developed BRET-based assay.[19] This assay relies on the observation that the degree of physical proximity between two receptor molecules within a dimer can be assessed in living cells by the level of energy transfer occurring between fusion proteins of the receptor tagged with the energy donor *Renilla* luciferase (RLuc) and another receptor tagged with a fluorescent acceptor, the yellow fluorescent protein (YFP) (Figure 4).[31,32] Ligand-induced conformational changes within preexisting dimers may be detected if the relative position and orientation of the Rluc and the YFP moieties within the dimer are altered.[33−35] Carboxy-terminal 5-$HT_4$R fusion proteins (5-$HT_4$R−RLuc, 5-$HT_4$R−YFP) were transiently coexpressed in CHO cells. All receptor constructs

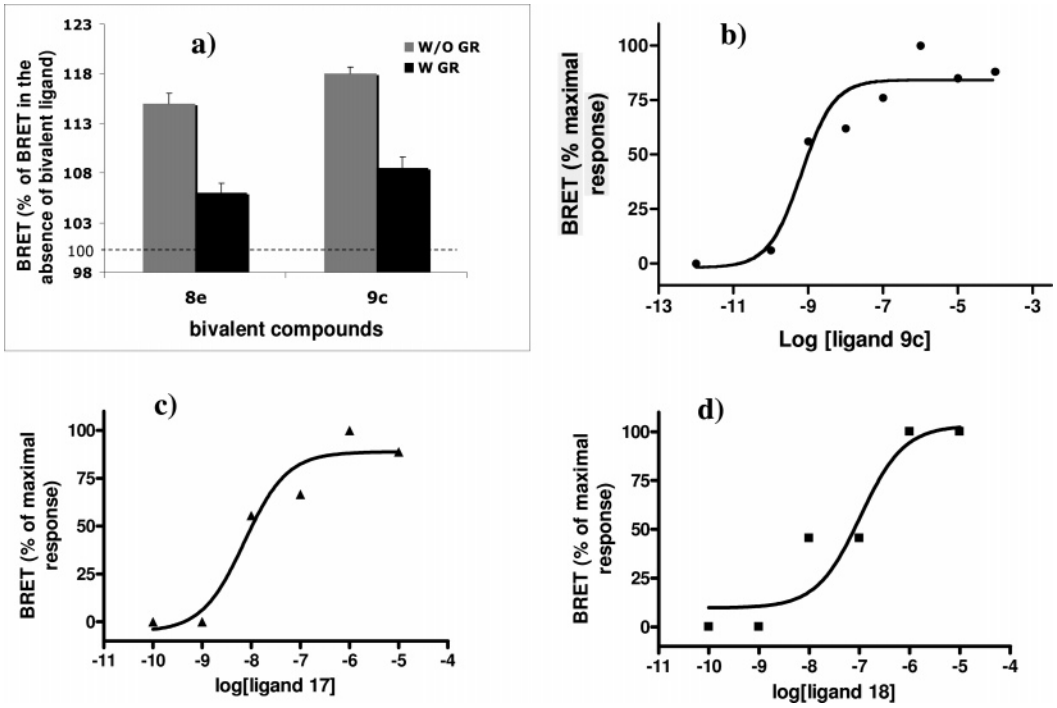



**Figure 5.** BRET assays. Membrane preparations from CHO cells expressing 5-HT$_4$R−RLuc and 5-HT$_4$R−YFP at equimolar amounts corresponding to nonsaturating conditions were incubated with different ligands: (a) effect of bivalent ligands **8e** and **9c** (1 $\mu$M) on the basal BRET signal in the presence (W, black) or absence (W/O, gray) of 1 $\mu$M monovalent antagonist GR113808; dose response curves of bivalent ligands **9c** (b), **17** (c), and **18** (d). Data are the mean of at least three experiments performed in duplicate.

were functional because the coupling of 5-HT$_4$R−RLuc and 5-HT$_4$R−YFP to adenylyl cyclase[19] and binding properties (Table 2) were identical to those of the wild type receptor expressed in C6 glial cells. BRET measurements were performed using saturating concentrations (1 $\mu$M) of newly synthesized and previously described ligands in cells expressing equimolar amounts of 5-HT$_4$R−RLuc and 5-HT$_4$R−YFP (Figure 5). Binding of ML10302 and its derivative **13**, which lacks the second pharmacophore but carries a long spacer, did not modify the basal BRET signal. In contrast, the bivalent ligands, in particular those with long spacers, induced a significant increase of the basal BRET signal (Table 2, **8e**, **9a**,**c**,**e**, **17**, **18**). This increase corresponds to a ligand-induced conformational change in preexisting 5-HT$_4$R dimers and not to a ligand-induced dimerization because previous studies have shown that all receptors exist already as covalent, disulfide-bridged dimers in the absence of ligand.[22]

Importantly, the ligand-promoted BRET of flexible (**9c**) and constrained (**8e**) ligands was abolished in the presence of the reference monovalent antagonist GR113808 (Figure 5a). To further confirm the specificity of the ligand-induced BRET, dose-response curves were generated for ligands **9c**, **17**, and **18**, which possess long flexible spacers with 22, 25, and 29 atoms, respectively. Half-maximal ligand-promoted BRET values of 0.7, 150, and 10 nM for ligands **9c**, **17**, and **18**, respectively, were in good agreement with $K_i$ values (Figure 5b−d). Bivalent ligands **14**−**16** with spacers containing less than 18 atoms did not significantly modify the basal BRET signal. Comparison of ligand-promoted BRET of bivalent ligands with flexible and constrained spacers revealed interesting differences. For the constrained molecules possessing a diyne structure (**8a**−**e**), only compound **8e** produced a significant change of the basal BRET signal. In contrast, for bivalent ligands with flexible spacers (**9a**−**e**, **15**−**18**) five compounds (**9a**, **9c**, **9e**, **17**, **18**) significantly modified the basal BRET signal. Intriguingly, in the series of bivalent ligands **9**, compounds **9b** and **9d** had no significant effect on basal BRET signals despite the presence of an apparently sufficiently long spacer. A closer look at the structure of compounds **9b** and **9d** revealed the presence of a 1,3-meta-substituted central cyclic core compared to a 1,4-para-substituted core present in **9a**, **9c**, and **9e**. This further highlights the crucial role not only of the length but also of the flexibility and the chemical structure of the spacer for the correct orientation of the two pharmacophores in bivalent ligands.

## Discussion

The concept of GPCR homo- and heterodimerization has an important impact on GPCR drug design. An obvious way to design new drug families for GPCRs is the synthesis of bivalent compounds that are expected to bind simultaneously to the two ligand binding sites present in GPCR dimers.[12,13,36] Ideally, bridging of the two protomers of the receptor dimer by bivalent ligands would generate unique functional properties, which are different from those of the monovalent pharmacophore. The successful design and characterization of bivalent ligands rely on the choice of the pharmacophore and the spacer linking both pharmacophores as well as the availability of adequate assay systems to determine their specific interaction on receptor dimers and their functional properties.

In the present study, we conserved the ML10302 compound as pharmacophore, as this compound has high affinity and specificity for the 5-HT$_4$R. Importantly, ML10302 is a partial agonist (~50% of maximal serotonin response) for 5-HT$_4$R.[27] Thus, differences in functional properties of the corresponding bivalent derivatives should be easily revealed and an increase and/or a decrease of the agonistic potential should be readily detected. On the basis of the optimized docking of the monovalent ML10302 molecule in our 5-HT$_4$R dimer model, position 4 of the piperidin ring of ML10302 was conserved as the most suitable attachment point for spacers. We prepared two series of bivalent ligands (**8** and **9**), one with constrained

spacers and the other with flexible ones. Both series possessed spacers ranging from 20 to 24 atoms of approximately 22 Å, the predicted optimal spacer length according to our own docking experiments and according to published results on opioid receptor dimers.[9,23]

We first determined the pharmacological properties of the bivalent compounds. [$^3$H]-GR113808 competition curves were monophasic for all bivalent compounds with a single high-affinity site for 5-HT$_4$R. Conservation of high affinity of bivalent compounds for 5-HT$_4$R is consistent with the binding of at least one pharmacophore of the bivalent ligands to 5-HT$_4$R. However, pharmacological studies did not provide any obvious evidence for the binding of the second pharmacophore. Indeed, we did not observe substantial changes in ligand affinity or the addition of a second, pharmacologically distinct binding site that might have indicated the existence of allosteric effects.[37] Although substantial differences in binding affinities have been reported for some bivalent ligands,[10,36,38−46] pharmacological evidence for the occupation of both binding sites in a dimer is generally difficult to obtain especially for the binding of two identical pharmacophores to receptor homodimers.

The functional activity of bivalent ligands was tested in cAMP accumulation assays. Despite the preservation of high affinity for 5-HT$_4$R, most bivalent compounds lost the agonistic properties of the ML10302 reference compound. This observation is particularly true for ligands with long spacers, which confirms our preliminary conclusion based on a limited set of previously described bivalent ligands. These functional differences are compatible with the hypothesis that both pharmacophores of bivalent ligands interact with 5-HT$_4$R dimers.

The docking studies of bivalent ligands on the 5-HT4R dimer model may provide a possible explanation for the loss of receptor activation in the presence of these bivalent ligands compared to the ML10302 reference compound. Since spacers of bivalent ligands most likely have to bypass the two helices III of the dimer, this may hinder the movement of this helix, which has been shown to be crucial for the activation process of GPCRs.[47]

In an effort to obtain evidence for the specific interaction of the second pharmacophore with 5-HT$_4$R, we tested bivalent ligands in a BRET assay (Figure 4). This assay has recently emerged as a sensitive approach to monitor conformational changes of a wide range of proteins in living cells including membrane receptors.[31,34] Whereas the ML10302 reference molecule was inactive in the BRET assay, several bivalent compounds significantly increased the basal BRET signal. The effect of compounds **9c**, **17**, and **18** was dose-dependent with half-maximal doses in agreement with the respective $K_i$ values. These ligand-induced BRET changes were inhibited in the presence of the 5-HT$_4$R-specific monovalent antagonist GR113808, further confirming the specificity of the signals. Importantly, the monovalent ML10302 derivate **13** was inactive in the BRET assay. This defines the specificity of the ligand-promoted BRET signal that exclusively relies on the properties of the second pharmacophore, which qualifies the BRET assay as an excellent tool to study the interaction of the second pharmacophore with 5-HT$_4$R.

Testing of the constrained family of bivalent ligands (**8a**−**e**) in the BRET assay revealed that only ligand **8e** significantly enhanced the basal BRET signal. The fact that only one 1 of 5 compounds was able to induce a significant BRET change may be explained by the rigidity introduced by the diyne central core in the spacer of this family preventing the binding of the second pharmacophore of these bivalent ligands. Interestingly, the active compound **8e** possesses the longest spacer with 24 atoms, suggesting that longer spacers are able to overcome the conformational constraints due to this type of spacer.

In the flexible family (**9a**−**e**), which complements the previously synthesized ligand family (**14**−**18**), most compounds induced a significant ligand-promoted BRET (**9a**,**c**,**e**). The high success rate for this ligand family in the BRET assay most likely resides in the flexibility of the spacer favoring the convenient orientation of the second pharmacophore.

However, in this flexible family, two ligands, **9b** and **9d**, were inactive in the BRET assay despite apparently adequate spacer length. The functional properties of these two ligands may be explained by a different type of position in the central cyclic core of the spacer that changes the relative orientation of the two pharmacophores. Whereas compounds **9b** and **9d** have a 1,3-meta-substituted central cyclic core, the other compounds have a 1,4-para0substituted core (Scheme 3). The absence of ligand-induced BRET for these 1,3-meta-substituted compounds further highlights the importance not only of spacer length but also of the orientation of the two pharmacophores, which is determined by the chemical nature of the spacer. The inability of compounds **9b** and **9d** to promote ligand-induced BRET changes further confirms the sensitivity and the specificity of the BRET signals. Altogether, the BRET results confirmed the prediction of our molecular docking studies. A spacer length of 20−24 atoms appears to be optimal for binding of the two pharmacophores of bivalent ligands to receptor dimers. Furthermore, additional structural constraints introduced by triple bonds (**8a**−**c**) or 1,3-meta-substituted aromatic rings (**9b** and **9d**) are equally important parameters to be considered for optimal docking of bivalent ligands. Interestingly, overlay of extended conformations of compounds with constrained (**8c)** and flexible (**9c**) linkers of the same size revealed a similar distance between the two ML10302 moieties. In contrast, the structure of the 5-HT$_4$R dimer plays a key role in the conformation adopted by the spacer because major obstacles such as helices III have to be bypassed.

To consolidate data obtained with mono- and bivalent 5-HT$_4$R-specific compounds, we propose the following model, where two monovalent ligands occupy both ligand binding sites of 5-HT$_4$R dimers with similar affinities (Figure 6a). Depending on the functional properties of each ligand, different conformational changes are induced leading to receptor activation or inactivation. None of these conformational changes are detectable in the BRET 5-HT$_4$R-dimerization assay. For bivalent ligands that promote significant ligand-induced BRET changes, interaction of both pharmacophores with the same receptor dimer occurs. These ligands bind to 5-HT$_4$R in a similar manner as monovalent ligands; however, the spacer imposes additional conformational constraints between the two protomers of the dimer (Figure 6b). This has two important consequences, a change of functional properties compared to the monovalent reference compound and the detection of ligand-promoted conformational change with the BRET assay. For bivalent ligands without detectable ligand-induced BRET changes, two alternative possibilities may be envisioned (Figure 6c,d). For ligands with short spacers and functional properties similar to those of the monovalent compound, binding of two single molecules to the receptor dimer appears likely (Figure 6d). For those with adequate spacers and modified functional properties, simultaneous binding of both pharmacophores to the same receptor dimer may be hypothesized (Figure 6c). However, the conformational constraints imposed by this type of spacer are not detectable in the BRET assay. Indeed, ligand binding to

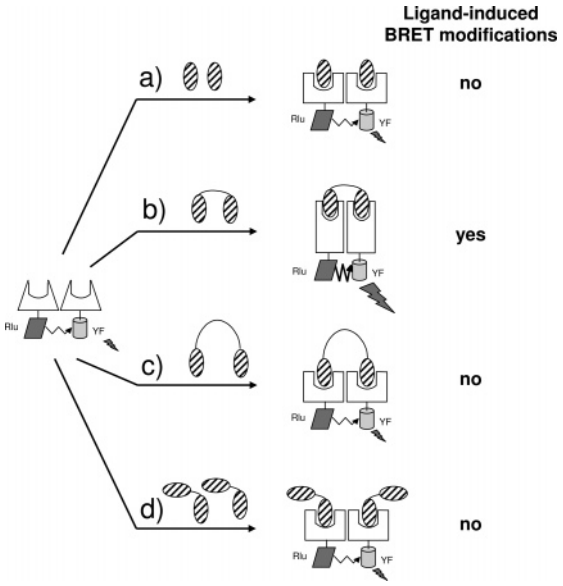



**Figure 6.** Proposed model: (a) binding of two monovalent ligands on both 5-HT$_4$R dimer binding sites; (b) binding of bivalent ligands inducing BRET modifications; (c) binding of bivalent ligands without detectable BRET modifications; (d) binding of short spacers bivalent ligands without detectable BRET modifications.

GPCRs does not systematically lead to detectable BRET changes because the conformational modification may not necessarily change the relative position of the energy donor (Rluc) and the energy acceptor (YFP).[8]

Taken together, we have synthesized two targeted series of bivalent ligands for 5-HT$_4$R dimers possessing spacers of 20−24 atoms corresponding to an optimized spacer length of approximately 22 Å. We applied the recently developed BRET GPCR dimerization assay for the first time to the study of bivalent ligands on GPCR dimers. Whereas classical competition binding experiments were unable to reveal differences in the binding properties of mono- and bivalent ligands, the BRET assay provided new insights in the interaction of bivalent ligands with 5-HT$_4$R dimers. We were able to provide evidence for the interaction of the two pharmacophores of several bivalent compounds with 5-HT$_4$R dimers. Having a validated molecular model of 5-HT$_4$R dimers, optimized spacers, and specific BRET assays at one's disposal, the development of heterobivalent ligands for 5-HT$_4$R homodimers or 5-HT$_4$R heterodimers can now be envisaged. Furthermore, our knowledge about the dimerization interface of 5-HT$_4$R dimers may lead in the future to the design of 5-HT$_4$R dimerization inhibitors. In conclusion, 5-HT$_4$R molecules able to stabilize or inhibit receptor dimerization may serve as a new basis for the synthesis of new drugs for 5-HT$_4$R-associated disorders.

## 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 $\delta$ 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 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 the usual 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.

**General Procedure for the Preparation of Compounds 2a and 2b: Preparation of 2-(*tert*-Butoxycarbonyl(prop-2-ynyl)amino)-acetic Acid (2a).** To a stirred solution of **1a** (3.0 g, 17.12 mmol, 1 equiv) in 40 mL of dry DMF at 0 °C was added NaH (60% in mineral oil, 2.12 g, 53.07 mmol, 3.5 equiv). After 1 h at this temperature, propargyl bromide (80% in toluene, 3.24 mL, 29.11 mmol, 1.7 equiv) was added, and the reaction mixture was allowed to reach room temperature and was stirred for 18 h. Water was added (20 mL), and the pH was adjusted to 3 with 1 N HCl. This aqueous solution was extracted with 3 × 20 mL of $CH_2Cl_2$, and the combined organic layers were dried with $Na_2SO_4$ and concentrated. The oil obtained was purified by flash chromatography using cHex/AcOEt (1/1) containing 0.1% AcOH to yield 2.20 g (66%) of **2a** as an amber solid. $R_f$(cHex/AcOEt (1/1) + 0.5% AcOH) = 0.67. $^1$H NMR (200 MHz) $\delta$ 10.84 (bs, 1H, O*H*), 4.18−4.07 (m, 4H), 2.25 (t, $J$ = 2.6 Hz, 1H), 1.41 (s, 9H). $^{13}$C NMR (50 MHz) $\delta$ 174.4, 154.6, 81.4, 78.2, 72.9, 47.0, 36.5, 28.0. $F$ = 118 °C.

**General Procedure for the Preparation of Compounds 3a, 3b, and 4: Preparation of 2,2′-(3,3′-(1,4-Phenylene)bis(prop-2-yne-3,1-diyl))bis(*tert*-butoxycarbonylazanediyl)diacetic Acid (3a).** A 30 mL solution of freshly distilled $NEt_3$ containing also 1,4-diiodobenzene (1.95 g, 5.92 mmol, 1 equiv), $PdCl_2(PPh_3)_2$ (0.165 g, 0.24 mmol, 0.05 equiv), and CuI (0.090 g, 0.47 mmol, 0.1 equiv) was stirred at room temperature. Then 30 mL of anhydrous DMF containing a solution of **2a** (3.0 g, 14.20 mmol, 2.4 equiv) was added dropwise. After the mixture was heated at 80 °C overnight, the solvents were removed. The crude product was taken up in 20 mL of water, and the mixture was acidified to pH 3 with 1 N HCl. This aqueous solution was extracted with 3 × 20 mL of $CH_2Cl_2$, and the combined organic layers were dried with $Na_2SO_4$ and concentrated. The resulting oil was purified by flash chromatography using cHex/AcOEt (6/4) containing 0.1% AcOH to yield 1.72 g (58%) of **3a** as an amber oil. $R_f$(cHex/AcOEt (6/4) + 0.5% AcOH) = 0.13. $^1$H NMR (200 MHz) $\delta$ 10.03 (bs, 2H), 7.30 (s, 4H), 4.40−4.03 (m, 8H), 1.41 (s, 18H). $^{13}$C NMR (50 MHz) $\delta$ 174.5, 154.6, 131.5, 122.5, 85.6, 84.1, 81.4, 47.2, 37.3, 28.1.

**General Procedure for the Preparation of Compounds 5a, 5b, and 6: Preparation of 2,2′-(3,3′-(1,4-Phenylene)bis(propane-3,1-diyl))bis(*tert*-butoxycarbonylazanediyl)diacetic Acid (5a).** To a stirring solution of **3a** (300 mg, 0.60 mmol) in 10 mL of MeOH at room temperature was added 30 mg of 10% Pd/C. The mixture was stirred under 1 bar of $H_2$ overnight and filtered through a pad of Celite. Evaporation of the solvent yielded 278 mg (91%) of **5a** as an amber oil. $^1$H NMR (200 MHz) $\delta$ ppm 10.17 (bs, 2H), 7.05 (s, 4H), 3.88 (bd, $J$ = 20.9 Hz, 4H), 3.54−3.09 (m, 4H), 2.55 (t, $J$ = 7.4 Hz, 4H), 1.99−1.62 (m, 4H), 1.32 (s, 18H). $^{13}$C NMR (50 MHz) $\delta$ 174.3 and 174.1 (two signals due to rotamers), 156.1 and 155.3 (two signals due to rotamers), 139.1, 128.4, 80.5, 49.1 and 48.8 (two signals due to rotamers), 48.3 and 48.1 (two signals due to rotamers), 32.7, 29. 9, 28.3.

**General Procedure for the Preparation of Dimers 8 and 9: Preparation of Dimer 8a.** To a solution of **3a** (595 mg, 1.19 mmol, 1 equiv), **7b** (986 mg, 2.38 mmol, 2 equiv),[20] HOBt·$H_2O$ (321 mg, 2.38 mmol, 2 equiv), and $NEt_3$ (1.5 mL, 10.7 mmol, 9 equiv) in 30 mL of anhydrous DMF at room temperature was added EDC·HCl (456 mg, 2.38 mmol, 2 equiv). The mixture was stirred overnight, and the solvent was removed. The crude product was dissolved in 30 mL of $CH_2Cl_2$ and washed with 20 mL of saturated $Na_2CO_3$ and 20 mL of brine. The organic layer was dried with $Na_2SO_4$ and concentrated in vacuo. Chromatography on silica gel using AcOEt/MeOH (90/10) followed by AcOEt/MeOH/$NH_4OH_{aq20\%}$ (87/10/3) afforded 330 mg (24% yield) of **8a** as white hygroscopic foam. $^1$H NMR (200 MHz) $\delta$ 7.79 (s, 2H), 7.32 (s, 4H), 6.28 (s, 2H), 4.49 (bs, 4H), 4.33 (m, 8H), 3.98 (s, 4H), 3.82 (s, 6H), 3.13 (t, $J$ = 6,0 Hz, 4H), 2.92 (m, 4H), 2.70 (t, $J$ = 6,0 Hz, 4H), 2.05−1.94 (m, 8H), 1.65−1.26 (m, 24H). $^{13}$C NMR (50 MHz) $\delta$ 171.7, 169.2,

164.4, 160.2, 147.9, 155.4, 133.1, 131.6, 122.5, 109.8, 98.2, 85.9, 81.5, 80.4, 61.8, 56.7, 55.9, 53.4, 50.9, 44.7, 38.7, 35.6, 29.6, 28.1. MS (ESI) $m/z = 1147$ $[M+H]^+$. Anal. ($C_{58}H_{76}N_8O_{12}Cl_2 \cdot 2.5H_2O$), C, H, N.

**4-(*tert*-Butoxycarbonyl(3-(4-iodophenyl)prop-2-ynyl)amino)-butanoic Acid (10).** To a stirring solution of 1,4-diiodobenzene (5.469 g, 16.59 mmol, 5 equiv), $PdCl_2(PPh_3)_2$ (117 mg, 0.17 mmol, 0.05 equiv), and CuI (63 mg, 0.33 mmol, 0.1 equiv) in 10 mL of freshly distilled $NEt_3$ at 0 °C was added dropwise a solution of **2b** (800 mg, 3.32 mmol, 1 equiv) in 20 mL of anhydrous DMF. The solution was stirred at 0 °C for 1 h and allowed to warm to room temperature overnight. After removal of the solvents, the crude mixture was treated with 70 mL of 5% $K_2CO_3$ and extracted with 150 mL of AcOEt. The aqueous layer was acidified to pH 3 with 1 M HCl and extracted with 100, 70, and 35 mL of $CH_2Cl_2$. The combined organic layers were washed with 100 mL of saturated NaCl and dried over $Na_2SO_4$. After concentration, purification by chromatography on silica gel using AcOEt/cHex (3/7) containing 0.5% AcOH afforded 719 mg (49% yield) of **10** as a brown oil. $R_f$(cHex/AcOEt (4/6) + 0.5% AcOH) = 0.53. $^1$H NMR (200 MHz) $\delta$ 7.62 (d, $J = 8.4$ Hz, 2H), 7.12 (d, $J = 8.4$ Hz, 2H), 4.23 (bs, 2H), 3.43 (t, $J = 6.9$ Hz, 2H), 2.40 (t, $J = 7.2$ Hz, 2H), 1.96 (m, 2H), 1.47 (s, 9H). $^{13}$C NMR (50 MHz) $\delta$ 178.7, 157.8, 155.3, 137.6, 133.3, 122.5, 86.7, 82.6, 80.7, 46.0, 37.3, 31.3, 28.5, 23.3.

***tert*-Butyl 4-(Cyclohexylamino)-4-oxobutyl(prop-2-ynyl)carbamate (11).** To a solution of **2b** (795 mg, 3.32 mmol, 1 equiv) in 30 mL of a 2/1 mixture of dry DMF and dry $CH_2Cl_2$ was added $NEt_3$ (1.39 mL, 9.95 mmol, 3 equiv), cyclohexylamine (270 $\mu$L, 3.98 mmol, 1.2 equiv), HOBt·$H_2O$ (539 mg, 3.98 mmol, 1.2 equiv), and EDC·HCl (764 mg, 3.98 mmol, 1.2 equiv). The mixture was stirred overnight at room temperature, and the solvents were removed under vacuum. The crude product was dissolved in 50 mL of AcOEt and washed with 25 mL of 1 M $KHSO_4$, 25 mL of saturated $Na_2CO_3$, and 25 mL of brine. The organic layer was dried over $Na_2SO_4$ and concentrated. Purification on silica gel, using AcOEt/cHex (4/6) as eluent, afforded 646 mg (61% yield) of **11** as a pale-yellow oil. $R_f$(cHex/AcOEt, 6/4) = 0.34. $^1$H NMR (200 MHz) $\delta$ 3.98 (bs, 2H), 3.74 (m, 1H), 3.35 (t, $J = 6.7$ Hz, 2H), 2.21−2.06 (m, 3H), 1.98−1.81 (m, 4H), 1.78−1.51 (m, 2H), 1.46 (s, 9H), 1.37−1.01 (m, 4H). $^{13}$C NMR (50 MHz) $\delta$ 177.3, 175.8, 161.6, 159.0, 155.8, 155.4, 131.6, 87.1, 83.0, 80.7, 48.4, 46.0, 37.3, 33.9, 33.1, 31.3, 28.3, 25.7, 24.9, 23.4.

**4-(*tert*-Butoxycarbonyl(3-(4-(3-(*tert*-butoxycarbonyl(4-(cyclohexylamino)-4-oxobutyl)amino)prop-1-ynyl)phenyl)prop-2-ynyl)amino)butanoic Acid (12).** To a stirring solution of **10** (656 mg, 1.48 mmol, 1 equiv), $PdCl_2(PPh_3)_2$ (53 mg, 0.074 mmol, 0.05 equiv), and CuI (30 mg, 0.15 mmol, 0.1 equiv) in 20 mL of a 1/1 mixture of freshly distilled $NEt_3$ and anhydrous DMF at room temperature was added dropwise a solution of **11** (497 mg, 1.55 mmol, 1.05 equiv) in 10 mL of anhydrous DMF. 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 (4/6) containing 0.5% AcOH, to give 503 mg (53% yield) of **12** as a yellow oil. $R_f$(cHex/AcOEt (1/1) + 0.5% AcOH) = 0.39. $^1$H NMR (200 MHz) $\delta$ 7.32 (s, 4H), 4.41 (bs, 2H), 4.23 (bs, 2H),3.70 (m, 1H), 3.41 (dd, $J = 11.8$ and 6.7 Hz, 4H), 2.38 (t, $J = 7.2$ Hz, 2H), 2.17 (t, $J = 7.2$ Hz, 2H), 1.90 (m, 4H), 1.64 (m, 4H), 1.56−1.39 (m, 22H), 1.17 (m, 2H). $^{13}$C NMR (50 MHz) $\delta$ 177.3, 175.8, 161.6, 159.0, 155.8, 155.4, 131.6, 87.1, 83.0, 80.7, 48.4, 46.0, 37.3, 33.9 and 33.1 (two signals due to rotamers), 28.5, 28.3, 25.7 and 24.9 (two signals due to rotamers), 23.4.

**2-(4-(4-(*tert*-Butoxycarbonyl(3-(4-(3-(*tert*-butoxycarbonyl(4-(cyclohexylamino)-4-oxobutyl)amino)propyl)phenyl)propyl)-amino)butanamido)piperidin-1-yl)ethyl 4-Amino-5-chloro-2-methoxybenzoate (13).** To a stirring solution of **12** (343 mg, 0.54 mmol) in 30 mL of MeOH at room temperature was added 36 mg of 10% Pd/C. The mixture was stirred under 1 bar of $H_2$ overnight and filtered through a pad of Celite. Evaporation of the solvent yielded 327 mg (94%) as a colorless oil. An amount of 230 mg (0.36 mmol, 1 equiv) of this oil was dissolved in 30 mL of dry DMF, and to this solution at room temperature was added **7a** (144 mg, 0.36 mmol, 1 equiv),[20] HBTU (145 mg, 0.38 mmol, 1.05 equiv), and $NEt_3$ (200 $\mu$L, 1.44 mmol, 4 equiv). After 5 h at room temperature, the solvents were removed, and the crude product was dissolved in 20 mL of AcOEt and washed with 10 mL of saturated $Na_2CO_3$ and 10 mL of brine. The organic layer was dried with $Na_2SO_4$ and concentrated in vacuum. Purification by chromatography on silica gel, using AcOEt/MeOH (95/5) followed by AcOEt/MeOH/$NH_4OH_{aq20\%}$ (92/5/3) afforded 280 mg (82% yield) of **13** as light-yellow foam. $R_f$(AcOEt/MeOH/$NH_4OH_{aq20\%}$, 92/5/3) = 0.21. $^1$H NMR (200 MHz) $\delta$ 7.76 (s, 1H), 7.05 (s, 4H), 6.72 (bs, 1H), 6.48 (bs, 1H), 6.27 (s, 1H), 4.62 (bs, 2H), 4.32 (t, $J = 5.9$ Hz, 2H), 3.72 (m, 5H), 3.19 (m, 8H), 2.89 (m, 2H), 2.70 (t, $J = 5.9$ Hz, 2H), 2.52 (m, 4H), 2.23 (m, 2H), 2.08 (m, 4H), 1.79 (m, 12H), 1.29−0.99 (m, 28H). $^{13}$C NMR (50 MHz) $\delta$ 172.0, 171.6, 164.6, 160.3, 156.2, 156.1, 148.1, 139.2, 133.3, 128.3, 109.9, 109.6, 98.3, 79.6, 62.3, 56.8, 56.1, 52.6, 48.2, 46.9, 46.3, 45.9, 33.8, 33.7, 33.1, 32.9, 32.2, 30.2, 28.5, 25.6, 24.9, 24.7. MS (ESI) $m/z = 978$ $[M + H]^+$. Anal. ($C_{51}H_{79}N_6O_9Cl \cdot 1.5H_2O$), C, H, N.

**Biological Methods. Cell Culture and Transient Transfection.** Chinese hamster ovary (CHO) cells were grown at 37 °C and 5% $CO_2$ in HamsF12 medium and Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum, 10 mM HEPES (pH 7.4), and antibiotics. Transient transfection experiments were performed using the transfection reagent Jet-PEI (Polyplus-Transfection, Illkirch, France) according to the manufacturer's instructions. Final concentration of DNA was adjusted to 8 $\mu$g per 100 mm Petri dish.

**Membrane Preparation and Radioligand Binding Assays.** 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 centrifugated 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 centrifugated at 40000*g* for 20 min at 4 °C. The resulting pellet was resuspended in 15 volumes of HEPES (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 250 $\mu$L of HEPES buffer (50 mM, pH 7.4), 20 $\mu$L of the studied ligand (seven concentrations), 20 $\mu$L of [$^3$H]-GR113808 at a concentration of 0.2 nM, and 50 $\mu$L of membranes preparation (100−200 $\mu$g of protein). Nonspecific binding was determined with 10 $\mu$M GR113808. Tubes were incubated at 25 °C for 30 min, and the reaction was terminated by filtration through Watman 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 LS6500C 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.

**cAMP Accumulation.** C6 glial cells stably transfected with h5-$HT_{4(e)}$ 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 $\mu$M pargyline. 5-HT (1 $\mu$M) 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 $\mu$L of ice-cold perchloric acid (20%). After a 30 min period, neutralization buffer was added (25 mM HEPES, 2 N KOH), supernatant was extracted after 5 min of centrifugation at 2000*g*, and cAMP was quantified using a radioimmunoassay kit (cAMP competitive radioimmunoassay, Beckman, France). The 5-HT concentration−effect curve was calculated using seven concentrations ($10^{-10}$−$10^{-6}$) alone or in the presence of compounds. The ligand concentration−effect curves were calculated using seven concentrations ($10^{-10}$−$10^{-5}$).

**Bioluminescence Resonance Energy Transfert (BRET) Assay.** The BRET experiment and the 5-$HT_4$R fusion proteins used for

BRET studies (5-$HT_4$R−RLuc, 5-$HT_4$R−YFP) have been previously described.[19] Two days after transfection, CHO cells transfected with 5-$HT_4$R fusion proteins were detached and washed with PBS. Membrane preparations were distributed in a 96-well optiplate (Packard) in the presence or absence of ligand at 25 °C. Coelenterazine h substrate (Molecular Probes, Eugene, OR) was added at a final concentration of 5 $\mu$M, and readings were performed with a lumino/fluorometer Fusion (Packard), which allows the sequential integration of luminescence signals detected with two filter settings (RLuc filter, 485 ± 10 nm; YFP filter, 530 ± 12.5 nm). Emission signals at 530 nm were divided by emission signals at 485 nm. The difference between this emission ratio obtained with cotransfected RLuc and YFP fusions proteins and that obtained with the RLuc fusion protein alone was defined as the BRET ratio. Results were expressed in milli-BRET units (mBU, with 1 mBU corresponding to the BRET ratio value multiplied by 1000).

**Molecular Modeling.** Manual docking of the ML10302 part is derived from our previously refined [5-$HT_4$R−ML10302] complex model.[26] After the different spacers were built, molecular dynamics at 1000 K followed by annealing to 300 K were carried out, the seven-transmembrane domain being frozen. The final structure was then energy-minimized using 10 000 steps of a conjugated gradient procedure.

Modeling was performed on an "Octane" Silicon Graphics computer, using software from Molecular Simulation Inc. (InsightII, Builder, and Discover). For all these calculations, a distance-dependent dielectric constant was used to avoid overevaluation of electrostatic interactions between charged groups. A cutoff of 2 nm was used to truncate the list of interactive atoms to a reasonable size in relation to the available central processing unit (CPU) time.

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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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