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Synthesis and pharmacological properties of benzamide derivatives as selective serotonin 4 receptor agonists

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Abstract—A series of 4-amino-5-chloro-2-methoxy-*N*-(piperidin-4-ylmethyl)benzamides with a polar substituent group at the 1-position of the piperidine ring was synthesized and evaluated for its effect on gastrointestinal motility. The benzoyl, phenylsulfonyl, and benzylsulfonyl derivatives accelerated gastric emptying and increased the frequency of defecation. One of them, 4-amino-*N*-[1-[3-(benzylsulfonyl)propyl]piperidin-4-ylmethyl]-5-chloro-2-methoxybenzamide (**13a**, **Y-36912**), was a selective 5-HT_4 receptor agonist offering potential as a novel prokinetic with reduced side effects derived from 5-HT_3- and dopamine D_2 receptor-binding affinity. In the oral route of administration, this compound enhanced gastric emptying and defecation in mice, and has a possibility as a prokinetic agent, which is effective on both the upper and the lower gastrointestinal tract.

1. Introduction

Serotonin (5-HT) is a neurotransmitter responsible for a wide range of pharmacological reactions. Serotonergic receptors are currently classified into four subtypes, 5-HT_1, 5-HT_2, 5-HT_3, and 5-HT_4, and clones for the subtypes, termed 5-HT_5, 5-HT_6, and 5-HT_7 receptors, have been identified.[1] Activation of the 5-HT_4 receptor mediates diverse effects in the central and peripheral nervous systems.[2] At the periphery, the receptors play an important role in the response functions of several organs including the gastrointestinal tract. Stimulation of the receptors present in the myenteric nerve causes the release of neurotransmitters from the nerve endings and finally bring about the contraction of the gastrointestinal smooth muscle. Many gastrointestinal prokinetics such as benzamides (e.g., metoclopramide,[3] cisapride[4]) have binding affinity for 5-HT_4 receptors and the pharmacological effect of these compounds is thought to be based on 5-HT_4 receptor agonism.

However, these benzamides are also reported to have binding affinity for dopamine D_2-, 5-HT_2-, and 5-HT_3-receptors.[5] Dopamine D_2 antagonism should cause adverse reactions in the central nervous system such as extrapyramidal syndrome, while 5-HT_3 receptor antagonism should reduce colonic transit in the lower gastrointestine.[6,7]

Accordingly, selective and potent 5-HT_4 receptor agonists would increase both upper and lower gastrointestinal motility and cause little adverse reaction, and are therefore seen as promising new gastroprokinetic candidates.

We have recently reported the pharmacological profile of a series of orally active 4-amino-5-chloro-2-methoxy-*N*-(piperidin-4-ylmethyl)benzamides with a benzoyl or phenylsulfonyl moiety in the side chain part at the

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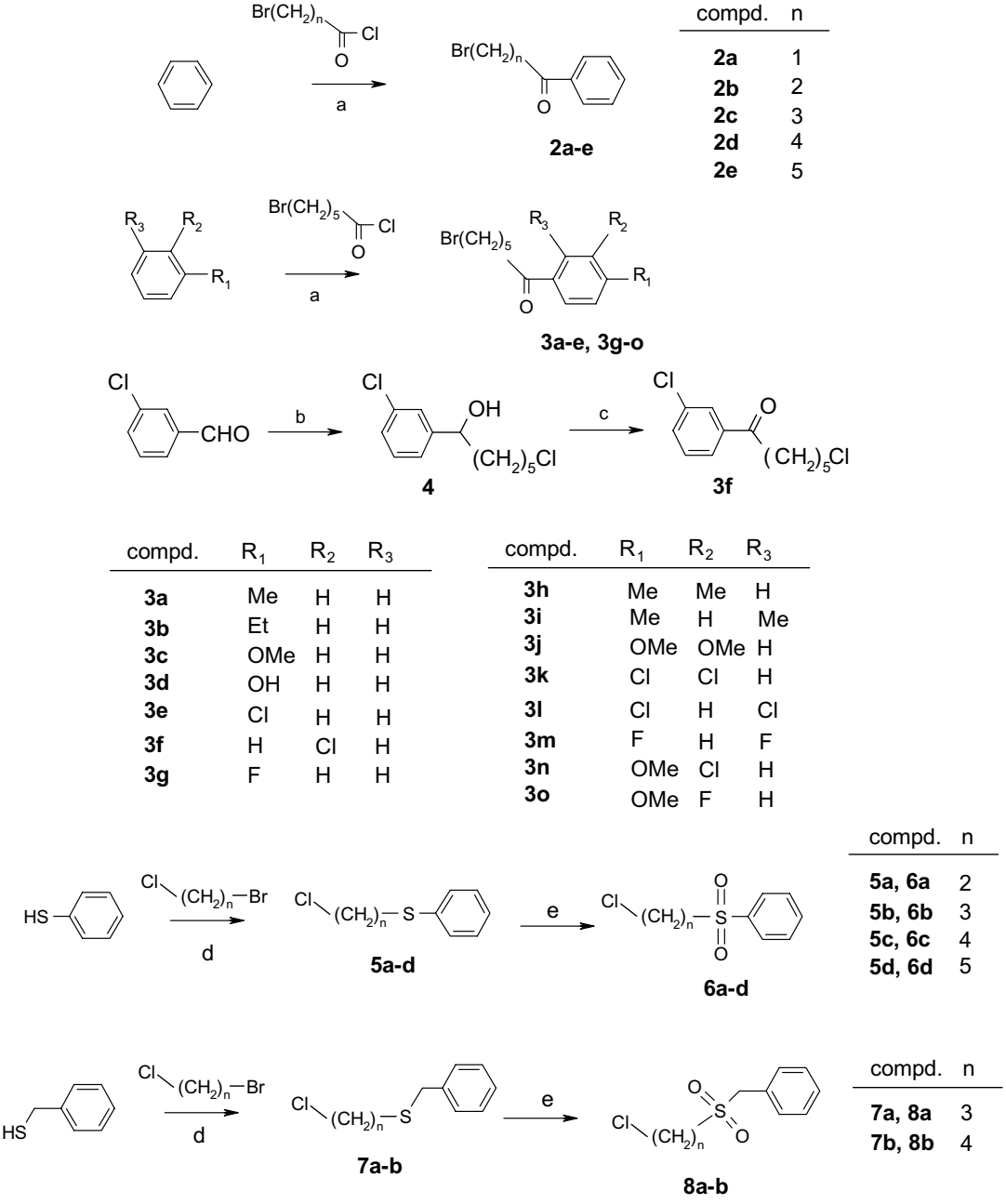
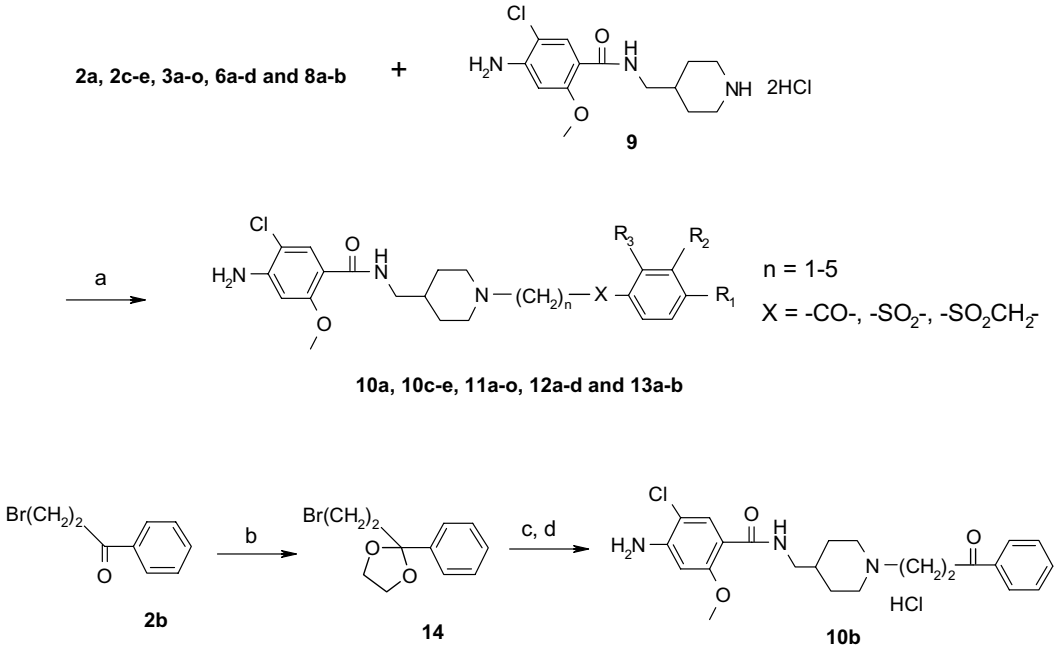
1-position of the piperidine.[8] These compounds were 5-HT_4 receptor agonists with no other receptor binding affinities.[6,7] The present paper describes further studies of benzoyl and phenylsulfonyl (or benzylsulfonyl) derivatives.

2. Chemistry

The general synthetic pathways used for preparation of the benzamides are illustrated in Schemes 1 and 2. The benzoyl derivatives **2a**–**e** and **3a**–**e**, **3g**–**o** were prepared by Friedel–Crafts reaction. The synthesis of the 3-chlorobenzoyl compound **3f** was carried out by oxidation using the MnO_2 of the benzyl alcohol derivative **4**, which was prepared by coupling reaction of 3-chlorobenzaldehyde with Grignard reagent. The phenyl sulfonyl (**6a**–**d**) and benzylsulfonyl (**8a**–**b**) derivatives were prepared by alkylation of benzenethiol or benzyl mercaptan followed by oxidation with H_2O_2/HCOOH. The key intermediate **9** was prepared by a method reported previously.[8] The coupling reaction of compound **9** with **2a**,**c**–**e** and **3a**–**o**, with **6a**–**d**, and with **8a**–**b** in K_2CO_3/DMF gave benzoyl (**10a**, **10c**–**e**, **11a**–**o**), phenylsulfonyl (**12a**–**d**), and benzylsulfonyl (**13a**–**b**) derivatives, respectively. (The preparation of **10e** and **11d** was described previously.[8]) The compound **2b** was protected with an acetal group (**14**) and reacted with the benzamide **9**. The



Scheme 1. Reagents and conditions: (a) $AlCl_3$, CH_2Cl_2, 25 °C; (b) Mg, $Br(CH_2)_5Cl$, 50 °C; (c) MnO_2, 25 °C, 5 days; (d) K_2CO_3/DMF, 50–60 °C; (e) 30% H_2O_2/HCOOH.



Scheme 2. Reagents and conditions: (a) K_2CO_3/DMF, 70–75 °C; (b) ethyleneglycol, *p*-TsOH/benzene, reflux; (c) **9**, K_2CO_3/DMF, 70–75 °C; (d) 1 N HCl.

derivative **10b** was obtained by treatment with hydrochloric acid.

3. Results and discussion

The synthesized compounds were evaluated for their 5-HT_4 receptor-binding affinity by use of [^{3}H]GR-113808 binding assay in guinea-pig striatum membranes[9] and for in vitro 5-HT_4 receptor-agonistic activity (EC_{50} value) by their ability to contract isolated guinea-pig ascending colon.[10] Evaluation for 5-HT_3 receptor-binding affinity was performed in rat cerebrocortical membranes by [^{3}H] Granisetron binding. The kinetic activity on the upper gastrointestinal tract was evaluated by determining the effect of the orally administered compounds on gastric emptying rates of phenol red semisolid meal through the stomach of rats or mice. The effect on lower gastrointestinal motility was evaluated by measuring the increase in defecation following oral administration of the derivatives in mice.

As the first step, we optimized the length of the straight alkyl chain at the 1-position of the piperidine ring of the derivatives with an unsubstituted benzoyl group. Compounds with a straight alkyl chain of from one to five methylenes were prepared; pharmacological data are listed in Table 1. Compounds **10a**–**e** showed high binding affinities for the 5-HT_4 receptor. Regarding effect on gastric emptying, compound **10a** ($n = 1$) was weaker than **10b**–**e** ($n = 2$–5). Compounds **10a** ($n = 1$) and **10c** ($n = 3$) had undesirable binding affinity toward the dopamine D_2 receptor ($K_i = 15$ and 21 nmol/L, respectively).

This observation suggests that compounds **10a** and **10c**, which have a short straight alkyl chain at the 1-position of the piperidine ring, possess dopamine D_2 binding affinity. Compound **10b** ($n = 2$) did not increase defecation in mice at oral doses of 3 mg/kg. Compound **10e** ($n = 5$) having the highest binding affinity ($K_i = 2.4$ nM) for the 5-HT_4 receptor showed the strongest effect on defecation (MED: 0.3 mg/kg). We therefore selected five methylenes as the composition of the straight alkyl chain at the 1-position of the piperidine ring.

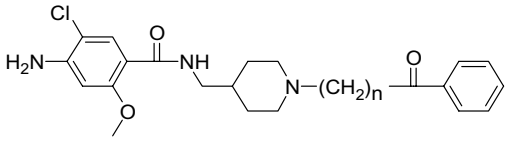
For the further improvement of the compound, we studied the influence of substitution in the benzoyl group. The derivatives were measured for the rate of increase in defecation induced in mice at oral doses of 1 mg/kg as shown in Table 2 (percentage increase in number, dry weight, and wet weight of fecal deposits).

In this evaluation system, compound **10e** significantly increased defecation (respective increases of 49%, 90%, 76% in the three items measured). Introduction of methyl (**11a**: 15%, 45%, 30%), ethyl (**11b**: 39%, 55%, 39%), and fluoro (**11g**: 44%, 59%, 50%) groups at the 4-position of the benzoyl group of **10e** somewhat reduced the effect on defecation.

Compounds substituted with chlorine at the 4-position (**11e**: −5%, 3%, 6%) and the 3,4-position (**11k**: −7%, 4%, 6%) produced almost no increase in defecation. The 3-chloro (**11f**: 23%, 31%, 29%) and 2,4-dichloro (**11l**: 15%, 46%, 47%) derivatives did increase defecation, but the effects were weaker than with compound **10e**. These results suggest that introduction of an electron-withdrawing group into the benzoyl group does not contribute to the effect on defecation.

A compound with a hydroxyl group at the 4-position of the benzoyl (**11d**: 79%, 88%, 68%), like compound **10e**,

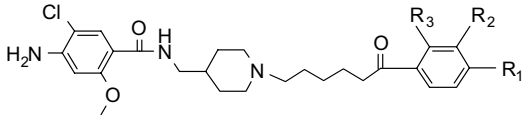
Table 1. Pharmacological data of benzoyl derivatives **10a–e**



Compound no	*n*	Binding affinities[a]			Potency[b]	Gastric emptying	Defecation[d]
		5-HT_4 K_i (nM)	5-HT_3 K_i (nM)	D_2 K_i (nM)	EC_{50} (nM)[c]	MED (mg/kg)	MED (mg/kg)
10a	1	3.0	270	15	NT	10[f]	NT
10b	2	6.1	360	>1000[e]	35	3[f]	>3
10c	3	2.5	>1000[e]	21	10	NT	NT
10d	4	4.0	>1000[e]	>1000[e]	9.1	3[g]	0.3
10e	5	2.4	>1000[e]	>1000[e]	10	3[g]	0.3

MED: minimum effective dose. NT: not tested.
[a] Each value is the mean from triplicate assay in single experiment.
[b] 5-HT_4 receptor agonistic activities; contractile effects in guinea-pig ascending colon.
[c] EC_{50} values were determined by linear regression.
[d] Increase in defecation following oral administration of the derivatives in mice.
[e] IC_{50} value.
[f] Effect of the orally administered compound on gastric emptying rates of phenol red semisolid meal through the stomach of mice.
[g] Effect of the orally administered compound on gastric emptying rates of phenol red semisolid meal through the stomach of rats.

Table 2. Pharmacological data of benzoyl derivatives **10e**, **11a–o**



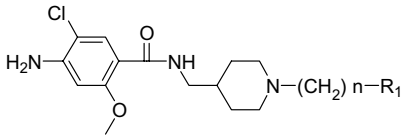
Compound no	R_1	R_2	R_3	Binding affinities[a]		Rate of increased defecation[b] (%)		
				5-HT_4 K_i (nM)	5-HT_3 IC_{50} (nM)	Number	Dry weight	Wet weight
11a	Me	H	H	3.9	>1000	15	45	30
11b	Et	H	H	3.6	>1000	39	55	39
11c	OMe	H	H	1.8	>1000	26	71	48
11d	OH	H	H	1.5	>1000	79	88	68
11e	Cl	H	H	4.6	>1000	−5	3	6
11f	H	Cl	H	2.7	>1000	23	31	29
11g	F	H	H	2.4	>1000	44	59	50
11h	Me	Me	H	2.8	370[c]	15	5	9
11i	Me	H	Me	4.4	>1000	15	15	20
11j	OMe	OMe	H	2.6	>1000	96	106	87
11k	Cl	Cl	H	2.0	>1000	−7	4	6
11l	Cl	H	Cl	2.5	>1000	15	46	47
11m	F	H	F	3.4	>1000	37	59	43
11n	OMe	Cl	H	1.8	>1000	24	67	48
11o	OMe	F	H	5.1	>1000	31	67	49
10e	H	H	H	2.4	>1000	49	90	76

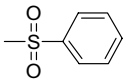
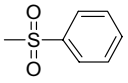
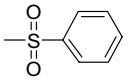
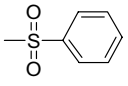
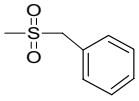
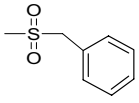
[a] Each value is the mean from triplicate assay in single experiment.
[b] Rate of increase in defecation induced in mice at oral doses of 1 mg/kg.
[c] K_i value.

increased the frequency of defecation, but the enhancing effect on gastric emptying was weaker (MED: 10 mg/kg po in rats) than with **10e** (MED: 3 mg/kg po in rats). The 4-methoxy compound (**11c**: 26%, 71%, 48%, respectively) showed moderate effect on defecation. Here, we speculated that an electron-donating group on the benzoyl group might contribute to the effect on defecation. Based on this speculation, we attempted to introduce a methoxy group at the 3,4-positions of the benzoyl group. As expected, the resulting compound (**11j**: 96%, 106%, 87%) powerfully enhanced defecation. Unfortunately, the effect of compound **11j** on gastric emptying in mice (MED: 10 mg/kg) was weaker than that of **10e** (MED: 3 mg/kg).

The above results indicate the important influence on gastric emptying and defecation of substituents in the benzoyl group of benzoyl derivatives.

Table 3. Pharmacological data of phenylsulfonyl (**12a–d**) and benzylsulfonyl (**13a–b**) derivatives



Compound no	R_1	n	Binding affinities[a]			Potency[b]	Gastric emptying[d]	Defecation[e]
			5-HT_4 K_i (nM)	5-HT_3 K_i (nM)	D_2 IC_{50} (nM)	EC_{50} (nM)[c]	MED (mg/kg)	MED (mg/kg)
12a		2	13	>1000[f]	>1000	NT	1	>10
12b		3	1.7	240	>1000	9.5	>10	NT
12c		4	3.0	>1000[f]	>1000	12	3	1
12d		5	1.4	>1000[f]	>1000	6.3	3	3
13a (Y-36912)		3	1.5	>1000[f]	>1000	10.8	3	0.3
13b		4	1.3	>1000[f]	>1000	NT	10	NT

MED: minimum effective dose. NT: not tested.
[a] Each value is the mean from triplicate assay in single experiment.
[b] 5-HT_4 receptor agonistic activities; contractile effects in guinea-pig ascending colon.
[c] EC_{50} values were determined by linear regression.
[d] Effect of the orally administered compounds on gastric emptying rates of phenol red semisolid meal through the stomach of mice.
[e] Increase in defecation following oral administration of the derivatives in mice.
[f] IC_{50} value.

Table 3 shows the pharmaceutical properties of the phenylsulfonyl (**12a–d**) and benzylsulfonyl (**13a–b**) derivatives. Although compound **12a** accelerated gastric emptying in mice at a dose of 1 mg, it did not increase defecation at a dose of 10 mg.

Prolongation of the straight alkyl chain (**12b–c**) enhanced 5-HT_4 receptor binding affinities ($K_i = 1.7$–3.0 nM) relative to **12a** ($K_i = 13$ nM), but reduced the effect on gastric emptying in mice. The benzylsulfonyl derivative **13a** showed a high affinity for the 5-HT_4 receptor, with a K_i value of 1.5 nM, but little or no affinity for other receptors. In the guinea-pig ascending colon, **13a** (**Y-36912**) induced contractions with an EC_{50} value of 10.8 nM and the concentration-effect curve was shifted rightward by a 5-HT_4 receptor antagonist (GR113808). This compound accelerated gastric emptying in mice at a dose of 3 mg/kg po and increased defecation in mice at a dose of 0.3 mg/kg po.[11] In contrast, prolongation (**13b**, $n = 4$) of the straight alkyl chain at the 1-position of the piperidine ring decreased the effect on gastric emptying.

Next, we studied the pharmacokinetics of compound **13a** (**Y-36912**). After oral administration in rats, the unchanged compound concentrations reached the C_{max} of 1.6 μg/mL at 3.3 h and the $t_{1/2}$ was 9.5 h. The AUC was 12.3 μg/mL and bioavailability was calculated to be 75.1% in oral administration (3 mg) in dogs.[11]

We therefore selected 4-amino-*N*-[1-[3-(benzylsulfonyl)propyl]piperidin-4-ylmethyl]-5-chloro-2-methoxybenzamide **13a** (**Y-36912**) as the candidate for additional biological and pharmaceutical investigation.

This compound might be clinically effective in the treatment of gastrointestinal motility disorders such as constipation-predominant irritable bowel syndrome and atonic constipation, and might improve postoperative

digestive function and the gastrointestinal symptoms caused by chronic gastritis.

4. Conclusion

A series of 4-amino-5-chloro-2-methoxy-*N*-(piperidin-4-ylmethyl)benzamides with a benzoyl, phenylsulfonyl, or benzylsulfonyl moiety in the side chain part at the 1-position of the piperidine was synthesized and their pharmacological properties evaluated. Structure–activity relationship studies gave useful information on the structures required for effect on gastric emptying and defecation. One of the series, the novel prokinetic agent 4-amino-*N*-[1-[3-(benzylsulfonyl)propyl]piperidin-4-ylmethyl]-5-chloro-2-methoxybenzamide (**13a**, **Y-36912**), was a selective 5-HT_4 receptor agonist and potential novel agent with reduced side effects due to 5-HT_3, and dopamine D_2 receptor binding. Compound **13a** (**Y-36912**) could be developed as a novel prokinetic, which can enhance the motor activity of both the upper and lower gastrointestinal tract with few side effects.

5. Experimental section

5.1. General procedure for preparation of intermediates (2a–e, 3a–e, 3g–o)

A cooled (5 °C) solution of benzene and 6-chlorohexanoyl chloride in CH_2Cl_2 was treated with $AlCl_3$ and stirred at 25 °C for 2–4 h. The reaction mixture was poured into ice water and extracted with CH_2Cl_2. The organic layer was concentrated in vacuo and purified by silica gel column chromatography ($CHCl_3$/MeOH) to give pure product **2e**.

5.2. 6-Chloro-1-(3-chlorophenyl)hexan-1-ol (4)

1-Bromo-5-chloropentane (7.00 g, 37.7 mmoL) in THF (30 mL) was added dropwise to a suspension of Mg turnings (920 mg, 37.7 mmoL) in THF (30 mL) at 40–50 °C under a nitrogen atmosphere. After 1 h 3-chlorobenzaldehyde (5.31 g, 37.7 mmoL) in THF (30 mL) was added dropwise and the mixture was stirred for 14 h at 25 °C. The reaction mixture was poured into aqueous NH_4Cl solution and extracted with $CHCl_3$. The organic phase was washed with brine, dried ($MgSO_4$), and evaporated to crude oil, which was purified by silica gel column chromatography (hexane/AcOEt: 90:10) to give **4** (32%).

5.3. 6-Chloro-1-(3-chlorophenyl)hexan-1-one (3f)

The mixture of **4** (1.50 g, 6.10 mmoL) and MnO_2 (7.99 g, 91.9 mmoL) in $CHCl_3$ (30 mL) was stirred for 5 days at 25 °C. The reaction mixture was filtered and purified by silica gel column chromatography (hexane/AcOEt: 9:1) to give **3f** (25%).

5.4. 2-(2-Bromoethyl)-2-phenyl-[1,3]dioxolane (14)

The compound **2b** (10.0 g, 46.9 mmoL) was dissolved in benzene (100 mL) and then ethylene glycol (2.90 g, 46.7 mmoL), *p*-TsOH were added. After refluxing for 70 h, the reaction mixture was washed with aqueous K_2CO_3 solution. The solution was dried over $MgSO_4$ and concentrated to afford crude product. Purification by silica gel column chromatography (hexane/AcOEt: 10:1) provided the title compound **14** (50%).

5.5. General procedure for preparation of intermediates (6a–d, 8a–b)

A solution of benzenethiol (or benzyl mercaptan) and bromochloroalkane in DMF was stirred at 50–60 °C for 3–4 h in the presence of K_2CO_3. The reaction mixture was poured into ice water and extracted with AcOEt. The combined organic extracts were concentrated in vaccuo and purified by silica gel column chromatography ($CHCl_3$/MeOH) to give thioether derivatives. 30% H_2O_2 was added to this compound in HCOOH and the reaction mixture was stirred at 25 °C for 12 h. The reaction mixture was poured into ice water and filtrated to afford the title compound **6a–d**, **8a–b**.

5.6. General procedure for preparation of benzamide derivatives (10a–e, 11a–o, 12a–d, and 13a–b)

The compound **9** was stirred with halide compounds (**2a–e**, **3a–o**, **6a–d**, and **8a–b**) at 70–75 °C in K_2CO_3/DMF for 5–12 h. The reaction mixture was poured into ice water and extracted with AcOEt. The combined extracts were washed with aqueous K_2CO_3 solution. The organic layer was dried over $MgSO_4$ and concentrated in vacuo. The residue was purified by silica gel column chromatography ($CHCl_3$/MeOH) and recrystallized with solvent, or added oxalate, hydrochloride to give pure crystals **10a–e**, **11a–o**, **12a–d**, and **13a–b**.

5.7. 4-Amino-5-chloro-2-methoxy-*N*-[1-(2-oxo-2-phenylethyl)piperidin-4-ylmethyl]benzamide oxalate (10a)

The general procedure was followed for reaction time of 6 h with compound **2a** as halide compound to give colorless oil (3.2 g, 57%). The pure oil was dissolved in ethanol, then oxalic acid was added, and the suspension was heated. After cooling, the resulting crystals were filtered to obtain **10a**; mp 183–185 °C; ^{1}H NMR (DMSO-d_6) δ 1.40–1.62 (2H, m), 1.65–1.91 (3H, m), 2.73–3.00 (2H, m), 3.10–3.20 (2H, m), 3.35–3.50 (2H, m), 3.83 (3H, s), 4.74 (2H, br s), 5.80–6.10 (2H, br s), 6.49 (1H, s), 7.53–7.80 (4H, m), 7.98–8.01 (3H, m); anal. calcd for $C_{22}H_{26}N_3O_3Cl{\cdot}C_2H_2O_4{\cdot}1/2H_2O$: C, 55.98; H, 5.68; N, 8.16. Found: C, 55.96; H, 5.47; N, 8.07.

5.8. 4-Amino-5-chloro-2-methoxy-*N*-[1-(3-oxo-3-phenylpropyl)piperidin-4-ylmethyl]benzamide hydrochloride (10b)

The general procedure was followed for reaction time of 3 h with compound **14** as halide compound to give colorless oil. The pure oil was treated with 1 mol/L hydrochloric acid to obtain compound **10b** (6.4 g, 80%); mp 141–143 °C; ^{1}H NMR (DMSO-d_6) δ 1.40–1.70 (2H, m), 1.72–2.02 (3H, m), 2.85–3.06 (2H, m), 3.15–3.40 (4H, m), 3.51–3.61 (2H, m), 3.62–3.72 (2H, m), 3.83 (3H, s), 5.93 (2H, br s), 6.49 (1H, s), 7.53–7.61 (2H, m), 7.65–7.72 (2H, m), 7.98–8.03 (3H, m), 10.03–10.40 (1H, m); anal. calcd for $C_{23}H_{28}N_3O_3Cl{\cdot}HCl{\cdot}2H_2O$: C, 54.98; H, 6.62; N, 8.36. Found: C, 55.09; H, 6.58; N, 8.40.

5.9. 4-Amino-5-chloro-2-methoxy-*N*-[1-(4-oxo-4-phenylbutyl)piperidin-4-ylmethyl]benzamide (10c)

The general procedure was followed for reaction time of 3 h with compound **2c** as halide compound to give colorless oil. The pure oil was crystallized with ethanol to obtain colorless crystals (1.8 g, 29%); mp 148–150 °C; ^{1}H NMR ($CDCl_3$) δ: 1.19–1.39 (2H, m), 1.50–2.02 (7H, m), 2.40 (2H, t, $J = 7.3$ Hz), 2.87–3.02 (4H, m), 3.30 (2H, t, $J = 6.0$ Hz), 3.88 (3H, s), 4.46 (2H, br s), 6.30 (1H, s), 7.42–7.60 (3H, m), 7.65–7.82 (1H, m), 7.95 (2H, dd, $J = 7.3$ Hz), 8.09 (1H, s); anal. calcd for $C_{24}H_{30}N_3O_3Cl{\cdot}1/10H_2O$: C, 64.67; H, 6.83; N, 9.43. Found: C, 64.55; H, 6.79; N, 9.43.

5.10. 4-Amino-5-chloro-2-methoxy-*N*-[1-(5-oxo-5-phenylpentyl)piperidin-4-ylmethyl]benzamide (10d)

The general procedure was followed for reaction time of 3 h with compound **2d** as halide compound to give colorless oil. The pure oil was crystallized with ethanol to obtain colorless crystals (1.3 g, 64%); mp 100–102 °C; ^{1}H NMR ($CDCl_3$) δ: 1.17 (2H, t, $J = 11$ Hz), 1.44–1.59 (4H, m), 1.51–1.66 (5H, m), 1.81 (2H, t, $J = 11$ Hz), 2.28 (2H, t, $J = 7.3$ Hz), 2.83 (2H, d, $J = 11$ Hz), 3.02 (2H, t, $J = 7.2$ Hz), 3.82 (3H, s), 5.90 (2H, s), 6.47 (1H, s), 7.49 (2H, dd, $J = 2.0, 7.7$ Hz), 7.60 (1H, dd, $J = 2.0, 7.7$ Hz), 7.66 (1H, s), 7.86 (1H, t, $J = 6.0$ Hz), 7.96 (2H, dd, $J = 1.6, 7.7$ Hz); anal. calcd for $C_{25}H_{32}N_3O_3Cl{\cdot}H_2O$: C, 63.08; H, 7.20; N, 8.83. Found: C, 63.48; H, 7.10; N, 8.83.

5.11. 4-Amino-5-chloro-2-methoxy-*N*-[1-(6-oxo-6-phenylhexyl)piperidin-4-ylmethyl]benzamide (10e)

The preparation, ^{1}H NMR and elemental analysis data of **10e** was described previously.[8]

5.12. 4-Amino-5-chloro-2-methoxy-*N*-[1-[6-(4-methylphenyl)-6-oxohexyl]piperidin-4-ylmethyl]benzamide (11a)

The general procedure was followed for reaction time of 3 h with compound **3a** as halide compound to give solid. The solid was recrystallized from EtOH/AcOEt to obtain colorless crystals (0.87 g, 45%); mp 130–131 °C; ^{1}H NMR ($CDCl_3$) δ 1.31–1.47 (4H, m), 1.52–1.81 (6H, m), 1.93–2.05 (2H, m) 2.28–2.38 (3H, m), 2.41 (3H, s), 2.80–3.02 (4H, m), 3.33 (2H, t, $J = 5.9$ Hz), 3.90 (3H, s), 4.38 (2H, br s), 6.29 (1H, s), 7.25 (2H, d, $J = 7.9$ Hz), 7.50–7.81 (1H, m), 7.84 (2H, d, $J = 7.9$ Hz), 8.11(1H, s); anal. calcd for $C_{27}H_{36}N_3O_3Cl{\cdot}1/2H_2O$: C, 65.51; H, 7.53; N, 8.49. Found: C, 65.55; H, 7.43; N, 8.44.

5.13. 4-Amino-5-chloro-*N*-[1-[6-(4-ethylphenyl)-6-oxohexyl]piperidin-4-ylmethyl]-2-methoxybenzamide (11b)

The general procedure was followed for reaction time of 5 h with compound **3b** as halide compound to give solid. The solid was recrystallized from EtOH/AcOEt to obtain colorless prisms (340 mg, 21%); mp 140–145 °C; ^{1}H NMR ($CDCl_3$) δ 1.26 (3H, t, $J = 7.2$ Hz), 1.34–1.90 (10H, m), 2.02–2.30 (2H, m), 2.42–2.60 (2H, m), 2.70 (2H, q, $J = 7.2$ Hz), 2.95 (2H, t, $J = 7.2$ Hz), 3.04–3.22 (2H, m), 3.29–3.41 (2H, m), 3.90 (3H, s), 4.42 (2H, br s), 6.31 (1H,s), 7.28 (2H, d, $J = 7.9$ Hz), 7.70–7.82 (2H, m), 7.87 (2H, d, $J = 7.9$ Hz), 8.09 (1H, s); anal. calcd for $C_{28}H_{38}N_3O_3Cl{\cdot}H_2O$: C, 64.91; H, 7.78; N, 8.11. Found: C, 64.73; H, 7.63; N, 8.24.

5.14. 4-Amino-5-chloro-2-methoxy-*N*-[1-[6-(4-methoxyphenyl)-6-oxohexyl]piperidin-4-ylmethyl]benzamide (11c)

The general procedure was followed for reaction time of 3 h with compound **3c** as halide compound to give solid. The solid was recrystallized from AcOEt to obtain colorless crystals (1.38 g, 69%); mp 133–135 °C; ^{1}H NMR ($CDCl_3$) δ 1.23–1.83 (11H, m), 1.89–2.08 (2H, m), 2.27–2.47 (2H, m), 2.86–3.04 (4H, m), 3.32 (2H, t, $J = 5.9$ Hz), 3.87 (3H, s), 3.90 (3H, s), 4.38 (2H, br s), 6.29 (1H, s), 6.92 (2H, d, $J = 7.9$ Hz), 7.68–7.88 (1H, m), 7.93 (2H, d, $J = 9.0$ Hz), 8.10 (1H, s); anal. calcd for $C_{27}H_{36}N_3O_4Cl{\cdot}1/4H_2O$: C, 64.02; H, 7.26; N, 8.30. Found: C, 63.95; H, 7.25; N, 8.32.

5.15. 4-Amino-5-chloro-*N*-[1-[6-(4-hydroxylphenyl)-6-oxohexyl]piperidin-4-ylmethyl]-2-methoxybenzamide (11d)

The general procedure was followed for reaction time of 6 h with compound **3d** as halide compound to give solid. The solid was recrystallized from EtOH/Et_2O to obtain colorless crystals (290 mg, 40%); mp 177–179 °C; ^{1}H NMR ($CDCl_3$/CD_3OD) δ 1.20–1.81 (12H, m), 1.91–2.09 (2H, m), 2.24–2.45 (2H, m), 2.82–3.02 (4H, m), 3.31–3.38 (2H, m), 3.78 (3H, s), 4.77 (2H, br s), 6.37 (1H, s), 6.85 (2H, d, $J = 9.2$ Hz), 7.85 (2H, d, $J = 9.2$ Hz), 7.88–7.99 (1H, m), 8.00 (1H, s); anal. calcd for $C_{26}H_{34}N_3O_4Cl{\cdot}1/2EtOH$: C, 63.46; H, 7.30; N, 8.22. Found: C, 63.24; H, 7.26; N, 8.33.

5.16. 4-Amino-5-chloro-*N*-[1-[6-(4-chlorophenyl)-6-oxohexyl]piperidin-4-ylmethyl]-2-methoxybenzamide (11e)

The general procedure was followed for reaction time of 8 h with compound **3e** as halide compound to give solid. After usual workup, title compound was obtained as

colorless powders (50%); mp 156–158 °C; ^{1}H NMR ($CDCl_3$) δ 1.30–1.80 (11H, m), 1.90–2.09 (2H, m), 2.28–2.48 (2H, m), 2.89–3.04 (4H, m), 3.32 (2H, t, $J = 6.4$ Hz), 3.90 (3H, s), 4.41 (2H, br s), 6.30 (1H, s), 7.43 (2H, d, $J = 8.6$ Hz), 7.68–7.82 (1H, m), 7.98 (2H, d, $J = 8.6$ Hz), 8.00 (1H, s); anal. calcd for $C_{26}H_{33}N_3O_3Cl_2 \cdot 1/2H_2O$: C, 60.58; H, 6.65; N, 8.15. Found: C, 60.82; H, 6.61; N, 8.11.

5.17. 4-Amino-5-chloro-*N*-[1-[6-(3-chlorophenyl)-6-oxo-hexyl]piperidin-4-ylmethyl]-2-methoxybenzamide (11f)

The general procedure was followed for reaction time of 8 h with compound **3f** as halide compound to give solid. The solid was recrystallized from EtOH to obtain colorless crystals (0.24 g, 31%); mp 128–132 °C; ^{1}H NMR ($CDCl_3$) δ 1.31–1.92 (14H, m), 2.09–2.30 (2H, m), 2.48–2.62 (2H, m), 2.95 (2H, t, $J = 7.0$ Hz), 3.33 (2H, q, $J = 6.0$ Hz), 3.91 (3H, s), 4.39 (2H, br s), 6.30 (1H,s), 7.36–7.43 (1H, m), 7.49–7.55 (1H, m), 7.76–7.84 (1H, m), 7.90–7.92 (1H, m), 8.09 (1H, s); anal. calcd for $C_{26}H_{33}N_3O_3Cl_2 \cdot 1.3H_2O$: C, 58.93; H, 6.77; N, 7.93. Found: C, 58.92; H, 6.63; N, 8.05.

5.18. 4-Amino-5-chloro-*N*-[1-[6-(4-fluorophenyl)-6-oxo-hexyl]piperidin-4-ylmethyl]-2-methoxybenzamide (11g)

The general procedure was followed for reaction time of 3 h with compound **3g** as halide compound to give solid. The solid was recrystallized from EtOH/AcOEt to obtain colorless crystals (1.0 g, 50%); mp 137–139 °C, ^{1}H NMR ($CDCl_3$) δ 1.25–1.82 (11H, m), 1.88–2.08 (2H, m), 2.25–2.45 (2H, m), 2.86–3.03 (4H, m), 3.33 (2H, t, $J = 6.3$ Hz), 3.90 (3H, s), 4.39 (2H, br s), 6.30 (1H, s), 7.04–7.20 (2H, m), 7.69–7.83 (1H, m), 7.92–8.03 (2H, m), 8.10 (1H, s); anal. calcd for $C_{26}H_{33}N_3O_3FCl \cdot 1/2H_2O$: C, 62.58; H, 6.87; N, 8.42. Found C, 62.77; H, 6.78; N, 8.44.

5.19. 4-Amino-5-chloro-*N*-[1-[6-(3,4-dimethylphenyl)-6-oxo-hexyl]piperidin-4-ylmethyl]-2-methoxybenzamide (11h)

The general procedure was followed for reaction time of 3 h with compound **3h** as halide compound to give solid. The solid was recrystallized from EtOH/AcOEt to obtain colorless crystals (0.82 g, 30%); mp 115–117 °C.

^{1}H NMR ($CDCl_3$) δ 1.21–1.47 (4H, m), 1.48–1.81 (9H, m), 1.82–2.01 (2H, m), 2.30 (6H, s), 2.31 (6H, s), 2.82–3.02 (4H, m), 3.32 (2H, q, $J = 6.0$ Hz), 3.90 (3H, s), 4.36 (2H, br s), 6.29 (1H, s), 7.20 (1H, d, $J = 7.9$ Hz), 7.62–7.81 (3H, m), 8.11 (1H, s); anal. calcd for $C_{28}H_{38}N_3O_3Cl_2$: C, 67.25; H, 7.66; N, 8.40. Found: C, 67.23; H, 7.73; N, 8.52.

5.20. 4-Amino-5-chloro-*N*-[1-[6-(2,4-dimethylphenyl)-6-oxo-hexyl]piperidin-4-ylmethyl]-2-methoxybenzamide (11i)

The general procedure was followed for reaction time of 7 h with compound **3i** as halide compound to give colorless solid. The solid was recrystallized from EtOH/AcOEt to obtain colorless crystals (0.63 g, 38%); mp 106–110 °C; ^{1}H NMR ($CDCl_3$) δ 1.30–1.89 (10H, m), 2.01–2.21 (2H, m), 2.34 (3H, s), 2.47 (3H, s), 2.42–2.52 (2H, m), 2.87 (2H, t, $J = 7.3$ Hz), 3.00–3.16 (2H, m), 3.33 (2H, t, $J = 6.3$ Hz), 3.90 (3H, s), 4.43 (2H, br s), 6.31 (1H, s), 7.01–7.09 (2H, m), 7.56 (1H, d, $J = 7.9$ Hz), 7.71–7.85 (1H, m), 8.09 (1H, s); anal. calcd for $C_{28}H_{38}N_3O_3Cl \cdot 5/4H_2O$: C, 63.26; H, 7.87; N, 7.90. Found: C, 63.19; H, 7.89; N, 7.91.

5.21. 4-Amino-5-chloro-*N*-[1-[6-(3,4-dimethoxylphenyl)-6-oxo-hexyl]piperidin-4-ylmethyl]-2-methoxybenzamide (11j)

The general procedure was followed for reaction time of 8 h with compound **3j** as halide compound to give colorless solid. The solid was recrystallized from EtOH/AcOEt to obtain colorless crystals (0.50 g, 29%); mp 102–105 °C; ^{1}H NMR ($CDCl_3$) δ 1.24–2.27 (13H, m), 2.28–2.45 (2H, m), 2.88–3.05 (4H, m), 3.32 (2H, t, $J = 6.3$ Hz), 3.89 (3H, s), 3.93 (3H, s), 3.94 (3H, s), 4.40 (2H, br s), 6.30 (1H, s), 6.88 (1H, d, $J = 8.6$ Hz), 7.52 (1H, d, $J = 2.0$ Hz), 7.57 (1H, dd, $J = 2.0$ Hz, 8.6 Hz), 7.68–7.85 (1H, m), 8.10 (1H, s); anal. calcd for $C_{28}H_{38}N_3O_5Cl \cdot 3/2H_2O$: C, 60.15; H, 7.39; N, 7.52. Found: C, 60.06; H, 7.60; N, 7.45.

5.22. 4-Amino-5-chloro-*N*-[1-[6-(3,4-dichlorophenyl)-6-oxo-hexyl]piperidin-4-ylmethyl]-2-methoxybenzamide hydrochloride (11k)

The general procedure was followed for reaction time of 3 h with compound **3k** as halide compound to give colorless oil (110 mg, 15%). The pure oil was converted into hydrochloride salt 10% HCl in EtOH to obtain colorless crystals; mp 203–205 °C; ^{1}H NMR (DMSO-d_6) δ 1.23–1.90 (11H, m), 2.70–3.55 (10H, m), 3.83 (3H, s), 5.93 (2H, br s), 6.48 (1H, s), 7.66 (1H, s), 7.81 (1H, d, $J = 8.5$ Hz), 7.92 (1H, dd, $J = 2.0$ Hz, 8.5 Hz), 7.95–8.05 (1H, m), 8.15 (1H, d, $J = 2.0$ Hz); anal. calcd for $C_{26}H_{32}N_3O_3Cl_3 \cdot HCl \cdot 1/4H_2O$: C, 53.67; H, 5.80; N, 7.22. Found: C, 53.69; H, 6.05; N, 7.10.

5.23. 4-Amino-5-chloro-*N*-[1-[6-(2,4-dichlorophenyl)-6-oxo-hexyl]piperidin-4-ylmethyl]-2-methoxybenzamide hydrochloride (11l)

The general procedure was followed for reaction time of 3 h min with compound **3l** as halide compound to give colorless oil. The pure oil was converted into hydrochloride salt with 10% HCl in EtOH to obtain colorless crystals (0.11 g, 33%); mp 150–155 °C; ^{1}H NMR (DMSO-d_6) δ 2.65–3.09 (17H, m), 3.10–3.22 (2H, m), 3.30–3.52 (2H, m), 3.83 (3H, s), 5.75–6.08 (2H, br s), 6.49 (1H, s), 7.55 (1H, dd, $J = 1.3$ Hz, 9.9 Hz), 7.68 (1H, d, $J = 9.9$ Hz), 7.73 (1H, d, $J = 1.3$ Hz), 7.92–8.07 (1H, m), 9.50–13.00 (1H, m); anal. calcd for $C_{26}H_{32}N_3O_3Cl_3 \cdot HCl \cdot H_2O$: C, 52.45; H, 5.80; N, 7.22. Found: C, 52.61; H, 6.06; N, 7.31.

5.24. 4-Amino-5-chloro-*N*-[1-[6-(2,4-difluorophenyl)-6-oxo-hexyl]piperidin-4-ylmethyl]-2-methoxybenzamide (11m)

The general procedure was followed for reaction time 5 h with compound **3m** as halide compound to give solid. The solid was recrystallized from EtOH/AcOEt to obtain colorless crystals (0.25 g, 18%); mp 115–117 °C; ^{1}H NMR ($CDCl_3$) δ 1.21–2.00 (13H, m), 2.21–2.40 (2H, m), 2.82–3.00 (4H, m), 3.32 (2H, t, $J = 6.3$ Hz), 3.90 (3H, s), 4.37 (2H, br s), 6.29 (1H, s), 6.80–6.99 (2H, m), 7.68–7.80 (1H, m), 7.86–7.92 (1H, m), 8.10 (1H, s); anal. calcd for $C_{26}H_{32}N_3O_3ClF_2$: C, 61.47; H, 6.35; N,8.27. Found: C, 61.15; H, 6.36; N, 8.22.

5.25. 4-Amino-5-chloro-*N*-[1-[6-(3-chloro-4-methoxyphenyl)-6-oxo-hexyl]piperidin-4-ylmethyl]-2-methoxybenzamide (11n)

The general procedure was followed for reaction time of 3 h with compound **3n** as halide compound to give colorless solid. The solid was recrystallized from EtOH/AcOEt to obtain colorless crystals (0.45 g, 33%); mp 104–106 °C; ^{1}H NMR ($CDCl_3$) δ 1.30–1.50 (2H, m), 1.51–1.83 (10H, m), 1.90–2.07 (2H, m), 2.31–2.43 (2H, m), 2.90 (2H, t, $J = 7.3$ Hz), 2.95–3.05 (2H, m), 3.32 (2H, t, $J = 6.3$ Hz), 3.90 (3H, s), 3.97 (3H, s), 4.40 (2H, br s), 6.29 (1H, s), 6.95 (1H, d, $J = 8.6$ Hz), 7.85 (1H, dd, $J = 2.0$ Hz, 8.6 Hz), 7.98 (1H, d, $J = 2.0$ Hz), 8.09 (1H, s); anal. calcd for $C_{27}H_{35}N_3O_4Cl_2{\cdot}H_2O$: C, 58.48; H, 6.73; N, 7.58. Found: C, 58.62; H, 6.66; N, 7.81.

5.26. 4-Amino-5-chloro-*N*-[1-[6-(3-fluoro-4-methoxyphenyl)-6-oxo-hexyl]piperidin-4-ylmethyl]-2-methoxybenzamide (11o)

The general procedure was followed for reaction time of 5 h with compound **3o** as halide compound to give colorless solid. The solid was recrystallized from EtOH/AcOEt to obtain colorless crystals (0.90 g, 64%); mp 112–113 °C; ^{1}H NMR ($CDCl_3$) δ 1.20–1.92 (10H, m), 1.93–2.04 (2H, m), 2.24–2.45 (2H, m), 2.80–3.02 (4H, m), 3.32 (2H, t, $J = 6.0$ Hz), 3.81 (3H, s), 3.95 (3H, s), 4.43 (2H, br s), 6.30 (1H, s), 6.99 (1H, d, $J = 8.3$ Hz), 7.64–7.89 (4H, m), 8.10 (1H, s); anal. calcd for $C_{27}H_{35}N_3O_4ClF{\cdot}1/2H_2O$: C, 61.30; H, 6.86; N, 7.94. Found: C, 61.46; H, 6.93; N, 7.85.

5.27. 4-Amino-5-chloro-2-methoxy-*N*-[1-(2-phenylsulfonylethyl)piperidin-4-ylmethyl]benzamide hydrochloride (12a)

The general procedure was followed for reaction time of 5 h with compound **6a** as halide compound to give colorless oil (2.74 g, 31%). The pure oil was converted to the hydrochloride salt with 1 N HCl in EtOH to obtain colorless crystals; mp 116–117 °C; ^{1}H NMR (DMSO-d_6) δ 1.35–2.05 (5H, m), 2.70–3.02 (2H, m), 3.03–3.60 (5H, m), 3.80 (3H, s), 3.88–4.15 (2H, m), 5.70–6.15 (2H, m), 6.50 (1H, s), 7.55–7.89 (4H, m), 7.90–8.09 (3H, m), 10.50–11.70 (1H, m); anal. calcd for $C_{22}H_{28}N_3O_4SCl{\cdot}HCl{\cdot}3/2H_2O$: C, 49.90; H, 6.09; N, 7.94. Found: C, 49.63; H, 6.04; N, 7.78.

5.28. 4-Amino-5-chloro-2-methoxy-*N*-[1-(3-phenylsulfonylpropyl)piperidin-4-ylmethyl]benzamide methanesulfonate (12b)

The general procedure was followed for reaction time of 4 h with compound **6b** as halide compound to give colorless oil (18 g, 71%). The pure oil was transformed into methanesulfonate and recrystallized from 2-propanol-diisopropylether to obtain title compound; mp 190–192 °C; ^{1}H NMR (DMSO-d_6) δ 1.25–1.50 (2H, m), 1.70–1.89 (3H, m), 1.90–2.11 (2H, m), 2.33 (3H, s), 2.73–3.01 (2H, m), 3.03–3.60 (5H, m), 3.82 (3H, s), 6.48 (1H, s), 7.56–8.04 (4H, m), 7.84 (4H, m), 7.88–8.05 (3H, m), 8.85–9.20 (1H, m); anal. calcd for $C_{23}H_{30}N_3O_4SCl{\cdot}CH_3SO_3H{\cdot}1/4H_2O$: C, 49.65; H, 5.99; N, 7.24. Found: C, 49.61; H, 5.99; N, 7.22.

5.29. 4-Amino-5-chloro-2-methoxy-*N*-[1-(4-phenylsulfonylbutyl)piperidin-4-ylmethyl]benzamide hydrochloride (12c)

The general procedure was followed procedure of compound **6c** as halide compound to give solid (9.2 g, 46%). The solid was converted to the hydrochloride salt with 1 N HCl in EtOH to obtain colorless crystals; mp 158–159 °C; ^{1}H-NMR (DMSO-d_6) δ: 1.52–1.63 (4H, m), 1.77–1.95 (5H, m), 2.77 (2H, dd, $J = 10$ Hz, 22 Hz), 2.90–3.02 (2H, m), 3.10–3.20 (3H, m), 3.83 (3H, s), 5.95 (2H, s), 6.51 (1H, s), 7.66 (2H, dd, $J = 2.0$ Hz, 6.6 Hz), 7.70 (1H, dd, $J = 2.0$ Hz, 6.6 Hz), 7.89 (2H, dd, $J = 2.0$, 6.6 Hz), 7.94 (1H, t, $J = 5.3$ Hz), 10.42 (1H, br s); anal. calcd for $C_{24}H_{32}ClN_3O_4S{\cdot}HCl{\cdot}H_2O$: C, 52.55; H, 6.43; N, 7.66. Found: C, 52.30; H, 6.45; N, 7.58.

5.30. 4-Amino-5-chloro-2-methoxy-*N*-[1-(5-phenylsulfonylpentyl)piperidin-4-ylmethyl]benzamide hydrochloride (12d)

The preparation, ^{1}H NMR and elemental analysis data of **12d** was described previously.[8]

5.31. 4-Amino-*N*-[1-[3-(benzylsulfonyl)propyl]piperidin-4-ylmethyl]-5-chloro-2-methoxybenzamide (13a, Y-36912)

The general procedure was followed for reaction time of 7.5 h with compound **8a** as halide compound to give solid. The resulting solid was recrystallized from ethanol to obtain colorless powders (45 g, 63%); mp 170–171 °C; ^{1}H NMR ($CDCl_3$) δ: 1.17–1.32 (2H, m), 1.50–1.63 (1H, m), 1.69 (2H, d, $J = 13$ Hz), 1.86–2.00 (4H, m), 2.37 (2H, t, $J = 6.9$ Hz), 2.82 (2H, d, $J = 12$ Hz), 2.88–2.94 (2H, m), 3.31 (2H, t, $J = 6.3$ Hz), 3.88 (3H, s), 4.22 (2H, s), 4.42 (2H, s), 6.30 (1H, s), 7.37–7.43(5H, m), 7.73 (1H, t, $J = 5.6$ Hz), 8.10 (1H, s). Anal. Calcd for $C_{24}H_{32}ClN_3O_4S$: C, 58.34; H, 6.53; N, 8.51. Found: C, 58.15; H, 6.56; N, 8.49.

5.32. 4-Amino-*N*-[1-[4-(benzylsulfonyl)butyl]piperidin-4-ylmethyl]-5-chloro-2-methoxybenzamide hydrochloride (13b)

The general procedure was followed for reaction time of 6 h with compound **8b** as halide compound to give colorless oil. The pure oil was converted into hydrochloride salt with 10% HCl/EtOH to obtain colorless crystals (0.96 g, 33%); mp 114–116 °C; ^{1}H NMR (DMSO-d_6) δ 1.45–2.02 (9H, m), 2.70–3.48 (12H, m), 3.83 (3H, s), 4.49 (2H, br s), 6.51 (1H, s), 7.30–7.51 (5H, m), 7.66 (3H, m), 7.91–8.09 (3H, m), 10.30–10.60 (1H, m); anal. calcd for $C_{25}H_{34}N_3O_4SCl\cdot HCl\cdot 5/4H_2O$: C, 52.95; H, 6.67; N, 7.41. Found C, 52.93; H, 6.72; N, 7.30.

5.33. 5-HT$_4$ receptor binding assay

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 volume 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 tube 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. Nonspecific binding was defined in the presence of unlabelled GR113808 to give a final concentration of 1 μmol/L. The IC50 value was determined by nonlinear regression of the displacement curve, and the K_i value was calculated according to the formula ($K_i = IC_{50}/(1 + L/K_d)$), where L is the concentration of radioligand and K_d is the dissociation constant of the radioligand.

5.34. 5-HT$_3$ receptor binding assay

[^{3}H]Granisetron binding assay was performed according to the method of Nelson and Thomas.[12] 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 1000×*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 suspension was incubated at 37 °C for 10 min, was 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 μmmol/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 μmmol/L) was used for the determination of nonspecific binding.

5.35. Dopamine D$_2$ receptor binding assay

[^{3}H]Spiperone binding assay was performed according to the method of Creese 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 re-centrifuged (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.

5.36. 5-HT$_4$ receptor agonistic activities in vitro contraction of isolated guinea-pig ascending colon

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 with 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.

5.37. Effect of compounds on gastric emptying of liquid meal in mice

Male Sea:ddY mice were deprived food for about 18 h before use and were orally administered test compounds. Half an hour later, mice were given 0.1 mL of test meal containing 0.05% phenol red in 1.5% hydroxypropyl methylcellulose solution. Animals were sacrificed 15 min after administration of test meal and the stomach was removed. Phenol red remaining in stomach was measured by colorimetric assay. Results are expressed as means ± SEM and were compared by Dunnett method.

5.38. Effect of compounds on gastric emptying of solid meal in rats

Female Crj:Wistar rats were orally administered test compounds. One hour later, rats were given 40 barium sulfate pellets coated with polystyrene through a polyethylene tube into stomach. Animals were sacrificed 1 h after administration of pellets and stomach were removed. The number of pellets remaining in stomach was counted. Results are expressed as means ± SEM and were compared by Dunnett method.

5.39. Effect on defecation in mice

Male Crj:CD-1(ICR) mice were orally or subcutaneously administered test compounds after being adapted to experimental surroundings in partition box for 30 min. The number, wet weight, and dry weight of feces excreted for 2 h from immediately after the administration were measured. Results are expressed as means ± SEM and were compared by Dunnett method.

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