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Ondansetron and Granisetron Binding Orientation in the 5-HT$_3$ Receptor Determined by Unnatural Amino Acid Mutagenesis

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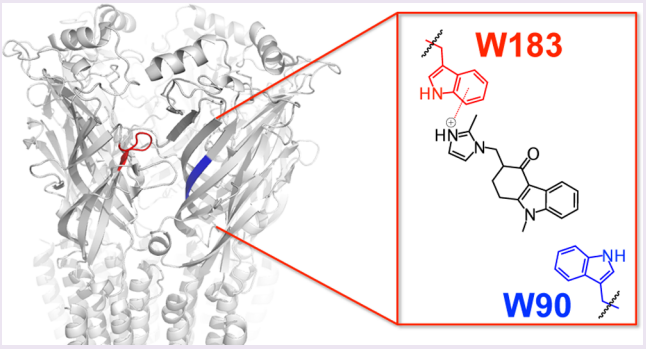
ABSTRACT: The serotonin type 3 receptor (5-HT$_3$R) is a ligand-gated ion channel found in the central and peripheral nervous systems. The 5-HT$_3$R is a therapeutic target, and the clinically available drugs ondansetron and granisetron inhibit receptor activity. Their inhibitory action is through competitive binding to the native ligand binding site, although the binding orientation of the drugs at the receptor has been a matter of debate. Here we heterologously express mouse 5-HT$_3$A receptors in *Xenopus* oocytes and use unnatural amino acid mutagenesis to establish a cation-π interaction for both ondansetron and granisetron to tryptophan 183 in the ligand binding pocket. This cation-π interaction establishes a binding orientation for both ondansetron and granisetron within the binding pocket.



The serotonin type 3 receptor (5-HT$_3$R)[1,2] is a ligand-gated ion channel in the Cys-loop (pentameric) family of receptors, which also includes GABA$_A$, glycine, and nicotinic acetylcholine (nACh) receptors.[3] The 5-HT$_3$ receptor is a cation selective channel found in the central and peripheral nervous systems. Conduction occurs through a central pore, formed by the pseudosymmetric assembly of five subunits (Figure 1). There are five known 5-HT$_3$R subunits (A–E),[4] with the best characterized receptors being the homomeric 5-HT$_3$A receptor (5-HT$_3$AR) and the heteromeric 5-HT$_3$AB receptors.[5]

The 5-HT$_3$ receptor has been validated as a therapeutic target; antagonists are currently used to control chemotherapy-induced nausea, as well as to treat irritable bowel syndrome.[6,7] Beyond current clinical uses, there is evidence that compounds targeting the 5-HT$_3$ receptor could be useful for the treatment of a variety of disorders including schizophrenia and substance abuse, as well as management of pain associated with, for example, fibromyalgia. Prototype antagonists of the 5-HT$_3$ receptor are ondansetron (Zofran) and granisetron (Kytril) (Figure 2a).

There is no high-resolution structure of the 5-HT$_3$ receptor. There is, however, structural information from a number of sources, including cryoelectron microscopy images of the nACh receptor,[8] high resolution structures of the homologous acetylcholine binding protein,[9] and the recently published X-ray structure of the glutamate-gated chloride channel from *C. elegans*, GluCl.[10] These data, along with homology modeling and biochemical studies, have provided a putative binding site for 5-HT$_3$ antagonists that coincides with the binding site of the native agonist, serotonin (5-HT, Figure 2b). This binding site is formed by a series of β-strands and connecting loops (labeled A–F), with loops A–C contributed by the "principal" and β-strands D–F contributed by the "complementary" subunit (Figure 1). Docking studies using homology models have found multiple energetically favorable poses of the antagonist granisetron within this binding site, and the orientation of the drug and the identities of the interacting residues are not constant across poses.[11−14] For example, Thompson *et al.* reported two classes of poses of granisetron.[13] In one class, the cationic ammonium was oriented between Trp183 (loop B) and Tyr234 (loop C), while the aromatic indazole was oriented between Trp90 (β-strand D) and Phe226 (loop C). In the second class of poses, this orientation was reversed, with the cationic ammonium toward Trp90 and Phe226. Experimentally, both Trp183 and Trp90 have been established to be important for granisetron binding. In other experimental work, Yan and White found that Trp90 is important for both ondansetron and granisetron binding, and they interpreted their results as providing evidence that the cationic ammonium of granisetron was oriented toward Trp90.[14]

The accepted pharmacophore model for 5-HT$_3$R includes an amine group on the ligand. Past studies in our laboratory have established the primary amine of 5-HT to make a cation-π interaction with a conserved tryptophan residue on loop B (Trp183).[15] Mutation of Trp183 (as well as Trp90, Glu129 (loop A), and Tyr234) to alanine abolishes binding of [^{3}H]granisetron.[13] Taken together, these results identify Trp183 as a prime candidate for more detailed studies. Moreover, the docking studies of Thompson *et al.* provide us

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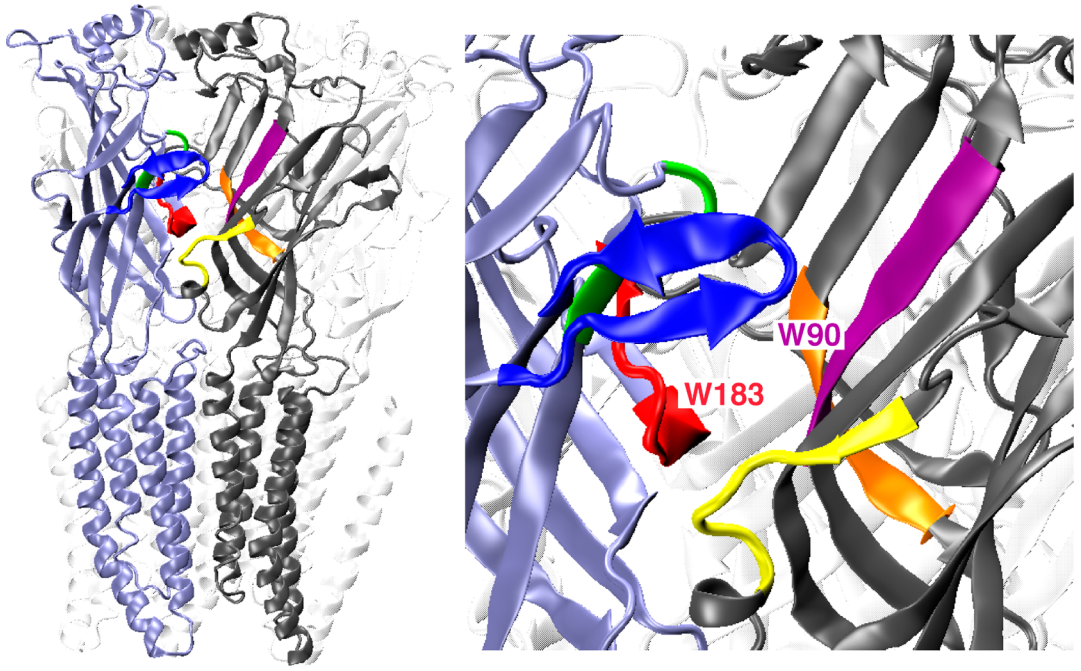

Figure 1. Basic layout of a Cys-loop (pentameric) receptor. The structure is that of the GluCl α subunit[10] (PDB: 3RIF). Left: The full receptor, with two subunits highlighted. One (blue) contributes principal agonist binding site residues, found on loops A (red), B (green), and C (blue). The complementary subunit (black) contributes loops D (purple), E (orange), and F (yellow). The α-helical region corresponds to the transmembrane domain; the region above it is extracellular. Right: Detail of agonist binding site, noting approximate locations of key residues considered here.

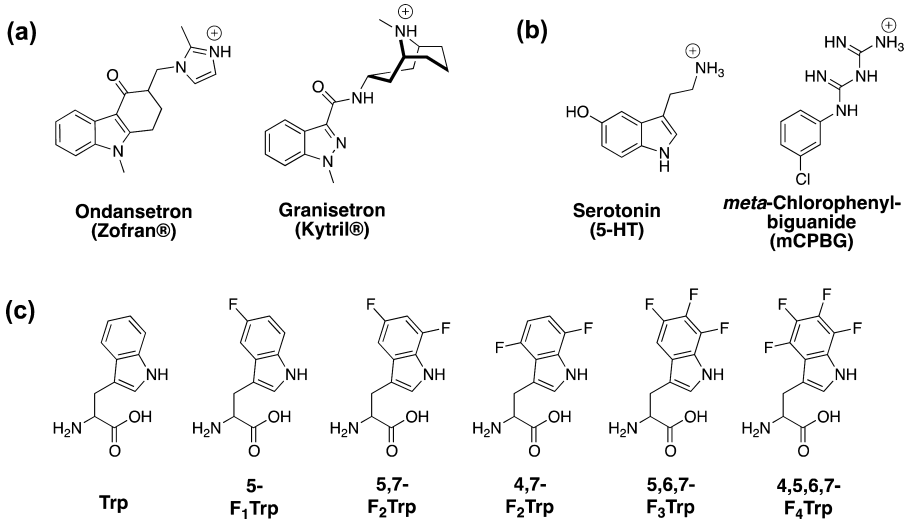

Figure 2. Chemical structures of drugs and amino acids used in this study. (a) 5-HT$_3$A receptor antagonists. (b) 5-HT$_3$A receptor agonists. (c) Tryptophan analogues.

with testable guidelines as to other possible interactions, specifically, with Trp90.[13] In the present work, we set out to better understand the binding of the high affinity antagonistic drugs ondansetron and granisetron to the 5-HT$_3$AR. The cationic center of granisetron is a tertiary ammonium ion in a granatane moiety (pK_a = 9.6), but ondansetron has a structurally distinct *N*-akylimidazolium moiety (pK_a = 7.4).[16] We sought to determine if this structural difference leads to different binding orientations for the two drugs.

RESULTS AND DISCUSSION

Ondansetron and Granisetron Schild Analysis. In examining the interaction of a competitive antagonist with a receptor, equilibrium dissociation constants, K_b, provide the most direct indicator of binding interactions.[17] Previous reports have indicated that granisetron and ondansetron act competitively with 5-HT at the 5-HT$_3$AR.[2,18] Both antagonists bind reversibly, in that after a several minute washout of either ondansetron or granisetron, agonist responses recovered completely. Previous studies have established dissociation rate constants of 0.58 min^{-1} for ondansetron and 0.13 min^{-1} for granisetron,[19] consistent with our observations. It has also been shown that granisetron and ondansetron directly compete with each other for the same binding site.[19] We attempted to determine K_b for ondansetron and granisetron using Schild (dose-ratio) analysis;[17] this requires measurements of dose–

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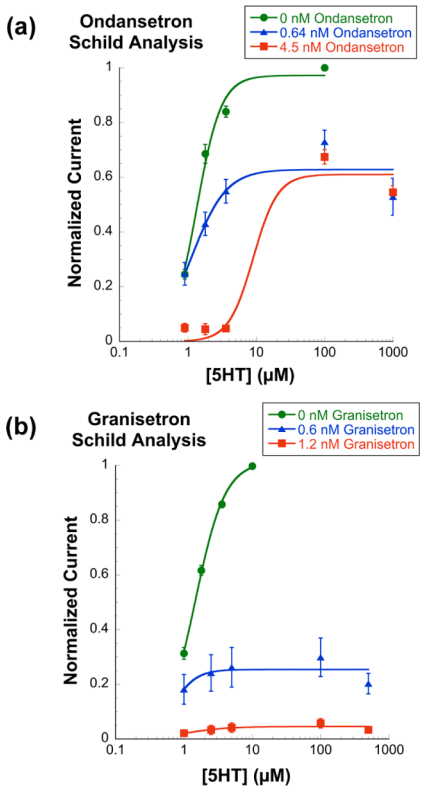

Figure 3. Dose–response curves of wild type 5-HT_3A receptor. Responses to 5-HT with increasing concentrations of (a) ondansetron and (b) granisetron. Data fit to the Hill equation. Fit parameters: (a) Mean maximal current: 8 ± 4 μA. [ondansetron] = 0 nM, EC_{50}: 1.3 ± 0.2 μM, n_H: 2.4 ± 0.6; [ondansetron] = 0.64 nM, Imax: 63%, EC_{50}: 1.1 ± 0.4 μM, n_H: 2 ± 1; [ondansetron] = 4.5 nM, Imax = 61%, EC_{50}: 9 ± 29 μM, n_H: 2 ± 8. (b) Mean maximal current: 9 ± 4 μA. [granisetron] = 0 nM, EC_{50}: 1.5 ± 0.1 μM, n_H: 2.0 ± 0.1; [granisetron] = 0.6 nM, Imax: 25%, EC_{50}: 0.7 ± 0.7 μM, n_H: 2 ± 7; [granisetron] = 1.2 nM, Imax: 4%, EC_{50}: 1 ± 1 μM, n_H: 1 ± 2.

response relationships (Figure 3). Schild analysis of ondansetron applied to preparations of rat vagus nerves gave parallel shifts, indicative of a competitive interaction, but efforts to perform similar studies with granisetron were not successful.[20]

As shown in Figure 3, during our standard 15 s agonist application (see Methods), the inhibition by ondansetron and granisetron was insurmountable by 5-HT: full receptor activity could not be restored even with high concentrations of 5-HT. We attribute the insurmountable inhibition to the slow off rates. The granisetron data are better behaved than the ondansetron data, but in either case we face a requirement of agonist applications several minutes in duration. However, the 5-HT_3AR desensitizes on this time scale before full equilibrium is achieved. Thus, for the high-affinity 5-HT_3AR antagonists ondansetron and granisetron, determination of true K_b by functional measurements is not possible.

Thus, the concentration required for 50% receptor inhibition (IC_{50}) was the only viable functional measurement, and so modified procedures were developed to render IC_{50} values a good direct measure of antagonist binding. The IC_{50} measurement is taken in the context of multiple equilibria, including both agonist and antagonist binding/dissociation, conformational changes in the protein, and the "gating" equilibria between the open and closed states of the receptor. We sought to reduce the contribution from changes in *agonist* potency, while retaining an index of *antagonist* potency. When evaluating competitive antagonists, it is useful to consider more than one agonist, so that one can be sure that IC_{50} determinations are not distorted by agonist-receptor interacts. To determine IC_{50} values for ondansetron and granisetron, we studied both 5-HT and an additional agonist, *m*-chlorophenylbiguanide (mCPBG, Figure 2b).[21]

Agonist Behavior at Trp183 and Trp90. The effective concentrations for 50% receptor activation (EC_{50}) were determined for both the native agonist 5-HT, as well as the potent partial agonist mCPBG. The EC_{50} values were also determined for a series of fluorinated tryptophan derivatives (Figure 2c) introduced by nonsense suppression at Trp183 (Table 1). The EC_{50} values confirm the previously reported[15] cation-π interaction for 5-HT at Trp183. Interestingly, we have recently found that mCPBG does not respond to fluorination at Trp183 in the same manner as 5-HT,[22] and the data of Table 2 produced for this work confirm that result. Detailed experiments and discussion concerning the activation of 5-HT_3 receptors by mCPBG has been presented in a separate

Table 1. EC_{50} Data for Wild Type and Mutant 5-HT_3AR in Response to 5-HT or mCPBG

agonist	mutation	N^a	EC_{50} (μM)^b	fold shift^c	Hill^d
5-HT	wild type	25	1.6 ± 0.1		2.1 ± 0.2
	W183F_1W (5)	18	3.4 ± 0.2	2.2	2.0 ± 0.2
	W183F_2W (5,7)	11	18 ± 1	11	2.9 ± 0.2
	W183F_2W (4,7)	19	36 ± 2	23	2.2 ± 0.3
	W183F_3W (5,6,7)	10	250 ± 10	160	5 ± 1
	W90F_3W (5,6,7)	20	0.23 ± 0.01	1/6.8	2.0 ± 0.2
	W90F_4W (4,5,6,7)	29	0.64 ± 0.03	1/2.4	2.3 ± 0.2
mCPBG	wild type	9	0.50 ± 0.02		2.2 ± 0.1
	W183F_1W (5)	14	5.8 ± 0.4	12	1.6 ± 0.1
	W183F_2W (5,7)	9	2.6 ± 0.1	5.3	1.8 ± 0.1
	W183F_2W (4,7)	19	0.34 ± 0.03	1/1.5	1.4 ± 0.1
	W183F_3W (5,6,7)	13	6.6 ± 0.2	13	2.6 ± 0.2
	W183F_4W (4,5,6,7)	14	8.4 ± 0.6	17	1.5 ± 0.1
	W90F_3W (5,6,7)	15	0.092 ± 0.004	1/5.4	3.2 ± 0.5
	W90F_4W (4,5,6,7)	7	0.20 ± 0.02	1/2.5	2.1 ± 0.3

[a]Number of oocytes averaged in EC_{50} determination. [b]The effective concentration for half-maximal receptor activation. [c]EC_{50}(mutant)/EC_{50}(wild type). [d]The Hill coefficient, n_H, as determined from fitting the Hill equation.

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Table 2. IC_{50} Data for Wild Type and Mutant 5-HT_3AR for Granisetron and Ondansetron in Response to 5-HT or mCPBG

antagonist	agonist[a]	mutation	N[b]	IC_{50} (nM)[c]	fold shift[d]	Hill[e]
granisetron	5-HT	wild type	27	0.78 ± 0.04		−1.4 ± 0.1
		W183F_1W (5)	17	2.9 ± 0.3	3.7	−1.4 ± 0.2
		W183F_2W (5,7)	14	100 ± 10	130	−1.7 ± 0.2
		W183F_2W (4,7)	11	3.4 ± 0.2	4.4	−1.6 ± 0.1
		W183F_3W (5,6,7)	9	61 ± 2	78	−1.6 ± 0.1
		W90F_3W (5,6,7)	11	0.20 ± 0.03	1/3.9	−1.1 ± 0.1
		W90F_4W (4,5,6,7)	12	0.64 ± 0.03	1/1.2	−1.8 ± 0.1
granisetron	mCPBG	wild type	27	1.5 ± 0.1		−1.5 ± 0.1
		W183F_1W (5)	8	3.3 ± 0.2	2.2	−1.4 ± 0.1
		W183F_2W (5,7)	16	140 ± 10	93	−1.5 ± 0.1
		W183F_2W (4,7)	13	2.4 ± 0.3	1.6	−1.3 ± 0.2
		W183F_3W (5,6,7)	9	41 ± 2	27	−1.2 ± 0.1
		W183F_4W (4,5,6,7)	14	110 ± 10	74	−0.9 ± 0.1
		W90F_3W (5,6,7)	7	0.20 ± 0.02	1/7.6	−1.2 ± 0.1
		W90F_4W (4,5,6,7)	9	0.38 ± 0.04	1/4	−1.3 ± 0.2
ondansetron	5-HT	wild type	21	0.87 ± 0.04		−1.6 ± 0.1
		W183F_1W (5)	16	6.2 ± 0.4	7.1	−1.5 ± 0.1
		W183F_2W (5,7)	11	140 ± 10	160	−2.2 ± 0.2
		W183F_2W (4,7)	9	67 ± 6	77	−1.7 ± 0.2
		W183F_3W (5,6,7)	9	160 ± 10	180	−1.8 ± 0.1
		W90F_3W (5,6,7)	13	0.40 ± 0.03	1/2.2	−1.3 ± 0.1
		W90F_4W (4,5,6,7)	11	1.3 ± 0.1	1.4	−1.7 ± 0.1
ondansetron	mCPBG	wild type	23	0.91 ± 0.04		−1.5 ± 0.1
		W183F_1W (5)	15	10 ± 1	11	−1.9 ± 0.2
		W183F_2W (5,7)	13	140 ± 10	160	−1.3 ± 0.1
		W183F_2W (4,7)	13	73 ± 6	80	−1.7 ± 0.2
		W183F_3W (5,6,7)	8	89 ± 5	99	−1.1 ± 0.1
		W183F_4W (4,5,6,7)	15	590 ± 80	650	−1.1 ± 0.1
		W90F_3W (5,6,7)	11	0.37 ± 0.03	1/2.4	−1.5 ± 0.2
		W90F_4W (4,5,6,7)	9	1.5 ± 0.1	1.6	−1.3 ± 0.1

[a]Agonist used in the presence of antagonist for channel activation. [b]Number of oocytes averaged in IC_{50} determination. [c]The effective concentration for half-maximal receptor inhibition. [d]IC_{50}(mutant)/IC_{50}(wild type). [e]The Hill coefficient, n_H, as determined from fitting the Hill equation.

publication.[22] For the present purposes, the key point is that mCPBG responds for fluorination at Trp183 differently than 5-HT.

When the fluorinated tryptophans F_3Trp and F_4Trp were installed at Trp90, both 5-HT and mCPBG show a *gain* of function. These data are consistent with no cation-π interaction for 5-HT or mCPBG with Trp90.

Ondansetron and Granisetron at Trp183 and Trp90. An important aspect of the complex nature of measuring IC_{50} in receptors is the concentration of agonist used for receptor activation. For a competitive interaction, a measured IC_{50} value will depend on the agonist concentration.[17] In order to make meaningful comparisons of IC_{50} values across mutant receptors, the concentration of agonist used was kept at a constant value of twice EC_{50}. The choice of a constant ratio of twice EC_{50} was made to ensure sufficient signal, even in cases of low receptor expression. We also emphasize that the series of mutations being introduced represents a much more subtle variation in structure than is possible with conventional mutagenesis. This provides further confidence that no dramatic changes in receptor−antagonist interactions are occurring in the study. We also exploited the difference in the binding modes of 5-HT and mCPBG to control for possible artificial trends in IC_{50} measurements.

Dose−response/inhibition relations were determined for ondansetron and granisetron, using both 5-HT and mCPBG as agonists, with representative voltage-clamp traces shown in Figure 4. These measurements were performed on the wild type 5-HT_3AR as well as for a series of fluorinated tryptophan derivatives at Trp183. Agonist concentrations were 2 × EC_{50} for each agonist at each receptor, and data were fit to the Hill equation (Figure 4). The resultant IC_{50} values are presented in Table 2. Inhibition data for F_4Trp could not be gathered using 5-HT as the agonist, because of channel block by high concentrations of 5-HT.

The effect of fluorine substitution in modulating a cation-π interaction has been well established.[15,23−25] For both ondansetron and granisetron, incremental substitutions of fluorine to Trp183 increased IC_{50}. As in previous studies of fluorination trends, IC_{50} fold-shift values were plotted against cation-π binding ability of fluorinated indoles, producing the "fluorination plots" shown in Figure 5. Ondansetron inhibition linearly correlates with the energy of cation-π binding, regardless of whether 5-HT or mCPBG was used as an agonist. Granisetron also displayed a strong correlation with respect to degree of fluorination, regardless of agonist identity.

We interpret these results as establishing a cation-π interaction between each drug and Trp183. The results of Figure 5 highlight the value of having two distinct agonists to evaluate an antagonist. With 5-HT as the agonist, we see linear fluorination plots for the antagonists. The agonist alone, 5-HT, shows a similar plot in a study of its EC_{50}. We corrected for this by always using a 5-HT does of 2 × EC_{50} for the particular fluorination mutant. Nevertheless, there could be concern

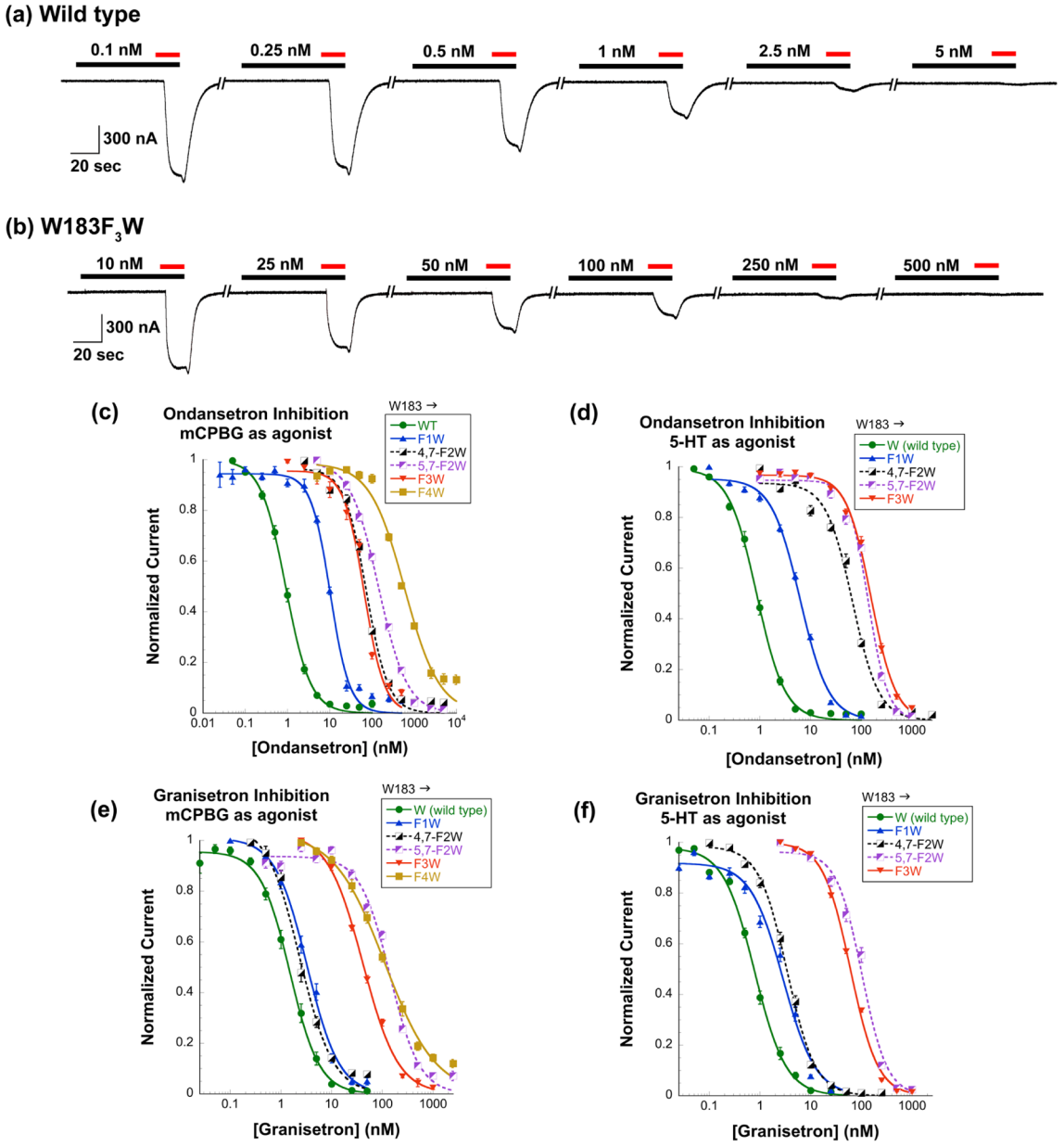

Figure 4. Representative voltage-clamp traces and dose-inhibition curves for antagonists for wild type and Trp183 mutant 5-HT$_3$A receptors. Representative traces for inhibition of (a) wild type and (b) W183F$_3$W receptors by increasing doses of granisetron. Time of application and concentration noted by black bars. Channels opened by addition of mCPBG (red bars) at (a) 1 μM and (b) 13 μM. Hashes indicate wash times. Ondansetron inhibition curves shown for receptors activated by (c) mCPBG and (d) 5-HT. Granisetron inhibition curves shown for receptors activated by (e) mCPBG and (f) 5-HT. Data fit to the Hill equation; fit parameters (IC_{50} and n_H) are in Table 2.

about deconvoluting the effect of fluorination on the agonist *versus* the antagonist. With mCPBG as the agonist, there is no such concern, as it does not respond to fluorination at Trp183 in a manner consistent with a cation-π interaction. Thus, the fluorination trends seen in Figure 5 can confidently be assigned as reflecting the response of the antagonist to the mutation. We also note that the fluorination effect remains regardless of the structural identity of the cation. Ondansetron, with a *N*-akylimidazolium moiety, and granisetron, with a tertiary ammonium, show similar trends at the same residue, which is evidence that their binding orientations are similar.

While the general trends in our data are clear, the detailed behaviors of the F_2Trp unnatural amino acids present interesting details. In the fluorination plots, 5,7-F_2Trp, a residue that we have used extensively, deviates from the line set by the other derivatives, especially for granisetron. The effect is evident, but much less pronounced, in studies of ondansetron. We considered the possibility that an additional unique, perhaps steric, feature of 5,7-F_2Trp was influencing the analysis.

As such, we prepared 4,7-F_2Trp (Scheme 1), which should have the same cation-π binding ability, but different steric requirements. Synthesis began by direct formation of 4,7-difluoroindole (**1**) in a Bartoli reaction between the appropriate nitrodifluorobenzene and vinyl magnesium bromide.[26,27] In a sequence similar to Gilchrist *et al.*[28] the difluoroindole (**1**) was then reacted with ethyl-3-bromo-2-hydroxyiminopropanoate to yield the oxime (**2**), which was then reduced using aluminum/mercury amalgam. The amine of the resulting amino acid ester was protected with the photocleavable 2-nitroveratryloxycarbonyl (NVOC), and the ester was hydrolyzed with sodium

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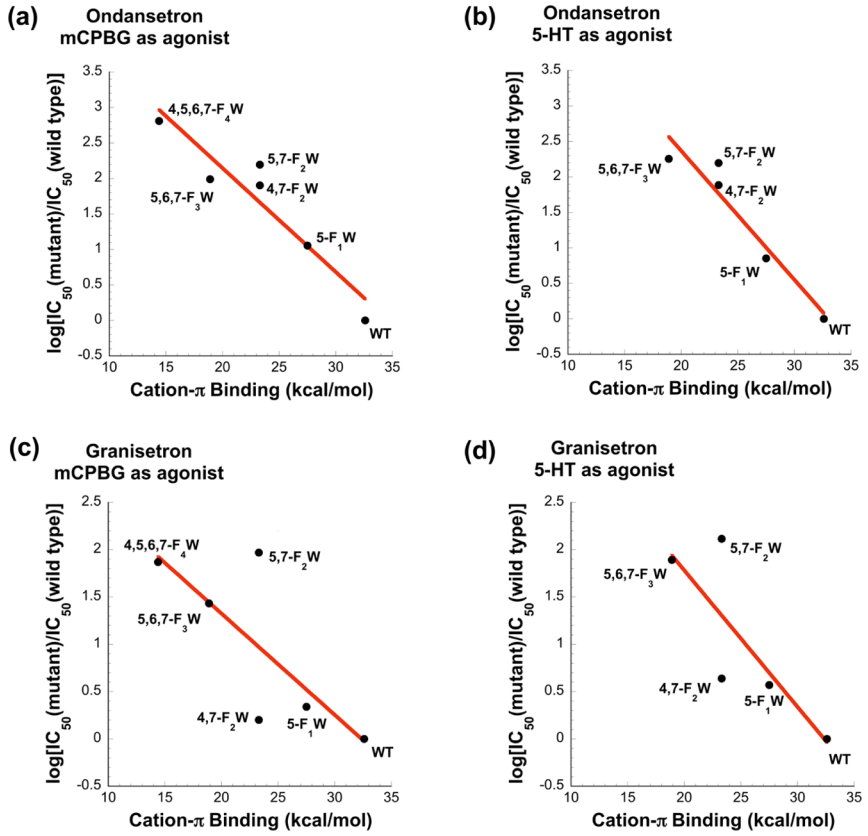
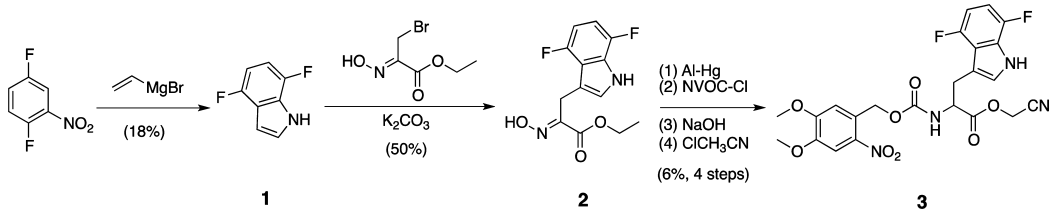


Figure 5. Fluorination plots. Calculated cation-π binding ability *versus* $\log[IC_{50}/IC_{50}(wt)]$ for a series of fluorinated tryptophan derivatives at Trp183. Ondansetron fluorination plots for receptors activated by (a) mCPBG and (b) 5-HT. Granisetron fluorination plots for receptors activated by (a) mCPBG and (b) 5-HT. Red lines are linear fits ($y = mx + b$) inclusive of all points. Fit parameters: (a) $m = -0.15 \pm 0.03$, $b = 5.1 \pm 0.6$, $R = 0.94$; (b) $m = -0.18 \pm 0.03$, $b = 6.0 \pm 0.8$, $R = 0.96$; (c) $m = -0.11 \pm 0.04$, $b = 3 \pm 1$, $R = 0.77$; (d) $m = -0.14 \pm 0.06$, $b = 5 \pm 2$, $R = 0.82$.

Scheme 1



hydroxide. Conversion to the cyanomethyl ester (**3**) gave material suitable for acylation of the dinucleotide dCA and for preparation of tRNA necessary for incorporation into the 5-HT_3R (methods described previously).[29] The unnatural amino acid prepared by this route is, of course, racemic, but only the natural L configuration will be incorporated; the ribosome of the *Xenopus* oocyte in effect performs a kinetic resolution.

From an electrostatic point of view, 5,7-F_2Trp and 4,7-F_2Trp are, to first order, indistinguishable, and so they should be equivalent in a cation-π interaction. This is born out in the EC_{50} data for serotonin (Table 1), where the two F_2Trp residues differ by only a factor of 2, while the full fluorination series spans more than a factor of 150. In contrast, the EC_{50} values for mCPBG differ by 8-fold for the two F_2Trps. Recall that mCPBG does not make a cation-π interaction to the Trp. This again suggests that specific steric interactions at Trp183 may be involved.

In the granisetron IC_{50} plots, 4,7-F_2Trp gives a quite different response than 5,7-F_2Trp. We have suggested the possibility of a special steric effect with 5,7-F_2Trp, but 5,6,7-F_3Trp and 4,5,6,7-F_4Trp both have fluorine atoms at the positions in 5,7-F_2Trp yet follow the trend indicative of electrostatics as the major determinant to binding. As such, we cannot provide a simple rationalization of the behaviors of the two difluoro-Trp residues. Nevertheless, the consistent linear trend of Trp, F_1Trp, F_3Trp, and F_4Trp (Figure 5) provides compelling evidence for a cation-π interaction to Trp183 for both granisetron and ondansetron.

Both ondansetron and granisetron either increase their potencies or retain their potencies when Trp90 is mutated to F_3Trp or F_4Trp, respectively. This holds for both 5-HT and mCPBG used as the agonist, which indicates no cation-π interaction at that site. A loss of potency would be expected if a cation-π interaction were present. We noted above that Yan and White concluded that Trp90 is important for binding of ondansetron and granisetron.[14] Based on the observation that granisetron and ondansetron responded differently to a W90F mutation, the authors concluded that the bicyclic amine of

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granisetron interacts with Trp90. Our results position this moiety in contact with Trp183, and we conclude that the importance of Trp90 is for reasons other than a cation-π interaction.

The present results provide evidence that the cationic centers of ondansetron and granisetron are oriented toward Trp183 and not toward Trp90. Establishing a cation-π interaction with ondansetron and granisetron at Trp183 determines a binding orientation for these antagonists. Docking studies of granisetron performed in other laboratories have generated a series of poses, some which are consistent with the cation pointed toward Trp183. Our data provide evidence that these poses are the most viable, while those with the cation pointed away from Trp183 are not likely to be relevant.

Summary. We have identified a cation-π interaction with the antagonists ondansetron and granisetron to Trp183 in the 5-HT_3AR. This interaction is consistent with the binding mode of 5-HT, but not mCPBG. The use of agonists with alternate binding modes validates our data as direct measurements of ondansetron and granisetron. Thus, the common antagonists follow the basic pharmacophore established by 5-HT and not the structurally dissimilar agonist mCPBG.

METHODS

Procedures for incorporating unnatural amino acids, expressing receptors in *Xenopus* oocytes, and characterization by electrophysiology followed established protocols.[29]

Protein Expression in *Xenopus* Oocytes. The mouse 5-HT_3A receptor in the pGEMHE vector was linearized with the restriction enzyme *Sbf* I (New England Biolabs). mRNA was prepared by *in vitro* transcription using the mMessage Machine T7 kit (Ambion). Unnatural mutations were introduced by the standard Stratagene QuickChange protocol using a TAG mutation at W183 and W90. Stage V–VI *Xenopus laevis* oocytes were injected with mRNA. Each cell was injected with 50 nL containing only mRNA (5 ng) for wild type 5-HT_3AR or a mixture of mRNA (5–32 ng, typically ~12 ng) and tRNA (18–30 ng, typically ~18 ng) for unnatural amino acid. Uncharged full length tRNA was injected as a negative control.

Electrophysiology. Electrophysiological experiments were performed 24–48 h after injection using the OpusXpress 6000A instrument (Axon Instruments) in two-electrode voltage clamp mode at a holding potential of −60 mV. The running buffer was Ca^{2+}-free ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.5). Serotonin hydrochloride (5-HT) was purchased from Alfa Aesar. 1-(3-Chlorophenyl)biguanide (mCPBG) was purchased from Sigma-Aldrich. Granisetron hydrochloride and ondansetron hydrochloride were purchased from Tocris Bioscience.

For EC_{50} determinations, oocytes were superfused with running buffer at 1 mL/min for 30s before application of 5-HT or mCPBG for 15 s followed by a 116 s wash with the running buffer. Data were sampled at 125 Hz and filtered at 50 Hz. Dose–response data were obtained for ≥9 concentrations of 5-HT or mCPBG on ≥9 cells. All EC_{50} and Hill coefficient values were obtained by fitting dose–response relations to the Hill equation (I_{norm} = 1/[1 + (EC_{50}/[agonist])^n]) and are reported as means ± standard error of the fit.

For IC_{50} determinations, oocyte response to either 5-HT or mCPBG at 2 × EC_{50} for each receptor was measured before application of antagonists by application of the agonist for 15 s followed by 116 s of wash with the running buffer. Granisetron or ondansetron doses were then preapplied, and the oocyte was allowed to incubate for 60 s, followed by application of a mixture of the antagonist dose with 5-HT or mCPBG at 2 fold EC_{50}. The oocytes were then washed with the running buffer for 116 s. Every 4 antagonist doses, the oocytes were washed for 10 min, and oocyte response reconfirmed using either 5-HT or mCPBG at 2 × EC_{50}. Oocytes that did not give consistent responses to 5-HT or mCPBG alone throughout the experiment were discarded. Dose–response data were obtained for ≥8 concentrations of granisetron or ondansetron on ≥8 cells. All EC_{50} and Hill coefficient values were obtained by fitting dose–response relations to the Hill equation (I_{norm} = 1/[1 + (EC_{50}/[antagonist])^n]) and are reported as means ± standard error of the fit.

For Schild analysis, the protocol for EC_{50} determinations was repeated with the following changes: during the course of the experiment after each minimal EC_{50} curve was determined, running buffer containing granisetron or ondansetron was used for the subsequent EC_{50} determinations. Agonist applications in the subsequent EC_{50} determinations contained the same concentration of antagonist as the running buffer.

Synthesis of 3 (Scheme 1). *4,7-Difluoroindole (1).* A solution of 3.5 mL (32.3 mmol) of 1,4-difluoro-2-nitrobenzene in 30 mL of dry THF was cooled in an acetone/dry ice bath to −78 °C under argon. A 1 M solution of vinylmagnesium bromide in THF (100 mL, 100 mmol, 3 equiv) was added *via* cannula over 20 min. The reaction was stirred for 1 h at −78 °C. Reaction quenched by the addition of 20 mL of saturated aq NH_4Cl. Upon warming to RT, 20 mL of water was added, which formed a thick emulsion. The reaction mixture was filtered through a layer of sand and washed copiously with ethyl acetate. The organic layer was separated and dried over Na_2SO_4. The solvent was removed under reduced pressure to yield a brown oil containing multiple compounds. Purification by silica gel chromatography using a gradient of 3% to 10% ethyl acetate in hexanes yielded a slightly volatile amber oil: 871 mg (18%). Silica TLC (4% EtOAc in hexanes) R_f = 0.26, stains red/pink using *p*-anisaldehyde. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.47 (br, 1H), 7.18 (t, J = 2.8 Hz, 1H), 6.78 (ddd, J = 10.3, 8.6, 3.5 Hz, 1H), 6.71–6.60 (m, 2H). ^{19}F NMR (282 MHz, $CDCl_3$): δ −124.1 to −129.8 (m), −139.4 to −142.3 (m). ^{13}C NMR (126 MHz, $CDCl_3$): δ 152.2 (dd, J = 239, 2.4 Hz), 145.8 (dd, J = 238.6, 3.0 Hz), 126.0 (dd, J = 15.9, 11.5 Hz), 124.7, 119.9 (dd, J = 24.9, 5.7 Hz), 106.4 (dd, J = 18.9, 8.2 Hz), 103.9 (dd, J = 18.9 8.2 Hz), 99.8. HRMS EI(+) m/z for $C_8H_5NF_2$ found 153.0395, calculated 153.0390 ($M^{+\bullet}$).

Ethyl 3-(4,7-Difluoro-1H-indol-3-yl)-2-(hydroxyimino)propanoate (2). A solution of 424 mg (2.8 mmol, 2 equiv) of 4,7-difluoroindole in 10 mL of CH_2Cl_2 was added to 290 mg (1.4 mmol, 1 equiv) of ethyl 3-bromo-2-(hydroxyimino)propanoate and 205 mg (1.9 mmol, 1.4 equiv) of Na_2CO_3. The mixture was stirred overnight under argon at RT. The reaction was diluted with 50 mL of CH_2Cl_2 and 50 mL of ethyl acetate and washed with 50 mL of water and 50 mL of brine. The organic phase was separated and dried over Na_2SO_4. Purification performed by silica chromatography, gradient 25% to 40% EtOAc in hexanes to yield a white solid: 195 mg (50%). ^{1}H NMR (300 MHz, CD_3CN): δ 9.93 (s, 1H), 9.64 (br, 1H), 7.01 (m, 1H), 6.81 (ddd, J = 10.5, 8.5, 3.5 Hz, 1H), 6.67 (ddd, J = 10.7, 8.5, 3.2 Hz, 1H), 4.21 (q, J = 7.2 Hz, 2H), 4.12 (d, J = 1.1 Hz, 2H), 1.23 (t, J = 7.1 Hz, 3H). ^{19}F NMR (282 MHz, CD_3CN): δ −130.99 to −131.20 (m), −141.02 to −141.19 (m). ^{13}C NMR (126 MHz, $CDCl_3$): δ 164.80, 153.77 (dd, J = 240.4, 2.3 Hz), 152.58, 146.84 (dd, J = 238.1, 2.9 Hz), 127.18 (dd, J = 16.4, 12.1 Hz), 125.12, 119.55 (dd, J = 22.1, 5.7 Hz), 110.05 (dd, J = 3.7, 1.6 Hz), 106.94 (dd, J = 19.7, 9.1 Hz), 104.42 (dd, J = 22.9, 7.5 Hz), 62.28, 21.96 (d, J = 3.1 Hz), 14.33. HRMS FAB(+) m/z for $C_{13}H_{13}O_3N_2F_2$ found 283.0888, calculated 283.0894 (M + H).

N-(2-Nitroveratryloxycarbonyl)-4,7-difluorotryptophan Cyanomethyl Ester (3). In a beaker 1.5 g of 8–20 mesh aluminum was stirred under 15 mL of 2 M NaOH for 5 min. After decanting, the aluminum was rinsed with water, and 15 mL of a 2% $HgCl_2$ solution was added and stirred slowly. The solution was decanted after formation of the Hg-Al (~10 min) and added to 195 mg (0.69 mmol) of **2** in 20 mL of 9:1 dioxane/water. The reaction was stirred slowly at RT overnight (~24 h). The reaction was filtered through fluted paper and then applied to a silica plug, eluting with EtOAc followed by 4% MeOH in EtOAc. After concentration under reduced pressure, the resulting oil was used directly. The oil was dissolved in 20 mL of 1:1 THF/water, and 151 mg of Na_2CO_3 (1.42 mmol) and 248 mg of 4,5-dimethoxy-2-nitrobenzyl chloroformate (0.9 mmol) were added. The reaction was stirred at RT for 3 h, followed by dilution with 20 mL of CH_2Cl_2 and 20 mL of 1 N HCl. The organic phase was separated, washed with brine, and dried over Na_2SO_4. Initial purification by silica

chromatography 20% to 40% EtOAc in hexanes did not separate the product from nitroveratryl side products. This mixture was dissolved in 3 mL dioxane and 3 mL of 2 N NaOH and stirred for 15 min. The reaction was quenched with 6 mL of 1 N HCl and diluted with 20 mL of EtOAc. The organic phase was separated, and the aqueous phase was washed with 20 mL of CH_2Cl_2. The combined organic phases were dried over Na_2SO_4, concentrated under reduced pressure, and filtered through a silica plug eluting with EtOAc followed by 0.5% acetic acid in EtOAc. This residue (~35 mg, 0.07 mmol) was dissolved in 1 mL of DMSO and added to a reaction flask containing 0.5 mL of chloroacetonitrile (7.9 mmol) and 0.5 mL of triethylamine (3.6 mmol). The reaction was allowed to stir at RT for 5 h. The reaction was poured onto a dry column of silica and eluted with EtOAc to recover 23 mg of a yellow solid (6%, 4 steps). ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.71 (s, 1H), 8.21 (d, J = 7.7 Hz, 1H), 7.68 (s, 1H), 7.23 (d, J = 2.0 Hz, 1H), 7.09 (s, 1H), 6.92−6.80 (m, 1H), 6.74−6.62 (m, 1H), 5.39−5.21 (m, 2H), 4.99 (s, 2H), 4.48−4.35 (m, 1H), 3.85 (s, 3H), 3.83 (s, 3H), 3.32−2.99 (m, 2H). ^{19}F NMR (282 MHz, DMSO-d_6): δ −130.18 (dd, J = 22.4, 9.9 Hz), −138.53 (ddd, J = 22.6, 10.6, 3.1 Hz). ^{13}C NMR (126 MHz, DMSO-d_6): δ 171.56, 156.03, 153.83, 152.62 (d, J = 239.6 Hz), 148.15, 146.10 (dd, J = 238.5, 2.2 Hz), 139.55, 128.09, 126.70 (dd, J = 16.1, 12.2 Hz), 126.36, 118.82−118.30 (m), 116.08, 110.61, 109.18, 108.59, 106.23 (dd, J = 18.8, 8.7 Hz), 103.90−103.23 (m), 63.15, 56.55, 55.30, 49.97, 27.84. HRMS FAB(+) m/z for $C_{23}H_{20}O_8N_4F_2$ found 518.1271, calculated 518.1249 ($M^{+\bullet}$).

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Notes

The authors declare no competing financial interest.

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