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# Toward Biophysical Probes for the 5-HT₃ Receptor: Structure–Activity Relationship Study of Granisetron Derivatives

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This report describes the synthesis and biological characterization of novel granisetron derivatives that are antagonists of the human serotonin (5-HT₃A) receptor. Some of these substituted granisetron derivatives showed low nanomolar binding affinity and allowed the identification of positions on the granisetron core that might be used as attachment points for biophysical tags. A BODIPY fluorophore was appended to one such position and specifically bound to 5-HT₃A receptors in mammalian cells.
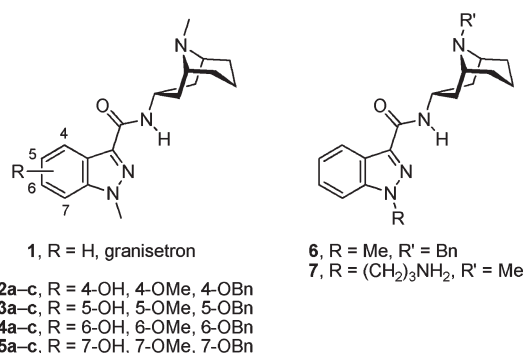
## Introduction

The serotonin (5-HT[a]) type 3 receptor (5-HT₃R) is a ligand-gated ion channel that is responsible for rapid transmission of nerve impulses at synapses of the central and peripheral nervous system.[1] It is a member of the Cys-loop family, which also includes nicotinic acetylcholine (nACh), γ-aminobutyric acid (GABA_A), and glycine receptors. These proteins consist of five pseudosymmetrically arranged subunits. Each subunit comprises a large extracellular N-terminal domain that is responsible for agonist binding, four transmembrane domains (M1–M4) that surround a central ion conducting pore, and a large intracellular loop between M3 and M4 that influences channel conductance and mediates the actions of intracellular messengers.

The 5-HT₃R is an attractive therapeutic target. Its antagonists are used to control chemotherapy-induced, radiotherapy-induced, and postoperative nausea and vomiting and for treatment of irritable bowel syndrome.[2] There is some evidence that 5-HT₃R antagonists might also be useful for treatment of psychiatric and neurological disorders such as anxiety, drug dependence, depression, bulimia nervosa, schizophrenia, and cognitive dysfunction.[3] Another potentially interesting widespread application is their capacity to reduce pain in certain conditions including rheumatoid arthritis, fibromyalgia, and migraine.[4]

Structural insight of Cys-loop receptors has been gained from high resolution structures of homologous acetylcholine binding proteins (AChBPs),[5] engineered nAChR subunits,[6] bacterial receptors,[7] and the cryoelectron microscopy data of

**Chart 1.** Reference and Title Compounds



**1**, R = H, granisetron
**2a–c**, R = 4-OH, 4-OMe, 4-OBn
**3a–c**, R = 5-OH, 5-OMe, 5-OBn
**4a–c**, R = 6-OH, 6-OMe, 6-OBn
**5a–c**, R = 7-OH, 7-OMe, 7-OBn

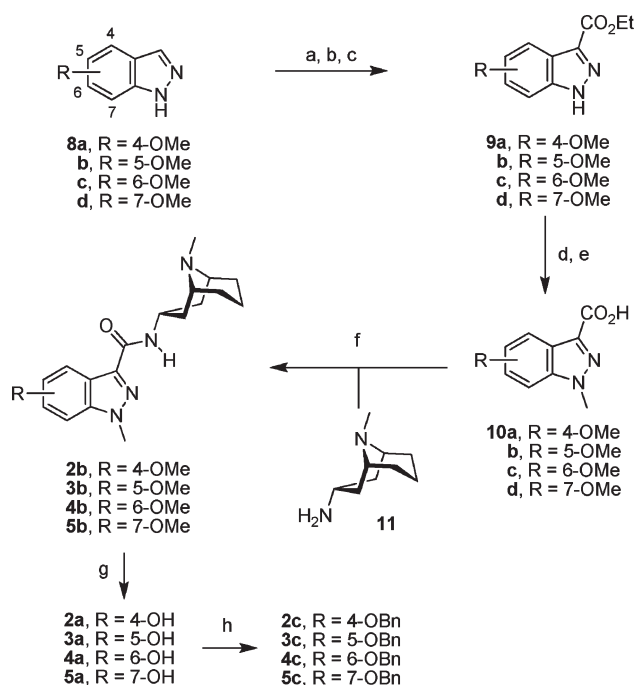**6**, R = Me, R' = Bn
**7**, R = (CH₂)₃NH₂, R' = Me

the nAChR.[8] This has allowed the construction of 5-HT₃R homology models.[9−12] However, such models may be too inaccurate to be useful for rational structure-based design and often require labor-intensive validation by experimental methods (i.e., receptor mutagenesis). Furthermore, despite structural information and homology modeling, the exact mechanism that couples agonist binding to channel opening in Cys-loop receptors is still poorly understood and cannot be elucidated by static high resolution structures alone.

Using high-affinity biophysical probes to investigate the structure and function of ligand-gated ion channels is a complementary strategy to traditional biological approaches such as radioligand binding and electrophysiology. In addition, such molecular probes, for example, utilizing fluorescent ligands as an alternative to radioligand binding or immunolabeling, can be used for assays that target specific ligand-gated ion channels.

The aim of the present study was to identify tethering positions for potentially bulky biophysical tags on the high-affinity 5-HT₃R antagonist granisetron[13] (**1**, Chart 1). Because of the lack of granisetron structure–activity data in the public domain, we substituted every synthetically possible position with differently sized functional groups on the granisetron core, which yielded 14 different granisetron derivatives (Chart 1).

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[a]Abbreviations: 5-HT, serotonin; 5-HT₃R, 5-HT₃ receptor; AChBP, acetylcholine binding protein; nAChR, nicotinic acetylcholine receptor; Boc, *tert*-butoxycarbonyl; CD₃OD, tetradeuteriomethanol; DCC, *N,N'*-dicyclohexylcarbodiimide; DMF, *N,N*-dimethylformamide; GABA, γ-aminobutyric acid; HOBt, 1-hydroxybenzotriazole; SEM, 2-(trimethylsilyl)ethoxymethyl; THF, tetrahydrofuran.

**Scheme 1.** Synthesis of Compounds **2**−**5**[a]



8a, R = 4-OMe
b, R = 5-OMe
c, R = 6-OMe
d, R = 7-OMe

9a, R = 4-OMe
b, R = 5-OMe
c, R = 6-OMe
d, R = 7-OMe

10a, R = 4-OMe
b, R = 5-OMe
c, R = 6-OMe
d, R = 7-OMe

2b, R = 4-OMe
3b, R = 5-OMe
4b, R = 6-OMe
5b, R = 7-OMe

2a, R = 4-OH
3a, R = 5-OH
4a, R = 6-OH
5a, R = 7-OH

2c, R = 4-OBn
3c, R = 5-OBn
4c, R = 6-OBn
5c, R = 7-OBn

[a] Reagents and conditions: (a) $^c$Hex$_2$NMe, SEM-Cl, THF, room temp, 65−88%; (b) $^n$BuLi, THF, −78 °C, CNCO$_2$Et, 66−75%; (c) 2 M HCl, EtOH, room temp, 76−98%; (d) KO$^t$Bu, THF, 0 °C, MeI, room temp, 78−95%; (e) 2 M NaOH, MeOH, room temp, 97−99%; (f) DCC, HOBt, **11**, 4:1 CH$_2$Cl$_2$/DMF, room temp, 85−94%; (g) [Me$_3$NH][Al$_2$Cl$_7$], CH$_2$Cl$_2$, reflux, or BBr$_3$, CH$_2$Cl$_2$, room temp, or NaSEt, DMF, 110 °C, 84−93%; (h) BnBr, Na$_2$CO$_3$, acetone, room temp, 63−94%.

**Scheme 2.** Synthesis of Compounds **6** and **7**[a]



12

14, R = Me
15, R = Bn

6

11, R = Me
13, R = Bn

7
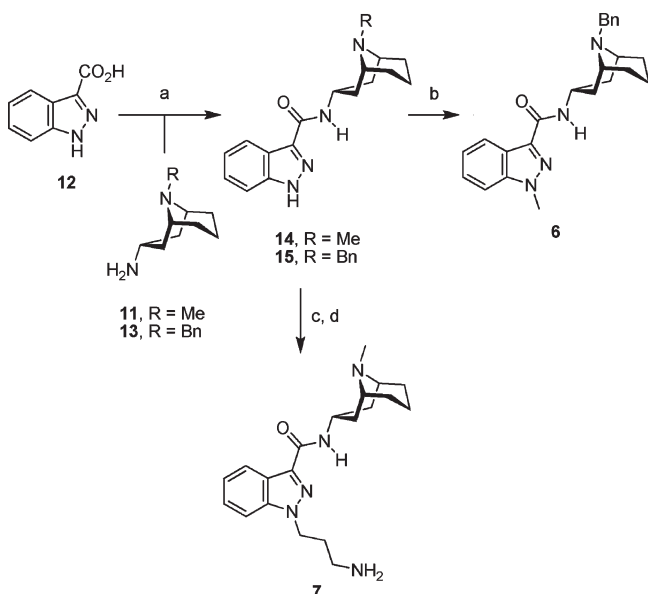
[a] Reagents and conditions: (a) DCC, HOBt, **11** or **13**, 4:1 CH$_2$Cl$_2$/DMF, room temp, 81−97%. (b) For **15**, R = Bn: KO$^t$Bu, THF, 0 °C; MeI, room temp, 90%. (c) For **14**, R = Me: KO$^t$Bu, 5:1 THF/DMF, 0 °C, Br(CH$_2$)$_3$NHBoc, room temp, 78%. (d) 1.2 M HCl in MeOH, room temp, 98%.

## Results and Discussion

**Chemistry.** The synthesis of **2**−**5** is shown in Scheme 1, and it was accomplished starting from indazoles **8a**−**d** which were
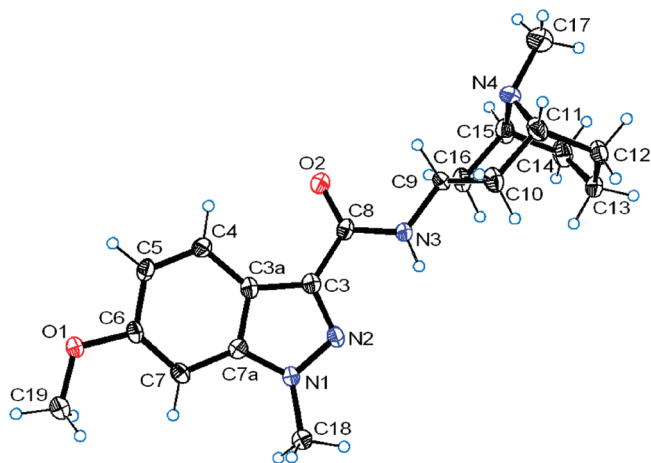


**Figure 1.** Crystal structure of **4b**. Thermal ellipsoids are shown at 50% probability.

prepared according to literature methods.[14,15] A SEM protecting group was selectively attached to N-2 of the indazoles which was followed by a SEM-directed C-3 lithiation and subsequent reaction with an ester group donor.[16] Protecting group cleavage, N-1 methylation, and subsequent ester hydrolysis furnished indazole carboxylic acids **10a**−**d** which were coupled with bicyclic amine **11**[17] to yield amides **2b**−**5b**.

The aromatic methoxy ethers were cleaved using either an excess of BBr$_3$ in CH$_2$Cl$_2$ or NaSEt in hot DMF. However, no product was obtained when these methods were used for the 7-methoxy derivative **5b**. Instead, when the latter compound was treated with a chloroaluminate ionic liquid[18] in refluxing CH$_2$Cl$_2$, the desired cleavage product **5a** was successfully produced in good yield (86%). Finally, hydroxyindazoles **2a**−**5a** were alkylated under basic conditions to give benzyl ethers **2c**−**5c**.

The synthesis of **6** and **7** started from commercially available 1*H*-indazole-3-carboxylic acid (**12**, Scheme 2). Coupling with bicyclic amines **11** and **13** afforded amides **14** and **15**. The amine **13** was synthesized from the corresponding bicyclic ketone, 9-benzyl-9-azabicyclo[3.3.1]nonan-3-one,[19] using an oxime formation and reduction sequence as described for **11** in ref 17. Amide **15** was methylated at N-1 to yield granisetron derivative **6**. Alkylation of amide **14** with 3-(Boc-amino)propyl bromide followed by deprotection under acidic conditions furnished **7**.

The granatane (9-methyl-9-azabicyclo[3.3.1]nonane) moiety of the synthesized granisetron derivatives **2**−**7** adopts a boat−chair conformation in solution, as confirmed by $^1$H NMR. In CD$_3$OD, the 3-proton of the azabicycle appears as a triplet of triplets and has a coupling constant of 11.5 Hz with the 2- and 4-*endo* protons and a coupling constant of 6.8 Hz with the 2- and 4-*exo* protons (e.g., for **5a**). A coupling constant of 11.0 Hz between the 2-*exo* and the bridgehead 1-proton was observed that is indicative of an eclipsed arrangement. Thus, the obtained $^1$H NMR data are in agreement with a 3-*exo* proton and a boat−chair conformation of the granatane.[13,20]

This particular structural aspect of granisetron derivatives **2**−**7** was further confirmed by the crystal structures of **4b** and **5a** (Figures 1 and 2). In both structures the indazole ring is coplanar with the amide bond and there appears to be flexibility around the bond between the amide nitrogen and the granatane. Intriguingly, the methyl group of the
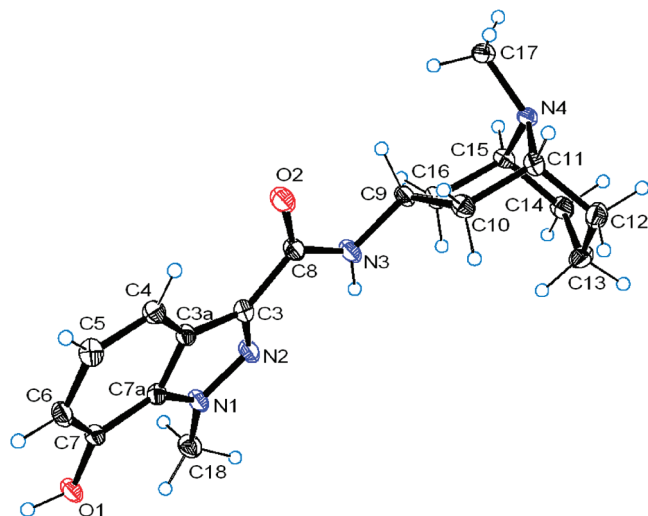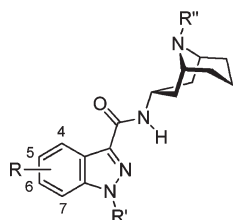
**Figure 2.** Crystal structure of **5a**. Thermal ellipsoids are shown at 50% probability.



**Figure 3.** Labeling of HEK293 cells stably transfected with h5-HT$_3$AR with **16**. Cells were incubated in 10 nM **16** for 10 min with (B) or without (A) 10 $\mu$M quipazine, washed, mounted, and then observed in a confocal microscope.[31] (A) Fluorescence is observed in all cells, with clusters of receptors clearly present in many cells. Unlabeled areas are nuclei. (B) Quipazine (a competitive 5-HT$_3$R ligand) has displaced **16** from its binding sites, leaving only weak autofluorescence. Scale bar represents 20 $\mu$m.

**Table 1.** Binding Affinities of Granisetron Derivatives **2**−**7** for the Human 5-HT$_3$A Receptor[a]



| compd | R | R′ | R″ | $K_i$ (nM) mean ± SEM | n |
|---|---|---|---|---|---|
| **1** | H | Me | Me | 1.45 ± 0.13[b] | 3 |
| **2a** | 4-OH | Me | Me | 0.17 ± 0.09 | 5 |
| **2b** | 4-OMe | Me | Me | 26.3 ± 7.4 | 4 |
| **2c** | 4-OBn | Me | Me | 375 ± 61 | 4 |
| **3a** | 5-OH | Me | Me | 7.3 ± 1.5 | 3 |
| **3b** | 5-OMe | Me | Me | 5306 ± 209 | 4 |
| **3c** | 5-OBn | Me | Me | 3029 ± 956 | 4 |
| **4a** | 6-OH | Me | Me | 279 ± 27 | 4 |
| **4b** | 6-OMe | Me | Me | 237 ± 51 | 3 |
| **4c** | 6-OBn | Me | Me | 749 ± 183 | 4 |
| **5a** | 7-OH | Me | Me | 0.67 ± 0.28 | 3 |
| **5b** | 7-OMe | Me | Me | 71.1 ± 8.0 | 6 |
| **5c** | 7-OBn | Me | Me | 0.23 ± 0.03 | 5 |
| **6** | H | Me | Bn | 59.3 ± 0.8 | 3 |
| **7** | H | (CH$_2$)$_3$NH$_2$ | Me | 1.89 ± 0.33 | 3 |

[a] $K_i$ determined from competition binding with [$^3$H]granisetron using HEK293 cell membranes stably expressing h5-HT$_3$AR. [b] Reference 23.

**Scheme 3.** Synthesis of Fluorescent Probe **16**[a]



[a] Reagents and conditions: (a) $^i$Pr$_2$EtN, BODIPY FL SE, DMF, room temp, 98%.

granatane is in an axial position with respect to the chair in **4b** whereas it is equatorial in **5a**. To the best of our knowledge, this is the first time crystal structures of granisetron derivatives have been reported.

**Biological Data.** The affinities of granisetron derivatives **2**−**7** were assessed by competition with radiolabeled [$^3$H]granisetron, an established 5-HT$_3$AR competitive antagonist. All the compounds, except 5-substituted derivatives **3b** and **3c**, exhibited affinities in the nanomolar range (Table 1).

4-Hydroxy compound **2a** displayed an 8.5-fold increase in affinity for 5-HT$_3$AR relative to **1**, consistent with an H-bond here. Siz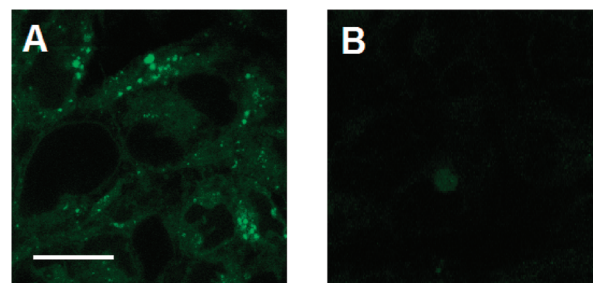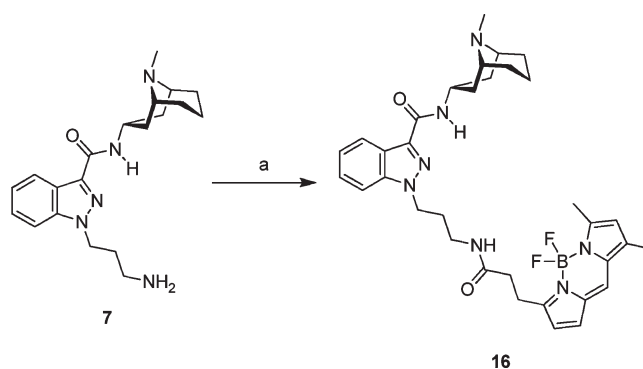e is important at this location; as bulk was increased (**2b** and **2c**), the affinity decreased ∼200-fold. However, at the 5-position size is more critical; substitution with large groups here resulted in a > 1000-fold decrease in affinity (**3a**−**c**). This is consistent with previous studies that showed that the presence of a 5-chloro substituent resulted in a marked reduction in potency relative to **1**.[13] Substitution of the 6-position of the indazole ring was also poorly tolerated, and all the compounds in this series showed > 100-fold decreases in affinities. 7-Hydroxy compound **5a** exhibited a similar affinity for the 5-HT$_3$AR when compared to **1**. The data for substitutions at the 7-position show no clear pattern. Compound **5a**, the major granisetron metabolite in humans,[21] is interestingly as potent as granisetron.[22] Affinity was decreased when the larger methoxy group was present at the 7-position but increased for the larger benzyl group, indicating that the $\pi$-system may interact with hydrophobic residues in the binding site. However, a benzyl group was less well tolerated at the bicycle 9-position: **6** exhibited a $K_i$ of 59 nM. The aminopropyl substituent at the indazole N1-position (**7**) did not significantly alter the binding affinity.

Thus, the data show that the N1- and 7-positions of indazole and the 9-position of the granatane of granisetron are the most tolerant regarding substitution. This is in agreement with established pharmacophore models,[24,25] and modifications of the regions we have identified have previously been well tolerated in structurally similar ligands, in particular the position occupied by groups located at a position similar to that of N1 of granisetron.[26−29] Given the

tolerance of the N1-position to addition of the aminopropyl linker, we consider this location the most appropriate to attach large biophysical tags.

To confirm the validity of this hypothesis, we attached a BODIPY FL fluorophore at this position (Scheme 3) to create fluorescent analogue **16** (BFL-GR). $K_i$ data show that **16** binds with high affinity to 5-HT$_3$AR ($K_i$ = 2.80 ± 0.72 nM, $n$ = 3) and can be visualized in HEK293 cells expressing 5-HT$_3$AR (Figure 3). Similar images have previously been obtained using fluorescein, rhodamine 6G, and cyanine Cy5 dyes attached via a linker at a similar position of ondansetron, another high-affinity 5-HT$_3$AR antagonist.[30]

## Conclusion

In summary, several novel derivatives of granisetron (**1**) have been discovered. Some derivatives have high affinities for the human 5-HT$_3$AR, and most notably derivatives **2a**, **5a**, **5c**, and **7** equal or exceed the affinity measured for **1**. The data of our systematic structure–activity study show that substitution at three positions of granisetron are well tolerated and that large functional biophysical tags can be attached at the N1-position.

## Experimental Section

**General.** Chemicals and solvents were either purchased from commercial suppliers or purified by standard techniques. All experiments involving air-sensitive reagents were performed under an inert atmosphere in oven-dried glassware. Thin-layer chromatography (TLC) was carried out on Merck silica gel 60 F254 plates, and compounds were visualized by irradiating with UV light, by exposing to I$_2$ vapors, and/or by staining with cerium molybdate stain (Hanessian's stain) followed by heating. Flash chromatography was carried out using Matrex silica gel 60 unless otherwise stated. Infrared spectra were recorded neat on a Nicolet AVATAR 320 FT-IR spectrometer. $^1$H and $^{13}$C NMR spectra were recorded on a Bruker DPX-300, DPX-400, and DRX-500. The chemical shifts are reported in $\delta$ (ppm), and the residual signal of the solvent was used as the internal standard. High resolution mass spectra were obtained using electrospray ionization mass (MS-ESI) technique on a Bruker MicroTOF instrument. Purity was determined by elemental analysis and/or HPLC; purity of key target compounds was ≥95%.

**1-(3-Aminopropyl)-*N*-[(3-*endo*)-9-methyl-9-azabicyclo-[3.3.1]-non-3-yl]-1*H*-indazole-3-carboxamide Dihydrochloride (7·2HCl).** Compound **14** (0.3 g, 1.0 mmol) was dissolved in anhydrous DMF/THF (1:5, 10 mL), cooled to 0 °C, and stirred for 5 min. Then a solution of KO$^t$Bu (0.135 g, 1.1 mmol) in anhydrous THF (2 mL) was added dropwise at 0 °C and stirred for 15 min, followed by the addition of a solution of *tert*-butyl *N*-(3-bromo-propyl)carbamate (0.27 g, 1.2 mmol) in anhydrous THF (2 mL). The mixture was warmed to room temperature and stirred for 10 h. The progress of the reaction was monitored by TLC. The solvents were removed under vacuo, and the residue was extracted with EtOAc (3 × 20 mL). The combined organic phases were dried over Na$_2$SO$_4$, filtered, and concentrated to give the crude product. The crude product was further purified by crystallization (CH$_2$Cl$_2$/Et$_2$O) to afford the carbamate (0.36 g, 0.79 mmol, 78%) as a white solid: mp 156–158 °C; $R_f$ = 0.23 (CH$_2$Cl$_2$/MeOH, 7:3); $^1$H NMR (CD$_3$OD, 400 MHz) $\delta$ 1.14–1.17 (m, 3H), 1.45 (s, 9H), 1.54–1.65 (m, 3H), 2.02–2.21 (m, 4H), 2.44–2.52 (m, 2H), 2.55 (s, 3H), 3.11–3.14 (m, 4H), 4.54 (t, $J$ = 7.7 Hz, 2H), 4.60 (tt, $J$ = 6.6 Hz, $J$ = 11.5 Hz, 1H), 7.29 (t, $J$ = 7.5 Hz, 1H), 7.47 (t, $J$ = 7.7 Hz, 1H), 7.64 (d, $J$ = 8.5 Hz, 1H), 8.25 (d, $J$ = 8.2 Hz, 1H); $^{13}$C NMR (CD$_3$OD, 100 MHz) $\delta$ 15.1, 25.7, 28.8, 33.3, 34.8, 38.8, 40.7, 41.6, 47.7, 52.8, 110.9, 123.2, 123.6, 124.0, 128.0, 137.5, 142.5, 166.8; IR

(neat) 3352, 3349, 2928, 1684, 1641, 1533, 1245, 1172, 1027, 748 cm$^{-1}$; HRMS-ESI(+) *m/z* calcd for C$_{25}$H$_{38}$N$_5$O$_3$ 456.2975 [M + H]$^+$, found 456.2990 [M + H]$^+$. Anal. Calcd for C$_{25}$H$_{37}$-N$_5$O$_3$: C 65.91%, H 8.19%, N 15.37%. Found C 65.52%, H 8.27%, N 15.04%.

To a solution of carbamate (0.16 g, 0.35 mmol) in MeOH (8 mL) was added dropwise a solution of 1.2 M HCl in MeOH (10 mL) and stirred for 12 h at room temperature. The progress of the reaction was monitored by TLC, and the solvents were removed in vacuo and 3 times coevaporated with toluene to get the crude product. The crude product was further purified by crystallization (CH$_2$Cl$_2$/Et$_2$O) to afford **7**·2HCl (0.15 g, 0.34 mmol, 98%) as a white solid: mp 296–298 °C; $^1$H NMR (CD$_3$OD, 400 MHz) $\delta$ 1.58–1.71 (m, 3H), 1.90–2.10 (m, 3H), 2.21–2.39 (m, 4H), 2.55–2.71 (m, 2H), 2.96–3.01 (m, 5H), 3.74 (d, $J$ = 10.2 Hz, 2H), 4.61–4.70 (m, 3H), 7.30 (t, $J$ = 7.5 Hz, 1H), 7.49 (t, $J$ = 7.7 Hz, 1H), 7.70 (d, $J$ = 8.5 Hz, 1H), 8.22 (d, $J$ = 8.2 Hz, 1H); $^{13}$C NMR (CD$_3$OD, 100 MHz) $\delta$ 15.2, 26.7, 31.1, 34.6, 40.9, 41.0, 41.9, 49.7, 57.8, 113.4, 125.6, 126.6, 127.1, 131.0, 137.5, 144.9, 166.8; IR (neat) 2884, 2749, 2676, 1630, 1553, 1208, 745 cm$^{-1}$; HRMS-ESI(+) *m/z* calcd for C$_{20}$H$_{30}$N$_5$O 356.2450 [M − 2HCl + H]$^+$, found 356.2445 [M − 2HCl + H]$^+$. Anal. Calcd for C$_{20}$H$_{31}$Cl$_2$N$_5$O: C 56.07%, H 7.29%, N 16.35%, Cl 16.55%. Found C 55.21%, H 7.35%, N 16.00%, Cl 16.31%.

**BFL-GR (16).** To a solution of amine hydrochloride **7**·2HCl (9 mg, 0.021 mmol) in anhydrous DMF (0.5 mL) was added $^i$Pr$_2$EtN (5.4 mg, 0.042 mmol) and stirred for 10 min. Then a solution of BODIPY FL SE (5 mg, 0.012 mmol) in DMF (1 mL) was added to the mixture and stirred at room temperature for 3.5 h. The progress of the reaction was monitored by TLC. The DMF was removed in vacuo and the crude product was purified by flash column chromatography (CH$_2$Cl$_2$ and then CH$_2$Cl$_2$/MeOH/Et$_3$N, 96:3:1) to afford **16** (7.9 mg, 0.012 mmol, 98%) as an orange solid: mp 190–192 °C (dec); $R_f$ = 0.42 (CH$_2$Cl$_2$/MeOH, 7:3); $^1$H NMR (CD$_3$OD, 400 MHz) $\delta$ 1.07–1.18 (m, 3H), 1.40–1.59 (m, 4H), 1.88–2.05 (m, 6H), 2.15 (s, 3H), 2.32–2.40 (m, 4H), 2.48–2.52 (m, 5H), 3.08–3.16 (m, 4H), 4.37 (t, $J$ = 6.8 Hz, 2H), 4.47 (tt, $J$ = 6.7 Hz, $J$ = 11.8 Hz, 1H), 6.10 (s, 1H), 6.23 (d, $J$ = 4.0 Hz, 1H), 6.86 (d, $J$ = 4.0 Hz, 1H), 7.17 (t, $J$ = 7.7 Hz, 1H), 7.26 (s, 1H), 7.33 (t, $J$ = 7.4 Hz, 1H), 7.47 (d, $J$ = 8.5 Hz, 1H), 8.10 (d, $J$ = 8.2 Hz, 1H); $^{13}$C NMR (CD$_3$OD, 100 MHz) $\delta$ 11.2, 14.6, 15.3, 25.6, 33.0, 36.2, 37.8, 40.4, 41.1, 47.6, 53.2, 110.9, 117.7, 123.0, 123.8, 125.8, 128.1, 129.6, 138.3, 139.6, 140.5, 145.2, 149.3, 164.5, 174.8; IR (neat) 2921, 1605, 1488, 1245, 1132, 1056, 974, 734 cm$^{-1}$; HRMS-ESI(+) *m/z* calcd for C$_{34}$H$_{43}$BF$_2$N$_7$O$_2$ 630.3539 [M + H]$^+$, found 630.3526 [M + H]$^+$; UV/vis/Fluo (MeOH) $\lambda_{max\ abs}$ = 506 nm, $\lambda_{max\ emiss}$ = 514 nm.

**Supporting Information Available:** Synthesis details and spectral data for compounds other than **7** and **16**, HPLC purity assessment for all target compounds, CIF files of **4b** and **5a**, experimental details for competition binding, and $K_i$ determination. This material is available free of charge via the Internet at http://pubs.acs.org.

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