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Development of Molecular Probes for the Human 5-HT$_6$ Receptor

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In this work we report the synthesis of a set of labeled ligands targeting the human 5-HT$_6$ receptor (h5-HT$_6$R). Among the synthesized compounds, fluorescent probe **10** (K_i = 175 nM and Φ_f = 0.21) and biotinylated derivative **15** (K_i = 90 nM) deserve special attention because they enable direct observation of the h5-HT$_6$R in cells. Thus, they represent the first molecular probes for 5-HT$_6$R visualization. These results are the starting point for introducing a variety of tags in these or other 5-HT$_6$R ligand scaffolds aimed at the development of optimized probes with tailored profiles in terms of fluorescence, affinity, or selectivity.

Introduction

Fluorescence-based molecular imaging facilitates visualization of specific biomolecules in their native environments. Current advances in this field include the development of fluorescent small molecules that allow detection of metabolites and ions,[1,2] imaging of glycan- and lipid-containing proteins,[3−5] and profiling of enzyme activities.[6] However, extension of these strategies to G-protein-coupled receptors (GPCRs[a]), which are the target of about the 50% of current marketed drugs, has proved challenging. Changes in levels and expression pattern of GPCRs have been associated with a variety of disease states including central nervous system (CNS) related pathologies and cancer.[7−9] Therefore, the ability to directly monitor GPCRs in the context of cells, tissues, and organisms would undoubtedly augment our understanding of their physiological significance and provide new clinical tools boosting drug development. Although antibodies against GPCRs enable their visualization in vitro or ex vivo, they have in vivo limited use. In addition, generation of GPCR antibodies is difficult because of their structure, poor immunogenicity, and low receptor density.[10] These technical challenges have hampered the general availability of GPCR antibodies and are associated with the restricted utility in many experimental formats of some of the available ones. Thus, the development of complementary methods for GPCR imaging is of great importance. Moreover, the introduction of alternative tags other than fluorophores to obtain additional information in a high-throughput manner comparable to activity-based protein profiling approaches in the field of enzymes is still lacking.[6]

With this broad objective in mind, we have initiated a project aimed at the search of fluorescent probes that enable direct visualization of GPCRs in complex biological systems. Although some GPCR fluorescent ligands have been recently described, their suitability for application in cell systems has proved challenging.[11] Ideally, excellent probes would be ligands with high affinity (i.e., K_i < 100 nM) and with fluorescence emission intensity as high as possible.

In this work, we have focused our efforts on the 5-HT$_6$ receptor (5-HT$_6$R), one of the most recent additions to the serotonin family of GPCRs. This receptor has been involved in the pathogenesis of CNS diseases related to cognitive or eating disorders,[12,13] and several 5-HT$_6$R ligands have entered clinical trials although none of them is on the market yet. In this regard, availability of agents able to directly monitor the 5-HT$_6$R, which to our knowledge has not been described so far, would aid the therapeutic validation of this receptor for the proposed or even new indications.

In this work we report the synthesis of a set of probes **1**−**15** based on the potent 5-HT$_6$R antagonist developed by SmithKlineBeecham SB-271046[14,15] (**16**, pK_i = 8.9; pA_2 = 8.7, Figure 1). Among the synthesized compounds, fluorescent probe **10** (K_i = 175 nM and fluorescence quantum yield, Φ_f, of 0.21) and biotinylated derivative **15** (K_i = 90 nM) deserve special attention, as they enable direct observation of the h5-HT$_6$R in cells. These results support the validity of our approach and represent the starting point for further probe optimization with tailored profiles in terms of tag incorporation, affinity, and selectivity, experiments that are underway in our laboratory.

Results and Discussion

Initially we based our structural exploration on the scaffold of **16**, in which we have studied different structural possibilities for the introduction of the fluorescent tag aiming to have a minimal influence on the biological activity of the parent compound. Accordingly, we considered small sets of probes where the tag was introduced (a) in the piperazine ring, (b) in

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[a] Abbreviations: 5-HT, serotonin; ACN, acetonitrile; a.u., arbitrary units; Ds, dansyl; DIPEA, *N*,*N*-diisopropylethylamine; Phth, phthalimide; BINAP, (±)-2,2′-bis(diphenylphosphino)-1,1′-binaphthalene; Em, emission; Ex, excitation; Φ_f, fluorescence quantum yield; GPCR, G-protein-coupled receptor; h5-HT$_6$R, type 6 of the human 5-HT receptor; HOBt, 1-hydroxybenzotriazole; *I*, intensity; SAR, structure−activity relationship.

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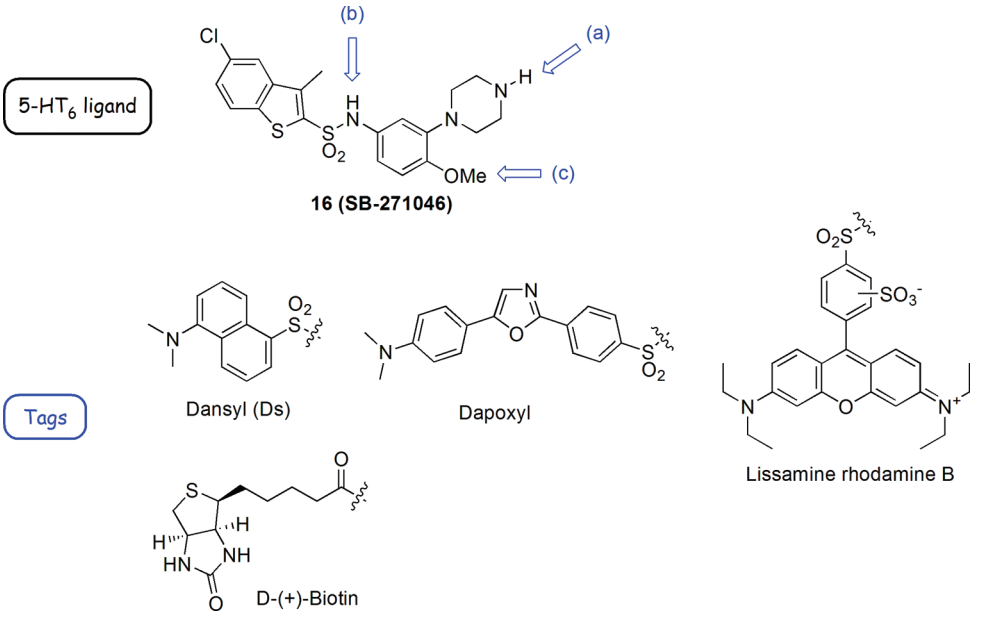
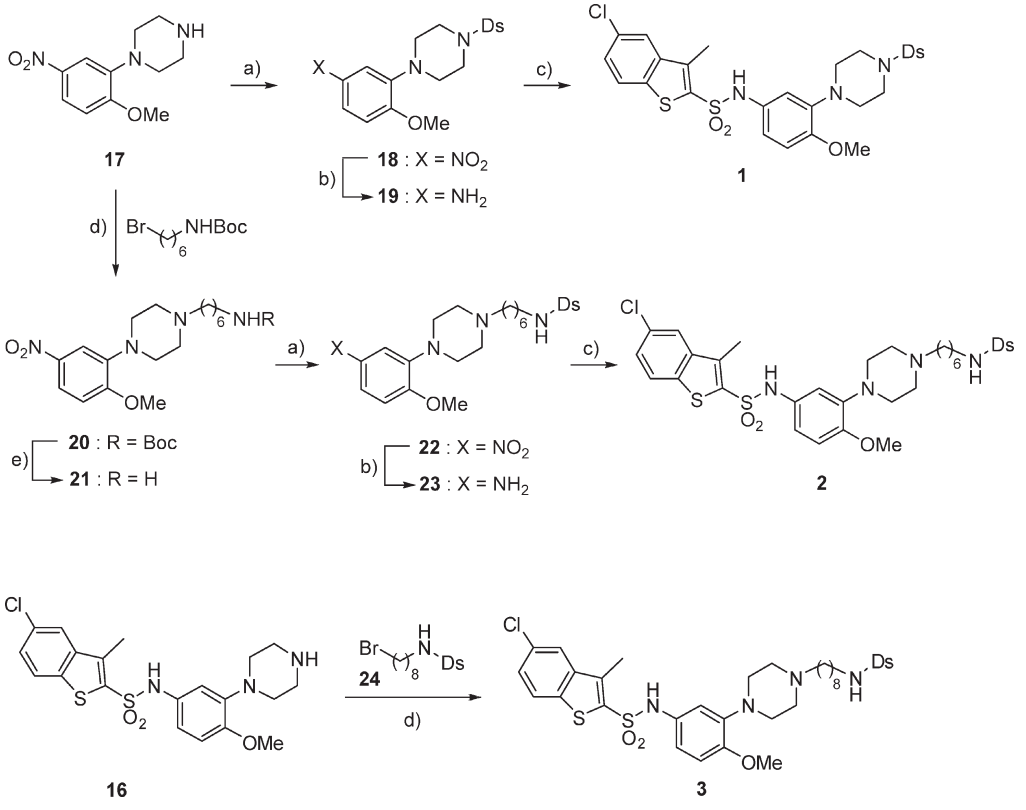


Figure 1. Labeled derivatives of the h5-HT_6R antagonist **16**.

Scheme 1. Synthesis of Dansylated Derivatives **1**−**3**[a]

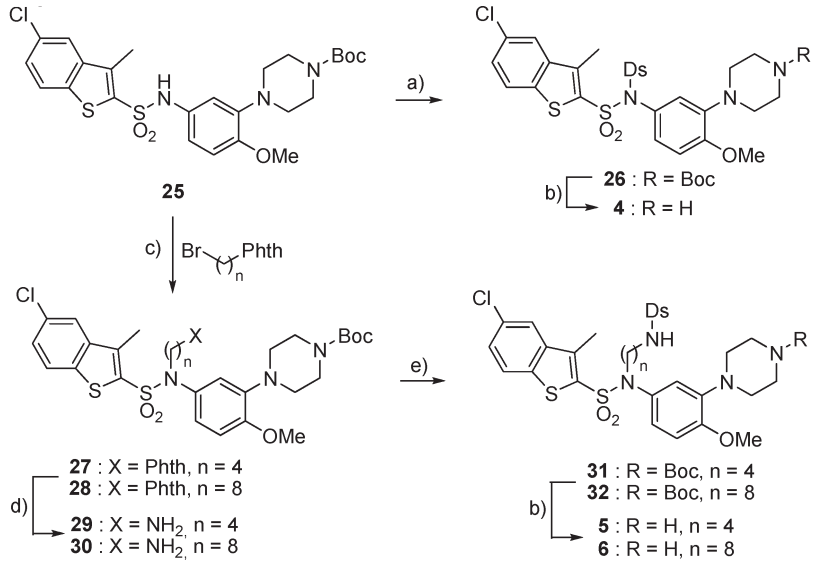


[a] Reagents and conditions: (a) DsCl (1.5 equiv), Et_3N (2 equiv), CH_2Cl_2, room temp, 12 h, 66−74%; (b) H_2 (40 psi), Pd/C (8% wt), EtOH/H_2O (200:1), room temp, 16 h, 94−96%; (c) 5-chloro-3-methylbenzo[*b*]thiophene-2-sulfonyl chloride (1.5 equiv), pyridine (2 equiv), CH_2Cl_2, room temp, 12 h, 62−66%; (d) NaI (2 equiv), ACN, reflux, 32 h, 79−89%; (e) TFA (20 equiv), CH_2Cl_2, room temp, 10 h, 99%.

the sulfonamide moiety, or (c) in the methoxy group (Figure 1). As tag, a dansyl ((5-dimethylamino)naphthalene-1-sulfonyl, Ds) group was originally selected by virtue of its synthetic versatility, small size, relatively large Stokes shift, the high degree of sensitivity of its emission wavelength and quantum yield to environmental changes, and its suitability for use in cellular environments.[16,17] In all cases, the tag was attached either directly or through a variable length aliphatic spacer that may provide some flexibility to the fluorophore in order to minimally affect probe−receptor affinity.

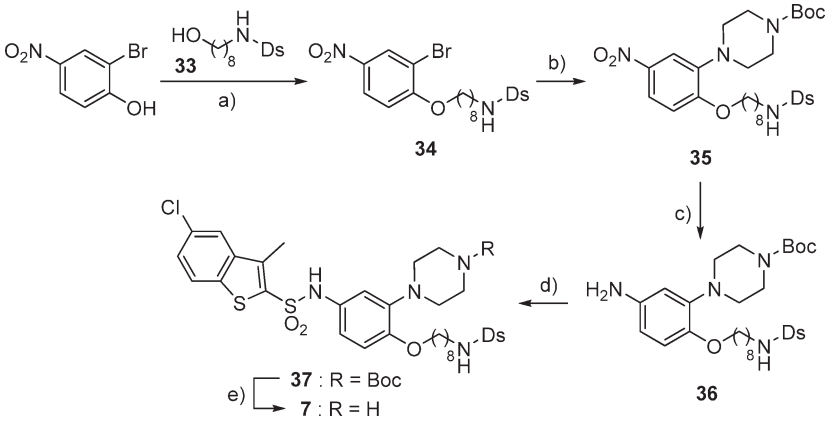
Synthesis of Probes Based on the Scaffold of 16. We prepared the first series of labeled compounds by attaching a dansyl group to the nitrogen of the piperazine ring of **16** either directly or through a spacer (Figure 1). The reaction

Scheme 2. Synthesis of Dansylated Derivatives **4**−**6**[a]



[a] Reagents and conditions: (a) DsCl (1.5 equiv), DIPEA (2 equiv), DMAP cat., CH_2Cl_2, room temp, 12 h, 85%; (b) TFA (20 equiv), CH_2Cl_2, room temp, 6 h, 81−97%; (c) NaH (1.8 equiv), DMF, 90 °C, 16 h, 95−96%; (d) $N_2H_4 \cdot H_2O$ (15 equiv), EtOH, 50 °C, 5 h, 86−98%; (e) DsCl (1.5 equiv), Et_3N (3 equiv), CH_2Cl_2, room temp, 16 h, 73−76%.

Scheme 3. Synthesis of Dansylated Derivative **7**[a]



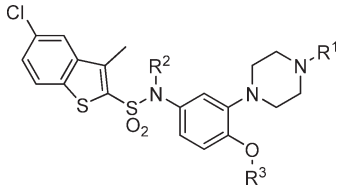
[a] Reagents and conditions: (a) DEAD (2 equiv), PPh_3 (2 equiv), THF, reflux, 34 h, 60%; (b) 1-Boc-piperazine (3 equiv), $Pd(OAc)_2$ (10 mol %), BINAP (15 mol %), Cs_2CO_3 (1.5 equiv), toluene, 100 °C, 24 h, 21%; (c) H_2 (40 psi), Pd/C (8% wt), EtOH/H_2O (100:1), room temp, 16 h, 99%; (d) 5-chloro-3-methylbenzo[*b*]thiophene-2-sulfonyl chloride (1.5 equiv), pyridine (2 equiv), CH_2Cl_2, room temp, 12 h, 80%; (e) TFA (20 equiv), CH_2Cl_2, room temp, 10 h, 88%.

of 1-(2-methoxy-5-nitrophenyl)piperazine **17** with dansyl chloride yielded intermediate **18**. Hydrogenation of the nitro group in **18** afforded the aniline derivative **19** that was then reacted with 5-chloro-3-methylbenzo[*b*]thiophene-2-sulfonyl chloride to afford the target compound **1**. Compound **2**, in which the dansyl group is attached to the piperazine through a hexamethylene chain, was prepared following a similar procedure starting with alkylation of **17** with *N*-Boc-6-bromohexylamine. Further increase of the spacer length (compound **3**) was achieved by direct alkylation of **16** with the dansylated bromooctylamine **24** (Scheme 1).

A second series of labeled ligands derived from **16** was synthesized by linking the dansyl group to the nitrogen atom of the sulfonamide group through alkyl spacers of variable length (Figure 1). Compound **4**, where the dansyl group is directly attached to the core structure, was prepared by reaction of the derivative **25** with dansyl chloride in the presence of DIPEA. Compounds **5** and **6**, in which the fluorophore is linked to the sulfonamide nitrogen through four and eight carbon alkyl chains, respectively, were prepared following an alkylation sequence (Scheme 2). Thus, reaction of **25** with the corresponding *N*-(bromoalkyl)phthalimide afforded the alkylated products **27** and **28**. Subsequent removal of the phthalimide followed by reaction with dansyl chloride led to the protected intermediates **31** and **32** that were finally transformed into the target compounds **5** and **6** by cleavage of the Boc group with TFA in dichloromethane.

Finally, we also explored whether it was possible to substitute the methoxy group of **16** by a dansyl tag attached through a spacer (see Figure 1). This compound was prepared following the synthetic route shown in Scheme 3. Mitsunobu reaction of 2-bromo-4-nitrophenol with the dansyl tagged alcohol **33** yielded compound **34**. Buchwald−Hartwig coupling of **34** with 1-Boc-piperazine led to **35** that was then transformed to the corresponding aniline derivative **36**. Its reaction with the sulfonyl chloride derivative followed by

Table 1. 5-HT_6R Affinity and Fluorescence Values for Probes **1**−**7**



Compd.	R^1	R^2	R^3	K_i (nM)[a]	Fluorescence[b]	
					λ_{em} (nm)	I_{em} (a.u.)[c]
1	Ds	H	Me	165±8	495	730
2		H	Me	266±7	498	>1000
3		H	Me	447±25	488	>1000
4	H	Ds	Me	27±2	488	19
5	H		Me	747±50	489	221
6	H		Me	215±19	487	>1000
7	H	H		741±42	468	700

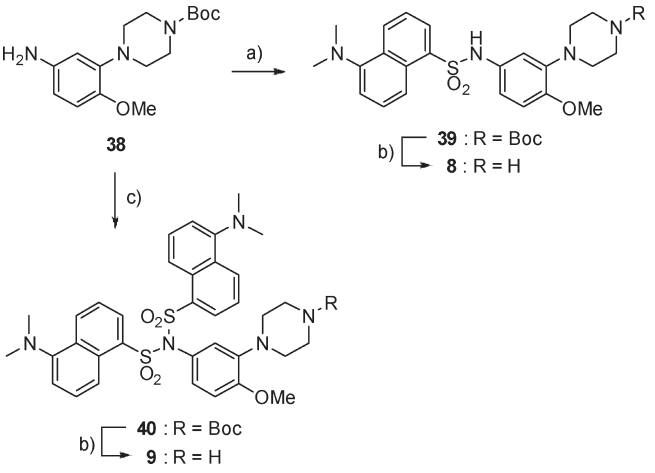
[a] The values are the mean ± SEM from two to four independent experiments performed in triplicate. [b] Fluorescence values were determined at 10 μM in binding assay buffer (50 mM Tris-HCl, 0.5 mM $MgSO_4$, pH 7.4) at 25 °C using an excitation slit of 2.5 nm. Excitation wavelength (λ_{ex}) was 350 nm for all the compounds except for **3** (λ_{ex} = 338 nm). [c] Emission slit of 5 nm.

cleavage of the Boc protecting group rendered the final compound **7**.

Biological Data and Fluorescent Properties of Probes 1−7. Table 1 shows the affinity values for the h5-HT_6R and the fluorescent properties of the probes **1**−**7**. Affinity values were determined by competition binding assays on membranes from h5-HT_6R stably expressing HEK-293 EBNA cells, using [^{3}H]LSD as radioligand. The fluorescent properties of compounds **1**−**7** were determined in 10 μM solutions of the corresponding compound in the buffer solution used for the binding assays. In general, the introduction of a dansyl group at any of the positions of **16** was detrimental for the h5-HT_6R affinity, which remained between moderate (compounds **1**, **2**, and **6**) and low (compounds **3**, **5**, and **7**). Only derivative **4** conserved high affinity (K_i = 27 nM), though in this case with a negligible fluorescence. The highest fluorescence values were shown by derivatives **2**, **3**, and **6**. Taken together, all these data indicate that modification of **16** by attachment of a fluorophore is not likely to yield a fluorescent high-affinity 5-HT_6R ligand.

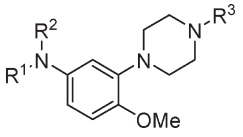
Therefore, we envisioned the possibility of replacing the benzothiophenesulfonyl moiety of **16** for the dansyl group. According to our previous pharmacophore model and structure−affinity relationship (SAR) studies,[18] this modification

Scheme 4. Synthesis of Compounds **8** and **9**[a]



[a] Reagents and conditions: (a) DsCl (1 equiv), pyridine (2 equiv), CH_2Cl_2, room temp, 15 h, 82%; (b) TFA (20 equiv), CH_2Cl_2, room temp, 6 h, 83−93%; (c) DsCl, DIPEA (2 equiv), DMAP cat., CH_2Cl_2, room temp, 12 h, 50%.

Table 2. 5-HT_6R Affinity and Fluorescence Values for Probes **8**−**12**



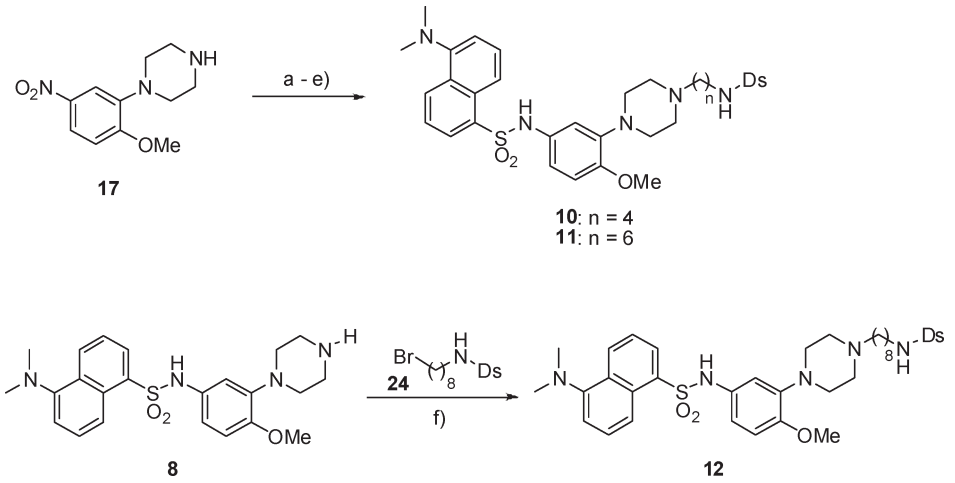
Compd.	R^1	R^2	R^3	K_i (nM)[a]	Fluorescence[b]	
					λ_{em} (nm)	I_{em} (a.u.)[c]
8	Ds	H	H	8.0±0.2	528	52
9	Ds	Ds	H	135±9	490	35
10	Ds	H		175±9	495	>1000
11	Ds	H		554±14	487	287
12	Ds	H		194±14	489	450

[a] The values are the mean ± SEM from two to four independent experiments performed in triplicate. [b] Fluorescence values were determined at 10 μM in binding assay buffer (50 mM Tris-HCl, 0.5 mM $MgSO_4$, pH 7.4 at 25 °C) using an excitation slit of 2.5 nm. λ_{ex} was 350 nm for **8** and **9**, 340 nm for **10** and **11**, and 338 nm for **12**. [c] Emission slit of 5 nm.

was likely to keep high 5-HT_6R affinity and we were confident in that this new scaffold would be fluorescent.

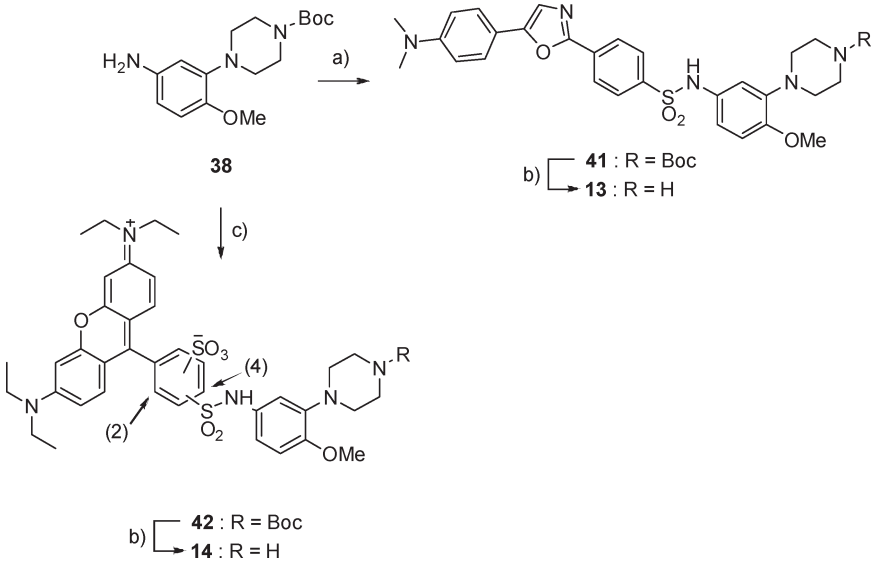
Dansyl-Based Probes. Initially, we synthesized an analogue of **16** in which the dansyl group replaces the benzothiophenesulfonyl group (Scheme 4). Thus, reaction of *N*-Boc-1-(5-amino-2-methoxyphenyl)piperazine **38** with dansyl chloride in the presence of pyridine yielded the expected compound **8** after deprotection of the piperazine ring by treatment with TFA. Although this compound showed a high 5-HT_6R affinity (K_i = 8 nM), it was not fluorescent (Table 2). Not even the introduction of a second dansyl moiety in the sulfonamide group (derivative **9**), obtained

Scheme 5. Synthesis of Compounds **10**−**12**[a]



[a] Reagents and conditions: (a) $Br(CH_2)_nNHBoc$, NaI (2 equiv), ACN, reflux, 32 h, 38−89%; (b) TFA (20 equiv), CH_2Cl_2, room temp, 10 h, 99%; (c) DsCl (1.5 equiv), Et_3N (3 equiv), CH_2Cl_2, room temp, 12 h, 74−79%; (d) H_2 (40 psi), Pd/C (8% wt), EtOH/H_2O (200:1), room temp, 16 h, 94%; (e) DsCl (1.5 equiv), pyridine (2 equiv), CH_2Cl_2, room temp, 16 h, 65−71%; (f) **24**, NaI (2 equiv), ACN, reflux, 32 h, 45%.

Scheme 6. Synthesis of Compounds **13** and **14**[a]

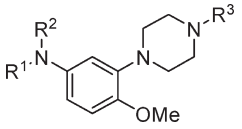


[a] Reagents and conditions: (a) dapoxyl chloride (1.1 equiv), pyridine (2 equiv), CH_2Cl_2, room temp, 16 h, 84%; (b) TFA (20 equiv), CH_2Cl_2, room temp, 6 h, 91−99%; (c) Lissamine rhodamine B sulfonyl chloride (1 equiv), Et_3N (5 equiv), THF, 80 °C, 24 h, 52%.

under the same conditions as **8** when diisopropylethylamine was used as base, allowed for obtaining significant fluorescence. Therefore, we focused our efforts on establishing whether the introduction of a second dansyl group attached to the piperazine by a methylene spacer of variable length would render simultaneous high 5-HT_6R affinity and fluorescence. Compounds **10** and **11** were synthesized following a route analogous to that used for compound **2** (see Scheme 1), attaching the second dansyl group to the aromatic ring in the last step of the sequence, whereas derivative **12**, with the longest spacer (n = 8), was synthesized by direct alkylation of the piperazine nitrogen of the parent compound **8** with the tagged chain **24** (Scheme 5).

Determination of the 5-HT_6R affinity and fluorescence (Table 2) of the synthesized compounds showed that **10**, with a K_i value of 175 nM, a fluorescence emission intensity (I_{em}) above 1000 a.u. for excitation and emission slits of 2.5 and 5 nm and a high Stokes shift, exhibited the best profile in

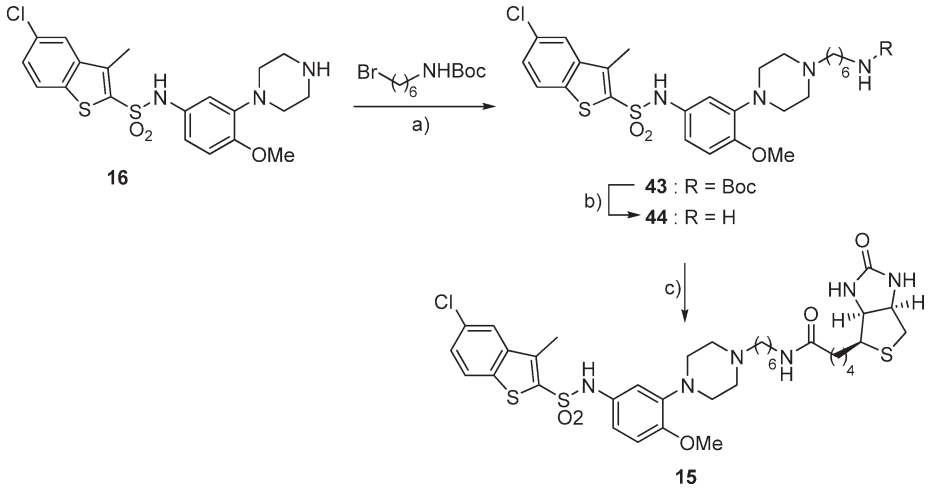
Table 3. 5-HT_6R Affinity and Fluorescence Values for Compounds **13** and **14**



					fluorescence[b]		
compd	R^1	R^2	R^3	K_i[a] (nM)	λ_{ex}[c] (nm)	λ_{em}[d] (nm)	I_{em} (a.u.)
13	dapoxyl	H	H	523 ± 22	370		*e*
14	lissamine	H	H	>1000	570	588	550

[a] The values are the mean ± SEM from two to four independent experiments performed in triplicate. [b] Fluorescence values were determined at 10 μM in binding assay buffer (50 mM Tris-HCl, 0.5 mM $MgSO_4$, pH 7.4 at 25 °C). Wavelengths for the maximal absorption and emission. [c] Excitation slit of 2.5 nm. [d] Emission slit of 5 nm. [e] Non-fluorescent compound.

Scheme 7. Synthesis of Biotin-Tagged Compound **15**[a]



[a] Reagents and conditions: (a) NaI (2 equiv), ACN, reflux, 36 h, 81%; (b) TFA (20 equiv), CH_2Cl_2, room temp, 6 h, 83%; (c) D-(+)-biotin (0.83 equiv), HOBt (0.2 equiv), DCC (1.1 equiv), DMAP (1 mol %), DMF, room temp, 24 h, 82%.

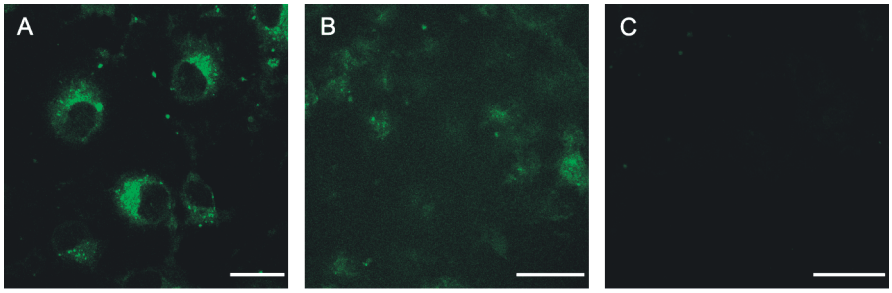


Figure 2. Labeling of COS7 cells transiently transfected with h5-HT_6R with fluorescent compound **10**. Cells were incubated in the presence of **10** (5 μM) for 10 min, washed, fixed, mounted, and then observed by confocal microscopy (A). To assess specificity, nontransfected cells were labeled under the same conditions (B) or transfected cells were incubated with **10** in the presence of an excess (50 μM) of **16** (C). Scale bar represents 25 μm. Preparations were visualized under a SP2 Leica confocal microscope with a 63× objective with constant laser beam and photodetector sensitivity and are representative of two or three independent experiments.

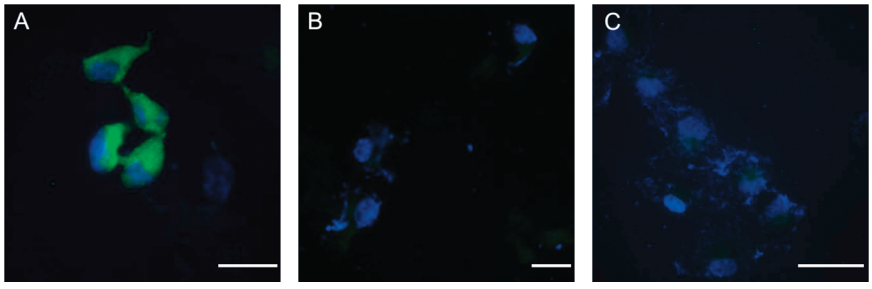


Figure 3. Labeling of COS7 cells transiently transfected with h5-HT_6R with biotinylated compound **15**. Cells were fixed, incubated in the presence of **15** (5 μM) for 10 min followed by streptavidin−Alexa Fluor 488 conjugate, mounted, and then observed by fluorescence microscopy. Nuclei were counterstained with Hoechst 33258 (A). To assess specificity, nontransfected cells were labeled under the same conditions (B) or in the absence of probe (C). Scale bar represents 25 μm. Preparations were visualized under a Zeiss Axioplan2 fluorescence microscope. Images are representative of two independent experiments.

terms of affinity and fluorescence and therefore could be a candidate to assess its potential as a probe for 5-HT_6R visualization in cellular systems. In spite of this encouraging data, we notice that even the best values obtained for simultaneous fluorescence and affinity (compound **10**, $K_i = 175$ nM; $I_{em} > 1000$ a.u., Table 2) are only moderate, so it was conceivable that further optimization of the probes would be highly desirable for their use in complex biological settings. However, the extensive structural variations addressed in derivatives **1**−**12** suggest that dansyl labeling of **16** does not allow for high affinity and fluorescent 5-HT_6R ligands. Therefore, we envisioned the possibility of incorporating other fluorophores or a versatile biotin subunit. Considering that substitution of the benzothiophenesulfonyl group in **16** for a dansyl moiety (compound **8**) kept 5-HT_6R affinity [K_i (**8**) = 8 nM], we tried to replace it for dapoxyl (4-{5-[4-(dimethylamino)-phenyl]-1,3-oxazol-2-yl}benzenesulfonyl) or lissamine rhodamine B (2-(chlorosulfonyl)-5-[6-(diethylamino)-3-(diethyliminio)-3*H*-xanthen-9-yl]benzenesulfonate), fluorescent moieties with higher extinction coefficients than dansyl group. Dapoxyl-labeled derivative **13** was synthesized by treatment of *N*-Boc-1-(5-amino-2-methoxyphenyl)piperazine **38** with dapoxyl chloride followed by cleavage of the Boc protecting group under standard conditions. On the other hand, reaction of **38** with

lissamine rhodamine B sulfonyl chloride (mixture of isomers) and subsequent deprotection of the piperazine ring yielded probe **14** as a mixture of isomers (Scheme 6).

Unfortunately, both compounds exhibited poor 5-HT_6R affinity and low fluorescence (Table 3). These results made us consider the piperazine ring as an attachment point of the biotin moiety. Thus, N-alkylation of **16** with *N*-Boc-6-bromohexylamine led to compound **43**. Cleavage of the Boc protecting group followed by coupling reaction with biotin afforded the tagged compound **15** (Scheme 7) which was characterized as 5-HT_6R ligand (K_i = 90 nM).

Cell Visualization. On the basis of all these results, we have selected two probes to carry out visualization experiments, dansyl-based derivative **10**, with the best balance between 5-HT_6R affinity and fluorescence (K_i = 175 nM; I_{em} > 1000 a.u. for excitation and emission slits of 2.5 and 5 nm; λ_{ex} = 340 nm; λ_{em} = 495 nm; Φ_f = 0.21), and the biotinylated probe **15** (K_i = 90 nM), which can be visualized using fluorophore-conjugated streptavidin. To assess whether these probes kept the binding profile of the parent compound **16**, we selected some representative metabotropic serotonin receptors (5-HT_{1A}, 5-HT_{5A}, and 5-HT_7) for which **16** displayed moderate affinity.[15] Both compounds **10** and **15** (at 1 μM) did not significantly bind (radioligand displacement < 15%) to any of the analyzed receptors, matching the binding affinities described for **16**.

In order to assess the full potential of these probes for visualization of the 5-HT_6R in cells, **10** and **15** were used in in vitro cell labeling experiments. Incubation of cells with probe **10** (5 μM) allowed direct observation of the h5-HT_6R in transiently transfected COS7 cells using conventional confocal microscopy (λ_{ex} = 405 nm) (Figure 2A). In order to assess specificity of the labeling, nontransfected cells were incubated with compound **10** under the same conditions (Figure 2B). In this case a diffuse fluorescence pattern that did not allow the clear distinction of the cells was obtained. In addition, incubation of transfected cells with probe **10** in the presence of an excess of **16** essentially eliminated fluorescent labeling (Figure 2C).

Although these results support that this probe enables 5-HT_6R visualization, biotinylated probe **15** might exhibit better properties considering its higher receptor affinity (K_i(**15**) = 90 nM vs K_i(**10**) = 175 nM) but especially its versatility. In this regard, there exists an array of easily available streptavidin−fluorophore conjugates optimized for fluorescence microscopy. Incubation of cells with probe **15** in similar conditions as above also allowed direct observation of the h5-HT_6R using fluorescence microscopy (Figure 3A). Supporting specificity, labeling is essentially absent in nontransfected cells (Figure 3B). Figure 3C shows cellular autofluorescence in the absence of probe. Considered together, all these results confirm the suitability of **10** and **15** as probes for cellular visualization of the h5-HT_6R.

Conclusions

In summary, the synthesized compounds **10** and **15** described herein represent the first fluorescent probes for the specific labeling of the h5-HT_6R in cells. These results are the starting point in order to introduce a variety of tags in the scaffold of other 5-HT_6R ligands different from **16**, aimed at the development of optimized probes with tailored profiles in terms of fluorescence, affinity, or selectivity. Availability of these probes should help boost the molecular imaging field focused on the GPCR superfamily by providing tools that facilitate direct monitoring of spatiotemporal changes associated with (patho)-physiological states. In addition to visualization, implementation of other chemical reporters with different tags that enable covalent binding or affinity pull-downs should provide new tools to interrogate GPCR states. These strategies should be extendable even to complex biological settings, a final goal worthy of pursuit given the enormous importance of the members of the GPCR superfamily in normal and disease states. Increase of the arsenal of tools for probing GPCRs belonging to different families and assessment of their biological potential are currently underway in our laboratory.

Experimental Section

Chemistry. Melting points (uncorrected) were determined on a Stuart Scientific electrothermal apparatus. Infrared (IR) spectra were measured on a Bruker Tensor 27 instrument equipped with a Specac ATR accessory of 5200−650 cm^{-1} transmission range. Frequencies (ν) are expressed in cm^{-1}. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance DPX 300 spectrometer (^{1}H, 300 MHz; ^{13}C, 75 MHz) or Bruker Avance 500 spectrometer (^{1}H, 500 MHz; ^{13}C, 125 MHz) at the UCM's NMR facilities or on a Varian INOVA-300 (^{1}H, 300 MHz; ^{13}C, 75 MHz) or Varian INOVA-400 (^{1}H, 400 MHz; ^{13}C, 100 MHz) at the CSIC's NMR facilities. Chemical shifts (δ) are expressed in parts per million relative to internal tetramethylsilane. Coupling constants (J) are in hertz (Hz). The following abbreviations are used to describe peak patterns when appropriate: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), br (broad), app (apparent). Two-dimensional NMR experiments (HMQC and HBQC) of representative compounds were carried out to assign protons and carbons of the new structures. High resolution mass spectrometry (HRMS) was carried out on a FTMS Bruker APEX Q IV (UCM) or Agilent QTOF 6520 (CSIC) spectrometer in electrospray ionization (ESI) mode. High pressure liquid chromatography−mass spectrometry (HPLC−MS) analysis was performed using an Agilent 1200LC-MSD VL instrument. LC separation was achieved with an Eclipse XDB-C18 column (5 μm, 4.6 mm × 15 mm) together with a guard column (5 μm, 4.6 mm × 12.5 mm). The gradient mobile phases consisted of A (95:5 water/acetonitrile) and B (5:95 water/acetonitrile) with 0.1% ammonium hydroxide and 0.1% formic acid as the solvent modifiers. The gradient started at 0% B (for 5 min) and increased linearly to 100% B over the course of 20 min, with a flow rate of 0.5 mL/min, and it was followed by an isocratic gradient of 100% B for 5 min before equilibrating for 5 min at 0% B. MS analysis was performed with an ESI source. The capillary voltage was set to 3.0 kV, and the fragmentor voltage was set at 70 eV. The drying gas temperature was 350 °C, the drying gas flow was 10 L/min, and the nebulizer pressure was 20 psi. Elemental analyses (C, H, N, S) were obtained on a LECO CHNS-932 apparatus at the UCM's or CSIC's analysis services and were within 0.5% of the theoretical values, confirming a purity of at least 95% for all tested compounds. Analytical thin-layer chromatography (TLC) was run on Merck silica gel plates (Kieselgel 60 F-254) with detection by UV light, ninhydrin solution, or 10% phosphomolybdic acid solution in ethanol. Flash chromatography was performed on glass column using silica gel type 60 (Merck, particle 230−400 mesh). Unless stated otherwise, starting materials, reagents, and solvents were purchased as high-grade commercial products from Sigma-Aldrich, Fluka, Lancaster, ABCR, Acros, Scharlab, or Panreac and were used without further purification. In general, solvents were obtained via elution through a Pure Solv column drying system from Innovative Technology, Inc. THF was distilled from sodium benzophenone ketyl and used immediately. Dichloromethane was distilled from calcium hydride.

Collected data for compounds **1**−**15** refer to free bases, and then hydrochloride salts were prepared prior to elemental analyses and biological assays. Spectroscopic data of all described compounds were consistent with the proposed structures. Satisfactory HPLC chromatograms were also obtained

for the final compounds **1**−**15** and for **16**. The data of final compounds **1**−**15** are described.

5-Chloro-*N*-[3-(4-{[5-(dimethylamino)-1-naphthyl]sulfonyl}-piperazin-1-yl)-4-methoxyphenyl]-3-methyl-1-benzothiophene-2-sulfonamide (1). Pyridine (42 μL, 0.52 mmol) was added to a solution of **19** (114 mg, 0.26 mmol) in anhydrous CH_2Cl_2 (2 mL) under an argon atmosphere at room temperature. A solution of 5-chloro-3-methylbenzo[*b*]thiophene-2-sulfonyl chloride (81 mg, 0.29 mmol) in anhydrous CH_2Cl_2 (1 mL) was added dropwise, and the mixture was stirred for 12 h at room temperature. The reaction was then quenched with a 1 M aqueous solution of HCl (5 mL), and the aqueous phase was extracted with CH_2Cl_2 (2 × 10 mL). The combined organic layers were washed with water (1 × 10 mL) and brine (1 × 10 mL), dried over Na_2SO_4, filtered, and evaporated under reduced pressure. Flash chromatography of the residue on silica gel (elution with CH_2Cl_2 to 500:1 CH_2Cl_2/MeOH) gave 210 mg (66% yield) of **1** as a pale yellow solid (mp = 161−163 °C). R_f = 0.40 (hexane/ethyl acetate, 1:1). IR (KBr) ν = 3439, 2924, 2855, 1597, 1508, 1453, 1393, 1334, 1242, 1154 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) δ = 2.26 (s, 3H, CH_3), 2.88 (m, 4H, CH_2), 2.97 (s, 6H, CH_3), 3.27 (m, 4H, CH_2), 3.73 (s, 3H, CH_3), 6.60 (s, 1H, Ar), 6.64 (d, J = 8.6 Hz, 1H, Ar), 6.70 (dd, J = 8.6, 2.3 Hz, 1H, Ar), 6.87 (s, 1H, NH), 7.20 (br d, J = 7.2 Hz, 1H, Ar), 7.41 (dd, J = 8.6, 2.0 Hz, 1H, Ar), 7.58 (app t, J = 7.8 Hz, 2H, Ar), 7.64 (d, J = 2.0 Hz, 1H, Ar), 7.68 (d, J = 8.6 Hz, 1H, Ar), 8.21 (dd, J = 7.3, 1.0 Hz, 1H, Ar), 8.53 (d, J = 8.7 Hz, 1H, Ar), 8.67 (d, J = 7.3 Hz, 1H, Ar) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 12.2 (CH_3), 45.8 ($2CH_3$), 45.9 ($2CH_2$), 50.1 ($2CH_2$), 55.7 (CH_3), 111.5 (CH), 115.6 (CH), 115.8 (CH), 119.5 (2CH), 123.5 (2CH), 123.9 (CH), 128.1 (CH), 128.2 (CH, C), 128.6 (C), 130.6 (CH), 130.8 (C), 130.9 (CH), 131.6 (C), 132.6 (C), 135.7 (C), 137.5 (C), 137.8 (C), 140.6 (2C), 151.1 (2C) ppm. HRMS (ESI) calcd for $C_{32}H_{32}ClN_4O_5S_3$ ($[M - H]^+$): 683.1223. Found: 683.1229. Elemental analysis calcd (%) for $C_{32}H_{33}ClN_4O_5S_3 \cdot 2HCl \cdot 2.5H_2O$: C 47.85, H 5.02, N 6.98, S 11.98. Found: C 47.80, H 4.63, N 6.80, S 11.69.

5-Chloro-*N*-(3-{4-[6-({[5-(dimethylamino)-1-naphthyl]sulfonyl}-amino)hexyl]piperazin-1-yl}-4-methoxyphenyl)-3-methyl-1-benzothiophene-2-sulfonamide (2). Pyridine (32 μL, 0.40 mmol) was added to a solution of **23** (110 mg, 0.20 mmol) in anhydrous CH_2Cl_2 (2 mL) under an argon atmosphere at room temperature. A solution of 5-chloro-3-methylbenzo[*b*]thiophene-2-sulfonyl chloride (62 mg, 0.22 mmol) in anhydrous CH_2Cl_2 (1 mL) was added dropwise, and the reaction mixture was stirred for 12 h at room temperature. The reaction was then quenched with a 10% aqueous solution of $NaHCO_3$ (3 mL), and the aqueous phase was extracted with CH_2Cl_2 (2 × 10 mL). The combined organic layers were washed with water (1 × 10 mL) and brine (1 × 10 mL), dried over Na_2SO_4, filtered, and evaporated under reduced pressure. Flash chromatography of the residue on silica gel (elution with 100:1 to 10:1 CH_2Cl_2/MeOH) gave 98 mg (62% yield) of **2** as a pale yellow solid (mp = 149−151 °C). R_f = 0.54 (CH_2Cl_2/MeOH, 9:1). IR (KBr) ν = 3290, 2929, 1508, 1322, 1158 cm^{-1}. ^1H NMR ($CDCl_3$, 500 MHz) δ = 1.10−1.28 (m, 4H, CH_2), 1.32−1.50 (m, 4H, CH_2), 2.22 (s, 3H, CH_3), 2.30−2.40 (m, 2H, CH_2), 2.54 (m, 4H, CH_2), 2.84 (m, 4H, CH_2), 2.80−2.98 (m, 8H, CH_3, CH_2), 3.80 (s, 3H, CH_3), 4.78 (t, J = 6.1 Hz, 1H, NH), 6.55 (d, J = 2.2 Hz, 1H, Ar), 6.67 (d, J = 8.6 Hz, 1H, Ar), 6.72 (dd, J = 8.6, 2.2 Hz, 1H, Ar), 7.18 (d, J = 7.6, 1H, Ar), 7.41 (dd, J = 8.6, 2.0 Hz, 1H, Ar), 7.52 (dd, J = 8.5, 7.3 Hz, 1H, Ar), 7.55 (dd, J = 8.6, 7.6 Hz, 1H, Ar), 7.65 (d, J = 1.7 Hz, 1H, Ar), 7.70 (d, J = 8.6 Hz, 1H, Ar), 8.25 (dd, J = 7.3, 1.2 Hz, 1H, Ar), 8.29 (d, J = 8.7 Hz, 1H, Ar), 8.54 (d, J = 8.5 Hz, 1H, Ar) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz) δ = 12.0 (CH_3), 25.8 (CH_2), 26.1 (CH_2), 26.7 (CH_2), 29.3 (CH_2), 43.2 (CH_2), 45.4 ($2CH_3$), 49.8 ($2CH_2$), 52.7 ($2CH_2$), 55.5 (CH_3), 58.0 (CH_2), 111.1 (CH), 115.1 (CH), 116.5 (CH), 118.7 (CH), 120.6 (CH), 123.1 (CH), 123.2 (CH), 123.7 (CH), 127.6 (CH), 128.1 (C), 128.3 (CH), 129.5 (CH), 129.6 (C), 129.8 (C), 130.3 (CH), 131.3 (C), 134.7 (C), 136.1 (C), 136.9 (C), 137.4 (C), 140.4 (C), 141.3 (C), 151.2 (C), 151.9 (C) ppm. MS (ESI) 784.5 $(M + H)^+$. Elemental analysis calcd (%) for $C_{38}H_{46}ClN_5O_5S_3 \cdot 2.5HCl \cdot 2.5H_2O$: C 49.57, H 5.86, N 7.61, S 10.45. Found: C 49.70, H 5.92, N 7.66, S 10.90.

5-Chloro-*N*-(3-{4-[8-({[5-(dimethylamino)-1-naphthyl]sulfonyl}-amino)octyl]piperazin-1-yl}-4-methoxyphenyl)-3-methyl-1-benzothiophene-2-sulfonamide (3). Sodium iodide (59 mg, 0.39 mmol) and **24** (100 mg, 0.23 mmol) were added to a solution of **16** (89 mg, 0.20 mmol) in anhydrous acetonitrile (10 mL) under an argon atmosphere, and the reaction mixture was refluxed for 34 h. Solvent was removed under reduced pressure and the residue was purified by flash chromatography on silica gel (elution with 150:1 to 20:1 CH_2Cl_2/MeOH) to afford pure **3** (126 mg, 79% yield) as a beige solid (mp = 158−160 °C). R_f = 0.45 (CH_2Cl_2/MeOH, 10:1). IR (KBr) ν = 3271, 2931, 2853, 1588, 1506, 1322, 1158 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) δ = 1.08−1.26 (m, 8H, CH_2), 1.31−1.44 (m, 2H, CH_2), 1.46−1.58 (m, 2H, CH_2), 2.24 (s, 3H, CH_3), 2.35−2.48 (m, 2H, CH_2), 2.64 (m, 4H, CH_2), 2.79−2.98 (m, 12H, CH_3, CH_2), 3.81 (s, 3H, CH_3), 4.73 (t, J = 6.1 Hz, 1H, NH), 6.60 (d, J = 2.2 Hz, 1H, Ar), 6.67 (d, J = 8.6 Hz, 1H, Ar), 6.73 (dd, J = 8.6, 2.2 Hz, 1H, Ar), 7.19 (d, J = 7.2 Hz, 1H, Ar), 7.40 (dd, J = 8.6, 2.0 Hz, 1H, Ar), 7.50−7.62 (m, 2H, Ar), 7.65 (dd, J = 1.8, 0.5 Hz, 1H, Ar), 7.70 (dd, J = 8.6, 0.5 Hz, 1H, Ar), 8.25 (dd, J = 7.3, 1.2 Hz, 1H, Ar), 8.29 (d, J = 8.7 Hz, 1H, Ar), 8.54 (d, J = 8.5 Hz, 1H, Ar) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 12.2 (CH_3), 26.4 (CH_2), 27.3 (CH_2), 28.9 (CH_2), 29.3 (CH_2), 29.6 (CH_2), 29.8 (CH_2), 43.4 (CH_2), 45.6 ($2CH_3$), 49.9 ($2CH_2$), 53.0 ($2CH_2$), 55.7 (CH_3), 58.6 (CH_2), 111.3 (CH), 115.3 (CH), 116.1 (CH), 118.8 (CH), 119.9 (CH), 123.3 (CH), 123.5 (CH), 123.9 (CH), 127.9 (CH), 128.4 (C), 128.5 (CH), 129.7 (C), 129.8 (CH), 130.0 (C), 130.5 (CH), 131.5 (C), 134.8 (C), 136.0 (C), 137.3 (C), 137.7 (C), 140.6 (C), 141.4 (C), 151.3 (C), 152.1 (C) ppm. HRMS (ESI) calcd for $C_{40}H_{51}ClN_5O_5S_3$ $(M + H)^+$: 812.2582. Found: 812.2589. Elemental analysis calcd (%) for $C_{40}H_{50}ClN_5O_5S_3 \cdot 2.5HCl \cdot 3H_2O$: C 50.16, H 6.16, N 7.31, S 10.04. Found: C 50.16, H 6.12, N 6.89, S 9.73.

5-Chloro-*N*-{[5-(dimethylamino)-1-naphthyl]sulfonyl}-*N*-(4-methoxy-3-piperazin-1-ylphenyl)-3-methyl-1-benzothiophene-2-sulfonamide (4). TFA (0.21 mL, 2.8 mmol) was added to a solution of **26** (110 mg, 0.14 mmol) in anhydrous CH_2Cl_2 (5 mL) under an argon atmosphere, and the mixture was stirred for 6 h at room temperature. The mixture was then neutralized with a 10% aqueous solution of $NaHCO_3$, and the aqueous phase was extracted with CH_2Cl_2 (2 × 10 mL). The combined organic layers were washed with water (1 × 10 mL) and brine (1 × 10 mL), dried over Na_2SO_4, filtered, and evaporated under reduced pressure. Flash chromatography of the residue on silica gel (elution with 50:1 to 10:1 CH_2Cl_2/MeOH) gave 75 mg (81% yield) of **4** as a pale yellow solid (mp = 135−137 °C). R_f = 0.14 (CH_2Cl_2/MeOH, 9:1). IR (KBr) ν = 3435, 2939, 2833, 1588, 1504, 1372, 1355, 1250, 1166 cm^{-1}. ^1H NMR ($CDCl_3$, 500 MHz) δ = 2.29 (s, 3H, CH_3), 2.59−2.63 (m, 2H, CH_2), 2.67−2.70 (m, 2H, CH_2), 2.89 (s, 6H, CH_3), 2.91 (m, 4H, CH_2), 3.86 (s, 3H, CH_3), 6.49 (d, J = 2.5 Hz, 1H, Ar), 6.75 (d, J = 8.5 Hz, 1H, Ar), 6.92 (dd, J = 8.5, 2.5 Hz, 1H, Ar), 7.16 (d, J = 7.5 Hz, 1H, Ar), 7.37 (dd, J = 8.5, 7.5 Hz, 1H, Ar), 7.48 (dd, J = 8.5, 2.0 Hz, 1H, Ar), 7.52 (dd, J = 8.0, 7.5 Hz, 1H, Ar), 7.74 (d, J = 7.0 Hz, 1H, Ar), 7.76 (s, 1H, Ar), 8.05 (d, J = 9.0 Hz, 1H, Ar), 8.36 (dd, J = 7.5, 1.5 Hz, 1H, Ar), 8.59 (d, J = 8.5 Hz, 1H, Ar) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz) δ = 12.4 (CH_3), 45.4 ($2CH_3$), 45.9 ($2CH_2$), 51.5 ($2CH_2$), 55.6 (CH_3), 110.7 (CH), 115.3 (CH), 119.2 (CH), 122.1 (CH), 123.2 (CH), 123.7 (CH), 123.8 (CH), 125.5 (C), 126.9 (CH), 128.4 (CH), 128.6 (CH), 129.7 (C), 129.8 (C), 131.5 (C), 131.9 (CH), 132.7 (CH), 134.6 (C), 134.8 (C), 138.7 (C), 139.8 (C), 140.4 (C), 141.8 (C), 151.9 (C), 153.7 (C) ppm. MS (ESI) 685.3 $(M + H)^+$. Elemental analysis calcd (%) for $C_{32}H_{33}ClN_4O_5S_3 \cdot 2.5HCl \cdot H_2O$: C 48.38, H 4.76, N 7.05, S 12.11. Found: C 48.31, H 5.01, N 6.80, S 11.73.

General Procedure for the Synthesis of Final Compounds 5 and 6. TFA (20 equiv) was added to a solution of the corresponding *N*-Boc protected compound (**31** and **32**) in anhydrous CH_2Cl_2 (20 mL/mmol) under an argon atmosphere, and the mixture was stirred for 6 h at room temperature. The mixture was then neutralized with a 10% aqueous solution of $NaHCO_3$, and the aqueous phase was extracted with CH_2Cl_2 (2×). The combined organic layers were washed with water (1×) and brine (1×),

dried over Na_2SO_4, filtered, and evaporated under reduced pressure. Flash chromatography of the residue on silica gel (elution with 50:1 to 10:1 CH_2Cl_2/MeOH) afforded pure **5** and **6**.

5-Chloro-*N*-[4-({[5-(dimethylamino)-1-naphthyl]sulfonyl}amino)-butyl]-*N*-(4-methoxy-3-piperazin-1-ylphenyl)-3-methyl-1-benzothiophene-2-sulfonamide (5). General procedure was followed using **31** (200 mg, 0.23 mmol) to afford 170 mg of final compound **5** (96% yield) (mp = 145−147 °C). R_f = 0.29 (CH_2Cl_2/MeOH, 10:1). IR (KBr) ν = 3317, 2927, 2851, 1586, 1506, 1452, 1346, 1320, 1240, 1153 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) δ = 1.34−1.56 (m, 4H, CH_2), 2.04 (s, 3H, CH_3), 2.73 (m, 4H, CH_2), 2.82−2.88 (m, 8H, CH_3, CH_2), 2.94 (m, 4H, CH_2), 3.54 (t, J = 6.6 Hz, 2H, CH_2), 3.83 (s, 3H, CH_3), 5.19 (br s, 1H, NH), 6.40 (d, J = 2.2 Hz, 1H, Ar), 6.62 (dd, J = 8.6, 2.2 Hz, 1H, Ar), 6.69 (d, J = 8.6 Hz, 1H, Ar), 7.16 (d, J = 7.5 Hz, 1H, Ar), 7.41 (dd, J = 8.6, 2.0 Hz, 1H, Ar), 7.47−7.55 (m, 2H, Ar), 7.65 (d, J = 1.8 Hz, 1H, Ar), 7.71 (d, J = 8.6 Hz, 1H, Ar), 8.20 (dd, J = 7.3, 1.0 Hz, 1H, Ar), 8.26 (d, J = 8.6 Hz, 1H, Ar), 8.51 (d, J = 8.5 Hz, 1H, Ar) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 12.1 (CH_3), 25.4 (CH_2), 26.4 (CH_2), 42.7 (CH_2), 45.5 ($2CH_3$), 46.0 ($2CH_2$), 50.9 (CH_2), 51.6 ($2CH_2$), 55.7 (CH_3), 111.1 (CH), 115.2 (CH), 118.7 (CH), 118.8 (CH), 123.3 (CH), 123.4 (2CH), 123.8 (CH), 127.8 (CH), 128.4 (CH), 129.6 (CH), 129.7 (C), 129.9 (C), 130.4 (CH), 130.8 (C), 131.5 (C), 134.8 (2C), 137.1 (C), 137.6 (C), 140.6 (C), 142.1 (C), 152.0 (C), 152.3 (C) ppm. HRMS (ESI) calcd for $C_{36}H_{43}ClN_5O_5S_3$ $(M + H)^+$: 756.2106. Found: 756.2109. Elemental analysis calcd (%) for $C_{36}H_{42}ClN_5O_5S_3 \cdot 4.5HCl \cdot 5H_2O$: C 42.79, H 5.64, N 6.93, S 9.52. Found: C 42.65, H 5.41, N 6.70, S 9.86.

5-Chloro-*N*-[8-({[5-(dimethylamino)-1-naphthyl]sulfonyl}amino)-octyl]-*N*-(4-methoxy-3-piperazin-1-ylphenyl)-3-methyl-1-benzothiophene-2-sulfonamide (6). General procedure was followed using **32** (140 mg, 0.15 mmol) to afford 120 mg of final compound **6** (97% yield) (mp = 165−167 °C). R_f = 0.10 (CH_2Cl_2/MeOH, 10:1). IR (KBr) ν = 3320, 2925, 2855, 1587, 1506, 1455, 1241, 1154 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz; mixture of conformers) δ = 1.11−1.42 (m, 12H, CH_2), 2.14 (s, 1.3H, CH_3), 2.18 (s, 1.6H, CH_3), 2.80−2.85 (m, 6H, CH_2), 2.93 (s, 2.8H, CH_3), 2.95 (s, 3.2H, CH_3), 3.18 (m, 2H, CH_2), 3.34 (m, 2H, CH_2), 3.62 (t, J = 6.7 Hz, 2H, CH_2), 3.84 (s, 1.6H, CH_3), 3.86 (s, 1.4H, CH_3), 5.74 (br s, 1H, NH), 6.48−6.62 (m, 1H, Ar), 6.75 (d, J = 9.6 Hz, 2H, Ar), 7.22 (m, 1H, Ar), 7.43 (dd, J = 8.5, 1.9 Hz, 1H, Ar), 7.50−7.61 (m, 2H, Ar), 7.67−7.76 (m, 2H, Ar), 8.23 (dd, J = 7.3, 1.0 Hz, 1H, Ar), 8.37 (app t, J = 7.8 Hz, 0.4H, Ar), 8.45 (d, J = 8.5 Hz, 0.6H, Ar), 8.62 (app t, J = 7.8 Hz, 1H, Ar) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 12.2 (CH_3), 26.0 (CH_2), 26.2 (CH_2), 28.6 (CH_2), 28.7 (CH_2), 28.8 (CH_2), 29.4 (CH_2), 43.3 (CH_2), 43.8 ($2CH_2$), 45.7 ($2CH_3$), 50.4 (CH_2), 51.5 ($2CH_2$), 55.8 (CH_3), 111.2 (CH), 115.5 (CH), 119.4 (CH), 119.9 (CH), 123.4 (CH), 123.8 (CH), 124.0 (CH), 124.1 (CH), 127.8 (CH), 128.2 (CH), 129.5 (CH), 129.7 (CH), 129.9 (C), 130.1 (C), 131.2 (C), 131.5 (C), 135.0 (C), 135.1 (C), 136.8 (C), 137.6 (C), 139.9 (C), 140.6 (C), 152.1 (2C) ppm. HRMS (ESI) calcd for $C_{40}H_{51}ClN_5O_5S_3$ $(M + H)^+$: 812.2735. Found: 812.2729. Elemental analysis calcd (%) for $C_{40}H_{50}ClN_5O_5S_3 \cdot 3HCl \cdot 2H_2O$: C 50.15, H 6.00, N 7.31, S 10.04. Found: C 50.13, H 5.92, N 6.96, S 9.94.

5-Chloro-*N*-(4-{[8-({[5-(dimethylamino)-1-naphthyl]sulfonyl}-amino)octyl]oxy}-3-piperazin-1-ylphenyl)-3-methyl-1-benzothiophene-2-sulfonamide (7). To a solution of **37** (30 mg, 0.033 mmol) in anhydrous CH_2Cl_2 (1 mL) under an argon atmosphere, TFA (51 μL, 0.66 mmol) was added, and the mixture was stirred for 6 h at room temperature. Then the reaction mixture was neutralized with a 10% aqueous solution of $NaHCO_3$ and the aqueous phase was extracted with CH_2Cl_2 (2 × 10 mL). The combined organic layers were washed with water (1 × 10 mL) and brine (1 × 10 mL), dried over Na_2SO_4, filtered, and evaporated under reduced pressure. Flash chromatography of the residue on silica gel (elution with 50:1 to 10:1 CH_2Cl_2/MeOH) gave 23 mg (88% yield) of the final compound **7** (mp = 139−141 °C). R_f = 0.28 (CH_2Cl_2/ethyl acetate, 1:1). IR (KBr) ν = 3315, 2925, 2857, 1584, 1505, 1422, 1241, 1154 cm^{-1}. ^1H NMR ($CDCl_3$, 500 MHz) δ = 1.11−1.30 (m, 6H, CH_2), 1.31−1.44 (m, 4H, CH_2), 1.66−1.78 (m, 2H, CH_2), 2.29 (s, 3H, CH_3), 2.78−2.94 (m, 12H, CH_3, CH_2), 3.07 (m, 4H, CH_2), 3.87 (t, J = 6.2 Hz, 2H, CH_2), 4.36 (br s, 1H, NH), 6.51 (d, J = 2.3 Hz, 1H, Ar), 6.65 (d, J = 8.7 Hz, 1H, Ar), 6.75 (dd, J = 8.6, 2.4 Hz, 1H, Ar), 7.16 (d, J = 7.4 Hz, 1H, Ar), 7.40 (dd, J = 8.6, 2.0 Hz, 1H, Ar), 7.49 (dd, J = 8.4, 7.4 Hz, 1H, Ar), 7.52−7.57 (m, 1H, Ar), 7.66 (d, J = 1.8 Hz, 1H, Ar), 7.69 (d, J = 8.6 Hz, 1H, Ar), 8.21 (dd, J = 7.3, 1.1 Hz, 1H, Ar), 8.31 (d, J = 8.6 Hz, 1H, Ar), 8.52 (d, J = 8.5 Hz, 1H, Ar) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz) δ = 12.3 (CH_3), 26.2 (CH_2), 26.4 (CH_2), 29.0 (CH_2), 29.2 (CH_2), 29.4 (CH_2), 29.8 (CH_2), 43.5 (CH_2), 45.4 ($2CH_2$), 45.6 ($2CH_3$), 50.4 ($2CH_2$), 68.5 (CH_2), 112.5 (CH), 115.3 (CH), 115.4 (CH), 119.0 (CH), 119.2 (CH), 123.4 (CH), 123.5 (CH), 123.9 (CH), 127.9 (CH), 128.5 (CH), 128.7 (C), 129.5 (CH), 129.8 (C), 130.0 (C), 130.4 (CH), 131.6 (C), 135.2 (C), 136.2 (C), 137.2 (C), 137.8 (C), 140.7 (C), 141.7 (C), 150.6 (C), 152.0 (C) ppm. HRMS (ESI) calcd for $C_{39}H_{47}ClN_5O_5S_3$ $(M - H)^+$: 796.2428. Found: 796.2433. Elemental analysis calcd (%) for $C_{39}H_{48}ClN_5O_5S_3 \cdot 3.5HCl \cdot 4H_2O$: C 46.93, H 6.01, N 7.02, S 9.64. Found: C 46.63, H 6.19, N 7.28, S 9.91.

5-(Dimethylamino)-*N*-(4-methoxy-3-piperazin-1-ylphenyl)naphthalene-1-sulfonamide (8). TFA (0.31 mL, 4 mmol) was added to a solution of **39** (109 mg, 0.20 mmol) in anhydrous CH_2Cl_2 (4 mL) under an argon atmosphere, and the mixture was stirred for 6 h at room temperature. The mixture was then neutralized with a 10% aqueous solution of $NaHCO_3$, and the aqueous phase was extracted with CH_2Cl_2 (2 × 10 mL). The combined organic layers were washed with water (1 × 10 mL) and brine (1 × 10 mL), dried over Na_2SO_4, filtered, and concentrated. Flash chromatography of the residue on silica gel (elution with 25:1 to 5:1 CH_2Cl_2/MeOH) afforded final compound **8** (82 mg, 93% yield) as a pale yellow solid (mp = 160−161 °C). R_f = 0.10 (CH_2Cl_2/MeOH, 9:1). IR (KBr) ν = 3435, 2942, 2832, 1588, 1506, 1454, 1316, 1234, 1144 cm^{-1}. ^1H NMR (CD_3OD, 400 MHz) δ = 2.70 (m, 4H, CH_2), 2.84 (s, 6H, CH_3), 2.88 (m, 4H, CH_2), 3.35 (s, 1H, NH), 3.71 (s, 3H, CH_3), 6.41 (d, J = 2.4 Hz, 1H, Ar), 6.62 (dd, J = 8.6, 2.5 Hz, 1H, Ar), 6.67 (d, J = 8.7 Hz, 1H, Ar), 7.25 (d, J = 7.5 Hz, 1H, Ar), 7.43 (dd, J = 8.5, 7.4 Hz, 1H, Ar), 7.57 (dd, J = 8.7, 7.6 Hz, 1H, Ar), 8.06 (dd, J = 7.3, 1.2 Hz, 1H, Ar), 8.39 (d, J = 8.7 Hz, 1H, Ar), 8.49 (d, J = 8.5 Hz, 1H, Ar) ppm. ^{13}C NMR (CD_3OD, 100 MHz) δ = 45.8 ($2CH_3$), 46.3 ($2CH_2$), 52.0 ($2CH_2$), 56.2 (CH_3), 113.1 (CH), 115.3 (CH), 116.4 (CH), 119.2 (CH), 120.6 (CH), 124.2 (CH), 129.2 (CH), 131.1 (C), 131.3 (C), 131.4 (2CH), 131.6 (C), 136.2 (C), 142.8 (C), 151.8 (C), 153.3 (C) ppm. MS (ESI) 441.2 $(M + H)^+$. Elemental analysis calcd (%) for $C_{23}H_{28}N_4O_3S \cdot 2.5HCl \cdot H_2O$: C 50.25, H 5.96, N 10.19, S 5.83. Found: C 50.16, H 6.19, N 10.04, S 5.44.

5-(Dimethylamino)-*N*-(5-(dimethylamino)naphthalen-1-ylsulfonyl)-*N*-(4-methoxy-3-(piperazin-1-yl)phenyl)naphthalene-1-sulfonamide (9). To a solution of **40** (155 mg, 0.20 mmol) in anhydrous CH_2Cl_2 (4 mL) under an argon atmosphere, TFA (0.31 mL, 4 mmol) was added, and the mixture was stirred for 6 h at room temperature. The reaction mixture was then neutralized with a 10% aqueous solution of $NaHCO_3$, and the aqueous phase was extracted with CH_2Cl_2 (2 × 10 mL). The combined organic layers were washed with water (1 × 10 mL) and brine (1 × 10 mL), dried over Na_2SO_4, filtered, and evaporated under reduced pressure. Flash chromatography of the residue on silica gel (elution with 50:1 to 10:1 CH_2Cl_2/MeOH) gave 111 mg (83% yield) of **9** as a solid (mp = 110−112 °C). R_f = 0.10 (CH_2Cl_2/MeOH, 9:1). IR (KBr) ν = 3435, 2941, 2832, 1587, 1572, 1504, 1350, 1250, 1176 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz) δ = 2.40 (m, 4H, CH_2), 2.78−2.86 (m, 16H, CH_3, CH_2), 3.81 (s, 3H, CH_3), 6.15 (d, J = 2.4 Hz, 1H, Ar), 6.68 (d, J = 8.7 Hz, 1H, Ar), 6.99 (dd, J = 8.6, 2.2 Hz, 1H, Ar), 7.06−7.08 (m, 4H, Ar), 7.47 (app t, J = 8.0 Hz, 2H, Ar), 7.75−7.81 (m, 2H, Ar), 8.30 (d, J = 7.5 Hz, 2H, Ar), 8.56 (d, J = 8.5 Hz, 2H, Ar) ppm. ^{13}C NMR ($CDCl_3$, 100 MHz) δ = 45.4 ($4CH_3$), 45.9 ($2CH_2$), 51.4 ($2CH_2$), 55.6 (CH_3), 110.6 (CH), 115.1 (2CH), 119.3 (2CH), 122.0 (CH), 123.2 (2CH), 125.4 (C), 127.3

(CH), 128.0 (2CH), 129.4 (2C), 130.0 (2C), 131.7 (2CH), 132.8 (2CH), 134.0 (2C), 141.3 (C), 151.6 (2C), 153.4 (C) ppm. MS (ESI) 674.5 $(M + H)^+$. Elemental analysis calcd (%) for $C_{35}H_{39}N_5O_5S_2 \cdot 2.5HCl \cdot 5H_2O$: C 49.16, H 6.07, N 8.19, S 7.50. Found: C 49.56, H 6.39, N 7.89, S 7.67.

5-(Dimethylamino)-*N*-(3-{4-[4-({[5-(dimethylamino)-1-naphthyl]sulfonyl}amino)butyl]piperazin-1-yl}-4-methoxyphenyl)naphthalene-1-sulfonamide (10). Pyridine (23 μL, 0.28 mmol) was added to a stirred solution of **50** (111 mg, 0.21 mmol) in anhydrous CH_2Cl_2 (1 mL) under an argon atmosphere at room temperature. A solution of dansyl chloride (117 mg, 0.43 mmol) in anhydrous CH_2Cl_2 (1 mL) was added dropwise, and the reaction mixture was stirred at room temperature for 16 h. Then the reaction was quenched with a 10% aqueous solution of $NaHCO_3$ (3 mL) and the aqueous phase was extracted with CH_2Cl_2 (2 × 10 mL). The combined organic layers were washed with water (1 × 10 mL) and brine (1 × 10 mL), dried over Na_2SO_4, filtered, and evaporated under reduced pressure. Flash chromatography of the residue on silica gel (elution with 150:1 to 20:1 CH_2Cl_2/MeOH) gave 105 mg (65% yield) of **10** as a yellow solid (mp = 169−171 °C). R_f = 0.40 (CH_2Cl_2/MeOH, 10:1). IR (KBr) ν = 3441, 2929, 2842, 1616, 1581, 1506, 1456, 1314, 1237, 1145 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) δ = 1.43−1.56 (m, 4H, CH_2), 2.24−2.35 (m, 2H, CH_2), 2.49 (m, 4H, CH_2), 2.75 (m, 4H, CH_2), 2.84 (s, 6H, CH_3), 2.88 (s, 6H, CH_3), 2.90−2.99 (m, 2H, CH_2), 3.72 (s, 3H, CH_3), 6.29 (d, J = 2.2 Hz, 1H, Ar), 6.55 (d, J = 8.7 Hz, 1H, Ar), 6.60 (dd, J = 8.6, 2.2 Hz, 1H, Ar), 7.16 (dd, J = 7.3, 3.6 Hz, 2H, Ar), 7.37 (dd, J = 8.4, 7.5 Hz, 1H, Ar), 7.45 (dd, J = 7.1, 6.1 Hz, 1H, Ar), 7.52 (dd, J = 15.2, 7.5 Hz, 2H, Ar), 8.08 (dd, J = 7.3, 1.1 Hz, 1H, Ar), 8.23 (dd, J = 7.3, 1.1 Hz, 1H, Ar), 8.35 (app t, J = 8.3 Hz, 2H, Ar), 8.45 (d, J = 8.5 Hz, 1H, Ar), 8.50 (d, J = 8.5 Hz, 1H, Ar) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 24.4 (CH_2), 28.5 (CH_2), 43.0 (CH_2), 45.5 ($2CH_3$), 45.6 ($2CH_3$), 49.8 ($2CH_2$), 53.0 ($2CH_2$), 55.6 (CH_3), 57.8 (CH_2), 111.3 (CH), 114.8 (CH), 115.2 (CH), 115.3 (CH), 118.4 (CH), 118.9 (CH), 119.4 (CH), 123.3 (2CH), 128.2 (CH), 128.6 (CH), 129.4 (C), 129.5 (CH), 129.8 (C), 129.9 (C), 130.0 (C), 130.1 (C), 130.2 (CH), 130.6 (CH), 130.7 (CH), 134.4 (C), 135.7 (C), 141.3 (C), 150.6 (C), 151.9 (C), 152.1 (C) ppm. MS (ESI) 745.3 $(M + H)^+$. Elemental analysis calcd (%) for $C_{39}H_{48}N_6O_5S_2 \cdot 3HCl \cdot 3H_2O$: C 51.57, H 6.32, N 9.25, S 7.06. Found: C 51.39, H 6.35, N 9.04, S 7.46.

5-(Dimethylamino)-*N*-(3-{4-[6-({[5-(dimethylamino)-1-naphthyl]sulfonyl}amino)hexyl]piperazin-1-yl}-4-methoxyphenyl)naphthalene-1-sulfonamide (11). Pyridine (97 μL, 1.2 mmol) was added to a solution of **23** (216 mg, 0.4 mmol) in anhydrous CH_2Cl_2 (4 mL) under an argon atmosphere at room temperature. A solution of dansyl chloride (129 mg, 0.48 mmol) in anhydrous CH_2Cl_2 (2 mL) was added over 5 min, and the reaction mixture was stirred at room temperature for 16 h. Then the reaction was quenched with a 10% aqueous solution of $NaHCO_3$ (3 mL) and the aqueous phase was extracted with CH_2Cl_2 (2 × 10 mL). The combined organic layers were washed with water (1 × 10 mL) and brine (1 × 10 mL), dried over Na_2SO_4, filtered, and evaporated under reduced pressure. Flash chromatography of the residue on silica gel (elution with 150:1 to 20:1 CH_2Cl_2/MeOH) gave 220 mg (71% yield) of **11** as pale yellow solid (mp = 155−157 °C). R_f = 0.48 (CH_2Cl_2/MeOH, 9:1). IR (KBr) ν = 3277, 2926, 2855, 1579, 1506, 1457, 1314, 1145 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) δ = 1.14−1.32 (m, 6H, CH_2), 1.36−1.47 (m, 2H, CH_2), 2.38−2.48 (m, 2H, CH_2), 2.65 (m, 4H, CH_2), 2.78−3.05 (m, 18H, CH_3, CH_2), 3.74 (s, 3H, CH_3), 4.83 (br s, 1H, NH), 6.30 (s, 1H, Ar), 6.55−6.65 (m, 2H, Ar), 7.19 (dd, J = 7.4, 4.7 Hz, 2H, Ar), 7.38 (dd, J = 8.4, 7.5 Hz, 1H, Ar), 7.52 (dd, J = 8.4, 7.4 Hz, 1H, Ar), 7.57 (app t, J = 8.1 Hz, 2H, Ar), 8.05 (d, J = 7.3 Hz, 1H, Ar), 8.24 (dd, J = 7.3, 1.1 Hz, 1H, Ar), 8.32 (d, J = 8.7 Hz, 1H, Ar), 8.36 (d, J = 8.7 Hz, 1H, Ar), 8.46 (d, J = 8.5 Hz, 1H, Ar), 8.53 (d, J = 8.5 Hz, 1H, Ar) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 25.9 (CH_2), 26.4 (CH_2), 29.2 (CH_2), 29.3 (CH_2), 43.3 (CH_2), 45.5 ($4CH_3$), 49.2 (br, $2CH_2$), 52.8 ($2CH_2$), 55.7 (CH_3), 57.9 (CH_2), 111.4 (CH), 114.9 (CH), 115.3 (2CH), 118.5 (CH), 118.9 (CH), 119.0 (CH), 123.3 (CH), 123.4 (CH), 128.5 (CH), 128.7 (CH), 129.5 (C), 129.6 (CH), 129.8 (2C), 129.9 (C), 130.0 (C), 130.4 (CH), 130.6 (CH), 130.7 (CH), 134.3 (C), 135.1 (C), 140.6 (C), 150.4 (C), 152.1 (C), 152.2 (C) ppm. MS (ESI) 773.2 $(M + H)^+$. Elemental analysis calcd (%) for $C_{41}H_{52}N_6O_5S_2 \cdot 3.5HCl \cdot 3.5H_2O$: C 51.10, H 6.54, N 8.72, S 6.65. Found: C 51.11, H 6.23, N 9.01, S 6.36.

5-(Dimethylamino)-*N*-(8-{4-[5-({[5-(dimethylamino)-1-naphthyl]sulfonyl}amino)-2-methoxyphenyl]piperazin-1-yl}octyl)naphthalene-1-sulfonamide (12). Sodium iodide (16 mg, 0.11 mmol) and **24** (24 mg, 0.055 mmol) were added to a solution of **8** (22 mg, 0.05 mmol) in anhydrous acetonitrile (3 mL) under an argon atmosphere, and the reaction mixture was refluxed for 32 h. Solvent was removed under reduced pressure and the residue was purified by flash chromatography on silica gel (elution with 150:1 to 20:1 CH_2Cl_2/MeOH) to afford pure **12** (20 mg, 45% yield) as a pale yellow solid (mp = 151−153 °C). R_f = 0.50 (CH_2Cl_2/MeOH, 9:1). IR (KBr) ν = 3438, 2926, 2854, 1625, 1506, 1456, 1404, 1314, 1237, 1146 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) δ = 1.00−1.26 (m, 8H, CH_2), 1.28−1.39 (m, 2H, CH_2), 1.40−1.56 (m, 2H, CH_2), 2.30−2.46 (m, 2H, CH_2), 2.59 (m, 4H, CH_2), 2.79 (m, 4H, CH_2), 2.85−2.88 (m, 14H, CH_3, CH_2), 3.71 (s, 3H, CH_3), 4.80 (t, J = 5.9 Hz, 1H, NH), 6.30 (d, J = 2.2 Hz, 1H, Ar), 6.55 (d, J = 8.7 Hz, 1H, Ar), 6.60 (dd, J = 8.6, 2.2 Hz, 1H, Ar), 7.17 (dd, J = 7.2, 5.3 Hz, 2H, Ar), 7.36 (dd, J = 8.4, 7.4 Hz, 1H, Ar), 7.46−7.60 (m, 3H, Ar), 8.05 (dd, J = 7.3, 1.1 Hz, 1H, Ar), 8.24 (dd, J = 7.3, 1.2 Hz, 1H, Ar), 8.30 (d, J = 8.7 Hz, 1H, Ar), 8.34 (d, J = 8.7 Hz, 1H, Ar), 8.45 (d, J = 8.5 Hz, 1H, Ar), 8.53 (d, J = 8.5 Hz, 1H, Ar) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 26.2 (CH_2), 26.4 (CH_2), 27.3 (CH_2), 28.9 (CH_2), 29.2 (CH_2), 29.6 (CH_2), 43.4 (CH_2), 45.6 ($4CH_3$), 49.7 ($2CH_2$), 53.1 ($2CH_2$), 55.6 (CH_3), 58.6 (CH_2), 111.2 (CH), 114.9 (CH), 115.3 (2CH), 118.7 (CH), 118.8 (CH), 118.9 (CH), 123.3 (CH), 123.4 (CH), 128.5 (CH), 128.6 (CH), 129.2 (C), 129.7 (2CH), 129.8 (C), 130.0 (2C), 130.5 (CH), 130.6 (CH), 130.7 (C), 134.2 (C), 134.9 (C), 141.1 (C), 150.5 (C), 152.0 (C), 152.1 (C) ppm. MS (ESI) 801.2 $(M + H)^+$. Elemental analysis calcd (%) for $C_{43}H_{56}N_6O_5S_2 \cdot 4HCl \cdot 3H_2O$: C 51.60, H 6.65, N 8.40, S 6.41. Found: C 51.71, H 6.92, N 8.02, S 6.16.

4-{5-[4-(Dimethylamino)phenyl]-1,3-oxazol-2-yl}-*N*-(4-methoxy-3-piperazin-1-ylphenyl)benzenesulfonamide (13). TFA (66 μL, 0.86 mmol) was added to a solution of **41** (27 mg, 0.043 mmol) in anhydrous CH_2Cl_2 (1 mL) under an argon atmosphere, and the mixture was stirred for 6 h at room temperature. Then the mixture was neutralized with a 10% aqueous solution of $NaHCO_3$, and the aqueous phase was extracted with CH_2Cl_2 (2 × 10 mL). The combined organic layers were washed with water (1 × 10 mL) and brine (1 × 10 mL), dried over Na_2SO_4, filtered, and evaporated under reduced pressure. Flash chromatography of the residue on silica gel (elution with 25:1 to 5:1 CH_2Cl_2/MeOH) gave compound **13** (21 mg, 91% yield) as a yellow solid (mp = 189 °C, dec.). R_f = 0.24 (CH_2Cl_2/MeOH, 1:1). IR (KBr) ν = 3315, 2925, 2855, 1671, 1605, 1510, 1456, 1281, 1164 cm^{-1}. ^1H NMR (CD_3CO, 500 MHz) δ = 2.84−2.97 (m, 8H, CH_2), 3.02 (s, 6H, CH_3), 3.76 (s, 3H, CH_3), 6.72−6.81 (m, 3H, Ar), 6.83 (d, J = 9.0 Hz, 2H, Ar), 7.47 (s, 1H, Ar), 7.68 (d, J = 9.0 Hz, 2H, Ar), 7.85 (d, J = 8.7 Hz, 2H, Ar), 8.18 (d, J = 8.6 Hz, 2H, Ar) ppm. ^{13}C NMR (CD_3CO, 125 MHz) δ = 40.3 ($2CH_3$), 46.5 ($2CH_2$), 51.8 ($2CH_2$), 55.9 (CH_3), 113.0 (CH), 113.1 (2CH), 114.3 (CH), 116.2 (C), 117.3 (CH), 122.1 (CH), 126.5 (2CH), 126.7 (2CH), 128.8 (2CH), 131.3 (C), 132.1 (C), 141.5 (C), 143.4 (C), 151.3 (C), 151.9 (C), 154.3 (C), 158.8 (C) ppm. HRMS (ESI) calcd for $C_{28}H_{32}N_5O_4S$ $(M + H)^+$: 534.2169. Found: 534.2163. Elemental analysis calcd (%) for $C_{28}H_{31}N_5O_4S \cdot 3HCl \cdot 7H_2O$: C 43.72, H 6.29, N 9.11, S 4.17. Found: C 43.42, H 5.95, N 8.77, S 4.06.

Mixture of 2-[6-(Diethylamino)-3-(diethyliminio)-3*H*-xanthen-9-yl]-5-{[(4-methoxy-3-piperazin-1-ylphenyl)amino]sulfonyl}benzenesulfonate and 5-[6-(Diethylamino)-3-(diethyliminio)-3*H*-xanthen-9-yl]-2-{[(4-methoxy-3-piperazin-1-ylphenyl)amino]sulfonyl}benzenesulfonate (14). TFA (65 μL, 0.85 mmol) was added to a solution of **42** (36 mg, 0.042 mmol) in anhydrous CH_2Cl_2 (1 mL) under an argon atmosphere, and the mixture was stirred for 6 h at room temperature. Afterward, the solvent was evaporated to afford the trifluoroacetate salt of compound **14** (mixed isomers; 36 mg, 100%

yield) as a red solid. IR (KBr) ν = 3402, 2928, 2851, 1592, 1456, 1339, 1151 cm^{-1}. ^1H NMR (CD_3OD, 500 MHz; mixture of isomers) δ = 1.27−1.35 (m, 12H, CH_3), 3.25 (m, 4H, CH_2), 3.29 (m, 4H, CH_2), 3.50−3.77 (m, 8H, CH_2), 3.83 (s, 3H, CH_3), 6.79−6.82 (m, 1H, Ar), 6.87−7.16 (m, 8H, Ar), 7.39−7.46 (m, 1H, Ar), 7.97−8.00 (m, 1H, Ar), 8.57−8.58 (m, 1H, Ar) ppm. ^{13}C NMR (CD_3OD, 125 MHz) δ = 12.9 ($2CH_3$), 13.9 (CH_3), 14.9 (CH_3), 39.3 (CH_2), 45.1 ($2CH_2$), 46.9 (CH_2), 47.2 (CH_2), 47.4 (CH_2), 47.9 (CH_2), 48.7 ($2CH_2$), 56.4 (CH_3), 97.1 (CH), 97.3 (CH), 104.1 (C), 108.5 (C), 112.4 (CH), 113.6 (CH), 114.6 (CH), 115.1 (CH), 115.2 (CH), 115.6 (C), 115.7 (C), 116.1 (CH), 117.0 (CH), 117.1 (CH), 118.9 (CH), 119.2 (CH), 128.4 (CH), 129.8 (CH), 130.2 (CH), 131.8 (C), 132.4 (CH), 132.5 (CH), 133.5 (CH), 134.1 (CH), 134.2 (CH), 135.1 (C), 135.3 (C), 135.7 (C), 141.7 (C), 142.7 (C), 142.9 (C), 146.9 (C), 151.6 (C), 153.4 (C), 154.0 (C), 154.3 (C), 156.6 (C), 157.2 (C), 157.5 (C), 157.9 (C), 158.1 (C), 158.2 (C), 158.6 (C), 159.4 (C), 159.5 (C), 160.0 (C) ppm. HRMS (ESI) calcd for $C_{38}H_{46}N_5O_7S_2$ $(M + H)^+$: 748.2833. Found: 748.2827. Elemental analysis calcd (%) for $C_{38}H_{45}N_5O_7S_2 \cdot CF_3CO_2H \cdot H_2O$: C 54.72, H 5.28, N 7.98, S 7.30. Found: C 54.35, H 5.74, N 8.12, S 7.57.

***N*-{6-[4-(5-{[(5-Chloro-3-methyl-1-benzothien-2-yl)sulfonyl]-amino}-2-methoxyphenyl)piperazin-1-yl]hexyl}-5-[(3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl]pentanamide (15).** D-(+)-Biotin (49 mg, 0.20 mmol) and *N*-hydroxybenzotriazole (5.4 mg, 0.04 mmol) were suspended in anhydrous DMF (2 mL) with 4 Å molecular sieves under an argon atmosphere, and the mixture was heated until a clear solution was obtained (~45 °C). When the mixture was cooled, a solution of dicyclohexylcarbodiimide in CH_2Cl_2 (0.22 mL of a 1 M solution in CH_2Cl_2, 0.22 mmol) was added dropwise and the mixture was stirred at room temperature for 3 h. **44** (133 mg, 0.24 mmol) and DMAP (0.3 mg, 0.0024 mmol) were added, and the mixture was stirred at 60 °C for 4 h and then at room temperature for 24 h. The mixture was filtered and washed with CH_2Cl_2/MeOH (1/1 v/v, 5 mL), and the filtrate was concentrated and purified by flash chromatography on silica gel (elution with 100:1 to 10:1 CH_2Cl_2/MeOH) to afford pure **15** (128 mg, 82% yield) as an off-white solid (mp = 196−198 °C). $[\alpha]^{25}_D$ +23 (*c*, 36 mg/mL, ethanol). R_f = 0.17 (CH_2Cl_2/MeOH, 10:1). IR (KBr) ν = 3400, 3078, 2932, 2856, 1693, 1641, 1506, 1458, 1334, 1232, 1156 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) δ = 1.24−1.36 (m, 4H, CH_2), 1.38−1.57 (m, 6H, CH_2), 1.58−1.80 (m, 4H, CH_2), 2.21 (t, J = 7.2 Hz, 2H, CH_2), 2.32 (s, 3H, CH_3), 2.38 (dd, J = 12.4, 5.4 Hz, 2H, CH_2), 2.56 (m, 4H, CH_2), 2.72 (d, J = 12.8 Hz, 1H, CH_2), 2.79−2.97 (m, 5H, CH_2), 3.15 (dd, J = 11.7, 7.1 Hz, 1H, CH), 3.23 (dd, J = 12.5, 6.4 Hz, 2H, CH_2), 3.79 (s, 3H, CH_3), 4.34 (dd, J = 7.2, 5.0 Hz, 1H, CH), 4.52 (dd, J = 7.2, 5.0 Hz, 1H, CH), 5.27 (br s, 1H, NH), 5.84 (br s, 2H, NH), 6.62−6.68 (m, 2H, Ar), 6.78 (dd, J = 8.6, 2.1 Hz, 1H, Ar), 7.39 (dd, J = 8.7, 1.9 Hz, 1H, Ar), 7.66 (d, J = 1.9 Hz, 1H, Ar), 7.69 (d, J = 8.6 Hz, 1H, Ar) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz) δ = 12.2 (CH_3), 25.8 (CH_2), 26.3 (CH_2), 26.8 (CH_2), 27.1 (CH_2), 28.1 (CH_2), 28.2 (CH_2), 29.5 (CH_2), 36.1 (CH_2), 39.5 (CH_2), 40.7 (CH_2), 50.1 ($2CH_2$), 53.6 ($2CH_2$), 55.69 (CH), 55.7 (CH_3), 58.2 (CH_2), 60.4 (CH), 61.9 (CH), 111.3 (CH), 115.1 (CH), 118.6 (CH), 123.4 (CH), 123.9 (CH), 127.8 (CH), 129.1 (C), 131.4 (C), 136.3 (C), 137.0 (C), 137.7 (C), 140.7 (C), 141.6 (C), 150.8 (C), 164.1 (C=O), 173.4 (C=O) ppm. HRMS (ESI) calcd for $C_{36}H_{50}ClN_6O_5S_3$ $(M + H)^+$: 777.2687. Found: 777.2687. Elemental analysis calcd for $C_{36}H_{49}ClN_6O_5S_3 \cdot 2HCl \cdot 3H_2O$: C 47.81, H 6.35, N 9.29, S 10.64. Found: C 47.80, H 6.08, N 8.96, S 10.87.

Binding Assays. Membranes from HEK-293-EBNA (5-HT_{1A}) CHO-K1 (5-HT_{5A}), and HEK-293 (5-HT_6 and 5-HT_7) cells expressing the indicated human serotonin receptors were purchased from Perkin-Elmer and conserved at −80 °C in packaging buffer for subsequent use. Competitive inhibition assays were performed according to standard procedures detailed below.

5-HT_{1A} Receptor. Cell membranes (6.4 mg/mL) were homogenized in 7 volumes of assay buffer (50 mM Tris-HCl, 0.5 mM $MgSO_4$, pH 7.4 at 25 °C). Fractions of 20 μL of the membrane suspension were incubated at 37 °C for 120 min with 2 nM [^{3}H]-8-hydroxy-DPAT (170.2 Ci/mmol, Perkin-Elmer) in the presence or absence of the competing drug in a final volume of 200 μL of assay buffer. Nonspecific binding was determined by radioligand binding in the presence of a saturating concentration of 10 μM serotonin and represented less than 10% of total binding.

5-HT_{5A} Receptor. Cell membranes (6.0 mg/mL) were homogenized in 199 volumes of assay buffer (50 mM Tris-HCl, 0.3% BSA, pH 7.4 at 25 °C). Fractions of 500 μL of the membrane suspension were incubated at 27 °C for 60 min with 1 nM [^{3}H]LSD (82.7 Ci/mmol, Perkin-Elmer) in the presence or absence of the competing drug in a final volume of 550 μL of assay buffer. Nonspecific binding was determined by radioligand binding in the presence of a saturating concentration of 10 μM 5-carboxamidotriptamine and represented less than 10% of total binding.

5-HT_6 Receptor. Cell membranes (6.0 mg/mL) were homogenized in 7 volumes of assay buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 0.5 mM EDTA, pH 7.4 at 25 °C). Fractions of 20 μL of the membrane suspension were incubated at 37 °C for 60 min with 2.5 nM [^{3}H]LSD (79.2 Ci/mmol, Perkin-Elmer) in the presence or absence of the competing drug (ranging from 10^{-5} to 10^{-10} M) in a final volume of 200 μL of assay buffer. Nonspecific binding was determined by radioligand binding in the presence of a saturating concentration of 100 μM serotonin and represented less than 10% of total binding.

5-HT_7 Receptor. Cell membranes (6.8 mg/mL) were homogenized in 200 volumes of assay buffer (50 mM Tris-HCl, 10 mM $MgSO_4$, 0.5 mM EDTA, pH 7.4 at 25 °C). Fractions of 500 μL of the membranes suspension were incubated at 27 °C for 120 min with 3 nM [^{3}H]LSD (79.2 Ci/mmol, Perkin-Elmer) in the presence or absence of the competing drug in a final volume of 540 μL of assay buffer. Nonspecific binding was determined by radioligand binding in the presence of a saturating concentration of 25 μM clozapine and represented less than 15% of total binding.

For all binding assays, competing drug and nonspecific, total, and radioligand bindings were defined in triplicate. Incubation was terminated by rapid vacuum filtration through Wallac Filtermat A filters, presoaked in polyethylenimine (0.5% for 5-HT_{1A} and 5-HT_6 receptors and 0.3% for the 5-HT_7 receptor) or 0.5% BSA (for 5-HT_{5A}), using a FilterMate Unifilter 96-Harvester. The filters were then washed 9 times with 500 μL of ice-cold 50 mM Tris-HCl buffer (pH 7.4 at 25 °C) and dried. The radioactivity bound to the filters was measured by scintillation spectrometry, using a Microbeta TopCount instrument. The data were analyzed by an iterative curve-fitting procedure using GraphPad Prism program. K_i values were calculated from the Cheng−Prusoff equation[19] and are the mean values of two to four experiments performed in triplicate.

Fluorescence Spectroscopy. Absorption spectra of compounds **1**−**14** were determined in 10 μM solutions of the corresponding compound in buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 0.5 mM EDTA, pH 7.4) at 25 °C on a Shimadzu UV-2550 UV−vis spectrophotometer in 1 cm path length quartz cells. Spectra were recorded between 250 and 700 nm (0.5 nm increments and 0.1 s integration time), and they were corrected for background absorbance by subtracting a blank scan of the buffer solution.

Emission spectra of compounds **1**−**14** were determined for the same solutions on a PerkinElmer LS50B luminescence spectrometer in 1 cm path length quartz cells. The spectra were recorded between 340 and 690 nm (0.5 nm increments and 0.1 s integration time) with excitation set at the appropriate excitation wavelength. Slit widths were set to 2.5 nm for excitation and to 2.5, 5, or 10 nm for emission, depending on the observed emission intensity. All the spectra were corrected for background fluorescence by subtracting a blank scan of the buffer solution.

Fluorescent quantum yield (Φ_f) of compound **10** was calculated with respect to quinine sulfate (Aldrich) in 0.1 M H_2SO_4 as a standard (Φ_f = 0.54).[20] Solutions of both the sample and the

reference were prepared by dilution of stock solutions whose absorbance was below 0.1 at the same excitation wavelength (350 nm). Fluorescence measurements were taken for each solution with the same instrument parameters, and the fluorescence spectra were corrected for instrumental response before integration. The integrated corrected emission intensities were plotted against the absorbance, and data were least-squares-fitted to a straight line. The slopes of the lines are proportional to the quantum yield of the different samples. Absolute values are calculated using standard samples of known Φ_f, according the following equation:[21]

$$\Phi_{f,x} = \Phi_{f,s}\frac{F_x}{A_x}\frac{A_s}{F_s}\left(\frac{\eta_x}{\eta_s}\right)^2 = \Phi_{f,s}\frac{\text{slope}_x}{\text{slope}_s}\left(\frac{\eta_x}{\eta_s}\right)^2$$

where A is the absorbance at the excitation wavelength, F is the area under the corrected emission curve, η is the refractive index of the solvent, and the slopes refer to the integrated corrected emission vs absorbance plots for the sample (x) and the standard (s). The correction for the refractive index $(\eta_x/\eta_s)^2$ was found[21] to be very close to 1 (0.9997), as expected for diluted aqueous solutions. Therefore, it was considered to be of no significance.

Cell Visualization. COS-7 cells, kindly donated by Prof. I. Rodríguez-Crespo (Universidad Complutense de Madrid, Spain), were routinely grown in DMEM media (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Gibco), 1% penicillin−streptomycin solution (Gibco), 1% sodium pyruvate (Gibco), and 1% nonessential amino acids (Gibco) in a humidified atmosphere with 5% CO_2 at 37 °C. For labeling studies, cells were treated with 0.125% trypsin (Invitrogen) and plated onto gelatin-coated glass coverslips in 24-well tissue culture dishes at a density of 15 000 cells/well and cultured under the same conditions as above for an additional 24 h. Cells were transiently transfected with the HA-tagged-5-HT_6 receptor cDNA[18] using Escort IV transfection reagent (Sigma) and following the manufacturer's instructions.

Stock solutions of the compounds were prepared in DMSO and then diluted to the final concentration with PBS buffer so that DMSO content was $<1\%$.

For cell labeling with fluorescent derivative **10**, culture media were aspirated off and cells were washed with PBS and incubated in the presence or absence of the compound in PBS buffer for 10 min at ∼20 °C. Afterward, buffer was removed and cells were washed with PBS, fixed with 2% paraformaldehyde, washed again, and mounted on glass slides with Immu-mount (Thermo Scientific). Preparations were observed with a SP2 Leica confocal microscope with a 63×, 1.4 NA, oil immersion objective (excitation at 405 nm and emission window at 461−568 nm).

For cell labeling with biotinylated derivative **15**, culture media were aspirated off, cells were washed with PBS, fixed with 2% paraformaldehyde, and washed again, and endogenous biotin was blocked with the Molecular Probes kit. Cells were incubated in the presence or absence of the compound in PBS buffer for 10 min at ∼20 °C. Afterward, buffer was removed, cells were washed with PBS, incubated with the streptavidin−Alexa Fluor 488 conjugate (Invitrogen Molecular Probes) at 1:1000 for 1 h at room temperature, and washed again, and nuclei were counterstained with Hoechst-33258 (1:5000 of a 10 mg/mL solution, 10 min, room temperature). After removal of Hoechst solution, cells were washed with PBS and mounted on glass slides with Immu-mount (Thermo Scientific). Fluorescence images were acquired using Metamorph-Offline 6.2 software (Universal Imaging) and a Zeiss Axioplan 2 microscope.

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Supporting Information Available: Full synthetic procedures and analytical and spectral characterization data of intermediates **18**−**24**, **26**−**37**, **39**−**44**. This material is available free of charge via the Internet at http://pubs.acs.org.

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