Synthesis and Characterization of the First Fluorescent Antagonists for Human 5-HT$_4$ Receptors

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Received January 27, 2003

Fluorescent antagonists for human 5-HT$_4$ receptors were synthesized based on ML10302 **1**, a potent 5-HT$_4$ receptor agonist and on piperazine analogue **2**. These molecules were derived with three fluorescent moieties, dansyl, naphthalimide, and NBD (7-nitrobenz-2-oxa-1,3-diazol-4-yl), through alkyl chains. The synthesized molecules were evaluated in binding assays on the recently cloned human 5-HT$_{4(e)}$ receptor isoform stably expressed in C6 glial cells with [^{3}H]GR113808 as the radioligand. The affinity values depended upon the basal structure together with the alkyl chain length. The derivatives based on ML10302 were more potent ligands than the derivatives based on piperazine analogue. For ML10302-based ligands, dansyl and NBD derivatives attached through a chain length of one carbon atom **17a** and **32**, respectively, led to affinities close to the affinity of ML10302. The most potent compounds **17a**, **28**, and **32** produced an inhibition of the 5-HT stimulated cyclic AMP synthesis in the same cellular system with nanomolar K_b values. Fluorescent properties of **17a**, **28**, and **32** were more particularly studied. Interactions of the fluorescent ligand **28** with the h5-HT$_{4(e)}$ receptor were indicated using h5-HT$_{4(e)}$ receptor transfected C6 glial cell membranes and entire cells. Ligand **28** was also used in fluorescence microscopy experiments in order to label h5-HT$_{4(e)}$ receptor transfected C6 glial cells, and subcellular localization of these receptors was more precisely determined using confocal microscopy.

Introduction

Human 5-HT$_4$ receptors, members of the seven transmembrane-spanning G-protein-coupled receptors, are expressed in a wide variety of tissues: brain, heart, bladder, gut, and kidney.[1,2] They have been implicated in a number of pathological disorders: irritable bowel syndrome, gastroparesis, urinary incontinence,[2] cardiac atrial arrhythmia, learning, and memorization.[3] Recently, a large body of evidence has shown an intervention of these receptors in vitro in the secretion of the nonamyloidogenic soluble form of the amyloid precursor protein (sAPPα) implicated in Alzheimer disease.[4] Consequently, 5-HT$_4$ receptors constitute a valuable target for the design of new drugs. At a molecular level, many 5-HT$_4$ receptors were identified as C-terminal splice variants and one with an extra insertion of 14 amino acids in the second extracellular loop.[5−7] These isoforms from different species were recently cloned and transfected in different cells allowing their pharmacological characterization,[8,9] and the study of molecules as agonists or antagonists of serotonin.[10,11,3] To date, no information is available on the specific role of the different isoforms and, in pathological disorders, on the possible implication of the variability in tissue distribution. None of the agonists or antagonists showed any specifity for any given 5-HT$_4$ receptor isoforms. However, some agonists presented variable efficiency strongly dependent on the considered isoform. This observation seems to reside in a specific pattern of expression of the different 5-HT$_4$ receptor isoforms in a given tissue which may confer to the cell a unique mechanism rather than the allosteric influence of the C-terminal end on the rest of the isoforms. The elucidation of the structural factors in this particular phenomenon is a challenge for the medicinal chemist.

Fluorescent receptor ligands have proven to be useful tools for the investigation of the interactions of different receptors with their ligands in complement to classical methods such as radioligand binding and site-directed mutagenesis.[12] Indeed, they offer a multiplicity of information such as the mechanism of ligand binding,[13,14] the movement and internalization of receptors in living cells,[15,16] the distances between ligands and fluorescently labeled amino acids,[17,18] the physical nature of the binding pocket,[19,20] and the visualization of labeled receptors.[21,22] In addition, such ligands provide an attractive alternative to radioligands in receptor studies, circumventing several drawbacks associated with radioactivity such as high costs, potential health hazards, and waste disposal problems.

To our knowledge, no fluorescent agonists or antagonists for 5-HT$_4$ receptors have been described in the literature. However, fluorescent ligands for these receptors should represent valuable tools for the visualization and the study of binding and activation mechanisms of the isoforms in different cell types and different tissues. Moreover, the discovery of potent and selective 5-HT$_4$ receptor ligands implies high throughput drug screening which should be envisaged with fluorescent ligands by the suitable detection method of fluorescent polarization.[23,24]

Here we report the design, synthesis, and physical characterization of the first fluorescent 5-HT$_4$ antagonists and their pharmacological properties for the

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10.1021/jm0307887 CCC: $25.00
Published on Web 05/14/2003

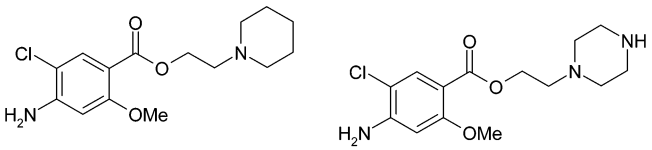


Figure 1.

human 5-HT$_{4(e)}$ receptor stably transfected in C6 glial cells. Some of them were tested for the labeling of these C6 glial cells. Spectrofluorimetric determination of the fluorescence bound to receptors in C6 glial cell membranes and entire C6 glial cells labeled with fluorescent antagonists was performed. We also report some results of visualization of fluorescent ligand-labeled cells using confocal microscopy imaging techniques.

Design and Synthesis of Fluorescent Ligands. The design of fluorescent ligands of the 5-HT$_4$ receptors was based on our previously reported agonist ML10302[11a] **1** and its piperazine analogue, 2-[*N*-piperazin-1-yl]ethyl 4-amino-5-chloro-2-methoxy-benzoate **2** (Figure 1).[11b] Previous mutagenesis and molecular modeling studies performing docking of different 5-HT$_4$ receptor ligands have shown that the aromatic moiety of the pharmacophore was buried into the transmembrane domain of the receptor in a relatively small pocket.[25] On the contrary, the basic amino group was located in a large favorable region into which voluminous substituents could be accommodated. Consequently, the piperidine or piperazine moiety could represent a favorable position to introduce fluorescent groups. Nevertheless, for these compounds two points of attachment appeared chemically obvious: the aromatic amino nitrogen and the atom in the 4-position of the piperidine or piperazine ring. Thus, for the fluorescent analogues of ML10302, these two positions were studied. For the molecules derived from the first point of attachment (the aromatic amino nitrogen), the fluorescent group was fixed through an aminohexane linker (compound **9**, Scheme 1) or directly (compound **33**, Scheme 6). Similarly, for the molecules derived from substitution on the 4-position of the piperidine ring, the fluorescent group was attached directly (compound **20**, Scheme 3) or through an aliphatic chain of one carbon atom (compound **17a**, Scheme 2; compound **28**, Scheme 5; compound **32**, Scheme 6) to four carbon atoms (compounds **17b**−**d**, Scheme 2; compound **29**, Scheme 5). For the molecules derived from the piperazine compound **2** (compounds **23a**−**g**, Scheme 4), the fluorescent moiety was attached on the secondary amino nitrogen through an aliphatic carbonyl chain containing one to ten carbon atoms. Whatever the derivative, the length of the linker was modified in order to introduce molecular flexibility for a possible more convenient positioning of the ligand into the 5-HT$_4$ receptor. Finally, we have introduced three known fluorescent labels possessing different structures and fluorescence properties. We selected fluorescent groups with relatively small molecular volume to introduce a weak perturbation on the affinity. Moreover, they are known to be strongly sensitive to the polarity of the medium with higher fluorescence in hydrophobic environment, and they possess different absorbance and fluorescence properties which is interesting in order to diversify fluorescence experiments. We used the fluorophores dansyl (5-(dimethylamino)-naphthalene-1-sulfonyl), NBD (7-nitrobenz-2-oxa-1,3-diazol-4-yl), and naphthalimide (6-amino-1,3-dioxo-1*H*,3*H*-benzo(de)isoquinolinyl), which are known to have different λ_{exc} (315 nm for the dansyl moiety, and 450−470 nm for the others).

Compound **9** was prepared as described in Scheme 1. In the first step, the weakly basic aromatic amine **3**[26] was alkylated through reductive amination with *N*-Boc-6-aminohexanal **4**[27] in the presence of sodium triacetoxyborohydride in dichloroethane according to a previously reported method.[28] Saponification of the resulting compound **5** with LiOH in H_2O/dioxane afforded the desired acid **6** with a good yield. After activation of **6** with 1,1′-carbonyldiimidazole (CDI) in dry THF, addition of commercial piperidineethanol to the isolated imidazolide in the presence of KOH provided compound **7**. Classical deprotection of **7** afforded the corresponding amine hydrochloride **8**, which upon condensation with dansyl chloride in CH_2Cl_2 in the presence of diisopropylethylamine at low temperature gave the desired fluorescent compound **9**.

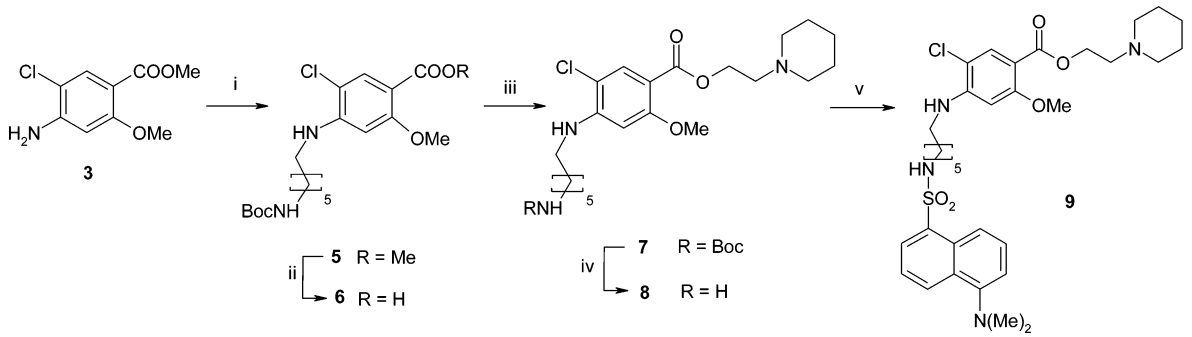
Preparation of compounds **17a**−**d** is outlined in Scheme 2. The not commercially available alcohol **10d** was prepared by reduction of 4-(3-ethoxycarbonylpropyl)piperidine-1-carboxylic acid benzyl ester[29] with $NaBH_4$ and MeOH in dry THF. The bromo compounds **11a**−**d**[30] were obtained by conversion of the corresponding alcohols **10a**−**d**[30,31] with CBr_4/PPh_3 in anhydrous THF. Condensation of **11a**−**d** with di-*tert*-butyliminocarbonate potassium salt[32] in dry DMF at 40 °C generated compounds **12a**−**d**. Classical deprotection with ammonium formate and Pd/C 10% in MeOH provided secondary amines **13a**−**d**. Coupling these amines with 2-bromoethyl 4-amino-5-chloro-2-methoxybenzoate **14**[11a] in the presence of diisopropylethylamine at reflux of dry CH_3CN yielded **15a**−**d**. Classical deprotection with MeOH/HCl afforded **16a**−**d** with good yield except for **16a**. Finally the target fluorescent molecules **17a**−**d** were synthesized by condensation of **16** with dansyl chloride in the presence of triethylamine as previously described.

An analogous route was used to synthesize compound **20** (Scheme 3). Condensation of *tert*-butyl *N*-(4-piperidine)carbamate with **14** in the presence of diisopropylethylamine at reflux of dry CH_3CN afforded **18**. Deprotection gave amine hydrochloride **19**, which upon coupling with dansyl chloride in the presence of triethylamine in CH_2Cl_2 at low temperature yielded the desired fluorescent derivative **20**.

The fluorescent compounds **23a**−**g** were synthesized as indicated in Scheme 4. Compounds **21a**−**g** were obtained by condensation of amine **2**[11b] with respective *N*-protected amino acids activated with BOP. Classical deprotection with MeOH/HCl gave the very hygroscopic amine hydrochlorides **22a**−**g** with moderate yields. Compounds **23a**−**g** were obtained as previously indicated in Scheme 2.

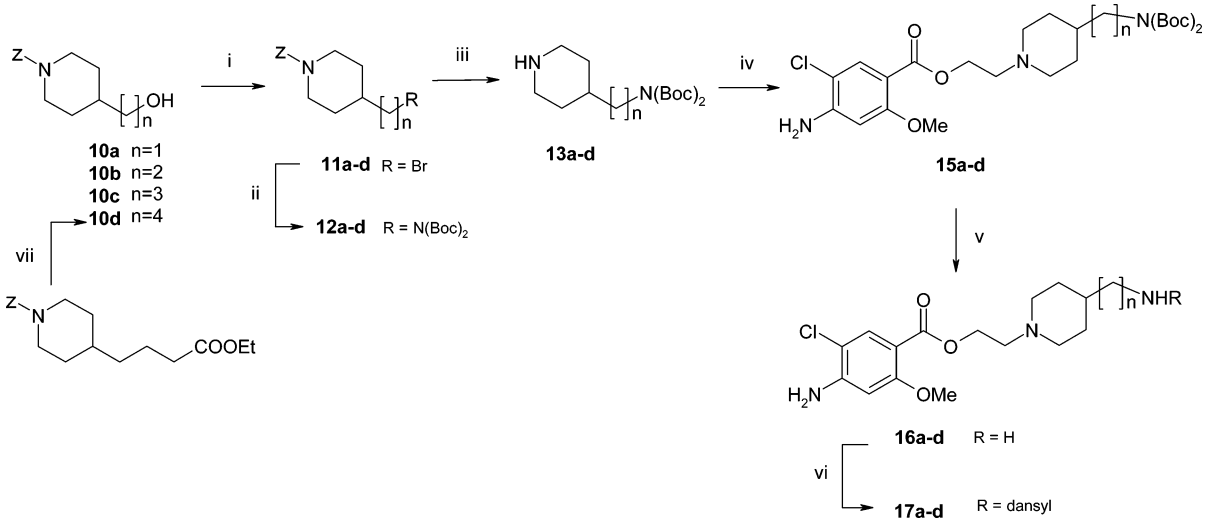
The fluorescent compounds **27b** and **28** were prepared according to Scheme 5. In a first step, molecules **25a**,**b** were prepared by reaction of the corresponding anhydrides **24a** or **24b**[33] with 1-Boc-4-aminomethylpiperidine[34] in ethanol under reflux. After deprotection, amines **26a**,**b** were condensed with **14** in dry CH_3CN or CH_3CN/CH_2Cl_2/DMF at reflux in the presence of

Scheme 1[a]



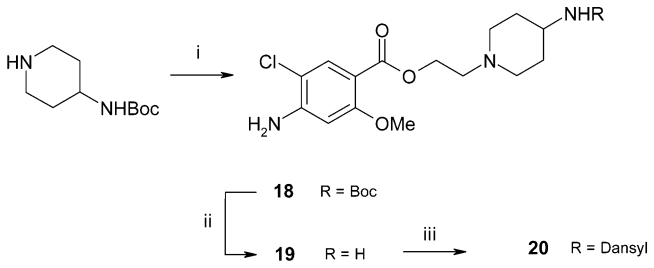
[a] Reagents and conditions: (i) *N*-Boc-6-aminohexanal **4**,[27] $NaBH(OAc)_3$, AcOH, dichloroethane, rt, 24 h; (ii) LiOH, dioxane/H_2O 3/1, reflux, 1 h, 10% $KHSO_4$; (iii) CDI, THF, piperidineethanol, KOH, reflux, 24 h; (iv) MeOH/HCl 3 N, 0 °C to rt, 2 h; (v) dansyl-Cl, CH_2Cl_2, DIEA, −10 °C to 0 °C, 1.5 h.

Scheme 2[a]



[a] Reagents and conditions: (i) PPh_3, CBr_4, THF, rt, 24 h; (ii) $KNBoc_2$,[32] DMF, 40 °C, 24 h; (iii) ammonium formate, Pd/C 10%, MeOH, reflux, 4−5 h; (iv) 2-bromoethyl 4-amino-5-chloro-2-methoxybenzoate **14**,[11a] DIEA, CH_3CN, reflux, 24 h; (v) MeOH/HCl 4N, rt, 1−4 h; (vi) dansyl-Cl, CH_2Cl_2, NEt_3, −10 to 0 °C, 6 h; (vii) $NaBH_4$, then MeOH, 2 h, 70−80 °C.

Scheme 3[a]



[a] Reagents and conditions: (i) **14**,[11a] DIEA, CH_3CN, reflux, 24 h; (ii) MeOH/HCl 4 N, rt,1−4 h; (iii) dansyl-Cl, CH_2Cl_2, NEt_3, 10 °C to 0 °C, 6 h.

diisopropylethylamine giving derivatives **27a**,**b**. Reduction of the nitro group of **27a** by catalytic hydrogenation was performed using a rapid and mild method with ammonium formate and Raney Ni.[35] Compound **28** was easily yielded, avoiding the hydrogenolysis of the sensitive reducible chlorine group of the aryl moiety. Product **29** was obtained by the same way as described for preparation of compounds **25a**,**b**.

Fluorescent compounds **32** and **33** containing the NBD moiety were prepared as indicated in Scheme 6. Condensation of 4-chloro-7-nitro-2,1,3-benzoxadiazole (NBD chloride) with 1-Boc-4-aminoethyl-piperidine in THF at 0 °C afforded **30** with a good yield. After deprotection, compound **31** was alkylated with **14** in dry DMF and diisopropylethylamine at 40 °C for 3 days to give the desired product **32** with a very low yield. Compound **33** was prepared under drastic conditions by reaction of NBD chloride with ML10302 in *n*-butanol with KI at 120 °C for 12 h, with a very low yield.

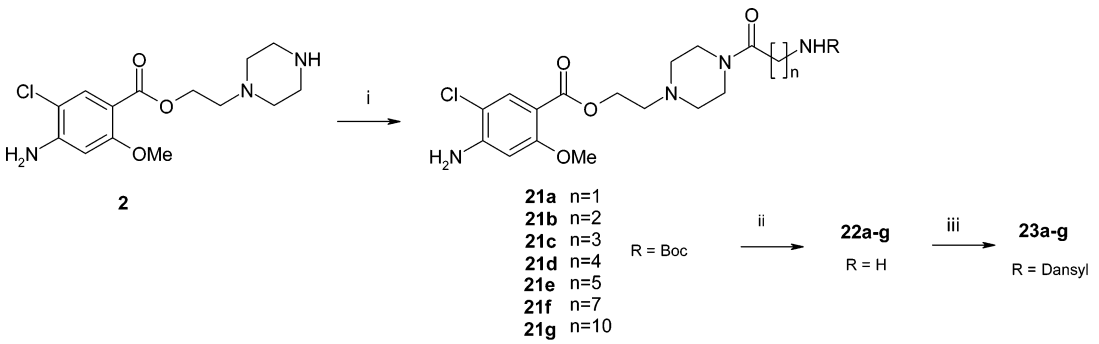
Biological Results

The affinity of labeled compounds was evaluated routinely by binding assays on the human 5-$HT_{4(e)}$ receptor stably expressed in C6 glial cells, using [^{3}H]GR113808 as the radioligand.

As expected, compounds **9** and **33**, where the fluorescent label was attached through the aromatic amino group of compound **1**, were found with no affinity for the h5-$HT_{4(e)}$ receptor. On the contrary, compounds **17a**−**d**, **20**, **27b**, **28**, **29**, and **32** in which the fluorescent group was fixed on the carbon atom in the 4-position of the piperidine ring of ML10302 were found to bind to the h5-$HT_{4(e)}$ receptor. These compounds presented a weak to good affinity for this receptor (Table 1). Similarly, compounds **23a**−**g** in which the fluorescent group was fixed on the nitrogen atom of the piperazine ring presented an affinity for the h5-$HT_{4(e)}$ receptor.

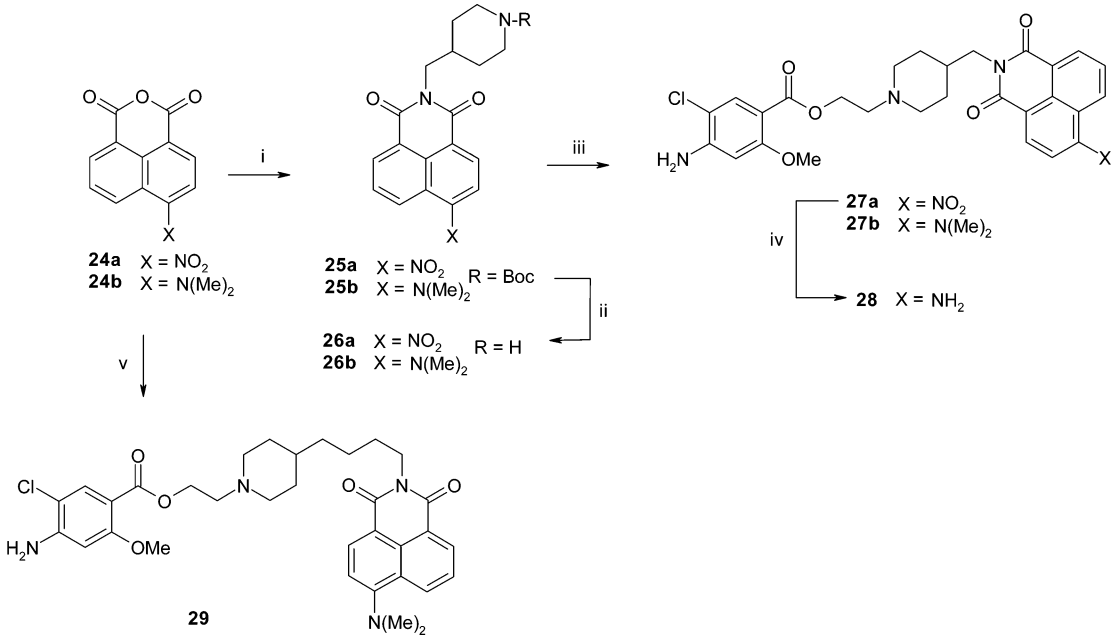
The influence of the chain size was clearly demonstrated for dansyl-labeled molecules **17**−**20** derived from

Scheme 4[a]



[a] Reagents and conditions: (i) BOP, HOOC(CH$_2$)$_n$NHBoc, NEt$_3$, CH$_3$CN, 0 °C to rt, 24 h; (ii) MeOH/HCl 4 N, rt, 2−3 h; (iii) dansyl-Cl, CH$_2$Cl$_2$, NEt$_3$, −10 °C to 0 °C, 6 h.

Scheme 5[a]



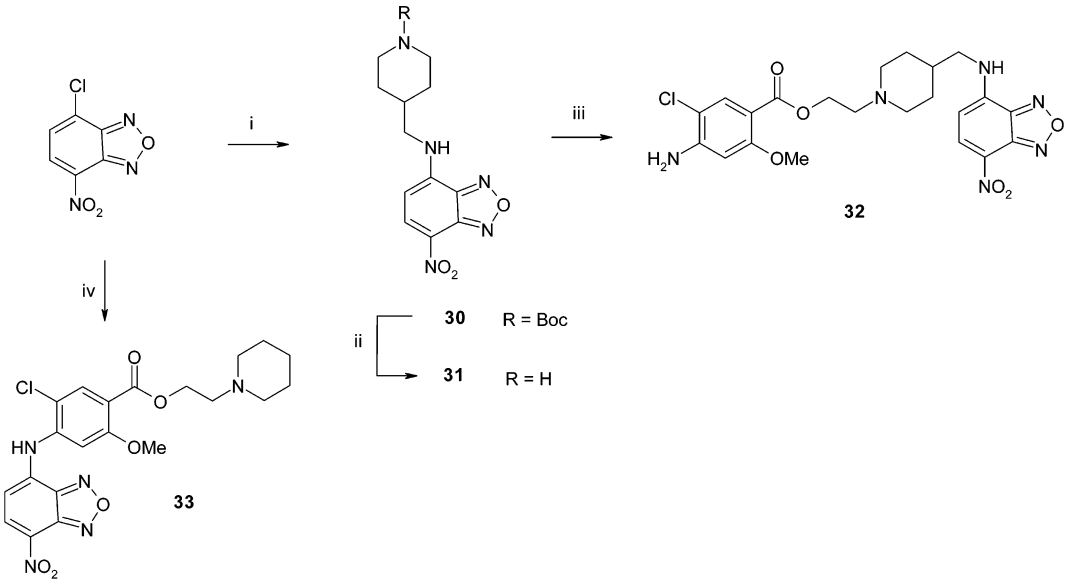
[a] Reagents and conditions: (i) 1-Boc-4-aminomethylpiperidine, EtOH, reflux, 24 h; (ii) MeOH/HCl 4 N, rt, 6 or 24 h; (iii) **14**, DIEA, CH$_3$CN or CH$_3$CN/CH$_2$Cl$_2$/DMF 30/10/10, reflux, 24 h; (iv) ammonium formate, Ra Ni, MeOH/dioxane 50/50, rt, 1 h; (v) **16d**, EtOH, NEt$_3$, reflux, 24 h.

1. In this series, the direct attachment of the fluorescent group to the 4-piperidine ring (compound **20**) was deleterious for the affinity (K_i = 264 ± 100 nM), since a link with one carbon atom (compound **17a**) is sufficient to restore a good affinity (K_i = 7 ± 5 nM) compared to the affinity of the reference molecule ML10302 (K_i = 1.07 ± 0.5 nM).[11a] However, the chain length increase of one more carbon atom (compound **17b**) affects the affinity of one order (K_i = 77 ± 24 nM), and modifying this chain length (**17c** and **17d**) did not change significantly this effect. This result confirmed the prediction of molecular modeling studies:[25] the large favorable region of the receptor seemed located around the basic amino group. Thus a fluorescent group can be allowed near this moiety but with a relative flexibility to permit a convenient positioning into the receptor. With a distance of two, three, or four carbons, the fluorescent label could leave the large pocket, going into a less favorable area which results in a decrease of affinity.

In the piperazine series (compounds **23**) where the labeled dansyl group was attached on the secondary amino nitrogen through an aliphatic carbonyl chain, we observed that the minimal chain length of two carbon atoms (compound **23a**, n = 1) induced a dramatic loss of affinity, compared to the affinity of molecules with bigger chain length (**23b**−**g**). Nevertheless, a fairly good affinity was restored for a minimum chain length of eight carbon atoms: K_i = 31 ± 9 nM for compound **23f** (n = 7) and 96 ± 29 nM for compound **23g** (n = 10). For a chain length of three to six carbon atoms, (compounds **23b**−**e**), the affinity was one order lower than compound **23f**. The general loss of affinity in this series could be explained by the relative rigidity brought by the amido junction of the chain with the piperazine moiety. Indeed, the piperidine compounds **17a**−**d**, more flexible for an equivalent chain length, possessed higher affinities. The optimal chain size for the family of piperazine ligands appeared to be over eight carbon atoms ($n \geq 7$), which would mean that the influence of the rigidity is canceled for this length or that the fluorescent group is situated outside the membrane. In this last case, these ligands might not be really of interest for the study of the binding site of 5-HT$_4$ receptors.

For naphthalimide labeled series, two different amino groups on the 4-position of the 1,8-naphthalimide ring were studied. Compound **28** possessed a primary amino

Scheme 6[a]



[a] Reagents and conditions: (i) 1-Boc-4-aminomethylpiperidine, THF, 0 °C, 2.5 h; (ii) MeOH/HCl 4 N, rt, 6 h; (iii) **14**,[11a] DIEA, DMF, 30 °C, 3 days; (iv) ML10302, KI, *n*-butanol, 120 °C, 12 h.

group on this position, since compounds **27b** and **29** possessed a tertiary dimethylamino group. For compound **28**, the good affinity obtained confirms that a link of one carbon atom is favorable to affinity (K_i = 64 ± 12 nM). Comparison of affinities between compound **29** (K_i = 59 ± 30 nM) and compound **27b** (K_i = 181 ± 62 nM) would indicate that a chain length of four carbon atoms was necessary for restoring the affinity obtained for primary amino group naphthalimide compound **28**. Nevertheless, compared to the dansyl label, the size of the naphthalimide label is apparently deleterious for affinity since compound **28** had a K_i one order higher than that of compound **17a**.

This hypothesis was validated by the affinity obtained for NBD labeled ligand **32** (K_i = 7.5 ± 3 nM). Indeed, with the small NBD group and a chain length of one carbon atom, the affinity of the molecule **17a** was restored.

Despite the steric bulkiness brought by the fluorescent label, most of the synthesized ligands presented an interesting affinity for the h5-$HT_{4(e)}$ receptor with two molecules at a nanomolar level, allowing these molecules for further fluorescent experiments. It is interesting to note that for each family of ligands, dansyl-, naphthalimide-, NBD-, a lead (**17a**, **28**, and **32**, respectively) could be selected.

The pharmacological profile of the labeled molecules was determined on the activity of the second messenger adenylate cyclase. Production of cAMP was measured in C6 glial cells stably expressing the human 5-$HT_{4(e)}$ receptor, using a radioimmunoassay technique as previously reported.[11b] In contrast to the homologous ligand 5-HT, which stimulates the synthesis of cAMP, all the labeled derivatives produced an antagonist effect on the 5-HT-stimulated cAMP synthesis. The competitive antagonist properties of the best compounds in each series **17a**, **28**, and **32** were evaluated on the 5-HT concentration-effect curves. In presence of a concentration of 10 K_i of each compounds (**17a**, **28**, and **32**), the curves were shifted to the right (Figure 2). These three labeled compounds were found to be potent antagonists of the h5-$HT_{4(e)}$ receptor, with nanomolar K_B, as calculated by Schild equation (Table 1).

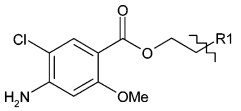
Fluorescence Properties of Ligands. For dansyl ligands, excitation and emission maxima were 315 and 540 nm, respectively. These values were 440/450 and 538 nm, respectively, for naphthalimide ligands, and 470 and 545 nm, respectively, for NBD ligands. The investigation of changes in properties of environment-sensitive fluorescent labeled ligands upon interaction with their receptors is a valuable approach to characterize binding sites. Thus, it is well-known that the quantum yields of dansyl- and NBD-fluorescent groups are dependent upon the polarity of their environment with higher quantum yields in less polar solvents. We found similar results for our dansyl and NBD ligands (**17a** and **32**) with, respectively, a 16-fold and a 6-fold increase in quantum yield by going from aqueous to 60% dioxane−water (v/v) HEPES buffers. This phenomenon was accompanied by a blue-shift of the emission maximum respectively from 540 to 524 nm and 545 to 539 nm.

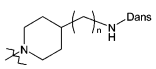
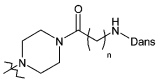
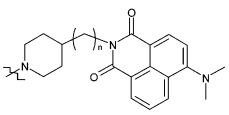
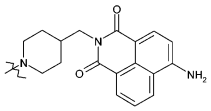
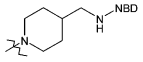
We observed the same phenomenon with naphthalimide fluorescent groups[36] which are less used in the literature than dansyl and NBD labels. For example, for compound **28**, the decrease in polarity (from aqueous buffer to 60% dioxane in HEPES) induced a 5-fold increase in quantum yield accompanied by a blue-shift of the emission maxima from 538 to 530 nm (Figure 3). These results show that the fluorescent naphthalimide ligands as well as dansyl and NBD ligands will constitute good probes for evaluating the nature of the binding site and thus valuable tools for the study of the ligand−receptor interactions.

Interaction of Fluorescent Ligands with the Human 5-$HT_{4(e)}$ Receptor. The three leads (**17a**, **28**, and **32**) were evaluated on h5-$HT_{4(e)}$ receptor stably transfected C6 glial cell membranes and on transfected entire C6 glial cells cultivated on well plates.

Interactions of the fluorescent ligands with the h5-$HT_{4(e)}$ receptor were indicated by fluorescence emission of ligands bound to receptor in a suspension of mem-

Table 1. Biological Properties of Fluorescent Compounds upon the h5-HT$_{4(e)}$ Receptor



Molecule	R1		Ki (nM)	Kb (nM)
ML10302			1 ± 0.5	-
17a		n = 1	7 ± 5	5.7
17b		n = 2	77 ± 24	n.d.
17c		n = 3	32 ± 16	n.d.
17d		n = 4	24 ± 7	n.d.
20		n = 0	264 ± 100	n.d.
23a		n = 1	1720 ± 1100	n.d.
23b		n = 2	116 ± 17	n.d.
23c		n = 3	183 ± 73	n.d.
23e		n = 5	180 ± 87	n.d.
23f		n = 7	31 ± 9	n.d.
23g		n = 10	96 ± 29	n.d.
27b		n = 1	181 ± 62	n.d.
29		n = 4	59 ± 30	n.d.
28			64 ± 12	9
32			7.5 ± 3	2.1

branes from stably transfected C6 glial cells. Small increases in fluorescence were observed for ligands **17a** and **32** with a few differences between total and nonspecific fluorescence. The naphthalimide labeled ligand **28** gave the best results. Figure 4 shows typical fluorescence curves representing the autofluorescence of cell membranes alone (3), the total observed fluorescence due to binding of ligand **28** with the receptor (1), and the nonspecific fluorescence determined in the presence of a 1000-fold excess of antagonist GR113808 (2). A clearly higher intensity of ligand-induced cellular fluorescence in cell membranes incubated in the absence of the 5-HT$_4$ antagonist GR113808 compared to the fluorescence measured in its presence was observed at 520 nM. The nonspecific binding of **28** in membranes was about 5% of the total fluorescence. In the inset, the comparison between the intensity of naphthalimide

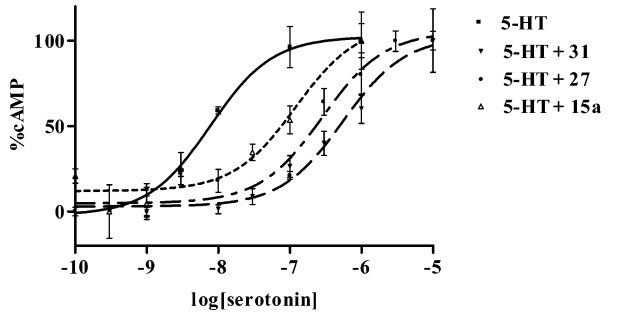


Figure 2. Concentration-effect curves for 5-HT on adenylyl cyclase activity in C6 glial cells stably transfected with the h5-HT$_{4(e)}$ receptor isoform: ■, 5-HT alone; ▲, 5-HT + **32** (100 nM); ♦, 5-HT + **28** (640 nM); △, 5-HT + **17a** (70 nM).

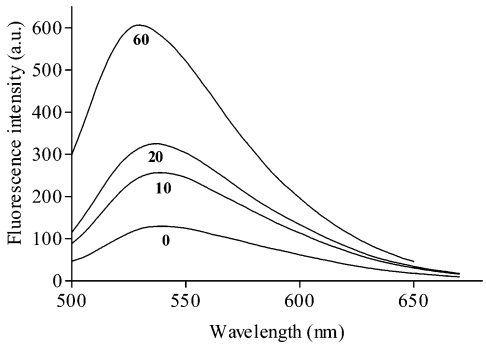


Figure 3. The influence of the polarity of the medium on the fluorescence of compound **28** was investigated by addition of dioxane (indicated in % v/v) in HEPES buffer (50 mM, pH 7.4) measured at a λ_{exc} of 450 nM.

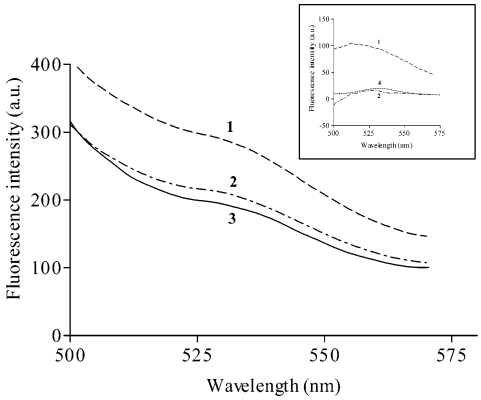


Figure 4. Spectrofluorimetric determination of the fluorescence of compound **28** bound to receptors in C6 glial cells stably transfected with the h5-HT$_{4(e)}$ receptor isoform. Excitation at 450 nm. 1 = total fluorescence in the presence of the fluorescent ligand (60 nM); 2 = nonspecific fluorescence in the presence of the fluorescent ligand (60 nM) and GR113808 (2 μM); 3 = autofluorescence of the cells in absence of ligand. The inset shows the total fluorescence (1) and the nonspecific fluorescence (2) after correction for the autofluorescence of cells and the fluorescence of **28** (60 nM) in solution in HEPES buffer (50 mM, pH 7.4) (4).

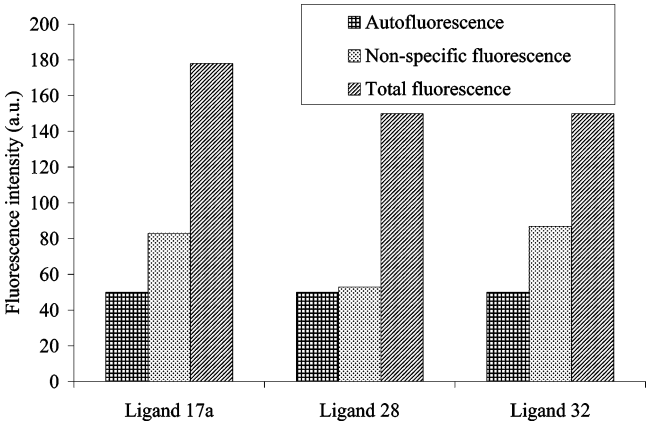


Figure 5. Spectrofluorimetric determination of the fluorescence of compounds **28**, **17a**, and **32** bound to receptors in C6 glial cells stably transfected with the h5-HT$_{4(e)}$ receptor isoform and cultivated on well plates (λ_{exc} = 450 nM). Cells were incubated alone (autofluorescence), with the fluorescent ligand (K_i), supplemented (nonspecific binding) or not (total binding) with 2 μM of GR113808, in HEPES buffer (50 mM, pH 7.4).

fluorescence emission, corrected for the autofluorescence of cells, when the antagonist ligand **28** was bound to the h5-HT$_4$ receptor and the fluorescence intensity of **28** in solution at the same concentration (K_i), reveals an increase about 5-fold accompanied by a blue-shift emission maximum from 540 to 520 nM. It indicates that the fluorophore in receptor-bound state was in a less polar environment than the unbound state. This result shows that ligand **28** represents an interesting candidate for further fluorescence experiments despite an affinity one order lower than the reference molecule ML10302.

Binding of the fluorescent ligands **17a**, **28**, and **32** to the h5-HT$_{4(e)}$ receptor was also measured on transfected C6 glial cells cultivated on well plates with the same procedure as for cell membranes. Results are given in Figure 5. Naphthalimide compound **28** induced the largest difference between total and nonspecific fluorescence. Indeed, the nonspecific binding of fluorescent ligands bound was about 3% for compound **28**, 25% for dansyl compound **17a**, and 37% for NBD compound **32**. It is interesting to note that the results obtained on entire cells compared to those obtained on cell membranes are similar and even better. Indeed, the work and the fluorescence measures on entire cells cultivated on well plates are much more easy to perform and permit, notably, the observation and studies of binding in real time.

Fluorescent Labeling of C6 Glial Cells Expressing the Human 5-HT$_{4(e)}$ Receptor. These labeling experiments were realized using confocal microscopy (Figure 6).

Ligand **28**, previously shown as the best fluorescent labeled ligand, was used for the labeling of C6 glial cells expressing the h5-HT$_{4(e)}$ receptor. The experiments were done at a λ_{exc} of 458 nm. Cells were incubated with ligand **28** in the presence or absence of GR113808 and observed at 515 nm. A clear labeling of cells was observed as shown in Figure 6. Panel b illustrates labeling of transfected cells. The cells were labeled but labeling intensity varied from one cell to another. The nonspecific labeling (panel c), obtained with an excess of GR113808, was relatively low and not different than the natural autofluorescence of cells (panel a). Panel d shows the same cells observed in transmitted light. Fluorescent labeling was mainly around the cells; however, a few labelings could be observed in the cytoplasmic area maybe resulting from internalization process, even if the ligands are antagonists[37] or because of the presence of 5-HT$_4$ receptors in maturation. But further experiments should be performed for the understanding of this phenomena.

The same experiments realized on wild-type C6 glial cells revealed no fluorescence intensity increase in the presence of fluorescent ligand **28**, showing that the binding of this compound is specific of the h5-HT$_{4(e)}$ receptor.

Taken together, these results show that compound **28** constitutes a novel fluorescent probe for h5-HT$_4$ receptors. It should be noted that all these experiments were also evaluated on cells or cell membranes after an overnight treatment of 5 mM sodium butyrate in order to increase receptor expression.[38] Nevertheless, the results were not better particularly because of the dramatic increase of the autofluorescence of the cells without any fluorescent ligand.

Conclusion

We have reported here the synthesis of the first fluorescent ligands of 5-HT$_4$ receptors on the basis of the structural framework of ML10302 **1** and of its piperazine analogue **2**. High affinity compounds for the human 5-HT$_{4(e)}$ receptor isoform were obtained, showing antagonist profile in adenylyl cyclase assay using C6 glial cells stably expressing this receptor isoform. The more potent fluorescent compound (**28**), possessing a naphthalimide label, was shown to be a good molecular probe for labeling the h5-HT$_{4(e)}$ receptor expressed in cellular systems. Studies are now in course to evaluate this new fluorescent probe for the labeling of other h5-HT$_4$ receptor isoforms on different cellular systems, as well as different tissues, and to perform binding studies.

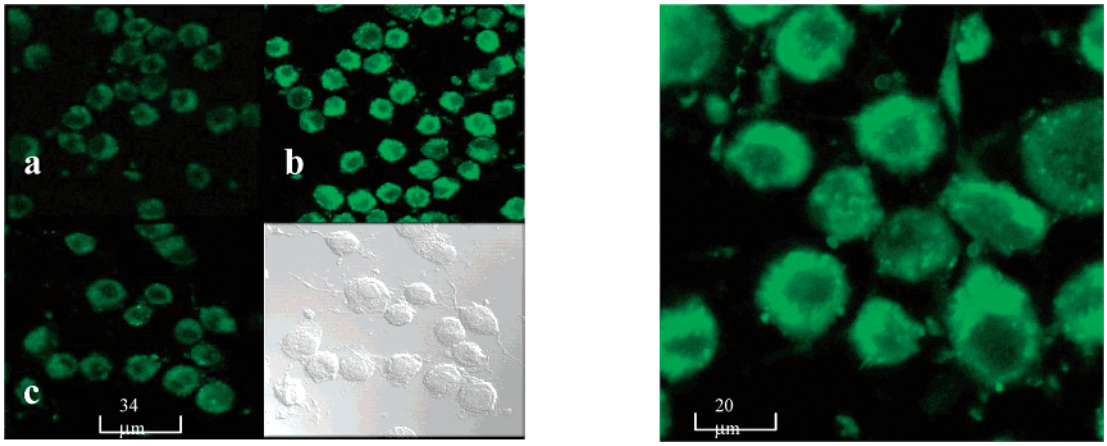


Figure 6. Labeling of C6 glial cells with 60 nM of compound **28** was observed on confocal microscopy. C6 glial cells were incubated with 60 nM of **28** (panel b) and without (panel a) or with 2 μM of GR113808 (panel c). Panel c shows nonspecific labeling, and panel d corresponds to the same cells as panel c, observed in transmitted light.

Moreover, this probe possesses an original naphthalimide fluorescent group, which appeared as a good label, for the design of fluorescent agonist ligands in order to visualize and finely study the internalization process of h5-HT$_4$ receptors.

Experimental Section

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

Materials. Tetrahydrofuran (THF) distilled from sodium/benzophenone, acetonitrile, dimethylformamide, and usual solvents were purchased from SDS (Paris, France). Liquid chromatography was performed on Merck silica gel 60 (70/30 mesh), and TLC was performed on silica gel, 60F-254 (0.26 mm thickness) plates. *N*-Piperidineethanol, dansyl chloride, 4-chloro-7-nitro-2,1,3-benzoxadiazole (NBD chloride), 4-nitro-1,8-naphthalic anhydride **24a**, Boc-Gly-OH, Boc-β-Ala-OH, Boc-4-aminobutyric acid, Boc-5-aminovaleric acid, Boc-6-aminocaproic acid, Boc-8-aminocaprylic acid, and Boc-11-aminoundecanoic acid were purchased from commercial sources. 4-Amino-5-chloro-2-methoxybenzoate **3**, *N*-Boc-6-aminohexanal **4**, benzyl 4-(hydroxymethyl)piperidine-carboxylate **10a**,[31] benzyl 4-(2-hydroxyethyl)piperidine-carboxylate **10b**,[31] benzyl 4-(3-hydroxypropyl)piperidine-carboxylate **10c**,[31] potassium salt of di-*tert*-butyliminocarbonate,[32] and 4-*N*,*N*-dimethylaminonaphthalic anhydride **24b**[33] were prepared according to methods reported in the literature. Benzyl 4-(4-hydroxybutyl)-piperidinecarboxylate **10d** was obtained from benzyl 4-(4-ethoxy-4-oxobutyl)piperidinecarboxylate as previously described in the literature.[29]

Methyl 4-({6-[(*tert*-Butoxycarbonyl)amino]hexyl}amino)-5-chloro-2-methoxybenzoate (5). Ester **3** (0.9 g, 3.94 mmol), aldehyde **4**[27] (1.8 g, 7.78 mmol), and glacial acetic acid (1.43 mL) were mixed in 1,2-dichloroethane (30 mL). Then, sodium triacetoxyborohydride (2.65 g, 12.5 mmol) was added to the above solution, and the reaction mixture was stirred at room temperature under argon atmosphere for 24 h. After concentration, the product was taken up with AcOEt (30 mL), washed with saturated aqueous $NaHCO_3$ (3 × 30 mL) and with saturated aqueous NaCl (30 mL). The AcOEt layer was dried ($MgSO_4$) and concentrated. The crude product was chromatographed on silica gel (petroleum ether/AcOEt 70:30) to give 1.38 g (79%) of 5 as a colorless oil. R_f (petroleum ether/AcOEt 70:30) 0.44; ^{1}H NMR (200 MHz): δ 7.8 (s, 1H), 6.12 (s, 1H), 4.66 (bs, 1H), 4.5 (bs, 1H), 3.9 (s, 3H), 3.8 (s, 3H), 3.15 (m, 4H), 1.7 (m, 2H), 1.43 (m, 15H). Anal. ($C_{20}H_{31}ClN_2O_5$) C, H, N.

4-({6-[(*tert*-Butoxycarbonyl)amino]hexyl}amino)-5-chloro-2-methoxybenzoic Acid (6). To a solution of **5** (1.36 g, 3 mmol) in dioxane/H_2O (3:1) mixture (30 mL) was added at room-temperature lithium hydroxyde (0.34 g, 8.1 mmol). The reaction was then heated under reflux for 1 h and after cooling extracted with Et_2O (30 mL). The aqueous layer was acidified to pH 2 with 10% aqueous $KHSO_4$, and the precipitate formed was extracted with AcOEt (50 mL). The organic phase was dried ($MgSO_4$) and evaporated to give 1.18 g (92%) of **6** as a colorless oil. R_f (petroleum ether/AcOEt 50:50) 0.31; ^{1}H NMR (200 MHz): δ 8 (s, 1H), 6.12 (s, 1H), 4.84 (bt, 1H), 4.55 (bs, 1H), 4.03 (s, 3H), 3.21(m, 2H), 3.12 (m, 2H), 1.72 (m, 2H), 1.43 (m, 15H). Anal. ($C_{19}H_{29}ClN_2O_5$), C, H, N.

2-(Piperidin-1-yl)ethyl 4-({6-[(*tert*-Butoxycarbonyl)-amino]hexyl}amino)-5-chloro-2-methoxybenzoate (7). To a solution of **6** (3 g, 7 mmol) in anhydrous THF (75 mL) was added 1,1′-carbonyldiimidazole (1.82 g, 11.2 mmol). The mixture was heated under reflux for 3 h. After cooling and concentration, the crude product was quickly chromatographed on silica gel (CH_2Cl_2 /MeOH 95:5) to afford 2.93 g (87%) of a colorless oil (R_f: 0.44) which was immediatly treated with piperidineethanol (0.98 g, 7.6 mmol) and KOH (2.32 g, 41.4 mmol) in dry THF (120 mL). The reaction mixture was stirred under reflux overnight, concentrated, and diluted with CH_2Cl_2 (100 mL). After washing with saturated aqueous NaCl, the organic phase was dried ($MgSO_4$) and concentrated in vacuo, and the residue chromatographed on silica gel (CH_2Cl_2/MeOH 90:10) to give 1.73 g (48%) of **7** as a colorless oil. R_f (CH_2Cl_2/MeOH 90:5) 0.51; ^{1}H NMR (200 MHz): δ 7.82 (s, 1H), 6.11 (s, 1H), 4.67 (bt, 1H), 4.5 (bs, 1H), 4.37 (t, J = 6.1 Hz, 2H), 3.88 (s, 3H), 3.21 (m, 2H), 3.12 (m, 2H), 2.72 (t, J = 6.1 Hz, 2H), 2.51 (m, 4H), 1.72 (m, 2H), 1.61 (m, 2H), 1.44 (m, 19H). Anal. ($C_{26}H_{42}ClN_3O_5$), C, H, N.

2-(Piperidin-1-yl)ethyl 4-[(6-Aminohexyl)amino)]-5-chloro-2-methoxybenzoate Hydrochloride (8). To a solution of **7** (0.6 g, 1.17 mmol) in MeOH (20 mL) was added 20 mL of 3 N HCl/MeOH solution at 0 °C. The reaction was stirred at room temperature for 2 h. After addition of anhydrous Et_2O (20 mL), the hydrochloride salt of **8** precipitated as an hygroscopic white powder 0.49 g (94%).^{1}H NMR (200 MHz): free base δ 7.81 (s, 1H), 6.11 (s, 1H), 4.67 (bt, 1H), 4.36 (t, J = 6.1 Hz, 2H), 3.88 (s, 3H), 3.20 (m, 2H), 2.72 (t, J = 6.1 Hz, 2H), 2.7 (m, 2H), 2.53 (m, 4H), 1.72 (m, 2H), 1.65−1.3 (m, 14H). Anal. ($C_{21}H_{34}ClN_3O_3$), C, H, N.

2-(Piperidin-1-yl)ethyl 5-Chloro-4-{[6-({[5-(dimethylamino)-1-naphthyl]sulfonyl}amino)hexyl]amino}-2-methoxybenzoate (9). Amine **8** (0.14 g, 0.29 mmol) was suspended in anhydrous CH_2Cl_2 (10 mL) under argon. The solution was cooled between −10 °C and 0 °C, then treated with DIEA (0.41 mmol) and solid dansyl chloride (92 mg, 0.34 mmol). Yellow green reaction mixture was stirred for 1.5 h. After concentration, the residue was purified by chromatography on silica gel (CH_2Cl_2/MeOH 98:2) to afford 96 mg (51%) of **9** as a pale green oil. R_f (CH_2Cl_2/MeOH 98:2) 0.25; ^{1}H NMR (400 MHz): δ 8.54 (d, J = 8.5 Hz, 1H), 8.29 (d, J = 8.6 Hz, 1H), 8.24 (dd, J = 1 Hz and J = 7.2 Hz, 1H), 7.81 (s, 1H), 7. 54 (m, 2H), 7.18 (d, J = 7.5 Hz, 1H), 6.80 (s, 1H), 4.65 (bt, 1H), 4.59 (bt, 1H), 4.37 (t, J = 6.1 Hz, 2H), 3.87 (s, 3H), 3.10 (m, 2H), 2.90 (m, 2H), 2.88 (s, 6H), 2.72 (t, J = 6.1 Hz, 2H), 2.50 (m, 4H), 1.60 (m, 4H), 1.53 (m, 2H), 1.44 (m, 4H), 1.25 (m, 4H). ^{13}C NMR (50 MHz): δ 164.7, 160.9, 152.1, 148.3, 134.9, 132.6, 130.4, 129.9, 129.7, 129.5, 128.3, 123.3, 118.8, 115.2, 109.8, 107.5, 94.2, 62.0, 57.5, 56.2, 54.8, 45.4, 43.0, 30.9, 29.5, 28.8, 26.4, 25.9, 25.8, 24.2. ESI: m/z 645.4 (M + H$^+$), 667.3 (M + Na$^+$). Anal. ($C_{33}H_{45}ClN_4O_5S$), C, H, N.

Synthesis of Bromo Compounds 11a−d: General Procedure. Benzyl 4-(Bromomethyl)piperidinecarboxylate (11a). Triphenylphosphine (22.1 g, 84 mmol) was added in portions to a solution of carbon tetrabromide (26.6 g, 80 mmol) and alcohol **10a** (10 g, 40 mmol) in anhydrous THF (300 mL) at 0 °C. The reaction mixture was stirred at room temperature for 24 h. The solution was filtered and concentrated to give an oil which was purified by chromatography on silica gel (CH_2Cl_2) to afford 10 g (80%) of **11a** as a beige solid. R_f (CH_2Cl_2) 0.43; mp 64 °C; ^{1}H NMR (200 MHz): δ 7.35 (s, 5H), 5.13 (s, 2H), 4.20 (m, 2H), 3.35 (d, J = 6.2 Hz, 2H), 2.77 (m, 2H), 1.92−1.63 (m, 3H), 1.31−1.05 (m, 2H). Anal. ($C_{14}H_{18}BrNO_2$), C, H, N.

Benzyl 4-(2-Bromoethyl)piperidinecarboxylate (11b). R_f (CH_2Cl_2) 0.97; 80% yield; pale yellow oil; ^{1}H NMR (200 MHz): δ 7.30 (s, 5H), 5.11 (s, 2H), 4.20 (m, 2H), 3.48 (t, J = 6.6 Hz, 2H), 2.80 (m, 2H), 1.87−1.65 (m, 5H), 1.27−1.00 (m, 2H). Anal. ($C_{15}H_{20}BrNO_2 \cdot 0.25H_2O$), C, H, N.

Benzyl 4-(3-Bromopropyl)piperidinecarboxylate (11c). R_f (CH_2Cl_2) 0.44; 86% yield; pale yellow oil; ^{1}H NMR (200 MHz): δ 7.35 (s, 5H), 5.14 (s, 2H), 4.17 (m, 2H), 3.41 (t, J = 6.6 Hz, 2H), 2.77 (m, 2H), 1.98−1.74 (m, 2H), 1.72−1.55 (m, 2H), 1.51−1.40 (m, 3H), 1.38−1 (m, 2H). Anal. ($C_{16}H_{22}BrNO_2 \cdot 0.25H_2O$), C, H, N.

Benzyl 4-(4-Bromobutyl)piperidinecarboxylate (11d). R_f (CH_2Cl_2) 0.6; 77% yield; pale yellow oil; ^{1}H NMR (200 MHz): δ 7.42 (s, 5H), 5.12 (s, 2H), 4.19 (m, 2H), 3.42 (t, J = 6.6 Hz, 2H), 2.75 (m, 2H), 1.92−1.73(m, 2H), 1.72−1.52 (m, 2H), 1.50−1.32 (m, 5H), 1.12−1 (m, 2H). Anal. ($C_{17}H_{24}BrNO_2$), C, H, N.

Synthesis of Boc-Protected Compounds 12a−d: General Procedure. Benzyl 4-{[Bis(*tert*-butoxycarbonyl)amino]methyl}piperidinecarboxylate (12a). To a vigorously stirred slurry of well dried, finely ground potassium salt of di-*tert*-butyl iminodicarbonate (1.6 g, 6.3 mmol) in dry DMF (40 mL) was added dropwise under argon a solution of **11a** (2.15 g, 6.9 mmol) in dry DMF (20 mL). The reaction mixture was heated at 40 °C for 24 h. After evaporation, the residue was taken up with CH_2Cl_2 (40 mL), the organic phase was washed with aqueous $NaHCO_3$ 10%, dried ($MgSO_4$), and evaporated. Purification by chromatography on silica gel (CH_2Cl_2/Et_2O 95:5) afforded 1.37 g (49%) of **12a** as a pale yellow oil. R_f (CH_2Cl_2/Et_2O 95:5) 0.42; ^{1}H NMR (200 MHz): δ 7.35 (s, 5H), 5.11 (s, 2H), 4.13 (m, 2H), 3.52 (t, J = 7 Hz, 2H), 2.75 (m, 2H), 1.81(m, 1H), 1.75−1.45(m, 20H), 1.30−1.02 (m, 2H). Anal. ($C_{24}H_{36}N_2O_6 \cdot 0.75H_2O$), C, H, N.

Benzyl 4-{2-[Bis(*tert*-butoxycarbonyl)amino]ethyl}piperidinecarboxylate (12b). R_f (CH_2Cl_2/Et_2O 95:5) 0.25; 74% yield; yellow oil; ^{1}H NMR (200 MHz): δ 7.35 (s, 5H), 5.12 (s, 2H), 4.17 (m, 2H), 3.62 (t, J = 7 Hz, 2H), 2.78 (m, 2H), 1.81−1.57(m, 3H), 1.50 (m, 20H), 1.25−1.05 (m, 2H). Anal. ($C_{25}H_{38}N_2O_6 \cdot 0.25H_2O$), C, H, N.

Benzyl 4-{3-[Bis(*tert*-butoxycarbonyl)amino]propyl}piperidinecarboxylate (12c). CH_2Cl_2/Et_2O/MeOH 50:45:5; R_f (CH_2Cl_2/ Et_2O/MeOH 50:45:5) 0.97; 92% yield; yellow oil;^{1}H NMR (200 MHz): δ 7.32 (s, 5H), 5.12 (s, 2H), 4.15 (m, 2H), 3.58 (t, J = 7 Hz, 2H), 2.74 (m, 2H), 1.83−1.45(m, 23H), 1.45−1 (m, 4H). Anal. ($C_{26}H_{40}N_2O_6 \cdot 0.75H_2O$), C, H, N.

Benzyl 4-{4-[Bis(*tert*-butoxycarbonyl)amino]butyl}piperidinecarboxylate (12d). CH_2Cl_2/ Et_2O 80:20; R_f (CH_2Cl_2/ Et_2O 80:20) 0.82; 86% yield; yellow oil; ^{1}H NMR (200 MHz): δ 7.35 (s, 5H), 5.13 (s, 2H), 4.12 (m, 2H), 3.55 (t, J = 7 Hz, 2H), 2.73 (m, 2H), 1.75−1.5 (m, 27H), 1.25−1 (m, 2H). Anal. ($C_{27}H_{42}N_2O_6 \cdot 0.125H_2O$), C, H, N.

Synthesis of Amino Compounds 13a−d: General Procedure. 4-{[Bis(*tert*-butoxycarbonyl)amino]methyl}piperidine (13a). A solution of **12a** (1.37 g, 3 mmol), ammonium formate (0.77 g, 12.2 mmol), and 10% Pd /C (0.25 g) in MeOH (30 mL) was heated to reflux for 4−5 h. The reaction mixture was filtered through Celite and concentrated in vacuo to give a residue which was taken up by CH_2Cl_2 (30 mL). The solution was washed with 20% aqueous NH_4OH (0.5 mL), dried ($MgSO_4$), and evaporated to give 0.77 g (82%) of a pale yellow oil used without further purification in next step. ^{1}H NMR (200 MHz): δ 3.53 (d, J = 6.6 Hz, 2H), 3.12 (m, 2H), 2.60 (m, 2H), 1.83−1.55 (m, 3H), 1.50 (s, 18H), 1.31−1 (m, 2H).

4-{2-[Bis(*tert*-butoxycarbonyl)amino]ethyl}piperidine (13b). 96% yield: yellow oil; ^{1}H NMR (200 MHz): δ 3.55 (t, J = 7.5 Hz, 2H), 3.03 (m, 2H), 2.55 (m, 2H), 1.75−1.60 (m, 2H), 1.50 (m, 21H), 1.25−1.05 (m, 2H).

4-{3-[Bis(*tert*-butoxycarbonyl)amino]propyl}piperidine (13c). 86% yield: yellow oil;^{1}H NMR (200 MHz): δ 4.29 (bs, 1H), 3.50 (t, J = 7.5 Hz, 2H), 3.12 (m, 2H), 2.55 (m, 2H), 1.75−1.40 (m, 5H), 1.51 (m, 18H), 1.31−1.08 (m, 4H).

4-{4-[Bis(*tert*-butoxycarbonyl)amino]butyl}piperidine (13d). 89% yield: yellow oil;^{1}H NMR (200 MHz): δ 3.51 (t, J = 7.5 Hz, 2H), 3.13 (m, 2H), 2.52 (m, 2H), 2.05 (bs, 1H), 1.77−1.58 (m, 2H), 1.50 (m, 21H), 1.48−1.16 (m, 4H), 1.14−0.8 (m, 2H).

Synthesis of Compounds 15a−d, 18: General Procedure. 2-(4-{[Bis(*tert*-butoxycarbonyl)amino]methyl}piperidin-1-yl)ethyl 4-Amino-5-chloro-2-methoxybenzoate (15a). A mixture of **14** (0.89 g, 2.9 mmol), N-Boc-protected amine **13a** (0.7 g, 2.23 mmol), and DIEA (0.81 mL, 4.96 mmol) in dry CH_3CN (30−40 mL) was refluxed for 24 h under argon. The resulting mixture was concentrated in vacuo and taken up in CH_2Cl_2. The organic layer was washed with brine, dried ($MgSO_4$), and concentrated. Column chromatography (CH_2Cl_2/ Et_2O 50:50 then CH_2Cl_2/MeOH 95:5) gave 0.93 g (59%) of **15a** as a beige solid. R_f (CH_2Cl_2/MeOH 95:5) 0.59; mp 126 °C; ^{1}H NMR (200 MHz): δ 7.76 (s, 1H), 6.27 (s, 1H), 4.42 (bs, 2H), 4.36 (t, J = 6.1 Hz, 2H), 3.83 (s, 3H), 3.47 (d, J = 6.1 Hz, 2H), 2.96 (m, 2H), 2.74 (t, J = 6.1 Hz, 2H), 2.12 (m, 2H), 1.65 (m, 2H), 1.81 (m, 1H), 1.49 (s, 18H), 1.31 (m, 2H). Anal. ($C_{26}H_{40}ClN_3O_7 \cdot 0.5H_2O$), C, H, N.

2-(4-{2-[Bis(*tert*-butoxycarbonyl)amino]ethyl}piperidin-1-yl)ethyl 4-Amino-5-chloro-2-methoxybenzoate (15b). CH_2Cl_2/Et_2O 50:50 then CH_2Cl_2/MeOH 95:5; R_f (CH_2Cl_2/MeOH 95:5) 0.12; mp 116.5 °C; 84% yield; beige solid;^{1}H NMR (200 MHz): δ 7.79 (s, 1H), 6.27 (s, 1H), 4.47 (bs, 2H), 4.35 (t, J = 6.1 Hz, 2H), 3.82 (s, 3H), 3.57 (m, 2H), 2.96 (m, 2H), 2.70 (t, J = 6.1 Hz, 2H), 2.20 (m, 2H), 1.7−1.67 (m, 3H), 1.49 (m, 20H); 1.37−1.25 (m, 2H). Anal. ($C_{27}H_{42}ClN_3O_7 \cdot 0.5H_2O$), C, H, N.

2-(4-{3-[Bis (*tert*-butoxycarbonyl)amino]propyl}piperidin-1-yl)ethyl 4-Amino-5-chloro-2-methoxybenzoate (15c). CH_2Cl_2/Et_2O 50:50 then CH_2Cl_2/MeOH 90:10; R_f (CH_2Cl_2/ MeOH 90:10) 0.60; mp 120 °C; 45% yield; white solid;^{1}H NMR (200 MHz): δ 7.82 (s, 1H), 6.28 (s, 1H), 4.41 (bs, 2H), 4.33 (t, J = 6.1 Hz, 2H), 3.84 (s, 3H), 3.48 (m, 2H), 2.95 (m, 2H), 2.70 (t, J = 6.1 Hz, 2H), 2.05 (m, 2H), 1.8−1.5 (m, 3H), 1.5 (m, 20H), 1.35−1.1 (m, 4H). Anal. ($C_{28}H_{44}ClN_3O_7$ .H_2O), C, H, N.

2-(4-{4-[Bis(*tert*-butoxycarbonyl)amino]butyl}piperidin-1-yl)ethyl 4-Amino-5-chloro-2-methoxybenzoate (15d). CH_2Cl_2/Et_2O 50:50 then CH_2Cl_2/MeOH 95:5 R_f (CH_2Cl_2/MeOH 95:5) 0.58; 71% yield; pale brown oil;^{1}H NMR (200 MHz): δ 7.79 (s, 1H), 6.28 (s, 1H), 4.48 (bs, 2H), 4.35 (t, J = 6.1 Hz,

2H), 3.81(s, 3H), 3.52 (m, 2H), 2.95 (m, 2H), 2.71 (t, J = 6.1 Hz, 2H), 2.05 (m, 2H), 1.69−1.49 (m, 5H), 1.49 (bs, 18H), 1.42−1.12 (m, 6H). Anal. ($C_{29}H_{46}ClN_3O_7 \cdot 0.75H_2O$), C, H, N.

2-{4-[(*tert*-Butoxycarbonyl)amino]piperidin-1-yl}-ethyl 4-Amino-5-chloro-2-methoxybenzoate (18). CH_2Cl_2/Et_2O 50:50 then CH_2Cl_2/Et_2O/MeOH 50:45:5; R_f (CH_2Cl_2/Et_2O/MeOH 50:45:5) 0.44; 39% yield; white foam solid; mp 88−89 °C; ^{1}H NMR (200 MHz): δ 7.80 (s, 1H), 6.27 (s, 1H), 4.36 (bs, 1H), 4.43 (bs, 2H), 4.35 (t, J = 6.1 Hz, 2H), 3.83 (s, 3H), 3.49 (m, 1H), 2.93 (m, 2H), 2.72 (t, J = 6.1 Hz, 2H), 2.22 (m, 2H), 1.95 (m, 2H), 1.65 (m, 2H), 1.44 (s, 9H). Anal. ($C_{20}H_{30}ClN_3O_5 \cdot 0.25H_2O$), C, H, N.

Synthesis of Compounds 16a−d, 19: General Procedure. 2-[4-(Aminomethyl)piperidin-1-yl]ethyl 4-amino-5-chloro-2-methoxybenzoate Hydrochloride (16a). 15a (0.91 g, 1.67 mmol) was dissolved in MeOH (25−30 mL), and 20 mL of MeOH/HCl 4 N was added. After 1−4 h at room temperature, 0.37 g (54%) of **16a** was obtained as a very hygroscopic beige powder after precipitation with Et_2O or iPr_2O. ^{1}H NMR (200 MHz): free base δ 7.77 (s, 1H), 6.23 (s, 1H), 4.58 (bs, 2H), 4.29 (t, J = 6.1 Hz, 2H), 3.76 (s, 3H), 2.97 (m, 2H), 2.75 (t, J = 6.1 Hz, 2H), 2.5 (d, J = 6.1 Hz, 2H), 2.04 (m, 2H), 1.63 (m, 2H), 1.21 (m, 5H). Anal. ($C_{16}H_{24}ClN_3O_3 \cdot 2HCl \cdot 2H_2O$), C, H, N.

2-[4-(2-Aminoethyl)piperidin-1-yl]ethyl 4-Amino-5-chloro-2-methoxybenzoate Hydrochloride (16b). Very hygroscopic pearly amorphous solid; 71% yield; ^{1}H NMR (200 MHz): free base δ 7.80 (s, 1H), 6.27 (s, 1H), 4.45 (bs, 2H), 4.38 (t, J = 6.1 Hz, 2H), 3.82 (s, 3H), 2.95 (m, 2H), 2.71 (m, 4H), 2.07 (m, 2H), 1.66 (m, 2H), 1.45−0.6 (m, 7H). Anal. ($C_{17}H_{26}ClN_3O_3 \cdot 2HCl \cdot 0.75H_2O$), C, H, N.

2-[4-(3-Aminopropyl)piperidin-1-yl]ethyl 4-Amino-5-chloro-2-methoxybenzoate Hydrochloride (16c). Very hygroscopic beige amorphous solid; 77% yield; ^{1}H NMR (200 MHz): free base δ 7.77 (s, 1H), 6.24 (s, 1H), 4.53 (bs, 2H), 4.47 (t, J = 6.1 Hz, 2H), 3.78 (s, 3H), 2.94 (m, 2H), 2.67 (m, 4H), 2.12 (m, 2H), 1.63 (m, 2H), 1.50−0.85 (m, 9H). Anal. ($C_{18}H_{28}ClN_3O_3 \cdot 2HCl \cdot 1.25H_2O$), C, H, N.

2-[4-(4-Aminobutyl)piperidin-1-yl]ethyl 4-Amino-5-chloro-2-methoxybenzoate Hydrochloride (16d). Very hygroscopic beige amorphous solid; 61% yield; ^{1}H NMR (200 MHz): free base δ 7.78 (s, 1H), 6.27 (s, 1H), 4.52 (bs, 2H), 4.37 (t, J = 6.1 Hz, 2H), 3.81 (s, 3H), 2.94 (m, 2H), 2.72 (t, J = 6.1 Hz, 2H), 2.65 (t, J = 6.8 Hz, 2H), 2.08 (m, 2H), 1.61 (m, 2H), 1.50−0.95 (m, 11H). Anal. ($C_{19}H_{30}ClN_3O_3 \cdot 2HCl \cdot 1.25H_2O$), C, H, N.

2-(4-Aminopiperidin-1-yl)ethyl 4-Amino-5-chloro-2-methoxybenzoate Hydrochloride (19). Hygroscopic white powder; 85% yield; ^{1}H NMR (200 MHz): free base δ 7.78 (s, 1H), 6.25 (s, 1H), 4.50 (bs, 2H), 4.33 (t, J = 6.1 Hz, 2H), 3.80 (s, 3H), 2.93(m, 2H), 2.70 (t, J = 6.1 Hz, 2H), 2.68 (m, 1H), 2.18 (m, 2H), 1.82 (m, 2H), 1.41 (m, 2H), 1 (bs, 2H). Anal. ($C_{15}H_{22}ClN_3O_3 \cdot 2HCl \cdot 0.5H_2O$), C, H, N.

Synthesis of Dansyl Compounds 17a−d, 20: General Procedure. 2-{4-[({[5-(Dimethylamino)-1-naphthyl]sulfonyl}amino)methyl]piperidin-1-yl}ethyl 4-Amino-5-chloro-2-methoxybenzoate (17a). To a cold solution (−10 °C and 0 °C) of anhydrous CH_2Cl_2 (20−30 mL) under argon, containing **16a** (0.1 g, 0.24 mmol) and NEt_3 (0.168 mL), was added dansyl chloride (0.078 g, 0.29 mmol) in several portions (0.168 mL). The yellow green reaction mixture was stirred between −10 °C and 0 °C for 6 h. Then the solvent was evaporated, and the residue was purified by chromatography (CH_2Cl_2 then eluent system A CH_2Cl_2/Et_2O/MeOH 50:45:5) to give 93 mg (67%) of **17a** as a pale green foam. R_f (system A) 0.24; ^{1}H NMR (200 MHz): δ 8.55 (m, 1H), 8.23 (m, 2H), 7.74 (s, 1H), 7.56 (m, 2H), 7.12 (m, 1H), 6.26 (s, 1H), 4.6 (bt, 1H), 4.42 (bs, 2H), 4.27 (t, J = 6.1 Hz, 2H), 3.82 (s, 3H), 2.89 (s, 6H), 2.87 (m, 2H), 2.73 (m, 2H), 2.64 (t, J = 6.1 Hz, 2H), 1.85 (m, 2H), 1.7−1.45 (m, 3H), 1.27−0.95 (m, 2H). ^{13}C NMR (50 MHz): δ 164.4, 160.2, 151.9, 147.7, 134.6, 133.2, 130.3, 129.8, 129.5, 128.3, 123.1, 118.5, 115.1, 109.7, 98.2, 61.9, 56.7, 55.9, 53.3, 48.7, 45.3, 35.7, 29.5. Anal. ($C_{28}H_{35}ClN_4O_5S$), C, H, N.

2-{4-[2-({[5-(Dimethylamino)-1-naphthyl]sulfonyl}-amino)ethyl]piperidin-1-yl}ethyl 4-Amino-5-chloro-2-methoxybenzoate (17b). CH_2Cl_2 then CH_2Cl_2/Et_2O/iPrOH 50:45:5; R_f CH_2Cl_2/Et_2O/iPrOH 50:45:5) 0.17; 88% yield; pale green foam; ^{1}H NMR (200 MHz): δ 8.55 (m, 1H), 8.25 (m, 2H), 7.79 (s, 1H), 7.56 (m, 2H), 7.19 (m, 1H), 6.28 (s, 1H), 4.47 (bt, 1H,), 4.26 (bs, 2H), 4.30 (t, J = 6.1 Hz, 2H), 3.83 (s, 3H), 2.95 (m, 2H), 2.86 (s, 6H), 2.80 (bd, 2H), 2.64 (t, J = 6.1 Hz, 2H), 1.85 (m, 2H), 1.41−1.15 (m, 4H), 1.15−0.95 (m, 3H). ^{13}C NMR (50 MHz): δ 164.5, 160.3, 152.1, 147.9, 134.7, 133.3, 130.5, 129.7, 128.4, 123.2, 118.7, 115.3, 109.9, 98.3, 62.1, 56.9, 56.1, 53.8, 45.4, 42.9, 40.7, 35.9, 32.4, 31.8. Anal. ($C_{29}H_{37}ClN_4O_5S \cdot 0.5H_2O$), C, H, N.

2-{4-[3-({[5-(Dimethylamino)-1-naphthyl]sulfonyl}-amino)propyl]piperidin-1-yl}ethyl 4-Amino-5-chloro-2-methoxybenzoate (17c). CH_2Cl_2 then CH_2Cl_2/Et_2O/MeOH 50:40:10; R_f CH_2Cl_2/Et_2O/MeOH 50:40:10) 0.20; 59% yield; pale green foam; ^{1}H NMR (200 MHz): δ 8.55 (m, 1H), 8.29 (m, 2H), 7.75 (s, 1H,), 7.53 (m, 2H,), 7.11 (m, 1H,), 6.28 (s, 1H), 4.57 (bt, 1H), 4.42 (bs, 2H), 4.27 (t, J = 6.1 Hz, 2H), 3.80 (s, 3H), 2.82 (m, 10H), 2.62 (t, J = 6.1 Hz, 2H), 1.92 (m, 2H), 1.65 (m, 2H), 1.45−1.15 (m, 3H), 1.1−0.8 (m, 4H). ^{13}C NMR (50 MHz): δ 164.5, 160.3, 152.1, 148.0, 134.9, 133.3, 130.4, 129.6, 128.4, 123.2, 118.7, 115.2, 109.9, 98.3, 62.1, 56.9, 56.1, 54.0, 45.4, 43.4, 34.9, 33.1, 31.9, 26.7. Anal. ($C_{30}H_{39}ClN_4O_5S \cdot 0.5H_2O$), C, H, N.

2-{4-[4-({[5-(Dimethylamino)-1-naphthyl]sulfonyl}-amino)butyl]piperidin-1-yl}ethyl 4-Amino-5-chloro-2-methoxybenzoate (17d). CH_2Cl_2 then eluent system A; R_f (system A) 0.14; Yield = 70%; pale green foam; ^{1}H NMR (200 MHz): δ 8.47 (m, 1H), 8.22 (m, 2H), 7.75 (s, 1H), 7.51 (m, 2H), 7.09 (m, 1H), 6.28 (s, 1H), 4.55 (bt, 1H), 4.40 (bs, 2H), 4.29 (t, J = 6.1 Hz, 2H), 3.80 (s, 3H), 2.88 (m, 10H), 2.60 (t, J = 6.1 Hz, 2H), 1.95 (m, 2H), 1.8−0.85 (m, 11H). ^{13}C NMR (50 MHz): δ 164.4, 160.2, 151.9, 147.8, 134.8, 133.2, 130.3, 129.8, 129.6, 129.5, 128.2, 123.0, 118.6, 115.0, 109.8, 109.6, 98.2, 62.0, 56.9, 55.9, 54.0, 45.3,43.2, 35.7, 35.1, 32.0, 29.6, 23.5. Anal. ($C_{31}H_{41}ClN_4O_5S$), C, H, N.

2-[4-({[5-(Dimethylamino)-1-naphthyl]sulfonyl}amino)-piperidin-1-yl]ethyl 4-Amino-5-chloro-2-methoxybenzoate (20). CH_2Cl_2 then eluent system A; R_f (system A) 0.5; 94% yield; pale green foam; ^{1}H NMR (200 MHz): δ 8.54 (m, 1H), 8.22 (m, 2H), 7.74 (s, 1H), 7.55 (m, 2H), 7.17 (m, 1H), 6.26 (s, 1H), 4.58 (bd, 1H), 4.43 (bs, 2H), 4.27 (t, J = 6.1 Hz, 2H), 3.79 (s, 3H), 2.89 (s, 6H), 2.72 (m, 2H), 2.64 (t, J = 6.1 Hz, 2H), 2.11 (m, 2H), 1.65 (m, 3H), 1.5−1.22 (m, 2H). ^{13}C NMR (50 MHz): δ 164.4, 160.2, 151.9, 147.9, 135.8, 133.2, 130.4, 129.9, 129.5, 129.2, 128.3, 123.1, 118.7, 115.1, 109.8, 109.4, 98.2, 61.8, 56.4, 55.9, 51.9, 45.3, 32.7. Anal. ($C_{27}H_{33}ClN_4O_5 \cdot 0.75H_2O$), C, H, N.

Synthesis of Piperazinyl Compounds 21a−g: General Procedure.2-(4-{2-[(*tert*-Butoxycarbonyl)amino]acetyl}piperazin-1-yl)ethyl4-Amino-5-chloro-2-methoxybenzoate(21a). A solution of Boc-gly-OH (0.6 g, 3.42 mmol) and BOP (1.81 g, 4.1 mmol) in dry CH_3CN (40 mL) was treated at 0 °C with NEt_3 (1.21 g, 11.9 mmol). The mixture was stirred for 0.5 h, and then hydrochloride salt of **2** (1.32 g, 3.42 mmol) was added. The reaction was continued at room temperature for 24 h. The solvent was removed and the residue taken up in CH_2Cl_2 (80 mL). The organic layer was washed with 10% aqueous $KHSO_4$ (80 mL), saturated $NaHCO_3$ (80 mL), and brine, dried ($MgSO_4$), and concentrated. The residue was purified by chromatography on silica gel (eluent system A) to afford 0.86 g (65%) of **21a** as a pale yellow oil which was used without further purification in the next step. R_f (system A) 0.22; ^{1}H NMR (200 MHz): δ 7.80 (s, 1H), 6.31 (s, 1H), 5.5 (bs, 1H), 4.55 (bs, 2H), 4.4 (t, J = 5.9 Hz, 2H), 3.93 (m, 2H), 3.8 (s, 3H), 3.61 (m, 2H), 3.4 (m, 2H), 2.75 (t, J = 5.9 Hz, 2H), 2.55 (m, 4H), 1.45 (s, 9H).

2-(4-{3-[(*tert*-Butoxycarbonyl)amino]propanoyl}piperazin-1-yl)ethyl4-Amino-5-chloro-2-methoxybenzoate(21b). From the reaction of **2** and Boc-β-alanine. Eluent system A; R_f (system A) 0.48; 99% yield; yellow oil; ^{1}H NMR (200 MHz): δ 7.80 (s, 1H), 6.30 (s, 1H), 5.25 (bs, 1H), 4.55 (bs, 2H), 4.35 (t, J = 5.9 Hz, 2H), 3.85 (s, 3H), 3.61 (m, 2H), 3.40 (m, 4H), 2.75 (t, J = 5.9 Hz, 2H), 2.51(m, 6H), 1.44 (s, 9H).

2-(4-{4-[(*tert*-Butoxycarbonyl)amino]butanoyl}piperazin-1-yl)ethyl 4-Amino-5-chloro-2-methoxybenzoate (21c). From the reaction of **2** and Boc-4-aminobutyric acid. Eluent system A; R_f (System A) 0.25; 88% yield; viscous orange oil; ^{1}H NMR (200 MHz): δ 7.78 (s, 1H), 6.27 (s, 1H), 4.8 (bs, 1H), 4.48 (bs, 2H), 4.30 (t, J = 5.9 Hz, 2H), 3.82 (s, 3H), 3.60 (m, 2H), 3.45 (m, 2H), 3.15 (m, 2H), 2.75 (t, J = 5.9 Hz, 2H), 2.52 (m, 4H), 2.35 (t, J = 7.2 Hz, 2H), 1.81 (quintet, J = 7.2 Hz, 2H), 1.41 (s, 9H).

2-(4-{5-[(*tert*-Butoxycarbonyl)amino]pentanoyl}piperazin-1-yl)ethyl 4-Amino-5-chloro-2-methoxybenzoate (21d). From the reaction of **2** and Boc-4-aminovaleric acid. Eluent system A; R_f (system A) 0.23; 61% yield; viscous yellow oil; ^{1}H NMR (200 MHz): δ 7.78 (s, 1H), 6.29 (s, 1H), 4.63 (bs, 1H), 4.53 (bs, 2H), 4.35 (t, J = 5.9 Hz, 2H), 3.82 (s, 3H), 3.61 (m, 2H), 3.45 (m, 2H), 3.08 (m, 2H), 2.74 (t, J = 5.9 Hz, 2H), 2.51 (m, 4H), 2.32 (t, J = 7.2 Hz, 2H), 1.75−1.5 (m, 4H), 1.45 (s, 9H).

2-(4-{6-[(*tert*-Butoxycarbonyl)amino]hexanoyl}piperazin-1-yl)ethyl 4-Amino-5-chloro-2-methoxybenzoate (21e). From the reaction of **2** and Boc-4-aminocaproic acid. Eluent system A; R_f (system A) 0.53; 45% yield; colorless oil; ^{1}H NMR (200 MHz): δ 7.80 (s, 1H), 6.25 (s, 1H), 4.52 (bs, 1H), 4.50 (bs, 2H), 4.35 (t, J = 5.9 Hz, 2H), 3.80 (s, 3H), 3.61 (m, 2H), 3.45 (m, 2H), 3.10 (m, 2H), 2.75 (t, J = 5.9 Hz, 2H), 2.51 (m, 4H), 2.30 (t, J = 7.2 Hz, 2H), 1.75−1.3 (m, 15H).

2-(4-{8-[(*tert*-Butoxycarbonyl)amino]octanoyl}piperazin-1-yl)ethyl 4-Amino-5-chloro-2-methoxybenzoate (21f). From the reaction of **2** and Boc-4-aminocaprylic acid. Eluent system A; R_f (system A) 0.55; 95% yield; colorless oil; ^{1}H NMR (200 MHz): δ 7.78 (s, 1H), 6.28 (s, 1H), 4.53 (bs, 3H), 4.38 (t, J = 5.9 Hz, 2H), 3.82 (s, 3H), 3.63 (m, 2H), 3.46 (m, 2H), 3.05 (m, 2H), 2.74 (t, J = 5.9 Hz, 2H), 2.51 (m, 4H), 2.29 (t, J = 7.2 Hz, 2H), 1.75−1.20 (m, 19H).

2-(4-{8-[(*tert*-Butoxycarbonyl)amino]undecanoyl}piperazin-1-yl)ethyl 4-Amino-5-chloro-2-methoxybenzoate (21g). From the reaction of **2** and Boc-11-aminodecanoic acid. CH_2Cl_2/Et_2O/*i*PrOH 50:45:5 then eluent system A; R_f (system A) 0.14; 65% yield; colorless oil; ^{1}H NMR (200 MHz): δ 7.79 (s, 1H), 6.28 (s, 1H), 4.50 (bs, 3H), 4.38 (t, J = 5.9 Hz, 2H), 3.82 (s, 3H), 3.63 (m, 2H), 3.48 (m, 2H), 3.12 (m, 2H), 2.77 (t, J = 5.9 Hz, 2H), 2.55 (m, 4H), 2.33 (t, J = 7.2 Hz, 2H), 1.75−1.1 (m, 25H). Anal. ($C_{30}H_{49}ClN_4O_6S\cdot0.5H_2O$), C, H, N.

2-[4-(2-Aminoacetyl)piperazin-1-yl]ethyl 4-Amino-5-chloro-2-methoxybenzoate Hydrochloride (22a). Same procedure as described for **16a**. 79% yield; hygroscopic white solid; ^{1}H NMR (200 MHz): free base δ 7.78 (s, 1H), 6.27 (s, 1H), 4.51 (bs, 2H), 4.35 (t, J = 5.9 Hz, 2H), 3.81 (s, 3H), 3.63 (m, 2H), 3.40 (m, 2H), 3.12 (m, 2H), 2.74 (t, J = 5.9 Hz, 2H), 2.53 (m, 4H), 1.20 (bs, 2H). ESI: m/z 371.1 (M + H$^+$). Anal. ($C_{16}H_{23}ClN_4O_4\cdot2HCl\cdot2H_2O$), C, H, N.

2-[4-(3-Aminopropanoyl)piperazin-1-yl]ethyl 4-Amino-5-chloro-2-methoxybenzoate Hydrochloride (22b). Same procedure as described for **16a**. 60% yield; hygroscopic white solid; ^{1}H NMR (200 MHz): free base δ 7.75 (s, 1H), 6.25 (s, 1H), 4.56 (bs, 2H), 4.32 (t, J = 5.9 Hz, 2H), 3.79 (s, 3H), 3.58 (m, 2H), 3.42 (m, 2H), 2.95 (t, J = 6.1 Hz, 2H), 2.71 (t, J = 5.9 Hz, 2H), 2.49 (m, 4H), 2.41 (t, J = 6.1 Hz, 2H), 1.0 (bs, 2H). ESI: m/z 385.2 (M + H$^+$). Anal. ($C_{17}H_{25}Cl\ N_4O_4\cdot2HCl\cdot1.5H_2O$), C, H, N.

2-[4-(4-Aminobutanoyl)piperazin-1-yl]ethyl 4-Amino-5-chloro-2-methoxybenzoate Hydrochloride (22c). Same procedure as described for **16a**. 57% yield; hygroscopic white solid; ^{1}H NMR (200 MHz): free base δ 7.76 (s, 1H), 6.26 (s, 1H), 4.53 (bs, 2H), 4.31 (t, J = 5.9 Hz, 2H), 3.81 (s, 3H), 3.59 (m, 2H), 3.46 (m, 2H), 2.71 (m, 4H), 2.5 (m, 4H), 2.35 (t, J = 7.2 Hz, 2H), 1.73 (quintet, J = 7.2 Hz, 2H), 0.86 (bs, 2H). ESI: m/z 399.2 (M + H$^+$). Anal. ($C_{18}H_{27}Cl\ N_4O_4.2HCl\cdot3.5H_2O$), C, H, N.

2-[4-(5-Aminopentanoyl)piperazin-1-yl]ethyl 4-Amino-5-chloro-2-methoxybenzoate Hydrochloride (22d). Same procedure as described for **16a**. 53% yield; very hygroscopic white solid; ^{1}H NMR (200 MHz): free base δ 7.76 (s, 1H), 6.26 (s, 1H), 4.56 (bs, 2H), 4.33 (t, J = 5.9 Hz, 2H), 3.80 (s, 3H), 3.60 (m, 2H), 3.45 (m, 2H), 2.71 (m, 4H), 2.5 (m, 4H), 2.30 (t, J = 7.2 Hz, 2H), 1.63 (m, 2H), 1.48 (m, 2H), 1.10 (bs, 2H). ESI: m/z 413.1 (M + H$^+$). Anal. ($C_{19}H_{29}Cl\ N_4O_4\cdot2HCl\cdot3H_2O$), C, H, N.

2-[4-(6-Aminohexanoyl)piperazin-1-yl]ethyl 4-Amino-5-chloro-2-methoxybenzoate Hydrochloride (22e). Same procedure as described for **16a**. 42% yield; very hygroscopic white nacre solid; ^{1}H NMR (200 MHz): free base δ 7.76 (s, 1H), 6.26 (s, 1H), 4.56 (bs, 2H), 4.33 (t, J = 5.9 Hz, 2H), 3.80 (s, 3H), 3.59 (m, 2H), 3.43 (m, 2H), 2.71 (m, 4H), 2.50 (m, 4H), 2.28 (t, J = 7.2 Hz, 2H), 1.61 (m, 2H), 1.41 (m, 4H), 1.10 (bs, 2H). ESI: m/z 427.2 (M + H$^+$). Anal. ($C_{20}H_{31}ClN_4O_4\cdot2HCl\cdot2.5H_2O$) C, H, N.

2-[4-(8-Aminooctanoyl)piperazin-1-yl]ethyl 4-Amino-5-chloro-2-methoxybenzoate Hydrochloride (22f). Same procedure as described for **16a**. 48% yield; very hygroscopic nacre foam; ^{1}H NMR (200 MHz): free base δ 7.64 (s, 1H), 6.26 (s, 1H), 4.58 (bs, 2H), 4.33 (t, J = 5.9 Hz, 2H), 3.80 (s, 3H), 3.59 (m, 2H), 3.42 (m, 2H), 2.71 (t, J = 5.9 Hz, 2H), 2.63 (t, J = 7.2 Hz, 2H), 2.49 (m, 4H), 2.27 (t, J = 7.2 Hz, 2H), 1.58 (m, 2H), 1.50−1.2 (m, 8H), 1.21 (bs, 2H). ESI: m/z 455.2 (M + H$^+$). Anal. ($C_{22}H_{35}ClN_4O_4\cdot2HCl\cdot1.5H_2O$), C, H, N.

2-[4-(11-Aminoundecanoyl)piperazin-1-yl]ethyl 4-Amino-5-chloro-2-methoxybenzoate Hydrochloride (22g). Same procedure as described for **16a**. 66% yield; very hygroscopic beige foam; ^{1}H NMR (200 MHz): free base δ 7.75 (s, 1H), 6.25 (s, 1H), 4.62 (bs, 2H), 4.32 (t, J = 5.9 Hz, 2H), 3.78 (s, 3H), 3.57 (m, 2H), 3.42 (m, 2H), 2.70 (t, J = 5.9 Hz, 2H), 2.61 (t, J = 7.2 Hz, 2H), 2.49 (m, 4H), 2.25 (t, J = 7.2 Hz, 2H), 1.55 (m, 2H), 1.50−1.2 (m, 14H), 1.15 (bs, 2H). ESI: m/z 569.1 (M + H$^+$). Anal. ($C_{25}H_{41}ClN_4O_4\cdot2HCl\cdot3.5H_2O$), C, H, N.

2-{4-[2-({[5-(Dimethylamino)-1-naphthyl]sulfonyl}amino)acetyl]piperazin-1-yl}ethyl 4-Amino-5-chloro-2-methoxybenzoate (23a). Same procedure as described for **17a** except NEt_3 was used instead of DIEA as base. CH_2Cl_2 then eluent system A; R_f (system A) 0.45; 86% yield; pale green foam; ^{1}H NMR (200 MHz): δ 8.55 (m, 1H), 8.35 (m, 1H), 8.23 (m, 1H), 7.80 (s, 1H), 7.55 (m, 2H), 7.15 (m, 1H), 6.30 (s, 1H), 5.9 (t, J = 4.3 Hz, 1H), 4.47 (bs, 2H), 4.36 (t, J = 6.1 Hz, 2H), 3.81 (s, 3H), 3.71 (d, J = 4.3 Hz, 2H), 3.54 (m, 2H), 3.27 (m, 2H), 2.88 (s, 6H), 2.75 (t, J = 6.1 Hz, 2H), 2.5 (m, 4H). ^{13}C NMR (50 MHz, $CDCl_3$, CD_3OD): δ 165.2, 164.6, 160.3, 151.7, 148.5, 133.9, 132.9, 130.5, 129.8, 129.5, 129.1, 128.4, 122.9, 118.7, 115.3, 109.6, 108.4, 97.9, 61.3, 56.2, 55.7, 52.6, 52.4, 45.2,44.1, 43.5, 41.9. Anal. ($C_{28}H_{34}ClN_5O_6S$), C, H, N.

2-{4-[3-({[5-(Dimethylamino)-1-naphthyl]sulfonyl}amino)propanoyl]piperazin-1-yl}ethyl 4-Amino-5-chloro-2-methoxybenzoate (23b). Same procedure as described for **17a** except NEt_3 was used instead of DIEA as base. CH_2Cl_2/Et_2O 50:50 then CH_2Cl_2/Et_2O/MeOH 50:47:3; R_f (CH_2Cl_2/Et_2O/MeOH 50:47:7) 0.45; 92% yield; pale green foam; ^{1}H NMR (200 MHz): δ 8.52 (m, 1H), 8.24 (m, 2H), 7.80 (s, 1H), 7.55 (m, 2H), 7.20 (m, 1H), 6.29 (s, 1H), 5.84 (m, 1H), 4.47 (bs, 2H), 4.34 (t, J = 6.1 Hz, 2H), 3.84 (s, 3H), 3.48 (m, 2H), 3.20 (m, 4H), 2.87 (s, 6H), 2.73 (t, J = 6.1 Hz, 2H), 2.40 (m, 6H). ^{13}C NMR (50 MHz, $CDCl_3$, CD_3OD): δ 169.4, 164.6, 160.3, 151.7, 148.5, 135.1, 132.9, 130.2, 129.7, 129.4, 128.9, 128.2, 123.0, 118.7, 115.2, 109.6, 108.4, 97.9, 61.3, 56.3, 55.7, 52.7, 52.5, 45.2,44.7, 41.1, 38.8, 32.6. Anal. ($C_{29}H_{36}ClN_5O_6S\cdot1.5H_2O$), C, H, N.

2-{4-[4-({[5-(Dimethylamino)-1-naphthyl]sulfonyl}amino)butanoyl]piperazin-1-yl}ethyl 4-Amino-5-chloro-2-methoxybenzoate (23c). Same procedure as described for **17a** except NEt_3 was used instead of DIEA as base. CH_2Cl_2 then eluent system A; R_f (system A) 0.28; 32% yield; pale green foam; ^{1}H NMR (200 MHz): δ 8.52 (m, 1H), 8.24 (m, 2H), 7.80 (s, 1H), 7.51 (m, 2H), 7.16 (m, 1H), 6.28 (s, 1H), 5.45 (m, 1H), 4.48 (bs, 2H), 4.36 (t, J = 6.1 Hz, 2H), 3.83 (s, 3H), 3.56 (m, 2H), 3.26 (m, 2H), 2.95 (m, 2H), 2.88 (s, 6H), 2.73 (t, J = 6.1 Hz, 2H), 2.48 (m, 4H), 2.24 (t, J = 7 Hz, 2H), 1.76 (quintet, J = 7 Hz, 2H). ^{13}C NMR (50 MHz, $CDCl_3$, CD_3OD): δ 171.1, 164.6, 160.3, 151.7, 148.5, 134.8, 132.9, 129.9, 129.6, 129.4, 129.0, 127.9, 123.0, 118.7, 114.9, 109.5, 108.1, 97.8, 61.2, 56.2, 55.6, 52.9, 52.6, 45.1, 44.9, 42.3, 41.2, 29.7, 24.4. Anal. ($C_{30}H_{38}ClN_5O_6S.H_2O$), C, H, N.

2-{4-[(5-({[5-(Dimethylamino)-1-naphthyl]sulfonyl}amino)pentanoyl]piperazin-1-yl}ethyl 4-Amino-5-chloro-2-methoxybenzoate (23d). Same procedure as described for **17a** except NEt_3 was used instead of DIEA as base. CH_2Cl_2 then eluent system; R_f (sytem A) 0.4; 58% yield; pale green foam; ^{1}H NMR (200 MHz): δ 8.53 (m, 1H), 8.24 (m, 2H), 7.80 (s, 1H), 7.55 (m, 2H), 7.18 (m, 1H), 6.29 (s, 1H), 5.06 (m, 1H), 4.46 (bs, 2H), 4.38 (t, J = 6.1 Hz, 2H), 3.84 (s, 3H), 3.58 (m, 2H), 3.41 (m, 2H), 2.88 (m, 8H), 2.78 (t, J = 6.1 Hz, 2H), 2.55 (m, 4H), 2.20 (t, J = 7 Hz, 2H), 1.70−1.40 (m, 4H). ^{13}C NMR (50 MHz, $CDCl_3$, CD_3OD): δ 171.4, 164.6, 160.3, 151.6, 148.5, 134.9, 132.9, 129.9, 129.7, 129.5, 129.0, 127.9, 122.9, 118.8, 114.9, 109.6, 108.4, 97.9, 61.3, 56.3, 55.7, 53.0, 52.7, 45.2, 42.3, 41.2, 31.9, 28.8, 21.7. Anal. ($C_{31}H_{40}ClN_5O_6S\cdot1.5H_2O$), C, H, N.

2-{4-[(6-({[5-(Dimethylamino)-1-naphthyl]sulfonyl}amino)hexanoyl]piperazin-1-yl}ethyl 4-Amino-5-chloro-2-methoxybenzoate (23e). Same procedure as described for **17a** except NEt_3 was used instead of DIEA as base. CH_2Cl_2 then eluent sytem A; R_f (system A) 0.6; 81% yield; pale green foam; ^{1}H NMR (200 MHz): δ 8.54 (m, 1H), 8.26 (m, 2H), 7.80 (s, 1H), 7.53 (m, 2H), 7.17 (m, 1H), 6.29 (s, 1H), 4.82 (m, 1H), 4.45 (bs, 2H), 4.40 (t, J = 6.1 Hz, 2H), 3.84 (s, 3H), 3.60 (m, 2H), 3.40 (m, 2H), 2.88 (m, 8H), 2.75 (t, J = 6.1 Hz, 2H), 2.51 (m, 4H), 2.20 (t, J = 7 Hz, 2H), 1.45 (m, 4H), 1.25 (m, 2H). ^{13}C NMR (50 MHz, $CDCl_3$, CD_3OD): δ 171.6, 164.6, 160.2, 151.6, 148.5, 134.9, 132.9, 129.9, 129.6, 129.4, 128.1, 127.9, 122.9, 118.8, 114.9, 109.5, 108.3, 97.9, 61.3, 56.3, 55.7, 53.1, 52.7, 45.1, 42.4, 41.2, 32.5, 28.9, 25.8, 24.22. Anal. ($C_{32}H_{42}ClN_5O_6S\cdot H_2O$), C, H, N.

2-{4-[(8-({[5-(Dimethylamino)-1-naphthyl]sulfonyl}amino)octanoyl]piperazin-1-yl}ethyl 4-Amino-5-chloro-2-methoxybenzoate (23f). Same procedure as described for **17a** except NEt_3 was used instead of DIEA as base. CH_2Cl_2 then eluent system A; R_f (system A) 0.33; 88% yield; pale green foam; ^{1}H NMR (200 MHz): δ 8.53 (m, 1H), 8.26 (m, 2H), 7.80 (s, 1H), 7.53 (m, 2H), 7.18 (m, 1H), 6.29 (s, 1H), 4.60 (m, 1H), 4.46 (bs, 2H), 4.37 (t, J = 6.1 Hz, 2H), 3.80 (s, 3H), 3.61 (m, 2H), 3.45 (m, 2H), 2.88 (m, 8H), 2.75 (t, J = 6.1 Hz, 2H), 2.54 (m, 4H), 2.24 (t, J = 7 Hz), 1.7−1.1 (m, 10H). ^{13}C NMR (50 MHz): δ 171.4, 164.4, 160.2, 151.9, 148.0, 134.8, 133.1, 130.2, 129.8, 129.6, 129.4, 128.2, 123.1, 118.7, 115.0, 109.8, 109.3, 98.2, 61.6, 56.5, 55.9, 53.3, 52.9, 45.4, 45.3, 43.1, 41.4, 32.9, 29.3, 28.9, 28.5, 26.1, 24.9. Anal. ($C_{34}H_{46}ClN_5O_6S\cdot0.5H_2O$), C, H, N.

2-{4-[(11-({[5-(Dimethylamino)-1-naphthyl]sulfonyl}amino)undecanoyl]piperazin-1-yl}ethyl 4-Amino-5-chloro-2-methoxybenzoate (23g). Same procedure as described for **17a** except NEt_3 was used instead of DIEA as base. CH_2Cl_2 then eluent system A; R_f (System A) 0.5; 86% yield; pale green foam; ^{1}H NMR (200 MHz): δ 8.53 (m, 1H), 8.24 (m, 2H), 7.80 (s, 1H), 7.53 (m, 2H), 7.17 (m, 1H), 6.28 (s, 1H), 4.65 (m, 1H), 4.49 (bs, 2H), 4.39 (t, J = 6.1 Hz, 2H), 3.82 (s, 3H), 3.64 (m, 2H), 3.48 (m, 2H), 2.88 (m, 8H), 2.79 (t, J = 6.1 Hz, 2H), 2.58 (m, 4H), 2.28 (t, J = 7 Hz, 2H), 1.58 (m, 2H), 1.43−1.1 (m, 14H). ^{13}C NMR (50 MHz): δ 171.6, 164.4, 160.2, 151.9, 148.1, 134.9, 133.1, 130.2, 129.8, 129.6, 129.4, 128.2, 123.0, 118.7, 115.0, 109.8, 109.2, 98.2, 61.6, 56.5, 55.9, 53.4, 53.0, 45.5, 45.3, 43.2, 41.4, 33.1, 29.4, 29.2, 29.1 29.0, 28.7, 26.2, 25.2. Anal. ($C_{37}H_{52}ClN_5O_6S$), C, H, N.

Synthesis of compounds 25a, 25b, 29: General Procedure. *tert*-Butyl 4-{[6-Nitro-1,3-dioxo-1*H*-benzo[*de*]isoquinolin-2(3*H*)-yl]methyl}piperidine-1-carboxylate (25a). 4-Nitro-1,8-naphthalic anhydride **24a** (5 g, 0.021 mol) and 1-Boc-4-aminomethylpiperidine (4.28 g, 0.02 mol) in absolute EtOH (120 mL) was heated under reflux for 24 h. After concentration, the crude product was chromatographed (EtOAc/cyclohexane 40:60) and the solid obtained recrystallized from cyclohexane/EtOAc to provide 6.2 g (71%) of **25a** as a yellow solid; R_f (EtOAc/cyclohexane) 0.92; mp 140 °C; ^{1}H NMR (400 MHz): δ 8.84 (dd, J = 0.94 Hz and J = 8.64 Hz, 1H), 8.73 (dd, J = 0.86 Hz and J = 7.34 Hz, 1H), 8.68 (d, J = 8 Hz, 1H), 8.40 (d, J = 8 Hz, 1H), 7.99 (dd, J = 7.34 Hz and J = 8.64 Hz, 1H), 4.13 (d, J = 7.2 Hz, 2H), 4.10 (m, 2H), 2.66 (bt, 2H), 2.07 (m, 1H), 1.67 (m, 2H), 1.44 (s, 9H), 1.34 (m, 2H). Anal. ($C_{23}H_{25}N_3O_6$), C, H, N.

***tert*-Butyl 4-{[6-(Dimethylamino)-1,3-dioxo-1*H*-benzo[*de*]isoquinolin-2(3*H*)-yl]methyl}piperidine-1-carboxylate (25b).** From **24b**. CH_2Cl_2 then CH_2Cl_2/Et_2O 90:10; R_f (CH_2Cl_2/Et_2O) 0.21; 83% yield; yellow foam; ^{1}H NMR (200 MHz): δ 8.54 (dd, J = 1.17 Hz and J = 7.29 Hz, 1H), 8.45 (d, J = 8.26 Hz, 1H), 8.43 (dd, J = 1.17 Hz and J = 8.45 Hz, 1H), 7.64 (dd, J = 7.29 Hz and J = 8.45 Hz, 1H), 7.10 (d, J = 8.26 Hz, 1H), 4.09 (d, J = 7.2 Hz, 2H), 4.08 (m, 2H), 3.09 (s, 6H), 2.64 (m, 2H), 2.05 (m, 1H), 1.66 (m, 2H), 1.44 (s, 9H,), 1.34 (m, 2H). Anal. ($C_{25}H_{31}N_3O_4$), C, H, N.

2-(4-{4-[6-(Dimethylamino)-1,3-dioxo-1*H*-benzo[*de*]isoquinolin-2(3*H*)-yl]butyl}piperidin-1-yl)ethyl 4-Amino-5-chloro-2-methoxybenzoate (29). From **24b** and **16d** in the presence of NEt_3. CH_2Cl_2 then CH_2Cl_2/MeOH 90:10; R_f (CH_2Cl_2/Et_2O) 0.13; 23% yield; yellow foam; ^{1}H NMR (200 MHz): δ 8.56 (dd, J = 1.15 Hz and J = 7.34 Hz, 1H), 8.48 (d, J = 8.29 Hz,1H), 8.44 (dd, J = 1.15 Hz and J = 8.46 Hz, 1H), 7.82 (s, 1H), 7.65 (dd, J = 7.34 Hz and J = 8.46 Hz, 1H), 7.16 (d, J = 8.29 Hz, 1H), 6.27 (s, 1H), 4.41 (bs, 2H), 4.36 (t, J = 5.9 Hz, 2H), 4.16 (t, J = 7.5 Hz, 2H), 3.83 (s, 3H), 3.10 (s, 6H), 2.96 (m, 2H), 2.72 (t, J = 5.9 Hz, 2H), 2.08 (m, 2H), 1.80−1.1 (m, 11H). ESI: m/z 607.3 ($M + H^+$). Anal. ($C_{33}H_{39}ClN_4O_5\cdot0.5H_2O$), C, H, N.

6-Nitro-2-(4-piperidinemethyl)-1*H*-benzo[*de*]isoquinoline-1,3(2*H*)-dione Hydrochloride (26a). Same procedure as described for **17a**, except that compound **25a** was dissolved in dioxane and reacted for 6 h. 94% yield; pale yellow solid; ^{1}H NMR (200 MHz): free base δ 8.82 (dd, J = 1.1 Hz and J = 8.68 Hz, 1H), 8.73 (dd, J = 0.87 Hz and J = 7.35 Hz, 1H), 8.69 (d, J = 8 Hz, 1H), 8.38 (d, J = 8 Hz, 1H), 7.97 (dd, J = 7.35 Hz and J = 8.68 Hz, 1H), 4.10 (d, J = 7.12 Hz, 2H), 3.06 (m, 2H), 2.54 (m, 2H), 2.01 (m, 1H), 1.65 (m, 2H), 1.29 (m, 2H). Anal. (C_{18} $H_{17}N_3O_4$.HCl), C, H, N.

6-(Dimethylamino)-2-(4-piperidinemethyl)-1*H*-benzo[*de*]isoquinoline-1,3(2*H*)-dione Hydrochloride (26b). Same procedure as described for **17a**, except reaction time 24 h. 83% yield; hygroscopic yellow solid; ^{1}H NMR (200 MHz): free base δ 8.52 (dd, J = 1.19 Hz and J = 7.28 Hz, 1H), 8.43 (d, J = 8.23 Hz, 1H), 8.40 (dd, J = 1.19 Hz and J = 8.51 Hz, 1H), 7.61 (dd, J = 7.28 Hz and J = 8.51 Hz, 1H), 7.08 (d, J = 8.23 Hz, 1H), 4.03 (d, J = 7.12 Hz, 2H), 3.06 (s, 6H,), 3.03 (m, 2H), 2.49 (m, 2H), 2 (m, 1H), 1.63 (m, 2H), 1.28 (m, 2H). Anal. ($C_{20}H_{23}N_3O_2$ .HCl·0.25H_2O), C, H, N.

2-(4-{[6-Nitro-1,3-dioxo-1*H*-benzo[*de*]isoquinolin-2(3*H*)-yl]methyl}piperidin-1-yl)ethyl 4-Amino-5-chloro-2-methoxybenzoate (27a). Same procedure as described for **15a**. CH_2Cl_2/ Et_2O 50:50 then system eluent A; R_f (system A) 0.48; 13% yield; orange solid; mp 194 °C; ^{1}H NMR (200 MHz, $CDCl_3$, CD_3OD): δ 8.77 (dd, J = 1.10 Hz and J = 8.73 Hz, 1H), 8.66 (dd, J = 1.10 Hz and J = 7.29 Hz, 1H), 8.62 (d, J = 8.07 Hz, 1H), 8.35 (d, J = 8.07 Hz, 1H), 7.93 (dd, J = 7.29 Hz and J = 8.73 Hz, 1H), 7.73 (s, 1H), 6.24 (s, 1H), 4.29 (t, J = 5.9 Hz, 2H), 4.07 (d, J = 7 Hz, 2H), 3.75 (s, 3H), 2.95 (m, 2H), 2.68 (t, J = 5.9 Hz, 2H), 2.06 (m, 2H), 1.86 (m, 1H), 1.66 (m, 2H), 1.49 (m, 2H). ESI: m/z 567.1 ($M + H^+$). Anal. ($C_{28}H_{27}Cl$ N_4O_7·0.5H_2O), C, H, N.

2-(4-{[6-(Dimethylamino)-1,3-dioxo-1*H*-benzo[*de*]isoquinolin-2(3*H*)-yl]methyl}piperidin-1-yl)ethyl 4-Amino-5-chloro-2-methoxybenzoate Hydrochloride (27b). Same procedure as described for **15a** except reaction mixture CH_3CN/CH_2Cl_2/DMF 30:10:10 was used. CH_2Cl_2/MeOH 90:10 then CH_2Cl_2/Et_2O 50:50. R_f (CH_2Cl_2/Et_2O 50:50) 0.42; 14% yield; yellow solid. The resulted product was transformed into its hydrochloride salt with 4 N HCl methanol solution. ^{1}H NMR (200 MHz): free base δ 8.53 (dd, J = 1.15 Hz and J = 7.34 Hz, 1H), 8.46 (d, J = 8.29 Hz, 1H), 8.42 (dd, J = 1.15 Hz and J = 8.46 Hz, 1H), 7.77 (s, 1H), 7.63 (dd, J = 7.34 Hz and J = 8.46 Hz, 1H), 7.09 (d, J = 8.29 Hz, 1H), 6.25 (s, 1H), 4.45 (bs, 2H), 4.32 (t, J = 5.9 Hz, 2H), 4.09 (d, J = 7 Hz, 2H), 3.79 (s, 3H), 3.08 (s, 6H), 2.95 (m, 2H), 2.68 (t, J = 5.9 Hz, 2H), 2.06

(m, 2H), 1.87 (m, 1H), 1.66 (m, 2H), 1.50 (m, 2H). ESI: *m/z* 565.1 (M + H^+). Anal. ($C_{30}H_{33}ClN_4O_5 \cdot HCl \cdot 1.5H_2O$), C, H, N.

2-(4-{[6-Amino-1,3-dioxo-1*H*-benzo[*de*]isoquinolin-2(3*H*)-yl]methyl}piperidin-1-yl)ethyl 4-amino-5-chloro-2-methoxybenzoate Hydrochloride (28). A suspension of nitro compound **27a** (0.104 g, 0.18 mmol) and Raney nickel (~ 0.2 g) in MeOH/dioxane 50:50 (12 mL) was stirred with ammonium formate (0.2 g) at room temperature. After completion of the reaction (1 h), the mixture was filtered off through Celite. The organic layer was concentrated, and the residue was chromatographed on silica gel using CH_2Cl_2/MeOH 80:20 to give an orange solid R_f (CH_2Cl_2/MeOH 80:20) 0.44 which was transformed into its hydrochloride salt **28** with 4 N HCl methanol solution (56 mg, 50%); hygroscopic yellow solid; ^{1}H NMR (400 MHz, CD_3OD): δ 8.50 (bd, 2H), 8.26 (dd, J = 1.36 Hz and J = 8.42 Hz, 1H), 7.79 (s, 1H), 7.62 (t, J = 8.3 Hz, 1H), 6.87 (d, J = 8.42 Hz, 1H), 6.45 (s, 1H), 4.55 (m, 2H), 4.12 (d, J = 6.8 Hz, 2H), 3.80 (s, 3H), 3.71 (m, 2H), 3.50 (m, 2H), 3.05 (m, 2H), 2.24 (m, 1H), 2.03 (m, 2H), 1.76 (m, 2H). ESI: *m/z* 537.2 (M + H^+), 559.2 (M + Na^+). Anal. ($C_{28}H_{29}ClN_4O_4 \cdot 2HCl \cdot 0.5H_2O$), C, H, N.

***tert*-Butyl-4-{[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]methyl}piperidine-1-carboxylate (30).** A solution of NBD chloride (2.48 g, 12.4 mmol) in dry THF (120 mL) was slowly added to a solution of 1-Boc-4-aminomethylpiperidine (9.3 g, 43.4 mmol) in THF (120 mL) at 0 °C for 2.5 h. Then, the solvent was evaporated, and the residue was chromatographed on silica gel (CH_2Cl_2/Et_2O 50:50) to give 3.23 g (69%) of **30** as an orange solid. R_f (CH_2Cl_2/Et_2O 50:50) 0.75; mp > 260 °C; ^{1}H NMR (200 MHz): δ 8.46 (d, J = 8.7 Hz, 1H), 6.43 (bt, 1H), 6.18 (d, J = 8.7 Hz, 1H), 4.18 (m, 2H), 3.42 (t, J = 6.3 Hz, 2H), 2.73 (m, 2H), 1.97 (m, 1H), 1.84 (m, 2H), 1.45 (s, 9H), 1.27 (m, 2H). Anal. ($C_{17}H_{23}N_5O_5$), C, H, N.

7-Nitro-*N*-(4-piperidinemethyl)-2,1,3-benzoxadiazol-4-amine Hydrochloride (31). Same procedure as described for **16a**, except that compound **30** was dissolved in MeOH/dioxane 50:50 and reacted for 5 h. 85% yield; ^{1}H NMR (200 MHz): free base δ 8.43 (d, J = 8.7 Hz, 1H), 6.13 (d, J = 8.7 Hz, 1H), 3.34 (d, J = 6.7 Hz, 2H), 3.10 (m, 2H), 2.58 (m, 2H), 1.95 (m,1H), 1.8 (m, 2H), 1.25 (m, 2H), 0.8 (bs, 1H). Anal. ($C_{12}H_{15}N_5O_5.HCl \cdot 0.25H_2O$), C, H, N.

2-(4-{[(7-Nitro-2,1,3-benzoxadiazol-4-yl)amino]methyl}-piperidin-1-yl)ethyl 4-Amino-5-chloro-2-methoxybenzoate (32). Same procedure as described for **15a**, except that hydrochloride salt of amine **31** was reacted in dry DMF at 30 °C for 3 days. CH_2Cl_2/acetone 50:50; R_f (CH_2Cl_2/acetone 50:50) 0.16; mp 182−183 °C (dec); ^{1}H NMR (200 MHz): δ 8.47 (d, J = 8.7 Hz, 1H), 7.79 (s, 1H); 6.35 (m, 1H), 6.28 (s, 1H), 6.17 (d, J = 8.7 Hz, 1H), 4.46 (bs, 2H), 4.39 (t, J = 5.9 Hz, 2H), 3.83 (s, 3H), 3.39 (m, 2H), 3.05 (m, 2H), 2.76 (t, J = 5.9 Hz, 2H), 2.20 (m, 2H), 1.80 (m, 3H), 1.46 (m, 2H). ESI: *m/z* 505.1 (M + H^+). Anal. ($C_{22}H_{25}ClN_6O_6 \cdot 0.125H_2O$), C, H, N.

2-(Piperidin-1-yl)ethyl 5-Chloro-2-methoxy-4-[(7-nitro-2,1,3-benzoxadiazo-4-yl)amino]benzoate (33). A mixture of **14** (0.17 g, 0.54 mmol), NBD chloride (0.18 g, 0.9 mmol), KI (8 mg), and n-BuOH (17 mL) was heated at reflux under argon for 12 h. After cooling, the reaction mixture was diluted with Et_2O, and the precipitate obtained was filtered, washed several times with Et_2O, and chromatographed on silica gel (CH_2Cl_2, then CH_2Cl_2/Et_2O 50:50) to give 80 mg (30%) of **33** as a dark blue solid; R_f (CH_2Cl_2/Et_2O 50:50) 0.28; ^{1}H NMR (200 MHz): δ 8.26 (d, J = 8.7 Hz, 1H), 7.55 (s, 1H); 6.53 (d, J = 8.7 Hz, 1H), 5.91 (s, 1H), 4.53 (t, J = 5.9 Hz, 2H,), 4.31 (bs, 1H), 3.71 (s, 3H), 3.45 (m 2H), 2.46 (t, J = 5.9 Hz, 2H), 2.12 (m, 2H), 1.52 (m, 6H). ESI: *m/z* 474.1 (M + H^+), 496.0 (M + Na^+).

Biology. Membrane Preparation and Radioligand Binding Assays. Briefly, C6 glial cells stably transfected with the h5-$HT_{4(e)}$ receptor, grown to confluence, were incubated with serum-free medium for 4 h, washed twice with phosphate-buffered saline (PBS), and centrifuged at 300*g* for 5 min. The pellet was used immediately or stored at −80 °C. The pellet was resuspended in 10 volumes of ice-cold HEPES buffer (50 mM, pH 7.4) and centrifuged at 40 000*g* for 20 min at 4 °C. The resulting pellet was resuspended in 15 volumes of HEPES buffer (50 mM, pH 7.4). The protein concentration was determined by the method of Bradford using bovine serum albumin as the standard.

Radioligand binding studies were performed in 500 μL of HEPES buffer (50 mM, pH 7.4), 20 μL of the studied ligand (7 concentrations), and 20 μL of [^{3}H]GR113808 at a concentration of 0.2 nM and 50 μL of membranes preparation (100−200 μg of protein). Nonspecific binding was determined with 10 μM GR113808. Tubes were incubated at 25 °C for 30 min, and the reaction was terminated by filtration through Whatman GF/B Filter paper using the Brandel 48R cell harvester. Filters were presoaked in a 0.1% solution of polyethylenimine. Filters were subsequently washed with ice-cold buffer (50 mM Tris-HCl, pH 7.4) and placed overnight in 4 mL of ready-safe scintillation cocktail. Radioactivity was measured using a Beckman model LS 6500C liquid scintillation counter. Binding data (K_i) were analyzed by computer-assisted nonlinear regression analysis (Prism, Graphpad Software, San Diego, CA). The data are the results of two or three determinations in triplicate.

Measurement of cAMP. C6 glial cells stably transfected with the human 5-$HT_{4(e)}$ receptor were grown to confluence and incubated with serum-free medium for 4 h before the beginning of the assay. Then the cells were preincubated for 15 min with serum-free medium supplemented with 5 mM theophylline and 10 μM pargyline. 5-HT (1 μM) and/or compounds were added and incubated for an additional 15 min at 37 °C in 5% CO_2. The reaction was stopped by aspiration of the medium and addition of 50 μL of ice-cold perchloric acid (20%). After a 30 min period, neutralization buffer was added (HEPES 25 mM, KOH 2 N) and supernatant was extracted after centrifugation at 2000*g* for 5 min, cAMP was quantified using radioimmunoassay kit (cAMP competitive radioimmunoassay, Beckman, France). The 5-HT concentration-effect curve was calculated using seven concentrations (10^{-10} to 10^{-5}) alone or in the presence of compounds.

Fluorescent Labeling of C6 Glial Cell Membranes Expressing the Human 5-$HT_{4(e)}$ Receptor. Autofluorescence: C6 glial cell membranes were incubated alone in HEPES buffer (50 mM, pH 7.4) for 30 min at 25 °C. Total fluorescence: C6 glial cell membranes were incubated with either 60 nM (K_i) of **28** or 7 nM (K_i) of **32** or 7 nM (K_i) of **17a** in HEPES buffer (50 mM, pH 7.4) for 30 min at 25 °C. Nonspecific fluorescence: C6 glial cell membranes were incubated with 0.2 μM (1000 K_i) of GR113808 in HEPES buffer (50 mM, pH 7.4) for 30 min at 25 °C, then incubated with either 60 nM (K_i) of **28** or 7 nM (K_i) of **32** or 7 nM (K_i) of **17a** for 30 min at 25 °C. All the samples were centrifuged at 20 000*g* for 15 min at 4 °C, washed with HEPES buffer (50 mM, pH 7.4), centrifuged at 20 000*g* for 15 min at 4 °C. The same protocole was repeated one more time and the pellet was resuspended in HEPES buffer for the measure performed at 20 °C. Fluorescence measurements were done on a Perkin-Elmer fluorimeter LS50B. Excitation and emission slits were both 15-nm band-pass. Results were analyzed with Prism, Graphpad Software (San Diego, CA).

Fluorescent Labeling of C6 Glial Cells Expressing Human the 5-$HT_{4(e)}$ Reeceptor. C6 glial cells stably transfected with the h5-$HT_{4(e)}$ receptor were cultivated on 24 well plates and eight-well Lab-Tek chamber plates according to the experiments. They were grown to confluence and incubated with serum-free medium for 4 h before the beginning of the assay. Autofluorescence: C6 glial cells were incubated alone in HEPES buffer (50 mM, pH 7.4) for 30 min at 25 °C. Total fluorescence: C6 glial cells were incubated with either 60 nM (K_i) of **28** or 7 nM (K_i) of **32** or 7 nM (K_i) of **17a** in HEPES buffer (50 mM, pH 7.4) for 30 min at 25 °C. Nonspecific fluorescence: C6 glial cells were incubated with 0.2 μM (1000 K_i) of GR 113808 in HEPES buffer (50 mM, pH 7.4) for 30 min at 25 °C, then incubated with either 60 nM (K_i) of **28** or 7 nM (K_i) of **32** or 7 nM (K_i) of **17a** for 30 min at 25 °C. Cells were then washed twice with HEPES buffer and fluorescence was observed using a Fluostar plate reader. The data are the results of two or three determinations in triplicate.

For microscopy observation, cells were fixed with 1% paraformaldehyde for 15 min, washed with a NH_4Cl (50 mM)−glycine (20 mM) solution (pH 7.4) and rinsed twice with HEPES buffer. The cells were observed on a confocal microscope (LSM 510, Zeiss France, Le Pecq, France). Images of cells were acquired with a Plan-Achromat × 63 1.4 NA oil-immersion objective (Zeiss) in a median plan with an optical section thickness of 10 μm. Excitation was at 458 nm and fluorescence detection with a band-pass filter of 505−550 nm.

Acknowledgment. We thank Valerie Nicolas for technical assistance and realization of the confocal studies.

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JM0307887