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3-Benzhydryl-4-piperidones as novel neurokinin-1 receptor antagonists and their efficient synthesis

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ABSTRACT

A series of novel 3-benzhydryl-4-piperidone derivatives were identified as potent tachykinin neurokinin-1 (NK_1) receptor antagonists. An efficient and versatile synthesis of this series was achieved with a coupling reaction of 1-benzylpiperidones with benzhydryl bromides or benzhydrols in the presence of trifluoromethanesulfonate and a condensation reaction of piperidones with benzyl alcohols using ethyl *o*-phenylenephosphate. The 3-benzhydryl-4-piperidone skeleton, which has a 1,1-diphenylmethane moiety that is a known privileged substructure targeting G-protein coupled receptors, can be used for chemical library synthesis because of chemical accessibility and diversity.

1. Introduction

Tachykinin peptides, which belong to a neuropeptide family consisting of five common residues (Phe-X-Gly-Leu-Met-NH_2) located at the C-terminal of the amino acid sequence, include substance P (SP), neurokinin A (NKA), and neurokinin B (NKB) in mammals. SP, NKA, and NKB selectively bind the neurokinin-1 (NK_1), neurokinin-2 (NK_2), and neurokinin-3 (NK_3) receptors, respectively, to cause a variety of biological responses in both the central nervous system (CNS) and peripheral tissues.[1] Among them, SP is one of the oldest and most extensively investigated neuropeptides.[2] Animal studies with tachykinin NK_1 antagonists have resulted in several prospective disease treatments, such as overactive bladder, gastrointestinal disorders, emesis, pain, and CNS disorders.[3]

We previously reported the identification of TAK-637 (**19**, Fig. 1), a structurally distinct 1,7-naphthyridine-6-carboxamide derivative, which is a potent and orally active tachykinin NK_1 antagonist.[4] In the course of a research program to identify a novel series of tachykinin NK_1 antagonists whose structures were different from that of TAK-637, the 3-benzhydryl-4-piperidone derivative **13r** was found from high-throughput screening (HTS) of Takeda's chemical library.

We were interested in the structural diversity, as well as in the novelty, of the 3-benzhydryl-4-piperidone core. The benzhydryl (1,1-diphenylmethane) group is a known privileged substructure of the G-protein coupled receptor (GPCR).[5] We have already found that benzhydryl groups can be easily introduced to the piperidone core at the α-position of the carbonyl group.[6] On the basis of structural attractiveness and synthetic accessibility, we began optimization using **13r** as the lead compound. The aim of this study was to elucidate the chemical tractability of the 3-benzhydryl-4-piperidone derivatives of tachykinin NK_1 antagonists in order to explore drugs targeting GPCRs. Herein, we describe the efficient and versatile synthesis of 3-benzhydryl-4-piperidone derivatives and their biological evaluation.

2. Chemistry

The synthesis of the 3-benzhydryl-4-piperidone derivatives **13a**–**y** was accomplished as illustrated in Scheme 1. The commercially available 1-benzylpiperidone derivatives **1**–**3** were treated with benzhydryl bromide in the presence of trimethylsilyl trifluoromethanesulfonate (TMSOTf) and zinc bromide or with benzhydrol derivatives in the presence of TMSOTf and zinc bromide to afford the 3-benzhydryl-4-piperidone derivatives **4**–**10**. Compounds **4** and **7** were converted to the piperidone derivatives **11** and **12** by treatment with hydrochloric acid, which was followed by hydrogenation. The modifications at the 1-position in compounds **11** and **12** were carried out by condensation with various benzyl alcohol derivatives using ethyl *o*-phenylenephosphate (EPPA).[7] EPPA is a useful dehydrating reagent because it is easy to handle (simply mix an alcohol, a nucleophile, and EPPA without intensive care) and it can be used in a simple procedure for removing reacted reagents by washing with alkaline. When corresponding benzyl halides are commercially

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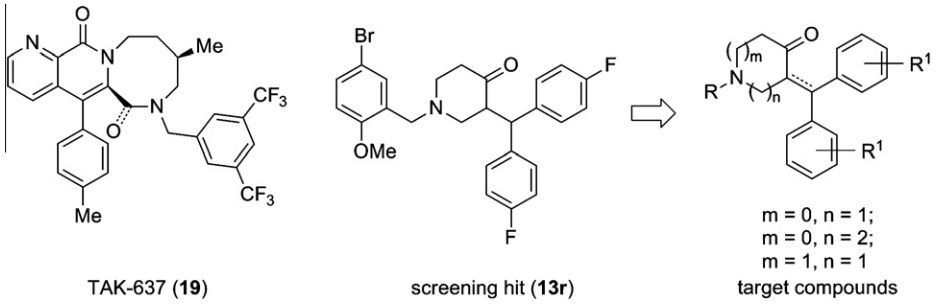
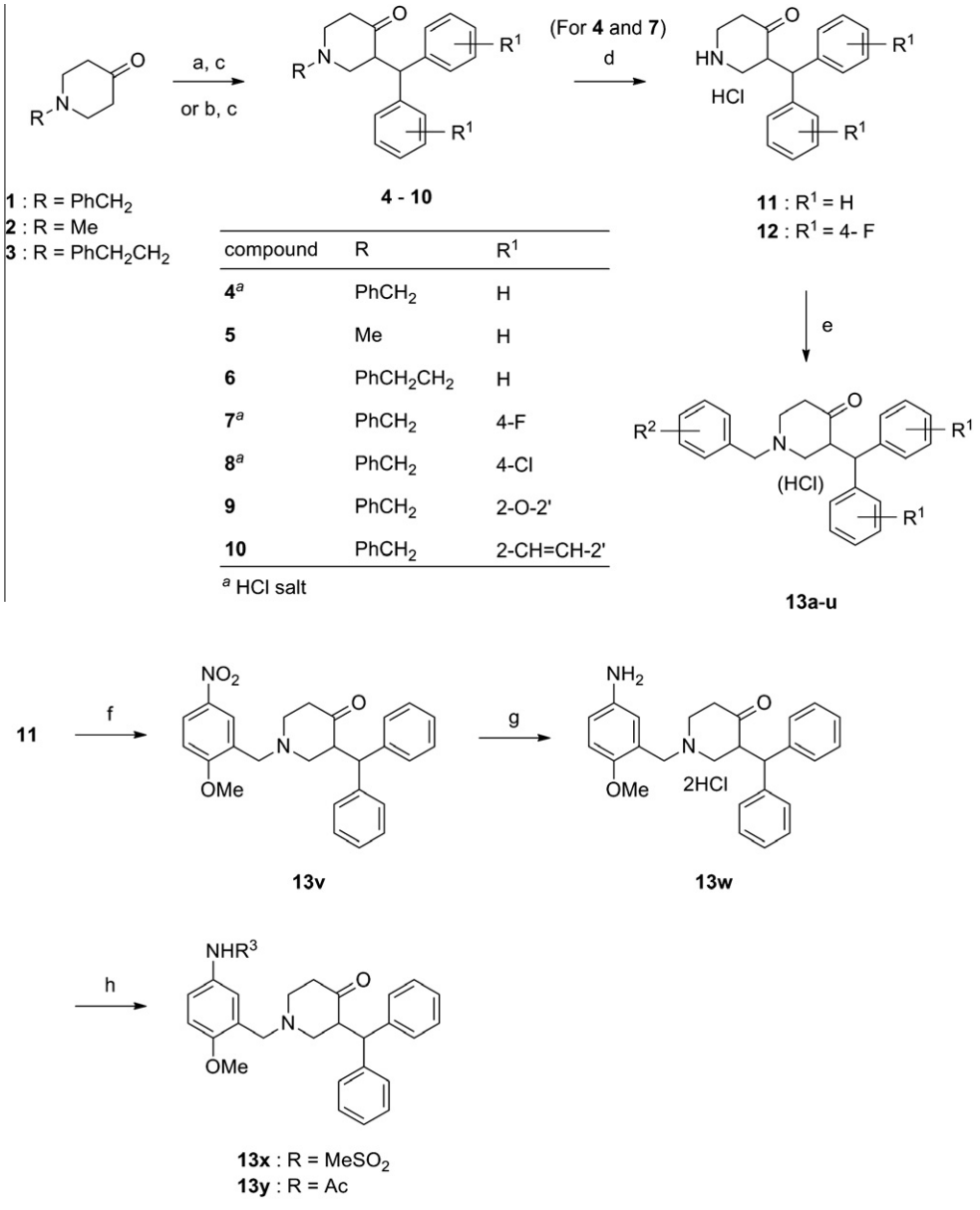


Figure 1. Chemical structure of TAK-637 (**19**), screening hit (**13r**), and target compounds.

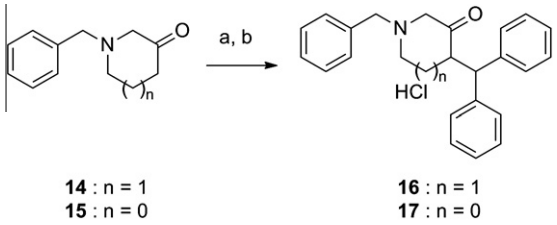


compound	R	R^1
4[a]	$PhCH_2$	H
5	Me	H
6	$PhCH_2CH_2$	H
7[a]	$PhCH_2$	4-F
8[a]	$PhCH_2$	4-Cl
9	$PhCH_2$	2-O-2'
10	$PhCH_2$	2-CH=CH-2'

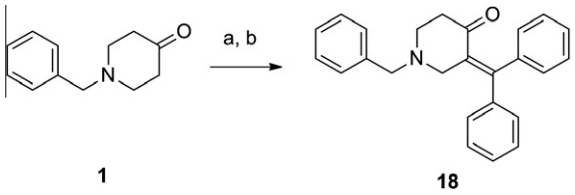
[a] HCl salt

Scheme 1. Synthesis of 3-benzhydryl-4-piperidone derivatives. Reagents and conditions: (a) (for **4**–**6**) benzhydryl bromide, trimethylsilyl trifluoromethanesulfonate, zinc bromide, CH_2Cl_2, rt; (b) (for **7**–**10**) benzhydrol derivatives, trimethylsilyl trifluoromethanesulfonate, zinc bromide, CH_2Cl_2, rt; (c) concentrated HCl, EtOH (then aqueous NaOH); (d) H_2, 5%Pd–C, MeOH, rt; (e) benzyl alcohol derivatives, ethyl *o*-phenylenephosphate, CH_2Cl_2, rt (then 4 M HCl/EtOAc); (f) 2-bromomethyl-4-nitroanisole, $NaHCO_3$, DMF, rt; (g) H_2, 5%Pd–C, THF, MeOH, rt then concentrated HCl, EtOH; (h) methanesulfonyl chloride or acetyl chloride, pyridine, rt.

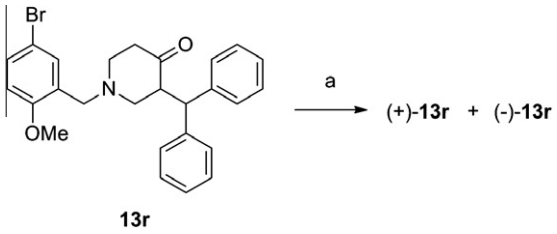
unavailable or chemically unstable, EPPA is suitable for this kind of reaction, such as for the synthesis of compound **13a** (R^2 = 2-OH). No protection/deprotection process is needed for synthesizing target products. Thus, this reaction will be applicable for library synthesis. Compounds bearing amino groups (**13x** and **13y**) on the *N*-benzyl group were synthesized by reaction of compound **11** with



Scheme 2. Synthesis of benzhydryl derivatives **16** and **17**. Reagents and conditions: (a) benzhydrol, trimethylsilyl trifluoromethanesulfonate, CH_2Cl_2, rt; (b) concentrated HCl, EtOH.



Scheme 3. Synthesis of diphenylmethylidene derivative **18**. Reagents and conditions: (a) dichlorodiphenylmethane, trimethylsilyl trifluoromethanesulfonate, zinc bromide, CH_2Cl_2, rt; (b) concentrated HCl, EtOH then aqueous NaOH.



Scheme 4. Optical resolution of **13r** by chiral column chromatography. Reagents: (a) Chiralpak AD, hexane and 2-propanol (9:1, v/v).

2-bromomethyl-4-nitroanisole in the presence of $NaHCO_3$, which was followed by reduction of the nitro group followed by sulfonylation or acylation.

The 3-piperidone derivatives **16** and the 3-pyrrolidone derivatives **17**, which are shown in Scheme 2, were also prepared by similar methods as those described in Scheme 1. Diphenylmethylidene derivative **18**, which is shown in Scheme 3, was synthesized by using dichlorodiphenylmethane instead of benzhydryl bromide as in Scheme 1.

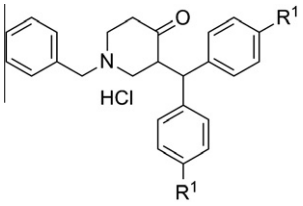
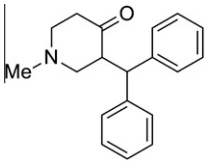
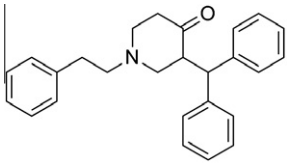
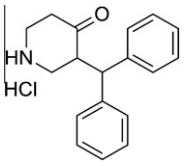
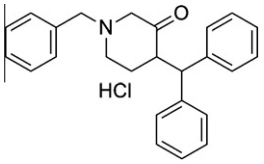
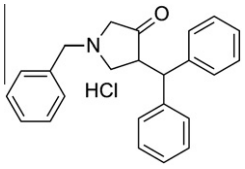
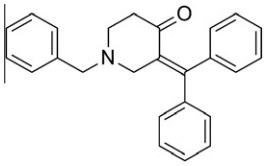
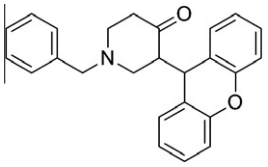
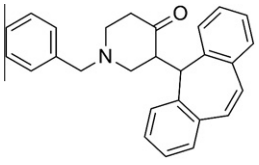
The optical resolution of the lead compound **13r** was performed by chiral column chromatography to assess the differences in biological activity between the enantiomers as shown in Scheme 4. Each of the enantiomers was successfully obtained with high optical purity. The racemization was not observed under physiological conditions (data not shown), and the absolute stereochemistry was not determined.

3. Results and discussion

The compounds thus synthesized were evaluated for their in vitro inhibitory activity against [^{125}I]Bolton–Hunter (BH)-SP binding in human IM-9 cells.[8] The results are listed in Tables 1–3 as % inhibition at 10^{-7} M and/or IC_{50} values.

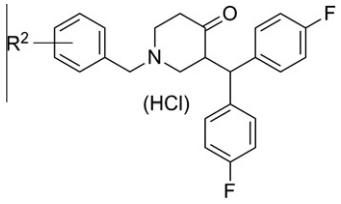
Since we assumed that the relative location of a benzhydryl group, a benzyl group, and a nitrogen atom would play an important role in the inhibitory activity, the effects of various benzhydryl derivatives were examined in order to confirm the requisite structure in compound **13r**. As shown in Table 1, *N*-benzyl piperidone **4** displayed inhibitory activity at 10^{-7} M, while the *N*-methyl

Table 1
Binding affinities of various benzhydryl derivatives for the NK_1 receptor in human IM-9 cells

Compound[a]	Structure	Binding affinity[b]	
		%Inh. (10^{-7} M)	IC_{50} (nM)
4, **7**, **8**		81.7	97
5		−16.2	NC[c]
6		54.3	NC[c]
11		17.6	NC[c]
16		9.7	NC[c]
17		−0.6	NC[c]
18		−2.0	NC[c]
9		29.8	NC[c]
10		31.1	NC[c]

[a] All of the compounds are racemic except for **18**.
[b] Inhibition of [^{125}I]Bolton–Hunter-SP binding in human IM-9 cells.
[c] NC means 'not calculable'.

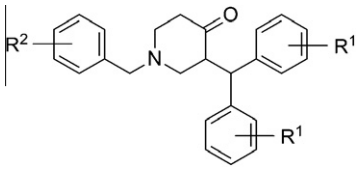
Table 2
Binding affinities of 1-benzyl-3-benzhydryl derivatives: effects of modification of *N*-benzyl group



Compound[a]	R^2	Binding affinity[b] IC_{50} (nM)
4	H	97
13a	2-OH	280
13b	2-MeO	6.6
13c	3-MeO	31
13d	4-MeO	77
13e	2-CF_3	4.9
13f	3-CF_3	270
13g	4-CF_3	220
13h	3,5-$(CF_3)_2$	32.9% inh. (10^{-7} M)
13i	2-F	46
13j	2-CN	6.1
13k	2-NO_2	18
131	2-Ph	14

[a] All of the compounds were racemic.
[b] See corresponding footnotes in Table 1

Table 3
Binding affinities of 1-(di-substituted)benzyl-3-benzhydryl derivatives



Compound[a]	R^1	R^2	Binding affinity[b] IC_{50} (nM)
13b	4-F	2-MeO	6.6
13m	4-F	2,3-$(MeO)_2$	55
13n	4-F	2,4-$(MeO)_2$	9.5
13o	4-F	2,5-$(MeO)_2$	3.1
13p	4-F	2,6-$(MeO)_2$	2.3
13q	4-F	2-MeO, 5-F	4.0
13r	4-F	2-MeO, 5-Br	1.1
13s	4-F	2-MeO, 5-CF_3O	0.81
13t	H	2-MeO, 5-CF3O	0.34
13u	H	2-MeO, 5-^{i}Pr	0.42
13v	H	2-MeO, 5-$N0_2$	2.6
13w	H	2-MeO, 5-NH_2	5.9
13x	H	2-MeO, 5-$NHSO_2Me$	0.26
l3y	H	2-MeO, 5-NHAc	15
(+)-13r	4-F	2-MeO, 5-Br	1.5
(−)-13r	4-F	2-MeO, 5-Br	0.62
TAK-637			0.45

[a] All of the compounds were racemic except for (+)-**13r** and (−)-**13r**.
[b] See corresponding footnotes in Table 1.

(**5**), *N*-phenylethyl (**6**), and N-unsubstituted (**11**) analogs showed low potencies. These results indicated that a benzyl group is the best substituent on piperidone nitrogen. The comparison of compounds **16**–**18** with **4** suggested that the position of the piperidone nitrogen, the ring size, and the linkage between the piperidine ring and the benzhydryl group were crucial for the activity. The effect of the substituents on the benzhydryl group was investigated. The introduction of a fluorine atom at the 4-position (**7**) enhanced the activity slightly, whereas the replacement of the benzhydryl group with 9*H*-xanthene (**9**) or a 5*H*-dibenzo[*a*,*d*][7]annulene (**10**) group resulted in reduced activity. The linkage of both phenyl rings prevents the compound from maintaining a preferable conformation between these phenyl groups and the *N*-benzyl analog.

On the basis of the results regarding the core structure and the benzhydryl group, we conducted further optimization by introducing various substituents onto the *N*-benzyl group (Table 2). The detailed structure–activity relationships were as follows. An introduction at the *ortho*-position was more preferable than at the *meta*- or *para*-position (**13b**–**g**). The *ortho* introduction of substituents with or without hydrogen bonding acceptors enhanced the activity (**13b**, **13e**, **13i**–**l**), whereas *ortho* substitution with a hydrogen bonding donor reduced the activity (**13a**). In addition, compound **13h** containing a 3,5-bis(trifluoromethyl) group showed a remarkably decreased activity even though the 3,5-bis(trifluoromethyl) group is a common substituent in tachykinin NK_1 antagonists.

Subsequently, the effects of additional substituents were examined for **13b**-related compounds (Table 3). The introduction of a methoxyl group at the 4-, 5- or 6-position gave more favorable results than that at the 3-position. Modification at the 5-position resulted in the discovery of the trifluoromethoxyl analog **13s**, which had subnanomolar affinity. Since we found that the corresponding phenyl compound **13t** exhibited more potency than **13s**, further investigation of the phenyl analogs **13u**–**y** was employed, showing that isopropyl (**13u**) and methylsulfonamide (**13x**) had a similar potency to that of **13t**.

Furthermore, to reveal the differences in affinity between the enantiomers, the evaluation of the optically active compounds for the screening hit **13r** was carried out (Table 3). Unexpectedly, the (−)-isomer ((−)-**13r**) was only 2.4 times more potent than the (+)-isomer ((+)-**13r**). To understand the reason for the small differences in the activities between the enantiomers, the conformational similarity was examined using a molecular modeling study with molecular operating environment (MOE) software (Fig. 2).[9] The analysis suggested that both enantiomers of **13r** ((*S*)-**13r** and (*R*)-**13r**) were well superimposed and shared a common pharmacophore.

Compounds with good affinity, such as **13r**, **13t**, **13u**, and **13x** were evaluated for the inhibition of capsaicin-induced plasma extravasation in the trachea of guinea pigs upon oral and intravenous administration.[10] Unfortunately, the in vivo activities were weaker than we expected. We suspect that the results involved pharmacokinetic issues due to the high lipophilicity and low solubility of the compounds. Further optimizations to resolve the issues are being carried out and will be reported in due course.

In addition, the series of compounds can be used as templates for chemical library synthesis because of their easy synthetic accessibility for broad modification. Furthermore, we found that several related compounds were active toward other GPCRs such as PAR1, PAR2, GPR100, and orexin receptor 2. Thus, the series of compounds may serve as drug-like leads for GPCRs because they have the benzhydryl (1,1-diphenylmethane) group, which is well known as a privileged substructure for GPCRs.

4. Conclusion

We revealed that a series of novel 3-benzhydryl-4-piperidone derivatives are potent tachykinin NK_1 receptor antagonists. A coupling reaction of 1-benzylpiperidones with benzhydryl bromides or benzhydrols in the presence of TMSOTf and a condensation reaction of piperidones with benzyl alcohols using EPPA are useful for the synthesis of the 3-benzhydryl-4-piperidone skeleton. The 3-benzhydryl-4-piperidone skeleton can be used for a chemical library synthesis of drug-like lead compounds targeting GPCRs.

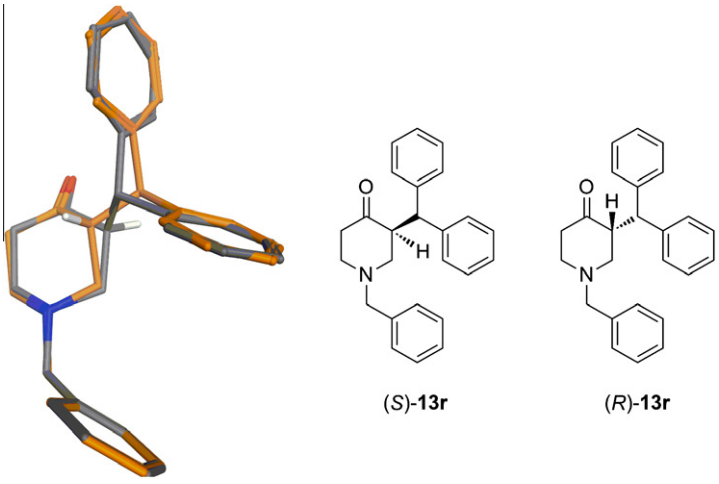


Figure 2. Overlapping between the most stable conformations of (*S*)-**13r** (gray) and (*R*)-**13r** (orange).

5. Experimental

5.1. Chemistry

Melting points were determined with a Yanagimoto melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a Varian Gemini-200 spectrometer. Chemical shifts are given in δ values (ppm) using tetramethylsilane as the internal standard. Reactions were followed by TLC on Silica Gel 60 F 254 precoated TLC plates (E. Merck) or NH TLC plates (Fuji Silysia Chemical Ltd). Chromatographic separations were carried out on Silica Gel 60 (0.063–0.200 or 0.040–0.063 mm, E. Merck) or basic silica gel (Chromatorex® NH, 100–200 mesh, Fuji Silysia Chemical Ltd) using the indicated eluents. Compounds **1**–**3** and solvents were commercially available and used as received. Yields are not optimized. Chemical intermediates were characterized by ^{1}H NMR. Elemental analysis (C, H, N) were carried out by the Analytical Department of Takeda Chemical Industries.

5.1.1. 3-Benzhydryl-1-benzyl-4-piperidone hydrochloride (4)

To a cooled solution of **1** (95 g, 0.50 mol) in CH_2Cl_2 (250 mL) was added trimethylsilyl trifluoromethanesulfonate (TMSOTf) (100 mL) portionwise in an ice water bath at below 25 °C. After stirring for 30 min, benzhydryl bromide (125 g, 0.50 mol) was added thereto, followed by zinc bromide (5.0 g). Then, the mixture was warmed to room temperature overnight and poured into ice (300 g) and sodium acetate (100 g). The organic layer was washed with water and concentrated in vacuo. The resultant oil was dissolved in EtOH (200 mL). Concentrated HCl (100 mL) was slowly added and then the mixture was concentrated. The residue was dissolved in EtOH (500 mL), and cooled in an ice bath. The precipitate was collected by filtration to give **4** (144 g, 74%) as crystals. Mp 205–207 °C. ^{1}H NMR ($CDCl_3$) δ: 2.20–2.40, 2.50–2.70 and 2.75–2.90 (m, 6H), 3.30–3.40 (m, 1H), 3.44 and 3.54 (q, J = 13.2 Hz, 2H), 4.64 (d, J = 11.2 Hz, 1H), 7.17–7.27 (m, 15H) (as free base). Anal. Calcd for $C_{25}H_{26}ClNO$: C, 76.61; H, 6.69; N, 3.57. Found: C, 76.52; H, 6.82; N, 3.50.

The following compounds **5** and **6** were prepared in a manner similar to that described for the synthesis of **4** using **2** or **3** instead of **1**. Compounds were obtained as free amine.

5.1.2. 3-(Diphenylmethyl)-1-methylpiperidin-4-one (5)

Crystals. Mp 127–128 °C. ^{1}H NMR ($CDCl_3$) δ: 2.28 (s, 3H), 2.45–2.60 (m, 4H), 2.70–2.80 (m, 2H), 3.40–3.55 (m, 2H), 4.56 (d, J = 11.4 Hz, 1H), 7.10–7.35 (m, 10H). Anal. Calcd for $C_{19}H_{21}NO$: C, 81.68; H, 7.58; N, 5.01. Found: C, 81.55; H, 7.70; N, 4.89.

5.1.3. 3-(Diphenylmethyl)-1-(2-phenylethyl)piperidin-4-one (6)

Crystals. Mp 73–76 °C. ^{1}H NMR ($CDCl_3$) δ: 2.30–3.05 (m, 10H), 4.57 (d, J = 11.4 Hz, 1H), 7.10–7.40 (m, 15H). Anal. Calcd for $C_{26}H_{27}NO$: C, 84.51; H, 7.37; N, 3.79. Found: C, 84.71; H, 7.44; N, 3.53.

5.1.4. 1-Benzyl-3-[bis(4-fluorophenyl)methyl]piperidin-4-one hydrochloride (7)

To a cooled mixture of **1** (10 g, 50 mmol) in CH_2Cl_2 (50 mL) was added TMSOTf (20 mL) in an ice bath, followed by 4,4′-difluorobenzhydrol (11 g, 55 mmol). The mixture was stirred at room temperature for 14 h. Iced water and sodium acetate (10 g) were added thereto. The organic layer was washed with water and concentrated in vacuo. The resultant oil was dissolved in EtOH (50 mL) and concentrated HCl (10 mL) and kept in a refrigerator overnight. The precipitate was collected by filtration and washed with cooled EtOH to give **7** (16.9 g, 79%) as crystals. Mp 180–182 °C. ^{1}H NMR ($CDCl_3$) δ: 2.30–2.75 (m, 5H), 2.80–2.94 (m, 1H), 3.20–3.30 (m, 1H), 3.42 and 3.57 (q, J = 13.0 Hz, 2H), 4.61 (d, J = 11.0 Hz, 1H), 6.81–7.30 (m, 13H) (as free base). Anal. Calcd for $C_{25}H_{24}ClF_2NO$: C, 70.17; H, 5.65; N, 3.27. Found: C, 70.08; H, 5.69; N, 3.42.

The following compounds from **8** to **10** were prepared in a manner similar to that described for the synthesis of **7** and desalting process if needed.

5.1.5. 1-Benzyl-3-[bis(4-chlorophenyl)methyl]piperidin-4-one hydrochloride (8)

Crystals. Mp 192–194 °C. ^{1}H NMR ($CDCl_3$) δ: 2.30–2.90 (m, 6H), 3.20–3.34 (m, 1H), 3.42 and 3.57(q, J = 12.9 Hz, 2H), 4.56 (d, J = 11.0 Hz, 1H), 7.00–7.32 (m, 13H) (as free base). Anal. Calcd for $C_{25}H_{24}Cl_3NO$: C, 65.16; H, 5.25; N, 3.04. Found: C, 64.76; H, 5.33; N, 3.25.

5.1.6. 1-Benzyl-3-(9*H*-xanthen-9-yl)piperidin-4-one (9)

Crystals. Mp 124–125 °C. ^{1}H NMR ($CDCl_3$) δ: 2.05–2.87 (m, 7H), 3.27 and 3.57(q, J = 13.0 Hz, 2H), 4.91 (d, J = 4.0 Hz, 1H), 6.90–7.35 (m, 13H). Anal. Calcd for $C_{25}H_{23}NO_2$: C, 81.21; H, 6.27; N, 3.79. Found: C, 81.11; H, 6.19; N, 3.97.

5.1.7. 1-Benzyl-3-(5*H*-dibenzo[*a*,*d*][7]annulen-5-yl)piperidin-4-one (10)

Crystals. Mp 167–169 °C. ^{1}H NMR ($CDCl_3$) δ: 2.08–3.20 (m, 7H), 3.39 and 3.47 (q, J = 13.2 Hz, 2H), 4.74 (d, J = 10.8 Hz, 1H), 6.86 (d, J = 11.8 Hz, 1H), 6.99 (d, J = 11.8 Hz, 1H), 7.10–7.40 (m, 13H). Anal. Calcd for $C_{27}H_{23}NO{\cdot}0.25H_2O$: C, 84.67; H, 6.45; N, 3.66. Found: C, 84.73; H, 6.58; N, 3.84.

5.1.8. 3-Benzhydryl-4-piperidone hydrochloride (11)

A mixture of **4** (210 g, 0.59 mol) and 5%Pd-C (21 g) in MeOH (2.8 L) and water (280 mL) was stirred under an atmosphere of H_2 (1 atm) at 30–40 °C for 3–4 h. The catalyst was removed by filtration and the filtrate was concentrated in vacuo. The resultant oil was dissolved in EtOH (300 mL). The solution was kept in a refrigerator for 14 h. The precipitate was collected by filtration with cooled EtOH to give **11** (150 g, 92%) as crystals. Mp 208–210 °C. ^{1}H NMR (DMSO-d_6) δ: 2.75–3.70 (m, 6H), 4.14 (m, 1H), 4.47 (d, J = 11.0 Hz, 1H), 7.35 (m, 10H), 9.63 (br s, 2H). Anal. Calcd for $C_{18}H_{20}ClNO$: C, 71.63; H, 6.68; N, 4.64. Found: C, 71.41; H, 6.60; N, 4.66.

The following compound **12** was prepared in a manner similar to that described for the synthesis of **11**.

5.1.9. 3-(4,4′-Difluorobenzhydryl)-4-piperidone hydrochloride (12)

Crystals. Mp 122–123 °C. ^{1}H NMR (DMSO-d_6) δ: 2.70–3.70 (m, 6H), 4.06 (m, 1H), 4.55 (d, J = 11.2 Hz, 1H), 7.00–7.50 (m, 8H), 9.56 (br s, 2H). Anal. Calcd for $C_{18}H_{18}F_2ClNO$: C, 64.00; H, 5.37; N, 4.15. Found: C, 63.81; H, 5.65; N, 4.15.

5.1.10. 3-[Bis(4-fluorophenyl)methyl]-1-(2-hydroxybenzyl) piperidin-4-one (13a)

A cooled mixture of **12** (6.7 g, 20.0 mmol), 2-hydroxybenxyl alcohol (2.6 g, 20.0 mmol) and diisopropylethylamine (10.2 mL, 58.6 mmol) in CH_2Cl_2 (50 mL) was added ethyl *o*-phenylenephosphate (EPPA) (4.8 mL) in an ice bath. The mixture was stirred at room temperature for 60 min, and poured into EtOAc. The solution was washed with water, dried over magnesium sulfate and concentrated in vacuo. The residue was crystallized from diethyl ether to give **13a** (6.9 g, 85%) as crystals. Mp 151–152 °C. ^{1}H NMR ($CDCl_3$) δ: 2.40–2.91 (m, 6H), 3.41 (m. 1H), 3.70 (s, 2H), 4.44 (d, J = 11.0 Hz, 2H), 6.74–7.28 (m, 12H). Anal. Calcd for $C_{25}H_{23}FNO_2$: C, 73.69; H, 5.69; N, 3.44. Found: C, 73.78; H, 5.72; N, 3.43.

The following compounds **13b**–**u** were prepared in a manner similar to that described for the synthesis of **13a** and salt formation process if needed.

5.1.11. 3-[Bis(4-fluorophenyl)methyl]-1-(2-methoxybenzyl) piperidin-4-one hydrochloride (13b)

Crystals. Mp 163–165 °C. ^{1}H NMR ($CDCl_3$) δ: 2.35–3.80 (m, 6H), 3.70 (s, 3H), 4.20–4.40 (m, 3H), 4.90 (m, 1H), 6.80–7.60 (m, 12H) (as free base). Anal. Calcd for $C_{26}H_{26}F_2ClNO_2$: C, 68.19; H, 5.72; N, 3.06. Found: C, 67.98; H, 5.72; N, 3.11.

5.1.12. 3-[Bis(4-fluorophenyl)methyl]-1-(3-methoxybenzyl) piperidin-4-one hydrochloride (13c)

Crystals. Mp 161–163 °C. ^{1}H NMR ($CDCl_3$) δ: 2.25–2.95 (m, 6H), 3.26 (m, 1H), 3.83 (s, 3H), 4.59 (d, J = 11.0 Hz, 1H), 6.75–7.85 (m, 12H) (as free base). Anal. Calcd for $C_{26}H_{26}F_2ClNO_2$: C, 68.19; H, 5.72; N, 3.06. Found: C, 68.05; H, 5.63; N, 2.94.

5.1.13. 3-[Bis(4-fluorophenyl)methyl]-1-(4-methoxybenzyl) piperidin-4-one hydrochloride (13d)

Crystals. Mp 187–190 °C. ^{1}H NMR ($CDCl_3$) δ: 2.25–2.40 (m, 1H), 2.45–2.70 (m, 4H), 2.80–2.90 (m, 1H), 3.19–3.27 (m, 1H), 3.34 (d, J = 12.9 Hz, 1H), 3.51 (d, J = 12.9 Hz, 1H), 3.82 (s, 3H), 4.58 (d, J = 11.4 Hz, 1H), 6.80–7.28 (m, 12H) (as free base). Anal. Calcd for $C_{26}H_{26}F_2ClNO_2$: C, 68.19; H, 5.72; N, 3.06. Found: C, 67.98; H, 5.72; N, 3.11.

5.1.14. 3-[Bis(4-fluorophenyl)methyl]-1-[2-(trifluoromethyl) benzyl]piperidin-4-one (13e)

Crystals. Mp 102–103 °C. ^{1}H NMR ($CDCl_3$) δ: 2.30–2.95 (m, 6H), 3.28(m, 1H), 3.59 (d, J = 14.0 Hz, 1H), 3.73 (d, J = 14.0 Hz, 1H), 4.59 (d, J = 11.2 Hz, 1H), 6.80–7.68 (m, 12H). Anal. Calcd for $C_{26}H_{22}F_5NO$: C, 67.97; H, 4.83; N, 3.05. Found: C, 67.93; H, 4.63; N, 2.95.

5.1.15. 3-[Bis(4-fluorophenyl)methyl]-1-[3-(trifluoromethyl) benzyl]piperidin-4-one (13f)

Crystals. Mp 150–151 °C. ^{1}H NMR ($CDCl_3$) δ: 2.25–3.00 (m, 6H), 3.20 (m, 1H), 3.51 (d, J = 13.0 Hz, 1H), 3.60 (d, J = 13.0 Hz, 1H), 4.59 (d, J = 11.0 Hz, 1H), 6.75–7.35 (12H, m). Anal. Calcd for $C_{26}H_{22}F_5NO{\cdot}0.1H_2O$: C, 67.34; H, 5.20; N, 3.14. Found: C, 67.19; H, 5.72; N, 2.90.

5.1.16. 3-[Bis(4-fluorophenyl)methyl]-1-[4-(trifluoromethyl) benzyl]piperidin-4-one (13g)

Crystals. Mp 126–127 °C. ^{1}H NMR ($CDCl_3$) δ: 2.30–2.95 (m, 6H), 3.26 (m, 1H), 3.45 (d, J = 13.2 Hz, 1H), 3.62 (d, J = 13.2 Hz, 1H), 4.56 (d, J = 11.2 Hz, 1H), 6.81–7.59 (m, 12H). Anal. Calcd for $C_{26}H_{22}F_5NO$: C, 67.97; H, 4.83; N, 3.05. Found: C, 67.75; H, 4.91; N, 3.01.

5.1.17. 3-[Bis(4-fluorophenyl)methyl]-1-[3,5-bis(trifluoromethyl)benzyl]piperidin-4-one (13h)

Crystals. Mp 205–206 °C. ^{1}H NMR ($CDCl_3$) δ: 2.33–2.61 (m, 2H), 2.52 (t, J = 6.0 Hz, 2H), 2.83 (t, J = 6.0 Hz, 2H), 3.31 (m, 1H), 3.52, (d, J = 13.7 Hz, 1H), 3.65(d, J = 13.7 Hz, 1H), 4.47 (d, J = 11.4 Hz, 1H), 6.78–7.79 (m, 11H). Anal. Calcd for $C_{27}H_{21}F_8NO$: C, 61.48; H, 4.01; N, 2.66. Found: C, 61.42; H, 4.15; N, 2.49.

5.1.18. 3-[Bis(4-fluorophenyl)methyl]-1-(2-fluorobenzyl) piperidin-4-one hydrochloride (13i)

Crystals. Mp 162–164 °C. ^{1}H NMR ($CDCl_3$) δ: 2.25–3.00 (m, 6H), 3.20 (m, 1H), 3.51 (d, J = 13.0 Hz, 1H), 3.60 (d, J = 13.0 Hz, 1H), 4.59 (d, J = 11.0 Hz, 1H), 6.75–7.35 (m, 12H) (as free base). Anal. Calcd for $C_{25}H_{23}F_3ClNO$: C, 67.34; H, 5.20; N, 3.14. Found: C, 67.19; H, 5.72; N, 2.90.

5.1.19. 2-({3-[Bis(4-fluorophenyl)methyl]-4-oxopiperidin-1-yl} methyl)benzonitrile (13j)

Crystals. Mp 83–84 °C. ^{1}H NMR ($CDCl_3$) δ: 2.26–3.10 (m, 6H), 3.20–3.30 (m, 1H), 4.63 (d, J = 11.4 Hz, 1H), 3.57 (d, J = 13.3 Hz, 1H), 3.78 (d, J = 13.3 Hz, 1H), 6.76–7.72 (m, 12H). Anal. Calcd for $C_{26}H_{22}F_2N_2O$: C, 74.98; H, 5.32; N, 6.73. Found: C, 74.99; H, 5.24; N, 6.51.

5.1.20. 3-[Bis(4-fluorophenyl)methyl]-1-(2-nitrobenzyl) piperidin-4-one (13k)

Crystals. Mp 151–152 °C. ^{1}H NMR ($CDCl_3$) δ: 2.15–2.97 (m, 6H), 3.15 (m, 1H), 3.63, (d, J = 13.6 Hz, 1H), 3.95 (d, J = 13.6 Hz, 1H), 4.44 (d, J = 11.4 Hz, 1H), 6.77–7.86 (m, 12H). Anal. Calcd for $C_{25}H_{22}F_2N_2O_3$: C, 68.80; H, 5.08; N, 6.42. Found: C, 68.66; H, 5.11; N, 6.41.

5.1.21. 1-(Biphenyl-2-ylmethyl)-3-[bis(4-fluorophenyl)methyl] piperidin-4-one hydrochloride (13l)

Crystals. Mp 144–146 °C. ^{1}H NMR ($CDCl_3$) δ: 2.20–2.70 (m, 6H), 3.12 (m, 1H), 3.36 (d, J = 13.2 Hz, 1H), 3.53 (d, J = 13.2 Hz, 1H), 4.48 (d, J = 11.0 Hz, 1H), 6.78–7.45 (m, 17H). (as free base). Anal. Calcd for $C_{31}H_{28}ClF_2NO$: C, 73.87; H, 5.60; N, 2.78. Found: C, 73.65; H, 5.44; N, 2.59.

5.1.22. 3-[Bis(4-fluorophenyl)methyl]-1-(2,3-dimethoxybenzyl) piperidin-4-one hydrochloride (13m)

Crystals. Mp 138–140 °C. ^{1}H NMR ($CDCl_3$) δ: 2.25–2.40 (m, 1H), 2.45–2.58 (m, 3H), 2.64–2.76 (m, 1H), 2.86–2.96 (m, 1H), 3.16–3.26 (m, 1H), 3.51 (d, J = 12.9 Hz, 1H), 3.58 (d, J = 12.9 Hz, 1H), 3.81 (3H,

s), 3.88 (3H, s), 4.60 (d, J = 11.1 Hz, 1H), 6.80–7.12 (m, 9H), 7.18–7.27 (m, 2H) (as free base). Anal. Calcd for $C_{27}H_{28}ClF_2NO_3{\cdot}H_2O$: C, 64.09; H, 5.98; N, 2.77. Found: C, 63.99; H, 6.15; N, 2.80.

5.1.23. 3-[Bis(4-fluorophenyl)methyl]-1-(2,4-dimethoxybenzyl)piperidin-4-one hydrochloride (13n)

Crystals. Mp 127–129 °C. ^{1}H NMR ($CDCl_3$) δ: 2.25–2.60 (m, 4H), 2.65–2.76 (m, 1H), 2.82–2.95 (m, 1H), 3.18–3.28 (m, 1H), 3.46 (d, J = 12.9 Hz, 1H), 3.51 (d, J = 12.9 Hz, 1H), 3.75 (s, 3H), 3.83 (s, 3H), 4.57 (d, J = 11.4 Hz, 1H), 6.38–6.50 (m, 2H), 6.80–7.00 (m,4H), 7.03–7.15 (m, 3H), 7.18–7.30 (m, 2H) (as free base). Anal. Calcd for $C_{27}H_{28}ClF_2NO_3{\cdot}0.5H_2O$: C, 65.25; H, 5.88; N, 2.82. Found: C, 65.20; H, 6.11; N, 2.70.

5.1.24. 3-[Bis(4-fluorophenyl)methyl]-1-(2,5-dimethoxybenzyl)piperidin-4-one hydrochloride (13o)

Crystals. Mp 195–198 °C. ^{1}H NMR ($CDCl_3$) δ: 2.35–2.41 (m, 1H), 2.44–2.35 (m, 2H), 2.58, 2.61 (dd, J = 12.0, 4.0 Hz, 1H), 2.72–2.78 (m, 1H), 2.86–2.90 (m, 1H), 3.23–3.29 (m, 1H), 3.53 (s, 2H), 3.73 (s, 3H), 3.80 (s, 3H), 4.59 (d, J = 10.8 Hz, 1H), 6.78 (s, 2H), 6.84–6.95 (m, 5H), 7.08, 7.10 (dd, J = 8.8, 5.2 Hz, 2H), 7.22–7.26 (m, 2H) (as free base). Anal. Calcd for $C_{27}H_{28}ClF_2NO_3{\cdot}H_2O$: C, 64.09; H, 5.98; N, 2.77. Found: C, 64.11; H, 6.18; N, 2.79.

5.1.25. 3-[Bis(4-fluorophenyl)methyl]-1-(2,6-dimethoxybenzyl)piperidin-4-one (13p)

Crystals. Mp 125–126 °C. ^{1}H NMR ($CDCl_3$) δ: 2.35–2.41 (m, 1H), 2.44–2.35 (m, 2H), 2.58, 2.61 (dd, J = 12.0, 4.0 Hz, 1H), 2.72–2.78 (m, 1H), 2.86–2.90 (m, 1H), 3.23–3.29 (m, 1H), 3.53 (s, 2H), 3.73 (s, 3H), 3.80 (s, 3H), 4.59 (d, J = 10.8 Hz, 1H), 6.78 (s, 2H), 6.84–6.95 (m, 5H), 7.08, 7.10 (dd, J = 8.8, 5.2 Hz, 2H), 7.22–7.26 (m, 2H). Anal. Calcd for $C_{27}H_{27}F_2NO_3$: C, 71.05; H, 6.19; N, 3.19. Found: C, 70.93; H, 5.93; N, 3.17.

5.1.26. 3-[Bis(4-fluorophenyl)methyl]-1-(5-fluoro-2-methoxybenzyl)piperidin-4-one (13q)

Amorphous power. ^{1}H NMR ($CDCl_3$) δ: 2.38–2.61 (m, 4H), 2.74–2.78 (m, 1H), 2.85–2.90 (m, 1H), 3.26–3.31 (m, 1H), 3.52 (s, 2H), 3.74 (s, 3H), 4.58 (d, J = 11.2 Hz, 1H), 6.75 (m, 1H), 6.86–6.96 (m, 5H), 7.09–7.12 (m, 3H), 7.23–7.26 (m, 2H).

5.1.27. 3-[Bis(4-fluorophenyl)methyl]-1-(5-bromo-2-methoxybenzyl)piperidin-4-one (13r)

Crystals. Mp 129–130 °C. ^{1}H NMR ($CDCl_3$) δ: 2.35–3.29 (m, 6H), 3.29 (m, 1H), 3.50 (s, 2H), 3.74(s, 3H), 4.55 (d, J = 11.0 Hz, 1H), 6.68–7.47 (m, 11H). Anal. Calcd for $C_{26}H_{24}BrF_2NO_2$: C, 71.05; H, 6.19; N, 3.19. Found: C, 70.93; H, 5.93; N, 3.17.

5.1.28. 3-[Bis(4-fluorophenyl)methyl]-1-[2-methoxy-5-(trifluoromethoxy)benzyl]piperidin-4-one hydrochloride (13s)

Amorphous powder. ^{1}H NMR ($CDCl_3$) δ: 2.40–2.45 (m, 2H), 2.49–2.61 (m, 4H), 3.30 (m, 1H), 3.53 (s, 2H), 3.77 (s, 3H), 4.56 (d, J = 11.2 Hz, 1H), 6.81 (d, J = 9.2 Hz, 1H), 6.87 (dd, J = 8.8, 8.6 Hz, 2H), 6.94 (dd, J = 8.8, 8.6 Hz, 2H), 7.07–7.12 (m, 3H), 7.22–7.25 (m, 3H) (as free base).

5.1.29. 3-(Diphenylmethyl)-1-[2-methoxy-5-(trifluoromethoxy)benzyl]piperidin-4-one hydrochloride (13t)

Crystals. Mp 174–175 °C. ^{1}H NMR ($CDCl_3$) δ: 2.39–2.55 (m, 3H), 2.63–2.66 (m, 1H), 2.72–2.78 (m, 1H), 2.82–2.86 (m, 1H), 3.39–3.45 (m, 1H), 3.53 (s, 2H), 3.76 (s, 3H), 4.59 (d, J = 11.2 Hz, 1H), 6.80 (d, J = 8.8 Hz, 1H), 7.08–7.32 (m, 12H) (as free base). Anal. Calcd for $C_{27}H_{27}ClF_3NO_3$: C, 64.09; H, 5.38; N, 2.77. Found: C, 64.11; H, 5.53; N, 2.65.

5.1.30. 3-(Diphenylmethyl)-1-[2-methoxy-5-(1-methylethyl)benzyl]piperidin-4-one (13u)

Amorphous powder. ^{1}H NMR ($CDCl_3$) δ: 1.23–1.25 (m, 6H), 2.41–2.52 (m, 3H), 2.64–2.68 (m, 1H), 2.77–2.90 (m, 3H), 3.41–3.42 (m, 1H), 3.56 (s, 2H), 3.74 (s, 3H), 4.59 (d, J = 10.8 Hz, 1H), 6.79 (d, J = 8.0 Hz, 1H), 7.09–7.31 (m, 12H).

5.1.31. 3-(Diphenylmethyl)-1-(2-methoxy-5-nitrobenzyl)piperidin-4-one (13v)

A mixture of **11** (9.1 g, 30 mmol), 2-methoxy-5-nitrobenzylbromide (7.4 g, 30 mmol), $NaHCO_3$ (5.0 g, 60 mmol) and DMF (40 mL) was stirred at room temperature for 30 h. After diluting with EtOAc (250 mL), hexane (50 mL) and H_2O (400 mL), the phases were separated. The organic phase was washed with H_2O, dried and concentrated. The residue was treated with EtOH to give **13v** as colorless crystals (12.0 g, 93%). Mp 119–120 °C. ^{1}H NMR ($CDCl_3$) δ: 2.42–4.50 (m, 2H), 2.54–2.67 (m, 2H), 2.78–2.82 (m, 2H), 3.44–3.50 (m, 1H), 3.56 (s, 2H), 3.87 (s, 3H), 4.56 (d, J = 11.2 Hz, 1H), 6.87 (d, J = 8.8 Hz, 1H), 7.05–7.32 (m, 10H), 8.14–8.17 (m, 1H), 8.33 (d, J = 2.8 Hz, 1H). Anal. Calcd for $C_{26}H_{26}N_2O_4$: C, 72.54; H, 6.09; N, 6.51. Found: C, 72.37; H, 5.92; N, 6.41.

5.1.32. 1-(5-Amino-2-methoxybenzyl)-3-(diphenylmethyl)piperidin-4-one dihydrochloride (13w)

To a solution of **13v** (1.8 g, 4.3 mmol) in THF (10 mL) was added a suspension of 5% Pd–C (520 mg) in MeOH (15 mL). The mixture was stirred under an atmosphere of hydrogen at room temperature. The catalyst was removed by filtration. After the filtrate was diluted with concentrated HCl (0.71 mL), toluene (40 mL), EtOH (50 mL), the mixture was evaporated. The residue was treated with EtOH–EtOAc (4:1, v/v, 50 mL) to give **13w** as colorless crystals (1.8 g, 88%). Mp 198–200 °C. ^{1}H NMR ($CDCl_3$) δ: 2.40–4.60 (m, 13H), 7.06–7.56 (m, 13H), 10.30 (br, 2H) (as free base). Anal. Calcd for $C_{26}H_{30}Cl_2N_2O_2{\cdot}0.5H_2O$: C, 64.73; H, 6.48; N, 5.81. Found: C, 64.90; H, 6.48; N, 5.71.

5.1.33. *N*-(3-{[3-(Diphenylmethyl)-4-oxopiperidin-1-yl]methyl}-4-methoxyphenyl)methanesulfonamide (13x)

To a solution of **13x** (473 mg, 1.0 mmol) in pyridine (5 mL) was added methanesulfonyl chloride (150 mg, 1.3 mmol) at room temperature, and then the mixture was stirred for 1 h and poured into H_2O (0.2 mL). The mixture was concentrated and the residue was diluted with EtOAc (40 mL) and aqueous $NaHCO_3$ (30 mL). The organic phase was dried and concentrated. The residue was purified by silica gel column chromatography using EtOAc–hexane (1:1, v/v) as an eluent to give **13x** as amorphous powder (408 mg, 85%). ^{1}H NMR ($CDCl_3$) δ: 2.36–2.43 (m, 1H), 2.54–2.63 (m, 3H), 2.71–2.77 (m, 1H), 2.85–2.91 (m, 1H), 2.94 (s, 3H), 3.39–3.44 (m, 1H), 3.49–3.59 (m, 2H), 3.77 (s, 3H), 4.64 (d, J = 11.2 Hz, 1H), 6.20 (br, 1H), 6.81 (d, J = 8.8 Hz, 1H), 7.11–7.31 (m, 12H).

The following compound **13y** was prepared in a manner similar to that described for the synthesis of **13x** using acetyl chloride instead of methanesulfonyl chloride.

5.1.34. *N*-(3-{[3-(Diphenylmethyl)-4-oxopiperidin-1-yl]methyl}-4-methoxyphenyl)acetamide (13y)

Amorphous powder. ^{1}H NMR ($CDCl_3$) δ: 2.19 (s, 3H), 2.37–2.43 (m, 1H), 2.49–2.55 (m, 2H), 2.62–2.66 (m, 1H), 2.72–2.78 (m, 1H), 2.82–2.88 (m, 1H), 3.38–3.43 (m, 1H), 3.49–3.59 (m, 2H), 3.74 (s, 3H), 4.62 (d, J = 11.2 Hz, 1H), 6.79 (d, J = 8.4 Hz, 1H), 7.01 (br, 1H), 7.11–7.42 (m, 12H).

5.1.35. (+)-3-[Bis(4-fluorophenyl)methyl]-1-(5-bromo-2-methoxybenzyl)piperidin-4-one ((+)-13r)

See Section 5.1.36.

5.1.36. (−)-3-[Bis(4-fluorophenyl)methyl]-1-(5-bromo-2-methoxybenzyl)piperidin-4-one ((−)-13r)

An optical resolution of **13r** (1.0 g) was carried out by preparative high-performance liquid chromatography (HPLC) using Chiralpak AD (50.0 mmID × 500 mmL, DAICEL Chemical Industries, Ltd, Japan) under detection at 254 nm with a mixture of hexane and 2-propanol (9:1, v/v) as the eluent at a flow rate of 70 mL/min at 28 °C to give (+)-**13r** (0.41 g, 41%, 99.9%*ee*) and (−)-**13r** (0.43 g, 43%, 99.9%*ee*) as colorless powders: (+)-**13r**, $[\alpha]_D$ = +93.0 (*c* 1.049, MeOH); (−)-**13r**, $[\alpha]_D$ = −94.3 (*c* 1.049, MeOH).

5.1.37. 1-Benzyl-4-(diphenylmethyl)piperidin-3-one hydrochloride (16)

Compound **14** (10 g, 44 mmol) was dissolved in CH_2Cl_2 (150 mL) and then saturated aqueous NaCl (50 mL) and potassium bicarbonate (6 g) were added thereto. The organic layer was dried over anhydrous magnesium sulfate and concentrated in vacuo. The resultant oil was dissolved in CH_2Cl_2 (150 mL). The mixture was cooled in an ice bath and TMSOTf (18 mL) was added, followed by benzhydrol (8.2 g, 44 mmol). The mixture was stirred at room temperature for 14 h. Cooled water (100 mL) and sodium acetate (20 g) were added thereto. The organic layer was washed with water and concentrated in vacuo. The resultant oil was dissolved in EtOH (5 mL) and concentrated HCl (5 mL) and kept in a refrigerator overnight. The precipitate was collected by filtration with cooled EtOH to give **16** (12.4 g, 72%) as crystals. Mp 169–171 °C. ^{1}H NMR ($CDCl_3$) δ: 1.50–3.30 (m, 6H), 2.92 and 3.16 (q, *J* = 13.0 Hz, 1H), 3.59 (s, 2H), 4.40 (d, *J* = 9.8 Hz, 1H), 7.28 (m, 15H). Anal. Calcd for $C_{25}H_{26}ClNO$: C, 76.61; H, 6.69; N, 3.57. Found: C, 76.52; H, 6.70; N, 3.33.

The following compound **17** was prepared in a manner similar to that described for the synthesis of **16**.

5.1.38. 1-Benzyl-4-benzhydryl-3-pyrrolidone hydrochloride (17)

Amorphous powder. ^{1}H NMR ($CDCl_3$) δ: 2.47–3.50 (m, 5H), 3.49 and 3.70 (q, *J* = 13.0 Hz, 2H), 4.50 (1H, d, *J* = 7.0 Hz), 7.00–7.36 (m, 15H). Anal. Calcd for $C_{24}H_{23}ClNO$: C, 76.28; H, 6.40; N, 3.71. Found: C, 76.51; H, 6.55; N, 3.27.

The following compound **18** was prepared in a manner similar to that described for the synthesis **4** using dichlorodiphenylmethane instead of benzhydryl bromide. The compound was obtained as free amine.

5.1.39. 1-Benzyl-3-(diphenylmethylidene)piperidin-4-one (18)

Crystals. Mp 135–137 °C. ^{1}H NMR ($CDCl_3$) δ: 2.67 (t, *J* = 5.8 Hz, 2H), 2.90 (t, *J* = 5.8 Hz, 2H), 3.50 (s, 2H), 3.61 (s, 2H), 7.06–7.25 (m, 15H). Anal. Calcd for $C_{25}H_{23}NO$: C, 84.93; H, 6.56; N, 3.96. Found: C, 83.93; H, 6.62; N, 3.94.

5.2. Biochemical evaluation

5.2.1. [^{125}I]Bolton–Hunter (BH) substance P binding in human IM-9 cells, preparation of receptors

The tachykinin NK_1 receptors from human lymphoblast cells (IM-9) were prepared according to the protocol in the literature with minor modification.[9] IM-9 cells (2×10^5 cells/mL) were inoculated and incubated for 3 days (1 L) and then subjected to centrifugation for 5 min at 500*g* to obtain a cell pellet. The pellet was washed once with PBS crushed using a Polytron homogenizer (Kinematika, Germany) in 30 mL of 50 mM Tris–HC1 buffer (pH 7.4) containing NaCl (120 mM), KC1 (5 mM), chymostatin (2 μg/mL), bacitracin (40 μg/mL), (*p*-amidinophenyl)methanesulfonyl fluoride (40 μg/mL), and ethylenediaminetetraacetic acid (EDTA) (1 mM), and then centrifuged at 40,000*g* for 20 min. The residue was suspended in 30 mL of a reaction buffer [50 mM Tris–HC1 buffer (pH 7.4), 0.02% bovine serum albumin, (*p*-amidinophenyl)methanesulfonyl fluoride (40 μg/mL), chymostatin (2 μg/mL), bacitracin (40 μg/mL) and $MnCl_2$ (3 mM)] and then preserved frozen (−80 °C) as a receptor specimen.

5.2.2. Radioligand binding assay

The above specimen was suspended in the reaction buffer, and a 50 μL portion of the suspension was used in the reaction. After addition of the sample and [^{125}I]BH-SP (final concentration 130 pM), the reaction was allowed to proceed in 0.2 mL of reaction mixture at room temperature for 30 min. The amount of nonspecific binding was determined by adding SP at a final concentration of 2×10^{-6} M. After the reaction, a cell harvester (Filtermate Harvester PerkinElmer, USA) was used, and the reaction was terminated by rapid filtration through a glass filter (GF/C) (PerkinElmer, USA). After washing three times with 50 mM Tris–HC1 buffer (pH 7.4) containing 0.02% bovine serum albumin, the radioactivity remaining on the filter was measured with TopCount Microplate Scintillation Counter (Packard BioScience). Before use, the filter was immersed in 0.3% poly(ethy1enimine) for 2–24 h.

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