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Original article

Synthesis and pharmacological evaluation of carboxamide derivatives as selective serotoninergic 5-HT_4 receptor agonists

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Abstract – A number of new carboxamide derivatives were synthesized. The affinity of these compounds for the serotoninergic 5-HT_4 receptor was evaluated by use of radioligand-binding techniques. The agonistic activity was evaluated as the contractile effect of the ascending colon isolated from guinea-pigs. Among these compounds, 4-amino-5-chloro-2-methoxy-*N*-[1-[2-[(methylsulfonyl)amino]ethly]-4-piperidinylmethyl]benzamide (**24**) showed a high affinity for the 5-HT_4 receptor (*Ki* = 9.6 nM). Compound **24** displayed a higher affinity for 5-HT_4 receptors than the other receptors, including, 5-HT_3 and dopamine D_2 receptors. In addition, compound **24** was confirmed to be a potent 5-HT_4 receptor agonist (ED_{50} = 7.0 nM). An interaction model between compound **24** and 5-HT_4 receptor was proposed. © Elsevier, Paris

5-HT_4 receptor / 5-HT_4 receptor agonist / structure–activity relationship / carboxamide / receptor model construction

1. Introduction

Serotonin (5-HT) is a neurotransmitter responsible for a wide range of pharmacological reactions. Serotonergic receptors are now classified into four broad subtypes such as 5-HT_1, 5-HT_2, 5-HT_3, and 5-HT_4 receptors, and clones for additional subtypes termed 5-HT_5, 5-HT_6, and 5-HT_7 receptors have been identified [1]. Among diverse 5-HT receptors, we have investigated 5-HT_4 function. Activation of the 5-HT_4 receptor mediates widespread effects in the central and peripheral nervous systems [2]. Therefore, selective 5-HT_4 receptor ligands would be useful for elucidating the physiological function of this receptor. Agonists for the 5-HT_4 receptor known to date are indole derivatives such as 5-methoxytryptamine, benzamide derivatives such as cisapride [3–5], benzimidazolone derivatives such as BIMU8 [6], and benzoate derivatives such as ML 10302 [7] *(figure 1)*. However, nonselective affinity or lability of these compounds prevents pure pharmacological characterization of this receptor in vivo. For example, 5-methoxytryptamine activates all 5-HT receptor subtypes except the 5-HT_3 receptor [8]. Cisapride and BIMU8 show obvious affinity for the 5-HT_3 receptor [9, 10]. While, ML 10302 is a selective 5-HT_4 receptor agonist, it shows limited pharmacological activities in vivo because of possible hydrolysis at its benzoate moiety. Recently, some ketone derivatives were reported to show a high affinity for the 5-HT_4 receptor and to be selective 5-HT_4 partial agonists [11]. In addition, the original 5-TH_4 receptor mapping by the active analogue approach by using several 5-HT_4 receptor antagonists and inactive ligands was reported [12].

One of our purposes is to supply a useful tool for elucidating the physiological function of the 5-HT_4 receptor. In the course of our synthetic study on 5-HT_3 receptor antagonists [13], it has been already clarified that a benzoxazine-8-carboxamide derivative **1** *(figure 2)* shows a weak affinity for the 5-HT_4 receptor. This result lead us to design selective 5-HT_4 receptor agonists having novel chemical structure and chemical stability. Thus, compound **1**, a leading compound for this study, could further be optimized at four points: optimization of (1) the structure of the cycloamine moiety, (2) the distance between the amide nitrogen in the carboxamide moiety and the basic nitrogen in the cycloamine moiety, (3) the variety and number of the substituents on the

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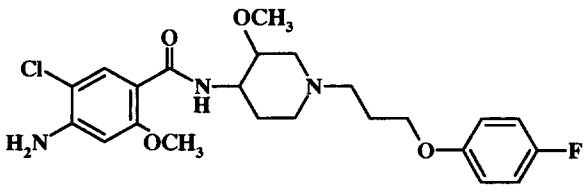
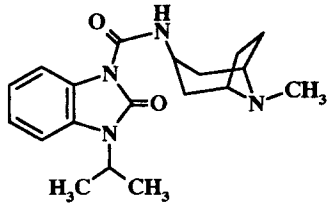


Cisapride



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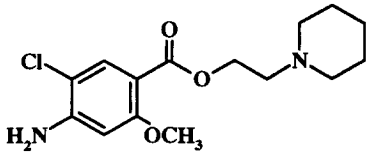


ML 10302

Figure 1. Chemical structures of 5-HT_4 receptor agonists.

aromatic moiety, and (4) the structure of the side chain on the cycloamine moiety. Herein, we describe the design, synthesis, and pharmacological evaluation of carboxamide derivatives as selective 5-HT_4 receptor agonists. Interaction between putative 5-HT_4 receptor models and agonistic ligand is also discussed here.

2. Chemistry

The general synthetic procedure used in this study is illustrated in *figure 3*. By the mixed anhydrides method (method A) or the 1-ethyl-3-[3-(dimethylamino)propyl] carbodiimide hydrochloride (WSC) method (method B), carboxylic acids (**2a–2h**) were coupled with appropriate amines (**4a**, **4b** and **6a–6f**) to afford target benzamides

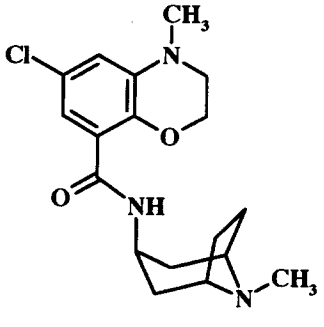


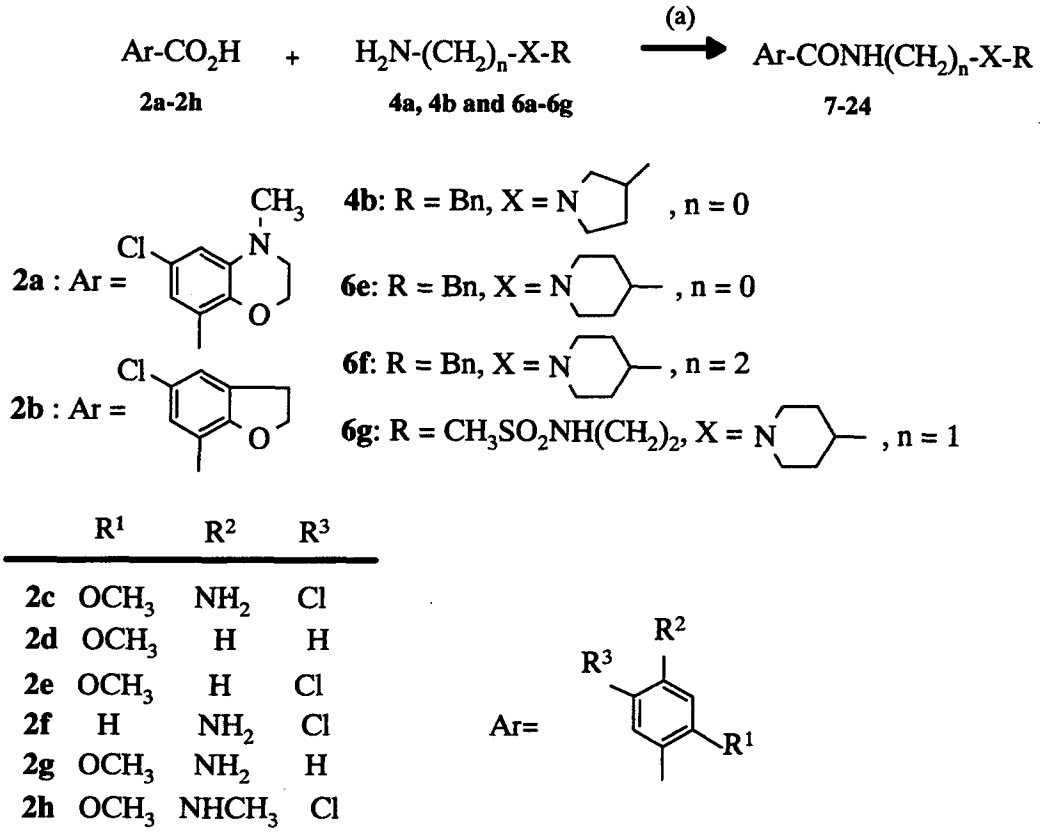
Figure 2. Chemical structure of compound **1**.

(**7–24**), on which the synthetic data are listed in *table I*. Among the acidic starting materials, 3,4-dihydro-1,4-benzoxazine-8-carboxylic acid (**2a**) was prepared by our method reported previously [14], and 2,3-dihydro-5-chlorobenzofuran-7-carboxlic acid (**2b**) [15] and 5-chloro-2-methoxy-4-methylaminobenzoic acid (**2h**) [16] were prepared by known procedures. As for the other group of starting materials, pyrrolidine-3-methanol (3) was condensed with phthalimide by the Mitsunobu reaction [17] to afford a corresponding phthalimide derivative, which gave 1-benzyl-3-pyrrolidinylmethylamine (**4a**) through a hydrazinolysis *(figure 4)*. 1-Substituted-4-piperidinylmethylamines (**6a–6d**) were prepared from their corresponding isonipecotamides (**5a–5d**) by lithium aluminium hydride reduction *(figure 5)*. 2-(1-Benzyl-4-piperidiyl)ethylamine (**6f**) and [1-(2-methansufonyl-amino)ethyl]-4-piperidylmethylamine (**6g**) were prepared using our procedures [18].

3. Pharmacological data and discussion

3.1. Structure–activity relationships

The affinity of compounds **7–24** for the 5-HT_4 receptor was determined as their ability to inhibit the binding of [^{3}H]GR113808 to the receptor. Their affinitiês for 5-HT_3 and dopamine D_2 receptors were similarly evaluated by using [^{3}H]granisetron and [^{3}H]spiperone as radioligand, respectively. Here, membrane preparations of the striatum of guinea-pigs, the cerebral cortex of rats, and the



	R^1	R^2	R^3
2c	OCH_3	NH_2	Cl
2d	OCH_3	H	H
2e	OCH_3	H	Cl
2f	H	NH_2	Cl
2g	OCH_3	NH_2	H
2h	OCH_3	$NHCH_3$	Cl

Figure 3. The general synthetic procedures used in this study. (a) method A: i-BuOCOCl, Et_3N, AcOEt or method B: WSC, HOBt, DMF.

striatum of rats were used for 5-HT_4, 5-HT_3, and D_2 receptor binding assays, respectively. Either agonistic or antagonistic activity of these compounds was evaluated as the contractile ability of the ascending colon of guinea-pigs.

Pharmacological data on compounds **1** and **7–24**, 4-amino-*N*-(1-benzyl-4-piperidinyl)-5-chloro-4-methoxybenzamide (clebopride), and 5-HT are listed in *table II*, where clebopride and 5-HT are reference compounds. As concerns the cycloamine part, we chose not the bicycloamine group (tropane) but the monocycloamine group (pyrrolidine and piperidine) to obtain the compounds having a higher affinity for 5-HT_4 receptor than for 5-HT_3

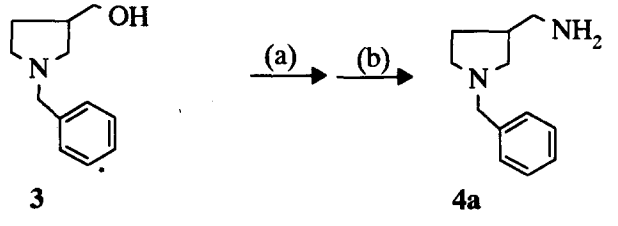


Figure 4. Condensation of **3** by the Mitsunobu reaction. (a) PPh_3, phthalimide, DEAD, THF; (b) $NH_2NH_2H_2O$, EtOH.

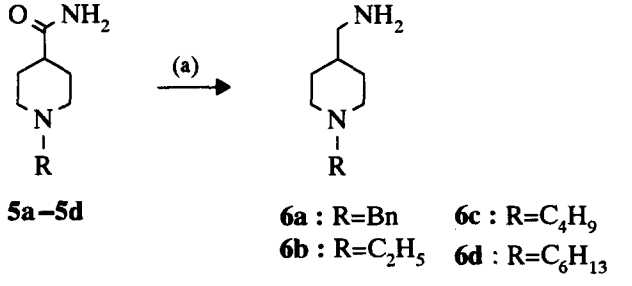
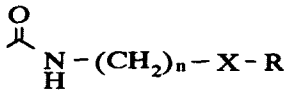


Figure 5. Lithium aluminium hydride reduction of **5a–5d.** (a) $LiAlH_4$, THF.

Table I. Physicochemical properties for compounds **7–24.**



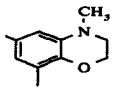
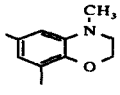
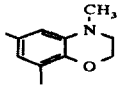
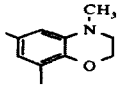
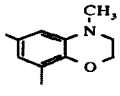
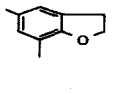
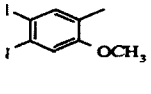
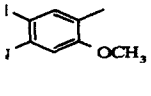
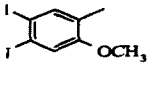
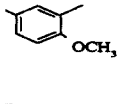
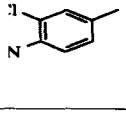
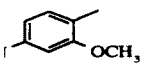
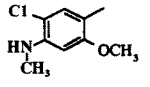
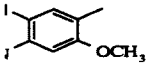
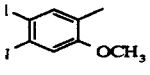
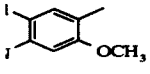
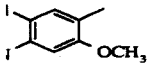
Compound	Ar	n	X	R	Method	Mp (°C)	Formula
7		0		Bn[a]	A	94–95	$C_{21}H_{24}ClN_3O_2$
8		0		Bn[a]	A	232–233	$C_{22}H_{26}ClN_3O_2C_4H_4O_4$[f]
9		1		Bn[a]	A	186–187	$C_{22}H_{26}ClN_3O_2C_2H_4O_4$[g]
10		1		Bn[a]	A	97–98	$C_{23}H_{28}ClN_3O_2$
11		2		Bn[a]	A	122–124	$C_{24}H_{30}ClN_3O_2C_2H_2O_4$1/2EtOH 1/2$H_2O$[g]
12		1		Bn[a]	B	176–178/DEC	$C_{22}H_{25}ClN_2O_2C_2H_2O_4$[g]
13		1		Bn[a]	B	161–163	$C_{20}H_{24}ClN_3O_2C_2H_2O_4$[g]
14		1		Bn[a]	B	155–156	$C_{21}H_{26}ClN_3O_2$
15		2		Bn[a]	B	135–137	$C_{22}H_{28}ClN_3O_2C_2H_2O_4$[g]
16		1		Bn[a]	B	169–170	$C_{21}H_{26}N_2O_2C_2H_2O_4$[g]
17		1		Bn[a]	B	90–93	$C_{21}H_{25}ClN_2O_2C_2H_2O_4$ 1/4H_2O[g]
18		1		Bn[a]	B	110–112/DEC	$C_{20}H_{24}ClN_3O_2C_2H_2O_4$ 4/5H_2O[g]

Table I. (Continued)

Compound	Ar	n	X	R	Method	Mp (°C)	Formula
19		1		Bn[a]	B	160–161	$C_{21}H_{27}N_3O_2C_2H_2O_4$[g]
20		1		Bn[a]	B	148–149	$C_{22}H_{28}ClN_3O_2C_4H_4O_4$[f]
21		1		Et[b]	B	115–116	$C_{16}H_{24}N_3O_2$
22		1		Bu[c]	B	179–181	$C_{18}H_{28}ClN_3O_2C_2H_2O_4$[g]
23		1		Hex[d]	B	113–115	$C_{20}H_{32}ClN_3O_2$
24		1		MSAE[e]	B	177–178/DEC	$C_{17}H_{27}ClN_4O_4S2C_2H_2O_4$[g]

[a]Bn, benzyl; [b]Et, ethyl; [c]Bu, butyl; [d]Hex, hexyl; [e]MSAE, (methylsulfonylamino)ethyl; [f]fumalate; [g]oxalate.

receptor [7]. As a result, monocycloamine derivatives (**7** and **8**) in benzoxazine series showed a low affinity for 5-HT_3 receptor. Furthermore, piperidine derivatives (**10** and **14**) showed a higher affinity for 5-HT_4 receptor than pyrrolidine derivatives (**9** and **13**).

Next, we investigated the distance requirements between the carbonyl group in the amide bond and the basic nitrogen in the cyclic amine of pyrrolidine and piperidine derivatives. Here, two benzoxazinecarboxamide derivatives (**7** and **8**) and clebopride, where the hydrogen atom on their carboxamide nitrogen is replaced by a 1-benzyl-3-pyrrolidinyl or 1-benzyl-4-piperidinyl group, showed affinities for 5-HT_3, 5-HT_4 and D_2 receptors. On the other hand, compounds **9**, **10** and **12–14**, where the hydrogen atom on their carboxamide nitrogen is replaced by a 1-benzyl-3-pyrrolidinylmethyl or 1-benzyl-4-piperidinylmethyl group, showed a higher affinity for the 5-HT_4 receptor than for the 5-HT_3 receptor, and showed a low affinity for the D_2 receptor. Compounds **11** and **15**, where the hydrogen atom on their carboxamide nitrogen is replaced by a 2-(1-benzyl-4-piperidinyl)ethyl group, exhibited only low affinities for these kinds of receptors. The results indicate that the optimum distance for selective affinity for the 5-HT_4 receptor was one carbon [9].

Compound **10** was confirmed to be a 5-HT_4 receptor antagonist (pK_B = 7.9). Compound **12**, a benzofuran analogue of benzoxazine derivative **10**, was also confirmed to behave as a 5-HT_4 receptor antagonist (pK_B = 8.2) although they showed a high affinity for the receptor. However, compounds **13** and **14**, both of which have a classic 4-amino-5-chloro-2-methoxybenzamide skeleton, were confirmed to be a 5-HT_4 receptor agonist. We next focused on the effect of each substituent (4-amino, 5-chloro and 2-methoxy group) on the phenyl ring in benzamide derivatives. A simple 2-methoxybenzamide derivative **16** showed a low affinity to the receptor. Although 5-chloro-2-methoxybenzamide derivative **17** exhibited a 5-HT_4 receptor affinity similar to compound **14**, the former never behaved as a agonist for the receptor. On the other hand, such an affinity was practically undetectable for the 4-amino-3-chlorobenzamide derivative **18**, a 2-desmethoxy analogue of compound **14**. The moderate affinity of compound **16** and the low affinity of compound **18** indicate that the 2-methoxy group is essential for binding to the 5-HT_4 receptor. Compound **17** showed a similar affinity to compound **14**. This result suggests that the 5-chloro group helped ligands to bind to the receptor complementarily. The 4-Amino-2-

Table II. Pharmacological data of compound **1**, compounds **7–24** and two reference compounds.

	Binding affinities, Ki (nM)[a]			Contractile effects in guinea pigs ascending colon	
Compound	5-HT_4	5-HT_3	D_2	EC_{50} (nM)[d]	Maximal response (%)[e]
1	280	0.27	> 1000[b]	NT[c]	–
7	160	10	12	NT[c]	–
8	530	21	67	NT[c]	–
9	19	95	> 1000[b]	> 10000	
10	0.93	120	> 1000[b]	> 10000	
11	530	> 1000[b]	> 1000[b]	NT	–
12	1.7	340	NT	> 10000	
13	20	160	> 1000[b]	47	21
14	6.7	290	> 1000[b]	20	18
15	> 1000[b]	470	> 1000[b]	NT[c]	–
16	66	> 1000[b]	880	NT[c]	–
17	8.1	> 1000[b]	NT[c]	> 10000	
18	> 1000[b]	> 1000[b]	NT[c]	NT[c]	–
19	110	> 1000[b]	NT[c]	150	20
20	100	> 1000[b]	NT[c]	900	8.9
21	28	> 1000[b]	NT[c]	61	16
22	8.8	> 1000[b]	NT[c]	13	15
23	6.7	> 1000[b]	NT[c]	130	20
24	9.6	> 1000[b]	> 1000[b]	7.0	27
clebopride	92	230	63	NT[c]	–
5-HT	130			35	21

[a]Each value is the mean from triplicate assays in a single experiment; [b]IC_{50} value; [c]not tested; [d]EC_{50} values were determined by linear regression; [e]% of contraction to methacoline at 30 μM.

methoxybenzamide derivative **19**, a 5-deschloro analogue of compound **14**, showed a potent 5-HT_4 receptor agonistic activity, but showed only a moderate affinity for the receptor. These results indicate that the 4-amino group played an important role in 5-HT_4 receptor agonistic activity. In addition, this consideration is supported by the facts, which 2,3-dihydro-4-amino-5-chlorobenzofuran carboxamides showed 5-HT_4 receptor agonistic activity [19], while 4-desamino-2,3-dihydro-5-chlorobenzofuran carboxamide **12** showed low 5-HT_4 receptor agonistic activity. Although 5-chloro-2-methoxy-4-methylaminobenzamide derivative **20**, an *N*-methyl analogue of compound **14**, also exhibited a moderate affinity, it showed low 5-HT_4 receptor agonistic activity. Consequently, all the three substituents on the phenyl ring are essential for both affinity and agonistic activity.

Finally, we examined the influence of the side chains of *N*-substituted piperidinylmethyl group on the interaction with the 5-HT_4 receptor. Ethyl derivative **21** showed a moderate affinity for the 5-HT_4 receptor. Butyl derivative **22** was almost equal to compound **14** in the affinity, hexyl derivative **23** was equal to compound **14** in the affinity, but with reduced 5-HT_4 receptor agonistic activity. When the substituent was a polar methylsulfonylamino group, agonistic activity increased. Compound **24** was a more potent agonist at the 5-HT_4 receptor than other compounds (**14**, **21–23**). Therefore, compound **24** was assayed for its binding ability for several kinds of receptors. The results were as follows (IC_{50} value (nM), ligand): α_1 > 1 000, [^{3}H]prazosin; 5-HT_{1A} > 1 000, [^{3}H]8-OH-DPAT; 5-HT_2 > 1 000, [^{3}H]ketanserin; MACh > 1 000, [^{3}H]QNB. Thus Compound **24** represented a selective 5-HT_4 receptor agonist.

3.2. Computational modelling study

3.2.1. 5-HT_4 receptor model construction

A 5-HT_4 receptor model was constructed in order to obtain rational structure–activity relationships and to clarify the interaction between ligands and the receptor. The 5-HT_4 receptor is a member of the G-protein coupled receptor (GPCR). Generally, GPCRs are assumed to consist of seven transmembrane helices and eight intra and extracellular loops and their models are constructed from coordinates of bacteriorhodopsin [20–27]. The credibility of the model is evaluated by the consistency with structure–activity relationships and mutational data. In this study, affinity for the guinea-pig 5-HT_4 receptor was measured. Therefore the receptor model should be constructed from the sequence of guinea-pig. But the guinea-

pig sequence has not been reported yet. Although sequences of receptors are not identical among animals, amino acids involved in ligand recognition are most likely to be conserved. Therefore, in the agonist recognition site, the conserved amino acids between human and rat 5-HT_4 receptors should be conserved in guinea-pig 5-HT_4 receptors. The 5-HT_4 receptor model was constructed with the residues in the human 5-HT_4 receptor and the conserved residues were used for speculation of receptor-ligand interaction. The seven transmembrane regions of the 5-HT_4 receptor was determined from a hydropathy plot [28]. To determine sequence alignment of bacteriorhodopsin and the 5-HT_4 receptor, over 70 GPCRs such as dopamine, adrenergic, muscarine receptor, and rhodopsin were included in the study.

3.2.2. Docking of compound **24** *into the 5-HT_4 receptor model*

Mutational studies for the 5-HT_4 receptor have not been reported yet. Therefore, the structure–activity relationships for this series of compounds and mutational data for the 5-HT_{1A} and 5-HT_{2A} receptors [29–33] were used for the docking study of compound **24** into the receptor model. From the structure–activity relationships, the 2-methoxy, 5-chloro, and 4-amino groups on the phenyl ring, and the methylsulfonylamino group in the piperidinomethyl moiety were defined to be essential for both affinity and agonistic activity. These groups are assumed to interact with certain residues in the 5-HT_4 receptor. From some mutational data, Asp308, Thr312, Ser507, Ala510, and Phe619 are defined to be important for agonist binding and receptor activation. Asp308 is supposed to form a salt bridge with the ammonium nitrogen of the ligand. Thr312 and Ser507 are supposed to form a hydrogen bond. Ala510 is supposed to perform a hydrophobic interaction with *N*-alkyl substituents in some tryptamine derivatives. Phe619 is assumed to recognise an aromatic ring of the ligand. From these structure–activity relationships and mutational data, an interaction model between the 5-HT_4 receptor and compound **24** was proposed : *(figure 6)* the ammonium nitrogen in the piperidine ring forms a salt bridge with Asp308. The 2-methoxy group forms an intramolecular hydrogen bond with the NH group in the benzamide and constrains the conformation of the benzamide group. The 5-chloro group performs a hydrophobic interaction with Ala510. The 4-amino group forms a hydrogen bond with Ser507. The methylsulfonylamino group forms a hydrogen bond with Thr312. Furthermore, aromatic residues such as Phe515, Phe612, Trp616, Phe619, and Phe620 are presumed to locate near the ligand and they are supposed to form an aromatic binding pocket or stabilize the positive charge of the cationic amine. As for Trp409, there may be a hydrogen bond to the CO group in the benzamide.

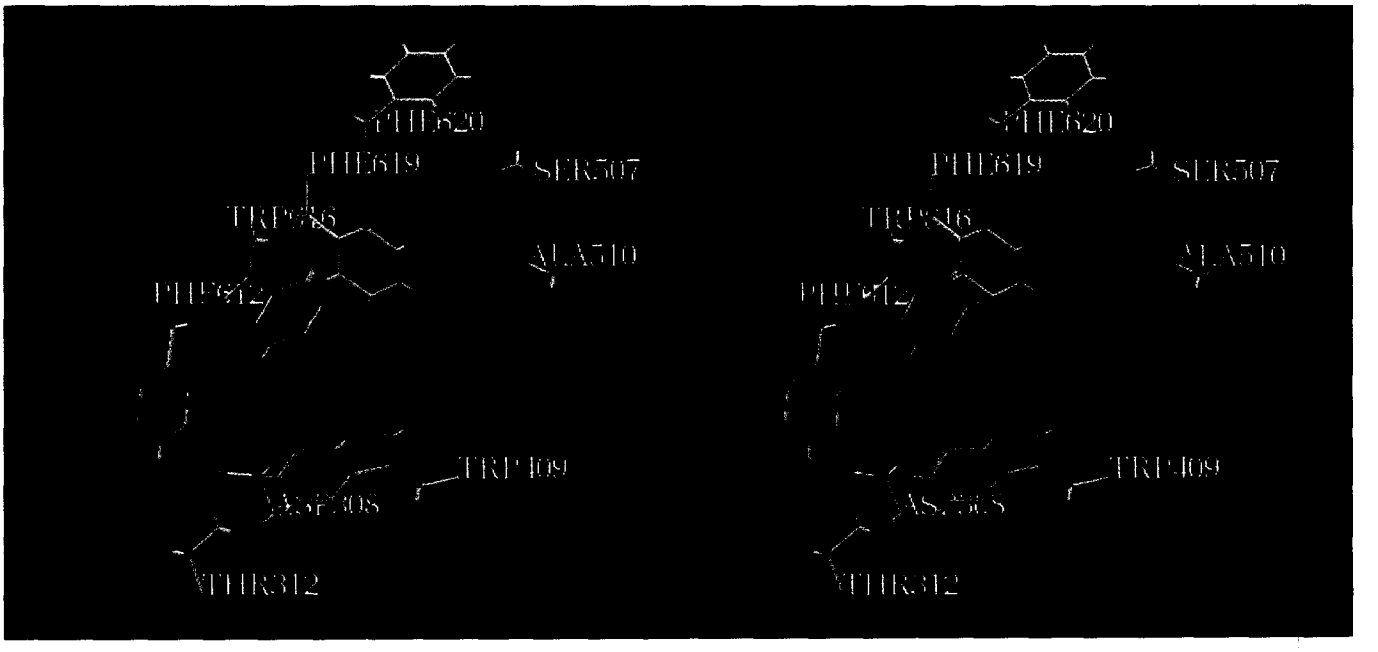


Figure 6. Stereoview of the interaction of compound **24** with the 5-HT_4 receptor.

4. Conclusion

We describe the synthesis and biological evaluation of a series of carboxamides as selective 5-HT_4 receptor agonists. Based on our results, we propose that 4-amino-5-chloro-2-methoxy-*N*-[(4-piperidinyl)methyl]benzamide with a polar group at the 1-position on piperidine moieties are necessary for the selective agonistic activity for 5-HT_4 receptors. Compound **24** represents a selective 5-HT_4 agonist and would be a useful tool for probing the 5-HT_4 receptor function in vivo. Detailed analyses of structure–activity relationships for the side chain moieties will be reported in due course.

5. Experimental protocols

5.1. Chemistry

All melting points were measured in open capillaries and uncorrected. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on Jeol JNM-EX270 spectrometers and chemical shifts are expressed in ppm with tetramethylsilane (TMS) as an internal standard. Signal multiplicities are represented by s (singlet), d (doublet), t (triplet), q (quartet), br-s (broad singlet) and m (multiplet). Mass spectra (MS) were taken on Jeol JMS-O1SG spectrometers. Elementary analysis was performed for C, H and N, and were within 0.4% of the calculated values. Silica-gel plates (Merck F254) and silica gel 60 (Merck, 70–230 mesh) were used for analytical and preparative column chromatography, respectively.

5.1.1. General procedure for the preparation of **7–11** *(method A)*

5.1.1.1. N-(1-Benzyl-3-pyrrolidinyl)-6-chloro-3,4-dihydro-4-methyl-2H-1,4-benzoxazine-8-carboxamide **7**

Isobutyl chloroformate (1.2 g, 9.7 mmol) was added to a mixture of **2a** (2.0 g, 8.8 mmol), triethylamine (2.0 g, 19 mol), and ethyl acetate (40 mL) at −10 °C. The mixture was stirred below −5 °C for 30 min and a solution of **4b** (1.5 g, 8.8 mmol) in ethyl acetate (10 mL) was added with stirring at −10 °C. Stirring was continued at the same temperature for 30 min. The resulting mixture was added to water and extracted with ethyl acetate. The extract was washed with brine, and dried over anhydrous magnesium sulfate. After evaporation in vacuo, the residue was chromatographed on silica gel ($CHCl_3$:MeOH = 10:1). Recrystallized from ethyl acetate/diisopropylether to give **7** (0.50 g, 15%); ^{1}H-NMR ($CDCl_3$) δ: 1.70–1.82 (2H, m), 2.64 (2H, d, J = 11 Hz), 2.90 (3H, s), 3.35 (2H, t, J = 5.6 Hz), 3.55 (1H, d, J = 11 Hz), 3.70 (1H, d, J = 11 Hz), 4.35 (2H, t, J = 5.6 Hz), 4.44–4.80 (1H, m), 6.62 (1H, d, J = 3.0 Hz), 7.20–7.30 (5H, m), 7.39 (1H, d, J = 3.0 Hz), 7.95 (1H, br-s); MS m/z: 385 (M^+).

5.1.1.2. N-(1-Benzyl-4-piperidinyl)-6-chloro-3,4-dihydro-4-methyl-2H-1,4-benzoxazine-8-carboxamide fumalate **8**

Similarly to **7**, **8** was prepared starting from **2a** (3.4 g, 15 mmol), triethylamine (3.3 g, 33 mmol), isobutyl chloroformate (2.3 g, 17 mmol), ethyl acetate (70 mL), and **6e** (3.0 g, 15 mmol). The resulting oil was transformed into fumalate and recrystallized from ethanol to give **8** (3.4 g, 55%); ^{1}H-NMR (DMSO-d_6) δ: 1.54 (2H, dd, J = 12, 24 Hz), 1.82 (2H, d, J = 12 Hz), 2.20–2.39 (2H, t, J = 12 Hz), 2.80 (2H, d, J = 12 Hz), 2.87 (3H, s), 3.29 (2H, t, J = 4.0 Hz), 3.50 (2H, s), 3.70–3.83 (1H, m), 4.29 (2H, t, J = 4.0 Hz), 6.60 (2H, s), 6.75 (1H, d, J = 3.0 Hz), 6.82 (1H, d, J = 3.0 Hz), 7.22–7.38 (5H, m), 7.98 (1H, d, J = 7.2 Hz); MS m/z: 399 (M^+).

5.1.1.3. N-[(1-Benzyl-3-pyrrolidinyl)methyl]-6-chloro-3,4-dihydro-4-methyl-2H-1,4-benzoxazine-8-carboxamide oxalate **9**

Similarly to **7**, **9** was prepared starting from **2a** (1.8 g, 7.9 mmol), triethylamine (1.6 g, 16 mmol), isobutyl chloroformate (1.1 g, 7.9 mmol), ethyl acetate (30 mL), and **4a** (1.5 g, 7.9 mmol). The resulting oil was transformed into oxalate and recrystallized from methanol to give **9** (2.4 g, 62%); ^{1}H-NMR (DMSO-d_6) δ: 1.60–2.22 (2H, m), 2.64–2.75 (2H, m), 2.80–2.92 (2H, m), 2.90 (3H, s), 3.17 (2H, d, J = 5.9 Hz), 3.55 (2H, s), 3.78–3.95 (1H, m), 4.21 (2H, t, J = 4.0 Hz), 4.29 (2H, t, J = 4.0 Hz), 6.74 (1H, d, J = 3.0 Hz), 6.78 (1H, d, J = 3.0 Hz), 7.25–7.60 (5H, m), 8.28 (1H, t, J = 6.0 Hz); MS m/z: 399 (M^+).

5.1.1.4. N-[(1-Benzyl-4-piperidinyl)methyl]-6-chloro-3,4-dihydro-4-methyl-2H-1,4-benzoxazine-8-carboxamide **10**

Similarly to **7**, **10** was prepared starting from **2a** (1.2 g, 5.3 mmol), triethylamine(0.80 g, 8.0 mmol), isobutyl chloroformate (0.67 g, 5.3 mmol), ethyl acetate (25 mL), and **6a** (1.0 g, 5.3 mmol). The resulting solid was recrystallized from ethyl acetate to give **10** (1.4 g, 64%); ^{1}H-NMR ($CDCl_3$) δ: 1.23–1.32 (2H, m), 1.55 (1H, br-s), 1.73 (2H, d, J = 11 Hz), 2.00–2.42 (2H, m), 2.80 (2H, d, J = 11 Hz), 2.90 (3H, s), 3.30 (2H, t, J = 5.6 Hz), 3.35 (2H, d, J = 11 Hz), 3.50 (2H, s), 4.35 (2H, t, J = 5.6 Hz), 6.64 (1H, d, J = 3.0 Hz), 7.20–7.30 (5H, m), 7.42 (1H, d, J = 3.0 Hz), 7.69 (1H, br-s); MS m/z: 413 (M^+).

5.1.1.5. N-[2-(1-Benzyl-4-piperidinyl)ethyl]-6-chloro-3,4-dihydro-4-methyl-2H-1,4-benzoxazine-8-carboxamide oxalate **11**

Similarly to **7**, **11** was prepared starting from **2a** (1.0 g, 4.6 mmol), triethylamine (1.0 g, 10 mmol), isobutyl chloroformate (0.70 g, 5.1 mmol), ethyl acetate (50 mL), and **6f** (1.0 g, 4.6 mmol). The resulting oil was transformed into oxalate and recrystallized from ethanol to give **11** (0.56 g, 24%); ^{1}H-NMR (DMSO-d_6) δ: 1.32–1.60 (4H, m), 1.84 (2H, d, J = 12 Hz), 2.80 (2H, d, J = 12 Hz), 2.84 (2H, t, J = 11 Hz), 2.87 (3H, s), 3.29 (5H, m), 4.17 (2H, s), 4.28 (2H, t, J = 4.6 Hz), 6.73 (1H, d, J = 2.7 Hz), 6.82 (1H, d, J = 2.7 Hz), 7.41–7.49 (5H, m), 8.12 (1H, t, J = 5.2 Hz); MS m/z: 427 (M^+).

5.1.2. General procedure for the preparation of **12–24** *(method B)*

5.1.2.1. 4-Amino-N-[(1-benzyl-3-pyrrolidinyl)methyl]-5-chloro-2-methoxybenzamide oxalate **13**

A mixture of **2c** (1.6 g, 7.9 mmol), **4a** (1.5 g, 7.9 mmol), 1-hydroxybenzotriazole (HOBt) (1.1 g, 8.1 mmol), and dimethylformamide (50 mL) was stirred under ice-cooling for 1 h and then 1-ethyl-3-[3-(dimethylamino)propyl] carbodiimide hydrochloride (WSC) (2.3 g, 8.4 mmol) was added at the same temperature. Stirring was continued overnight at room temperature. After evaporation, 5% aqueous sodium bicarbonate was added to the residue and extracted with ethyl acetate. The extraction was washed with brine, and was dried over anhydrous magnesium sulfate. After evaporation in vacuo, the residue was dissolved in ethanol, an alcoholic solution of oxalic acid (2.5 equiv.) was added. The precipitates were collected and recrystallized from ethanol to give **13** (1.9 g, 52%); ^{1}H-NMR (DMSO-d_6) δ: 1.60–2.22 (2H, m), 2.64–2.75 (2H, m), 2.80–2.92 (3H, m), 3.17 (2H, d, J = 5.9 Hz), 3.55 (2H, s), 3.80 (3H, s), 4.23 (2H, br-s), 6.48 (1H, s), 7.25–7.55 (5H, m), 7.62 (1H, s), 8.02 (1H, t, J = 6.0 Hz); MS m/z: 373 (M^+).

5.1.2.2. N-[(1-Benzyl-4-piperidinyl)methyl]-5-chloro-3,4-dihydro-1,4-benzofuran-7-carboxamide oxalate **12**

Similarly to **13**, **12** was prepared starting from **2b** (0.50 g, 2.5 mmol), **6a** (0.49 g, 2.5mmol), HOBt (0.33 g, 2.5 mmol), dimethylformamide (20 mL), and WSC (0.47g, 2.5 mmol). The resulting oil was transformed into oxalate and recrystallized from isopropanol to give **12** (0.89 g, 77%), ^{1}H-NMR ($CDCl_3$) δ: 1.65–2.02 (6H, m), 2.45–2.60 (2H, br-s), 3.16 (2H, t, J = 6.0 Hz), 3.30 (2H, br-s), 3.58 (2H, br-s), 4.18 (2H, br-s), 4.72 (2H, t, J = 6.0 Hz), 7.30–7.42 (5H, m), 7.62 (1H, t, J = 6.0 Hz), 8.82 (1H, d, J = 2.0 Hz); MS m/z: 384 (M^+).

5.1.2.3. 4-Amino-N-[(1-benzyl-4-piperidinyl)methyl]-5-chloro-2-methoxybenzamide **14**

Similarly to **13**, **14** was prepared starting from **2c** (1.0 g, 5.0 mmol), **6a** (1.0 g, 5.0 mmol), HOBt (0.86 g, 6.4 mmol), dimethylformamide (30 mL), and WSC (1.1 g, 5.7 mmol). The resulting solid was recrystallized from ethyl acetate to give **14** (1.4 g, 72%); ^{1}H-NMR ($CDCl_3$) δ: 1.35–1.55 (5H, m), 2.00 (2H, d, J = 12 Hz), 2.90 (2H, d, J = 12 Hz), 3.30 (2H, t, J = 5.6 Hz), 3.50 (2H, s), 3.92 (3H, s), 4.38 (2H, br-s), 7.22–7.38 (5H, m), 7.70 (1H, br-s), 8.10 (1H, s); MS m/z: 387 (M^+).

5.1.2.4. 4-Amino-N-[2-(1-benzyl-4-piperidinyl)ethyl]-5-chloro-2-methoxybenzamide oxalate **15**

Similarly to **13**, **15** was prepared starting from **2c** (0.93 g, 4.6 mmol), **6f** (1.0 g, 4.6 mmol), HOBt (0.65 g, 0.48 mmol), dimethylformamide (30 mL), and WSC (0.92 g, 4.8 mmol). The resulting oil was transformed into oxalate and recrystallized from ethanol to give **15** (0.56 g, 24%); ^{1}H-NMR (DMSO-d_6) δ: 1.35–1.55 (5H, m), 1.85 (2H, d, J = 12 Hz), 2.75 (2H, t, J = 6.8 Hz), 3.19–3.30 (4H, m), 3.88 (3H, s), 4.14 (2H, s), 5.91 (2H, br-s), 6.47 (1H, s), 7.41–7.47 (5H, m), 7.66 (1H, s), 7.89 (1H, t, J = *5.9Hz*); MS m/z: 401 (M^+).

5.1.2.5. N-[(1-Benzyl-4-piperidinyl)methyl]-2-methoxybenzamide oxalate **16**

Similarly to **13**, **16** was prepared starting from **2d** (0.74 g, 4.9 mmol), **6a** (1.0 g, 4.9 mmol), HOBt (0.73 g, 5.4 mmol), dimethylformamide (30 mL), and WSC (1.0 g, 5.4 mmol). The resulting oil was transformed into oxalate and recrystallized from ethanol to give **16** (1.5 g, 71%); ^{1}H-NMR (DMSO-d_6) δ: 1.44 (2H, dd, J = 12, 24 Hz), 1.70–1.80 (1H, m), 1.82 (2H, d, J = 12 Hz), 2.78 (2H, t, J = 12 Hz), 3.17–3.24 (4H, m), 3.85 (3H, s), 4.15 (2H, s), 6.76 (1H, d, J = 8.0 Hz), 7.01 (1H, d, J = 7.2 Hz), 7.43–7.50 (6H, m), 7.54 (1H, dd, J = 2.0, 7.2 Hz), 8.22 (1H, t, J = 6.0 Hz); MS m/z: 338 (M^+).

5.1.2.6. N-[(1-Benzyl-4-piperidinyl)methyl]-5-chloro-2-methoxybenzamide oxalate **17**

Similarly to **13**, **17** was prepared starting from **2e** (0.82 g, 4.4 mmol), **6a** (0.90 g, 4.4 mmol), HOBt (0.66 g, 4.8 mmol), dimethylformamide (30 mL), and WSC (0.93 g, 4.8 mmol). The resulting oil was transformed into oxalate and recrystallized from ethanol to give **17** (0.27 g, 16%); ^{1}H-NMR ($CDCl_3$-CD_3OD) δ: 1.69 (2H, dd, J = 12, 24 Hz), 1.93 (2H, d, J = 12 Hz), 2.00 (1H, m), 2.78 (2H, br-s), 3.32 (2H, d, J = 6.6 Hz), 3.48 (2H, d, J = 12 Hz), 3.96(3H, s), 4.24 (2H, s), 6.97 (1H, d, J = 10 Hz), 7.41 (1H, dd, J = 2.7, 10 Hz), 7.50–7.62 (5H, m), 8.00 (1H, d, J = 2.7 Hz), 7.93 (1H, br-s); MS m/z: 372 (M^+).

5.1.2.7. 4-Amino-N-[(1-benzyl-4-piperidinyl)methyl]-5-chlorobenzamide oxalate **18**

Similarly to **13**, **18** was prepared starting from **2f** (0.76 g, 4.4 mmol), **6a** (0.90 g, 4.4 mmol), HOBt (0.66 g, 4.8 mmol), dimethylformamide (30 mL), and WSC (0.93 g, 4.8 mmol). The resulting oil was transformed into oxalate and recrystallized from ethanol to give **18** (0.41 g, 21%); ^{1}H-NMR ($CDCl_3$-CD_3OD) δ: 1.74 (2H, dd, J = 12, 24 Hz), 1.90 (2H, d, J = 12 Hz), 1.98 (1H, m), 2.74 (2H, br-s), 3.24 (2H, d, J = 6.6 Hz), 3.44 (2H, d, J = 12 Hz), 4.26 (2H, s), 6.76 (1H, d, J = 10 Hz), 7.32–7.48 (5H, m), 7.54 (1H, dd, J = 2.7, 10 Hz), 7.79 (1H, d, J = 2.7 Hz), 7.93 (1H, br-s); MS m/z: 357 (M^+).

5.1.2.8. 4-Amino-N-[(1-benzyl-4-piperidinyl)methyl]-2-methoxybenzamide oxalate **19**

Similarly to **13**, **19** was prepared starting from **2g** (0.82 g, 4.9 mmol), **6a** (1.0 g, 4.9 mmol), HOBt (0.73 g, 5.4 mmol), dimethylformamide (30 mL), and WSC (1.0 g, 5.4 mmol). The resulting oil was transformed into oxalate and recrystallized from ethanol to give **19** (0.49 g, 23%); ^{1}H-NMR (DMSO-d_6) δ: 1.43 (2H, t, J = 12 Hz), 1.70 (1H, br-s), 1.77 (2H, d, J = 12 Hz), 2.78 (2H, t, J = 12 Hz), 3.18 (2H, t, J = 5.9 Hz), 3.25 (2H, d, J = 12 Hz), 3.80 (3H, s), 4.16 (2H, br-s), 6.16 (1H, dd, J = 2.0, 8.0 Hz), 6.22 (1H, J = 2.0 Hz), 7.46 (5H, m), 7.59 (1H, d, J = 8.0 Hz), 7.90 (1H, t, J = 6.0 Hz); MS m/z: 353 (M^+).

5.1.2.9. N-[(1-Benzyl-4-piperidinyl)methyl]-5-chloro-2-methoxy-4-methylaminobenzamide fumalate **20**

Similarly to **13**, **20** was prepared starting from **2h** (1.2 g, 5.6 mmol), **6a** (1.1 g, 5.6 mmol), HOBt (0.84 g, 6.2 mmol), dimethylformamide (30 mL), and WSC (1.2 g, 6.2 mmol). The resulting oil was transformed into fumalate and recrystallized from ethanol/acetone to give **20** (0.31 g, 14%); ^{1}H-NMR (DMSO-d6) δ: 1.27 (2H, dd, J = 12, 21 Hz), 1.59 (1H, m), 1.65 (2H, d, J = 12 Hz), 2.21 (2H, t, J = 11 Hz), 2.83 (3H, d, J = 4.7 Hz), 2.95 (2H, d, J = 11 Hz), 3.18 (2H, t, J = 6.0 Hz), 3.69 (2H, s), 3.93 (3H, s), 6.50 (1H, q, J = 4.7 Hz), 6.22 (1H, s), 6.59 (2H, s), 7.31–7.70 (5H, m), 7.93 (1H, t, J = 6.0Hz); MS m/z: 401 (M^+).

5.1.2.10. 4-Amino-5-chloro-N-[(1-ethyl-4-piperidinyl)methyl]-2-methoxybenzamide **21**

Similarly to **13**, **21** was prepared starting from **2c** (2.0 g, 9.9mmol), **6b** (1.4 g, 9.9 mmol), HOBt (1.7 g, 13 mmol), dimethylformamide (30 mL), and WSC (2.3 g, 1.2 mmol). The resulting solid was recrystallized from ethyl acetate to give **21** (2.0 g, 62%); ^{1}H-NMR ($CDCl_3$) δ: 1.14 (3H, t, J = 12 Hz), 2.20–3.05 (7H, m), 2.20 (2H, q, J = 12 Hz), 2.92 (2H, d, J = 12 Hz), 3.32 (2H, t, J = 6.6 Hz), 3.88 (3H, s), 4.38 (2H, br-s), 6.23 (1H, s), 7.68 (1H, br-s), 8.03 (1H, s); MS m/z: 325 (M^+).

5.1.2.11. 4-Amino-N-[(1-butyl-4-piperidinyl)methyl]-5-chloro-2-methoxybenzamide oxalate **22**

Similarly to **13**, **22** was prepared starting from **2c** (0.40 g, 2.0 mmol), **6c** (0.31 g, 2.0 mmol), HOBt (0.29 g, 2.2 mmol), dimethylformamide (10 mL), and WSC (0.42 g, 2.2 mmol). The resulting oil was transformed into oxalate and recrystallized from ethanol to give **22** (0.26 g, 29%); ^{1}H-NMR (CD_3OD) δ: 1.23 (3H, t, J = 8.5 Hz), 1.35–1.42 (2H, m), 1.62 (2h, br-s), 1.70–1.85(2H, m), 1.95 (1H, br-s), 1.97 (2H, d, J = 13Hz), 2.90 (2H, br-s), 3.07 (2H, t, J = 8.5 Hz), 3.31 (2H, br-s), 3.35 (2H, br-s), 3.91 (3H, s), 6.23 (1H, s), 7.78 (1H, s); MS m/z: 353 (M^+).

5.1.2.12. 4-Amino-5-chloro-N-[(1-hexyl-4-piperidinyl)methyl]-2-methoxybenzamide **23**

Similarly to **13**, **23** was prepared starting from **2c** (1.7 g, 8.4 mmol), **6d** (1.5 g, 8.4 mmol), HOBt (1.5 g, 11 mmol), dimethylformamide (30 mL), and WSC (2.1 g, 11 mmol). The resulting solid was recrystallized from ethyl acetate/diisopropylether to give **23** (2.1 g, 66%); ^{1}H-NMR ($CDCl_3$) δ: 0.83 (3H, t, J = 12 Hz), 1.20–2.05 (15H, m), 3.30 (2H, t, J = 12, 24 Hz), 3.92 (2H, d, J = 12 Hz), 3.33 (2H, t, J = 6.6 Hz), 3.90 (3H, s), 4.38 (2H, br-s), 6.28 (1H, s), 7.70 (1H, br-s), 8.10 (1H, s); MS m/z: 381 (M^+).

5.1.2.13. 4-Amino-5-chloro-N-[[2-[(methylsulfonyl)amino]ethyl]-4-piperidinyl]-2-methoxybenzamide oxalate **24**

Similarly to **13**, **24** was prepared starting from **2c** (2.0 g, 9.9 mmol), **6g** (2.3 g, 9.9 mmol), HOBt (1.6 g, 12 mmol), dimethylformamide (50 mL), and WSC (2.3 g, 12 mmol). The resulting oil was transformed into oxalate and recrystallized from ethanol to give **24** (1.3 g, 26%); ^{1}H-NMR (DMSO-d_6) δ: 1.20–2.00 (5H, m), 2.60–3.80 (13H, m), 2.96 (3H, s), 3.79 (3H, s), 4.38 (2H, br-s), 6.42 (1H, s), 7.60 (1H, s), 7.94 (1H, t, J = 6.0 Hz); MS m/z: 418 (M^+).

5.1.3. 1-Benzyl-3-pyrrolidinylmethylamine **4a**

A mixture of **3** (5.0 g, 23 mmol), triphenylphosphine (6.6 g, 25 mmol), phthalimide (3.7 g, 25 mmol) and tetrahydrofuran (30 mL) was stirred under ice-cooling and then diethyl azodicarboxylate (3.6 mL, 23 mmol) was added at the same temperature. Stirring was continued overnight at room temperature. After evaporation, the residue was dissolved in ethyl alcohol and then hydrazine monohydrate (3.3 mL, 68.4 mmol) was added. The mixture was refluxed for 2 h. After cooling, the reaction

mixture was filtered by suction though celite and to the filtrate was added diethylether. The precipitate was filtered off and filtrate was extracted with 10% hydrochloric acid. The aqueous layer was basified by 10% NaOH and extracted with chloroform. The extraction was washed with brine, and was dried over anhydrous magnesium sulfate. After evaporation in vacuo to give **4a** (2.8 g, 56%); ^{1}H-NMR ($CDCl_3$) δ: 1.10–1.29 (2H, m), 1.38–1.52 (1H, m), 1.55 (2H, t, J = 7.2 Hz), 1.63 (2H, d, J = 6.6 Hz), 1.92 (2H, dt, J = 1.0, 12 Hz), 2.50 (2H, br-s), 2.72 (2H, t, J = 7.2 Hz), 2.82 (2H, d, J = 12 Hz), 3.61 (2H, s), 7.12–7.23 (5H, m); MS m/z: 218 (M^+).

5.1.4. General procedure for the preparation of **6a–6d**

5.1.4.1. 4-Aminomethyl-1-benzylpipeidine **6a**

A solution of **5a** (5.7 g, 26 mmol) in tetrahydrofuran (50 mL) was added dropwise to a suspension of lithium aluminium hydride ($LiAlH_4$) (2.0 g, 52 mmol) in tetrahydrofuran (50 mL) at 0 °C, and the mixture was heated at 45 °C with stirring for 4 h. After cooling, water was added dropwise to the mixture at a temperature below 0 °C to destroy the excess $LiAlH_4$. After filtration, the filtrate was dried over anhydrous magnesium sulfate, and after evaporation in vacuo, the residue was distilled under reduced pressure to give **6a** (5.0 g, 94%), b.p. 125 °C/1.0 mmHg; ^{1}H-NMR ($CDCl_3$) δ: 1.28 (2H, dd, J = 4.0, 12 Hz), 1.30 (2H, br-s), 1.35–1.50 (1H, m), 1.72 (2H, d, J = 6.6 Hz), 1.92 (2H, dt, J = 2.0, 6.6 Hz), 2.64 (2H, d, J = 6.6 Hz), 2.87 (2H, d, J = 6.6Hz), 3.00 (2H, t, J = 6.6 Hz), 7.18–7.35 (5H, m); MS m/z: 142 (M^+).

The other piperidines (**6b–6d**) were prepared in a similar manner.

5.2. Pharmacology

5.2.1. Radioligand binding assays

5.2.1.1. 5-HT_4 receptor

Male Hartley guinea-pigs (Japan SLC, Ltd., Shizuoka, Japan) were sacrificed by cervical dislocation and the striatum was separated from each brain. The striatum was homogenized in 15 volumes of 50 mmol/L ice-cold HEPES buffer (pH 7.4) with Polytron PT-10 and then centrifuged at 35 000 × g for 20 min. The resulting pellet was resuspended in the HEPES buffer and finally diluted to the appropriate concentration for assay (6 mg wet weight per assay tube). This suspension was used as the tissue preparation. Assay tubes contained 50 μL of HEPES buffer or a solution of the test agents, 50 μL solution of [^{3}H]GR113808 (Amersham International, UK) to give a final concentration of 0.1 nmol/L and 900 μL of tissue preparation. Each tube was incubated for 30 min at 37 °C and the reaction was terminated by rapid filtration through a Whatmann GF/B filter (presoaked in 0.01% v/v polyethyleneimine) followed by washing with 1 × 4 mL of ice-cold HEPES buffer. Then the filter was placed in 3 mL of scintillator and the radioactivity was determined by scintillation counting in a Beckman model LS3801 scintillation counter. Non specific binding was defined in the presence of unlabelled GR113808 to give a final concentration of 1 μmol/L. The IC_{50} value was determined by non-linear regression of the displacement curve, and the Ki value was calculated according to the formula (Ki = IC_{50}/(1 + L/Kd)), where L is the concentration of radioligand and Kd is the dissociation constant of the radioligand.

5.2.1.2. 5-HT_3 receptor

[^{3}H]Granisetron binding assays were performed according to the method of Nelson and Thomas [34]. Male Wistar rat (Japan SLC, Ltd., Shizuoka, Japan) cerebral cortex was homogenized in 20 volumes of 0.32 mol/L sucrose and the centrifuged at 1 000 × g for 10 min. The supernatant was centrifuged at 40 000 × g for 15 min. The pellet was suspended in 20 volumes of HEPES buffer (50 mmol/L, pH 7.4) and the suspension was incubated at 37 °C for 10 min and centrifuged at 40 000 × g for 15 min. The pellet was washed and centrifuged (40 000 × g for 15 min). The final pellet was resuspended in 30 volumes of HEPES buffer and used as tissue homogenate. The binding assay consisted of 50 μmol/L of [^{3}H]Granisetron, 50 μL of displacing drugs and 900 μL of tissue homogenate. Following a 30 min incubation at 25 °C, the assay mixture was rapidly filtered under reduced pressure through Whatman GF/B glass filters which had been presoaked in 0.1% polyethyleneimie. Filters were washed immediately with 3 × 3 mL of ice-cold Tris-HCl buffer (50 mM, pH 7.4). ICS 205930 (100 mmol/L) was used for the determination of nonspecific binding.

5.2.1.3. D_2 receptor

[^{3}H]Spiperone binding assays were performed according to the method of Crees et al. Male Wistar rat (Japan SLC, Ltd., Shizuoka, Japan) striatal membrane was homogenized in 100 volumes of ice-cold Tris-HCl buffer (50 mmol/L, pH 7.7) and centrifuged (500 × g, 10 min, 0 °C). The supernatant was centrifuged at 50 000 × g for 15 min. The pellet was suspended in 100 volumes of ice-cold Tris-HCl buffer (50 mmol/L, pH 7.7) and recentrifuged (500 × g, 10 min, 0 °C). The final pellet was resuspended in 150 volumes (50 mmol/L, pH 7.7) containing 120 mmol/L NaCl, 5 mmol/L KCl, 2 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 1.1 mmol/L ascorbic acid and 10 μmol/L pargyline, and incubated at 37 °C for 10 min.

A portion of this membrane suspension (900 μmol/L) was placed in a tube, and 50 μmol/L of either test compound or vehicle solution was added, followed by 50 μL of [^{3}H]Spiperone (40 Ci/mmol) at a final concentration of 0.2 nmol/L. The tubes were incubated at 37 °C for 20 min and filtered through Whatman GF/B glass filters, which were then washed three times with 3 mL of Tris-HCl buffer (50 mmol/L, pH 7.7). Sulpiride (100 μmol/L) was used for the determination of nonspecific binding. The radioactivity trapped on the filters was measured by liquid scintillation spectrometry.

5.2.2. Contractile effects

5.2.2.1. 5-HT$_4$ receptor agonism

Male Hartley guinea-pigs (Japan SLC, Ltd., Shizuoka, Japan) were killed by cervical dislocation and the ascending colon (a 10 cm segment starting 1 cm from the caecum) was removed. The longitudinal muscle layer was separated from the underlying circular muscle and divided into four segments of about 2.5 cm. Four muscle strip preparations were individually mounted vertically for isotonic measurement into a tissue bath containing 10 mL Tyrode solution. Only 5-HT was tested in the Tyrode solution containing methysergide (1 μmol/L) and granisetron (1 μmol/L) to inhibit responses mediated by 5-HT$_2$ and 5-HT$_1$-like and 5-HT$_3$ receptors, respectively. This solution was kept at 37 °C and gassed with 95% O_2, 5% CO_2. The strips were subjected to a preload of 1 g and allowed to stabilize for 20 min. After stabilization, the response of the longitudinal muscle to 30 μmol/L methacholine was measured. Agonist concentration-effect curves were constructed using sequential dosing, leaving 15 min between doses. A 15 min dosing cycle was required to prevent desensitization. The agonist was left in contact with a preparation until the response had reached a maximum, the preparation was washed. Forty minutes was left between the determination of concentration-effect curves. GR113808 (10 nmol/L) were incubated for 10 min before repeating agonist concentration-effect curves. After each determination of concentration-effect curve, 30 μmol/L of methacholine was added to the tissue bath again. All responses were expressed as a percentage of the mean of the two contractions induced by 30 μmol/L methacholine. The EC_{50} value, the concentration causing 50% of the maximal response, was determined by linear regression analysis.

5.2.2.2. 5-HT$_4$ receptor antagonism

5-HT$_4$ receptor antagonism was expressed in the form of pK_B value on the contractile response to 5-MeOT.

5.3. Construction of receptor model

5.3.1. Sequence

The sequence in this study, except for the 5-HT$_4$ receptor, was from the Swiss-Prot Protein Sequence Data Bank [35]. The coordinates of Bacteriorhodopsin (entry 1BRD) [36] were from the Protein Data Bank [37] at Brookhaven National Laboratory. Modelling was achieved with the molecular package SYBYL 6.2 [38]. The interactive modelling and display were performed on a Silicon Graphics IRIS INDIGO/Elan 4 000 computer. Five main steps were used: stretch of α-helical structure where putative transmembrane region is longer than bacteriorhodopsin, amino acid substitution, local geometry optimization, docking of ligand, and side-chain rotation to minimize overlaps between helices. Some manual adjustments were made to remove bad steric interactions in geometry optimization. In Energy minimization procedure, the backbone was aggregated until the RMS gradient was less than 0.05 (kcal/mol Å^2). A dielectric constant of compound **24** was calculated where a cut off distance of 8 Å was used and no solvent molecules were included in the calculation.

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