Available online at www.sciencedirect.com
SCIENCE DIRECT
Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 16 (2006) 3886–3890

Synthesis and pharmacological characterization of novel fluorescent histamine H_2-receptor ligands derived from aminopotentidine

Sheng-Xue Xie,[a] Georgiana Petrache,[b] Erich Schneider,[b] Qi-Zhuang Ye,[a] Günther Bernhardt,[b] Roland Seifert[b,c] and Armin Buschauer[b,*]

[a]*High Throughput Screening Laboratory, The University of Kansas, Lawrence, KS 66045, USA*
[b]*Institute of Pharmacy, University of Regensburg, D-93040 Regensburg, Germany*
[c]*Department of Pharmacology and Toxicology, The University of Kansas, Lawrence, KS 66045, USA*

Received 24 April 2006; revised 11 May 2006; accepted 13 May 2006
Available online 30 May 2006

Abstract—In an effort to develop a non-radioactive alternative to the [^{3}H]tiotidine and [^{125}I]iodoaminopotentidine binding assays for the histamine H_2-receptor (H_2R), primary amines related to aminopotentidine were prepared and coupled with the succinimidyl esters of the bulky fluorescent dyes S0536 and BODIPY® 650/665-X. The primary amines exhibited different degrees of antagonistic potency at the human and guinea pig H_2R. Surprisingly, one compound (**5**) coupled to the cyanine dye S0536 acted as potent partial agonist/antagonist at the H_2R ($K_B \sim 50$ nM; $EC_{50} \sim 100$–150 nM). Compounds coupled to the BODIPY dye exhibited moderately high H_2R-affinity, too. Thus, the H_2R accommodates bulky fluorophores, probably through interaction with extracellular receptor domains. The compounds presented herein provide a starting point for the optimization of fluorescent H_2R ligands with respect to affinity and fluorescence as valuable tools to analyze the molecular mechanisms of H_2R activation.

The histamine H_2-receptor (H_2R) couples to G_s-proteins and mediates activation of adenylyl cyclase.[1] Functionally, the H_2R can be analyzed by measuring the high-affinity GTPase activity of fusion proteins of the H_2R with $G_{s\alpha}$, adenylyl cyclase activity or positive chronotropy in the spontaneously beating guinea pig right atrium.[2–4] All these assays yield robust signals and have provided the basis for the development of both potent H_2R agonists and antagonists. Unfortunately, in terms of direct analysis of the ligand-binding properties of the H_2R, the situation is more problematic. The tritiated radioligand [^{3}H]tiotidine suffers from a relatively low affinity ($K_d \sim 35$ nM) and labels only a subpopulation of the available H_2R molecules.[2] [^{125}I]aminopotentidine exhibits high affinity for the H_2R (K_d 0.3 nM),[5] but its high costs and the relatively short $t_{1/2}$ of ^{125}I (60 days) substantially limit its general use in H_2R ligand development.

In an effort to overcome these technical difficulties in ligand-binding analysis of the H_2R, we have initiated a program directed toward the development of fluorescent H_2R ligands. To this end, we have prepared aminopotentidine-derived compounds substituted with fluorescein, acridine, dansyl, nitrobenzoxadiazole or indolo[2,3-*a*]quinolizine at the primary amino group.[6] These compounds are H_2R antagonists with pA_2 values in the range of 7.5–8.0, that is, comparable to the affinity of tiotidine.[2,6] Meanwhile, according to the same strategy Malan et al.[7] prepared aminopotentidine-like H_2R ligands labeled with some of the aforementioned and several other fluorophores such as *N*-methylanthranilamide, 1-cyanoisoindole and 1-cyanoindolizine-2-carboxamide. Based on the experience with [^{3}H]tiotidine,[2] the affinity of most of the fluorescent H_2R ligands is not high enough for spectroscopic applications. In addition, the most important lesson from investigations with such fluorescent probes is that the optical properties of the previously prepared compounds are not optimal for H_2R analysis in intact cells and tissues.[6] Therefore, we prepared a novel series of ligands with bulky fluorescent dyes emitting light at wavelengths >650 nm in order to improve their signal-to-noise ratio.

Primary amines **2a**,**b** related to the H_2R antagonist aminopotentidine[5] were prepared from **1**[8] according to

Keywords: Fluorescent probes; Cyanine; BODIPY; Histamine H_2-receptor agonist; GTPase assay.
* Corresponding author. Tel.: +49 941 943 4827; fax: +49 941 943 4820; e-mail: armin.buschauer@chemie.uni-regensburg.de

0960-894X/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2006.05.039

a known synthetic route[6,9] (Scheme 1) and coupled to the succinimidyl esters **3** and **4** of the fluorescent dyes S0536 and BODIPY® 650/665-X according to standard procedures (Scheme 2), yielding the fluorescent H_2R ligands **5**, **6** and **7**.[10] The fluorescent products were isolated and purified on a C-18 reversed-phase high-performance liquid chromatography column (Fig. 1).

The primary amines **1**, **2a**, and **2b** derived from aminopotentidine and the fluorescent compounds **5**, **6**, and **7** were compared to the reference compound aminopotentidine in the H_2R antagonist mode measuring inhibition of histamine-stimulated high-affinity GTP hydrolysis in Sf9 insect cell membranes expressing fusion proteins of the human H_2R (hH_2R) or guinea pig H_2R (gpH_2R) and $G_{s\alpha}$ according to the previously described procedure.[2] The antagonist data are summarized in Table 1. We also examined the effects of the novel compounds in the absence of histamine in order to detect possible partial agonistic and inverse agonistic activity.[2] Those

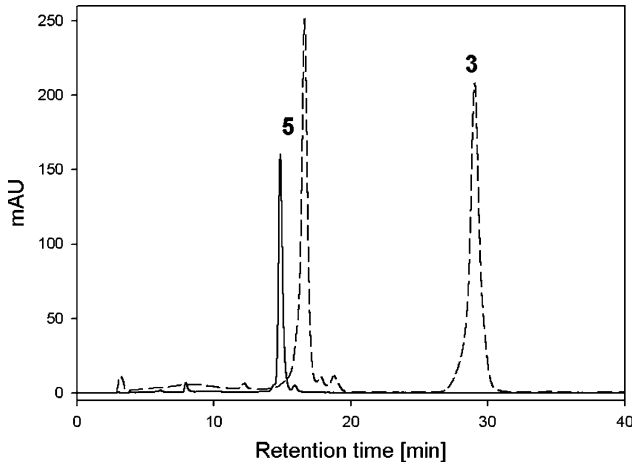
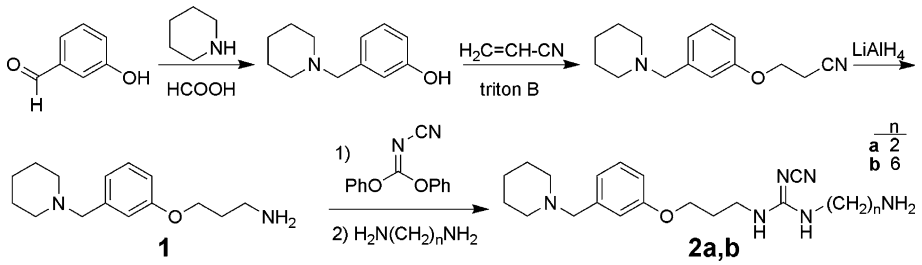
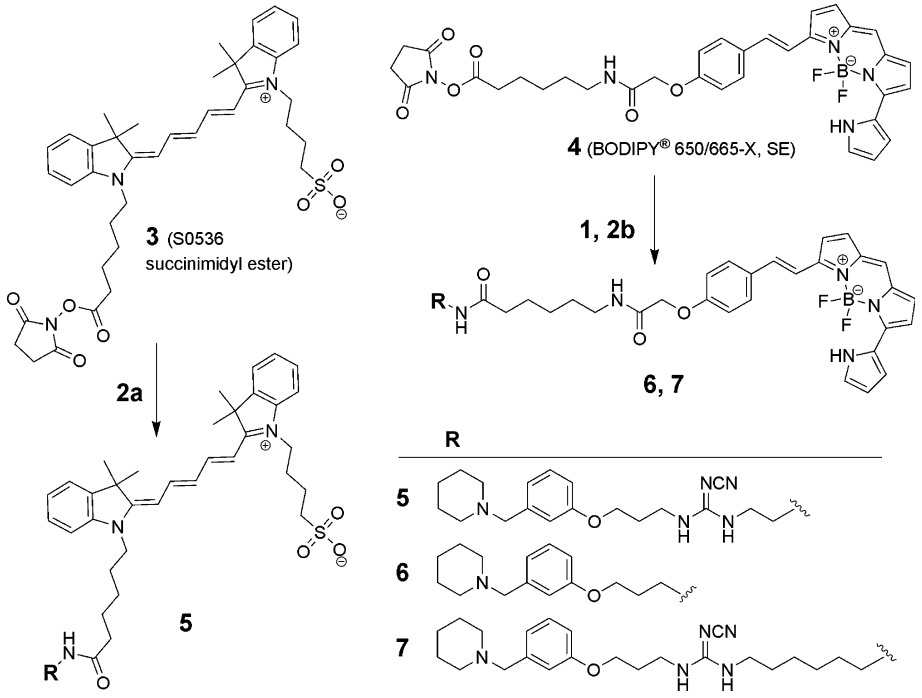


Figure 1. HPLC analysis of **5**. Dashed line, reaction mixture (injected as 1:10 dilution with mobile phase) after addition of the second portion of **2a**. Solid line, chromatogram of the purified compound **5** after vacuum concentration and dissolution in MeCN. Detection at 640 nm.



Scheme 1. Synthesis of the aminopotentidine-derived primary amines **2a** and **2b**.



Scheme 2. Fluorescence labeling of the primary amines **1**, **2a**, and **2b** with the succinimidyl ester of S0536 (**3**), yielding **5**, and with the succinimidyl ester of BODIPY® 650/665-X (**4**), yielding **6** and **7**.

Table 1. Antagonist potencies of aminopotentidine, **1**, **2a**, **2b**, **5**, **6** and **7** at hH_2R and gpH_2R

Compound	K_B hH_2R (nM)	K_B gpH_2R (nM)	K_B hH_2R/K_B gpH_2R
Aminopotentidine	220 ± 30	280 ± 40	0.79
1	15,000 ± 3500	6800 ± 1700	2.20
2a	9300 ± 2400	2300 ± 1200	4.04
2b	1800 ± 920	75 ± 18	24.0
5	47 ± 12	58 ± 10	0.81
6	640 ± 210	150 ± 95	4.27
7	260 ± 80	190 ± 70	1.37

Antagonist potencies were determined in the GTPase assay. Reaction mixtures contained 100 nM unlabeled GTP, 0.1 μCi of [γ-^{32}P]GTP, 10 μg of Sf9 insect cell membrane protein expressing hH_2R-$G_{s\alpha}$ or gpH_2R-$G_{s\alpha}$, 1 μM histamine, and antagonists at concentrations between 1 nM and 100 μM to obtain saturated inhibition curves. Data shown are means ± SD of three experiments. Non-linear curve fitting was performed using the Prism 4.0 program. K_B values were calculated as described.[2]

data are summarized in Figure 2. In order to confirm the data obtained with fusion proteins, we also performed some complementary adenylyl cyclase experiments with Sf9 membranes expressing non-fused hH_2R and gpH_2R.[11]

Expectedly, the piperidinomethylphenoxypropylamine building block **1** was a far less potent H_2R antagonist than the cyanoguanidine aminopotentidine (Table 1) since the cyanoguanidino group is important for receptor interaction.[2] Connection of the piperidinomethylphenoxypropylamine building block with a cyanoguanidino moiety linked to the reactant primary amine via an ethylene group (**2a**) moderately increased antagonist affinity. A hexamethylene linker between the cyanoguanidino moiety and the primary amine (**2b**) increased affinity much more substantially, particularly for the gpH_2R. In fact, the affinity of **2b** for gpH_2R-$G_{s\alpha}$ is 24-fold higher than for hH_2R-$G_{s\alpha}$. Adenylyl cyclase experiments with Sf9 membranes expressing non-fused hH_2R and gpH_2R confirmed the large affinity difference of **2b** for the two receptor isoforms (K_B of 2.5 μM vs 33 nM). These are the largest affinity differences for a ligand between hH_2R and gpH_2R described so far.[2,11] Compound **2b** possesses a longer linker between the cyanoguanidino group and the primary amine than **2a**, and previous studies already showed that gpH_2R exhibits higher conformational flexibility than hH_2R.[2] Thus, **2b** is a particularly useful probe to dissect the species-differences between hH_2R and gpH_2R.

It was taken into account that introduction of bulky fluorophores might result in a decrease in affinity of the labeled compounds for the H_2R since similar observations had been made for other biogenic amine receptors.[12] This presumption is also compatible with the suggestion that the ligand binding pocket in biogenic amine receptors is more constrained than in peptide receptors, allowing only for small structural changes without compromising affinity. However, to our surprise, the opposite was the case. Specifically, **5** was a 200-fold more potent antagonist at hH_2R than **2a**, and for gpH_2R the affinity difference was 40-fold. The linkage of **1** and **2b** with the BODIPY dye **4**, yielding **6** and **7**, resulted in antagonists that showed higher affinity for hH_2R than the corresponding building blocks **1** and **2b**. For gpH_2R, this difference was also observed for **1** and **6**. These data show that biogenic amine receptors can not only tolerate bulky fluorescent ligands as was shown already for some cases[6,13] but that bulky fluorescent groups can even substantially increase ligand-affinity.

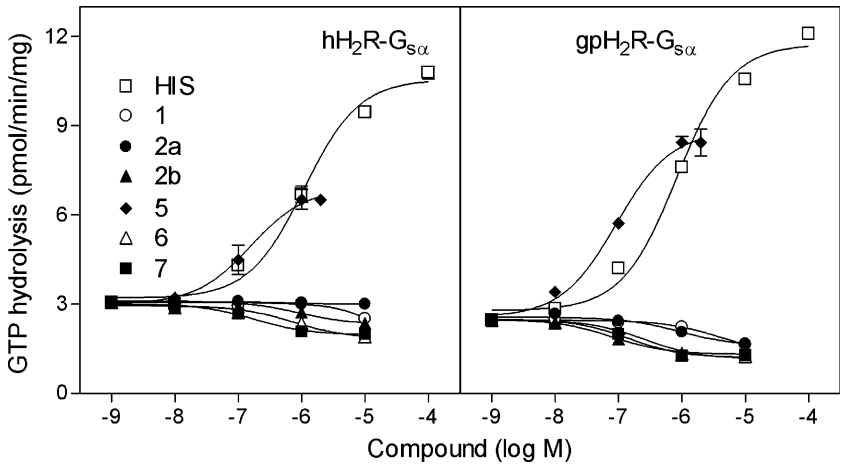


Figure 2. Effects of histamine, **1**, **2a**, **2b**, **5**, **6** and **7** on GTPase activity in Sf9 insect cell membranes expressing hH_2R-$G_{s\alpha S}$ or gpH_2R-$G_{s\alpha S}$. Reaction mixtures contained 100 nM unlabeled GTP, 0.1 μCi [γ-^{32}P]GTP, 10 μg of membrane protein expressing the fusion proteins, and ligands at the concentrations indicated on the abscissa. Data shown are means ± SD of three experiments. Non-linear curve fitting was performed using the Prism 4.0 program.

The results for compounds **1**, **2a**, **2b**, **5**, **6**, and **7** in the GTPase assay in the absence of histamine were striking, too (Fig. 2). Histamine was a full agonist at hH_2R-$G_{s\alpha}$ and gpH_2R-$G_{s\alpha}$ and served as a reference compound. All compounds except for **5** and **2a** at hH_2R-$G_{s\alpha}$ slightly reduced basal GTPase activity, indicating that the H_2R exhibits a certain degree of constitutive activity and that the compounds act as inverse agonists.[2] In marked contrast, **5** acted as partial agonist both at hH_2R and gpH_2R. At the hH_2R the EC_{50} of **5** was 160 nM, and the efficacy was 58% of that of histamine. The EC_{50} of **5** at gpH_2R was 90 nM, and the efficacy was 65% of that of histamine. The partial agonist effects of **5** on hH_2R and gpH_2R were clearly H_2R-mediated since the H_2R antagonists tiotidine, cimetidine, ranitidine, famotidine, and zolantidine inhibited the effects of **5** on GTP hydrolysis with very similar K_B values as for histamine (data not shown).[2] We also conducted adenylyl cyclase assays with Sf9 membranes expressing non-fused H_2R from human and guinea pig[11] and confirmed the partial agonistic activity of **5** (data not shown). Finally, we examined the potential antagonistic effects of the compounds at the human and guinea pig H_1R measuring inhibition of histamine-stimulated GTP hydrolysis in Sf9 membranes expressing recombinant H_1-receptors.[11] Compounds **1**, **2a**, **2b**, **5**, **6**, and **7** exhibited only very low affinity for the H_1R ($K_B > 30$ μM, data not shown).

It is unlikely that transmembrane domains are involved in conferring the relatively high affinity of the hH_2R and the gpH_2R for **5** for two reasons. First, the fluorescent group in compound **5** is too bulky to be inserted into the ligand binding pocket of the H_2R.[2,11] Second, cyanine dye moiety is charged, that is, it bears a negatively charged sulfate group and a resonance-stabilized cation (Scheme 2). Accordingly, it is reasonable to assume that the fluorophore of **5** interacts with extracellular domains of the H_2R, that is, the N-terminus and/or the extracellular loops, both of which contain positively and negatively charged amino acids.[2] It is conceivable that ionic interactions between the fluorescent group and extracellular portions of the receptor protein increase the affinity of the H_2R for fluorescent ligands. In addition, we assume that conformational changes in extracellular portions of the H_2R can result in at least partial activation. So far, conformational changes related to biogenic amine receptor activation were assumed to be restricted to the transmembrane domains.[14] Thus, fluorescent ligands, particularly **5**, may serve as probes to examine the molecular mechanisms underlying biogenic amine receptor activation. For actual fluorescence analysis of the H_2R, the affinity of **5** is still too low and should be increased by at least one order of magnitude.

It should be noted that the identification of **5** as a relatively high-affinity partial H_2R agonist was fortuitous. Our present data show that the H_2R also tolerates BODIPY dyes linked to cyanoguanidines (**6** and **7**) quite well. Accordingly, future studies will have to systematically combine various cyanoguanidines with various fluorophores emitting at wavelengths > 650 nm. We assume that more potent fluorescent H_2R ligands than **5** can be developed. Moreover, given the high degree of conservation between biogenic amine receptors,[14] the results of the present study suggest that it will ultimately also become possible to develop high-affinity fluorescent ligands for β-adrenergic receptors and muscarinic cholinoceptors.

Acknowledgments

We thank Dr. G. Georg (Department of Medicinal Chemistry, University of Kansas, KS) for continuous support and encouragement, and Ms. S. Bollwein for technical assistance.We are also grateful to the Deutscher Akademischer Austauschdienst (DAAD) for a predoctoral fellowship to G.P. This work was supported by the National Institutes of Health Center of Biomedical Research Excellence (COBRE) award 1 P20 RR15563 and matching support from the State of Kansas and the University of Kansas (R.S. and Q.-Z.Y.) and the Graduate Training Program (Graduiertenkolleg) GRK 760 'Medicinal Chemistry: Molecular Recognition—Ligand–Receptor Interactions' of the Deutsche Forschungsgemeinschaft.

References and notes

1. Hill, S. J.; Ganellin, C. R.; Timmerman, H.; Schwartz, J. C.; Shankley, N. P.; Young, J. M.; Schunack, W.; Levi, R.; Haas, H. L. *Pharmacol. Rev.* **1997**, *49*, 253.
2. Kelley, M. T.; Bürckstümmer, T.; Wenzel-Seifert, K.; Dove, S.; Buschauer, A.; Seifert, R. *Mol. Pharmacol.* **2001**, *60*, 1210.
3. Houston, C.; Wenzel-Seifert, K.; Bürckstümmer, T.; Seifert, R. *J. Neurochem.* **2002**, *80*, 678.
4. Black, J. W.; Duncan, W. A. M.; Durant, G. J.; Ganellin, C. R.; Parsons, M. E. *Nature* **1972**, *236*, 385.
5. Ruat, M.; Traiffort, E.; Bouthenet, M. L.; Schwartz, J. C.; Hirschfeld, J.; Buschauer, A.; Schunack, W. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 1658.
6. Li, L.; Kracht, J.; Peng, S.; Bernhardt, G.; Elz, S.; Buschauer, A. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 1717.
7. Malan, S. F.; van Marle, A.; Menge, W. M.; Zuliana, V.; Hoffman, M.; Timmerman, H.; Leurs, R. *Bioorg. Med. Chem.* **2004**, *12*, 6495.
8. Buschauer, A.; Postius, S.; Szelenyi, I.; Schunack, W. *Arzneim.-Forsch.* **1985**, *35*, 1025.
9. Hirschfeld, J.; Buschauer, A.; Elz, S.; Schunack, W.; Ruat, M.; Traiffort, E.; Schwartz, J.-C. *J. Med. Chem.* **1992**, *35*, 2231.
10. (a) Preparation of 4-[2-[5-[1-[6-[2-[2-cyano-3-[3-[3-(piperidin-1-ylmethyl)phenoxy]-propyl]guanidino]ethylamino]-6-oxohexyl]-3,3-dimethylindolin-2-ylidene]penta-1,3-dienyl]-3,3-dimethyl-3*H*-indolium-1-yl]butane-1-sulfonate (**5**). To a mixture of 20 μL of anhydrous pyridine and 200 μL of a 1 mM solution of 4-[2-[5-[1-[6-(2,5-dioxopyrrolidin-1-yloxy)-6-oxohexyl]-3,3-dimethylindolin-2-ylidene]penta-1,3-dienyl]-3,3-dimethyl-3*H*-indolium-1-yl]butane-1-sulfonate (**3**) (FEW Chemicals) in MeCN three portions of 5 μL of a 10 mM solution of **2a** were added under argon atmosphere within 2 h. After 17 h, the product was isolated by RP-HPLC: Thermo Separation Products (ThermoQuest), SN4000 controller, P4000 pump, AS3000 autosampler, UV–vis detector Spectra Focus, fluorescence detector FL3000, SCM400 solvent degasser; column: Luna 3 μm C18(2), (150 mm × 4 mm) equipped with a guard column

(Phenomenex); mobile phase: MeOH/aqueous TFA (0.1%), 62/38; flow rate: 0.5 mL/min; column temperature: 40 °C; injection: 20 μL push loop; detection of **5** (**2a**): absorbance 640 nm, fluorescence ex = 640 (275) nm; em = 664 (305) nm. For pharmacological investigations, the pooled fractions of compound **5** were aliquoted in portions of 1 nmol after photometric determination of the concentration (ε_{646} (in MeOH) = 127,000 M^{-1} cm^{-1}). After removal of the solvent in a vacuum concentrator at room temperature, the purity of **5** was confirmed by RP-HPLC under the aforementioned conditions (k' value = 2.70). Yield: 29%. ESI-MS (Finnigan Thermo Separations Q 7000) of compound **5** ($C_{54}H_{72}N_8O_5S$): m/z = 945 (MH^+, 100%). (b) Preparation of 5,5-difluoro-3-[4-[2-oxo-2-[6-oxo-6-[3-[3-(piperidin-1-ylmethyl)phenoxy]propylamino]hexylamino]-ethoxy]styryl]-7-(1*H*-pyrrol-2-yl)-5*H*-dipyrrolo[1,2-*c*:1′,2′-*f*][1,3,2]diazaborinin-4-ium-5-uide (**6**) and 3-[4-[18-(cyanoimino)-2,9-dioxo-22-[3-(piperidin-1-ylmethyl)phenoxy]-3,10,17,19-tetraazadocosyloxy]styryl]-5,5-difluoro-7-(1*H*-pyrrol-2-yl)-5*H*-dipyrrolo[1,2-*c*:1′,2′-*f*][1,3,2]diazaborinin-4-ium-5-uide (**7**). A solution of 2 μmol of the pertinent amine (**1** or **2b**) in 200 μL of a 1:1 mixture of pyridine and anhydrous DMF was added to 1.04 μmol (0.67 mg) of 6-[[[4,4-difluoro-5-(2-pyrrolyl)-4-bora-3a,4a-diaza-*s*-indacene-3-yl]styryloxy]acetyl]aminohexanoic acid succinimidyl ester (**4**, BODIPY® 650/665-X SE) (Invitrogen) and stirred for 2 day under argon atmosphere. The solvents were removed in a vacuum concentrator, the residue was dissolved in MeCN and purified by RP-HPLC: Kontron Instruments: HPLC pump 420, UV–vis detector: HPLC detector 430, fluorescence detector: Merck–Hitachi F1000; column (250 × 4 mm, 5 μm) and precolumn: Nucleosil 300-5 C18 (Macherey & Nagel); eluent: MeCN/0.1% TFA (**6**: 50/50; **7**: 60/40), flow rate: 1.0 mL/min; detection: 649 nm, fluorescence detection: λ_{ex}:275 nm, λ_{em}: 305 nm. k' values: **6**, 4.36; **7**, 1.40. Compound **6** ($C_{44}H_{51}BF_2N_6O_4$), yield 33%, EI-MS: m/z = 777 (MH^+, 100%); **7** ($C_{52}H_{65}BF_2N_{10}O_4$), yield 27%, EI-MS: m/z = 943 (MH^+, 100%). For pharmacological investigations, the pooled fractions of **6** and **7**, respectively, were aliquoted in portions of 1 nmol after photometric determination of the concentration (ε_{646} (in MeOH) = 106,600 M^{-1} cm^{-1}). (c) The fluorescence properties of compounds **5**–**7** in phosphate-buffered saline, pH 7.4 (Dulbecco's solution B), containing 5% (vol/vol) DMSO (corresponding to the conditions of the pharmacological assay) were analyzed with an Aminco Bowman AB2 spectrofluorometer at 20 °C. Both emission and excitation slits were set to 4 nm. λ_{max} (nm): **5**, ex 648, em 666; **6** and **7**, ex 651, em 670.

11. Xie, S. X.; Ghorai, P.; Ye, Q. Z.; Buschauer, A.; Seifert, R. *J. Pharmacol. Exp. Ther.* **2006**, *317*, 139.
12. Middleton, R. J.; Kellam, B. *Curr. Opin. Chem. Biol.* **2005**, *9*, 517.
13. Li, L.; Kracht, J.; Peng, S.; Bernhardt, G.; Buschauer, A. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 1245.
14. Gether, U. *Endocr. Rev.* **2000**, *21*, 90.