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Synthesis and separation of the enantiomers of the Neuropeptide S receptor antagonist (9R/S)-3-Oxo-1,1-diphenyl-tetrahydro-oxazolo[3,4-a]pyrazine-7-carboxylic acid 4-fluoro-benzylamide (SHA 68)

Claudio Trapella^{§,‡}, Michela Pela^{§,‡}, Luisa Del Zoppo[§], Girolamo Calo[#], Valeria Camarda[#], Chiara Ruzza[#], Alberto Cavazzini[¶], Valentina Costa[¶], Valerio Bertolasi^{¶,‡}, Rainer K. Reinscheid[‡], Severo Salvadori[§], and Remo Guerrini^{§,*}

[§]Department of Pharmaceutical Sciences and Biotechnology Center, University of Ferrara, via Fossato di Mortara 19, 44100 Ferrara, Italy

[#]Department of Experimental and Clinical Medicine, Section of Pharmacology and Neuroscience center, and National Institute of Neuroscience, University of Ferrara, via Fossato di Mortara 19, 44100 Ferrara, Italy

[¶]Department of Chemistry, University of Ferrara, via L. Borsari 46, 44100 Ferrara, Italy

[‡]Centre for Structural Diffractometry, University of Ferrara, via L. Borsari 46, 44100 Ferrara, Italy

[‡]Department of Pharmaceutical Sciences, University of California Irvine, 2214 Natural Sciences I, Irvine, CA 92697, USA

Abstract

This study reports the synthesis, chromatographic separation and pharmacological evaluation of the two enantiomers of the neuropeptide S receptor (NPSR) antagonist (9R/S)-3-oxo-1,1-diphenyl-tetrahydro-oxazolo[3,4-a]pyrazine-7-carboxylic acid 4-fluoro-benzylamide (SHA 68). The (9R)-3-oxo-1,1-diphenyl-tetrahydro-oxazolo[3,4-a]pyrazine-7-carboxylic acid 4-fluoro-benzylamide (compound **10**) and (9S)-3-oxo-1,1-diphenyl-tetrahydro-oxazolo[3,4-a]pyrazine-7-carboxylic acid 4-fluoro-benzylamide (compound **10a**) were synthesized and their purity assessed by chiral chromatography. The absolute configuration of the enantiomer **10** has been assigned from the crystal structure of the corresponding (S)-phenyl ethyl amine derivative **8**. Calcium mobilization studies performed on cells expressing the recombinant NPSR demonstrated that compound **10** is the active enantiomer while the contribution of **10a** to the NPSR antagonist properties of the racemic mixture is negligible.

Introduction

Neuropeptide S (NPS) is the last neuropeptide identified via the reverse pharmacology approach.¹ NPS selectively binds and activates a previously orphan GPCR receptor now referred to as NPSR.¹ NPSR is widely distributed in the brain while the expression of the NPS peptide precursor is limited to few discrete brain areas.^{1,2} The supraspinal

*To whom Correspondence should be addressed. Remo Guerrini, Phone: +39-0532-455-988; Fax: +39-0532-455953, r.guerrini@unife.it.

[‡]These authors contributed equally to this work

Supporting Information **Available**: monodimensional and bidimensional NMR spectra of compounds **8**, **8a**, and final products and crystal data of compound **8** (cif file) are available free of charge via the internet at <http://pubs.acs.org>

administration of NPS in rodents produces a rather unique pattern of actions: stimulation of wakefulness associated with anxiolytic-like effects.¹ In addition, NPS has been reported to inhibit food intake, facilitate memory, elicit antinociceptive effects, and recent evidence suggests an involvement of the NPS/NPSR system in drug addiction (see for a review³).

Potent and NPSR selective antagonists are now required for understanding the biological functions controlled by the NPS/NPSR system. As far as peptide antagonists are concerned, these molecules were recently discovered by replacing Gly⁵ in the natural peptide sequence with a D-amino acid. Examples of such compounds are [D-Cys(tBu)⁵]NPS⁴, [D-Val⁵]NPS⁵ and more recently [¹⁴Bu-D-Gly⁵]NPS.⁶ The first example of non-peptide molecules able to interact with the NPSR was reported in the patent literature by Takeda researchers.⁷ Among the different molecules described in the patent, the compound (9R/S)-3-oxo-1,1-diphenyl-tetrahydro-oxazolo[3,4-a]pyrazine-7-carboxylic acid 4-fluoro-benzylamide (SHA 68; compound **1**) has been pharmacologically characterized. In vitro, compound **1** behaves as a selective, potent ($pA_2 \cong 8$) and competitive antagonist at human⁸ and murine⁹ NPSR. In vivo, compound **1** has been reported to prevent the arousal promoting and anxiolytic-like effects elicited by NPS in mice and rats.^{8,9} In addition, compound **1** reversed the protective effect of NPS on the NMDA receptor antagonist MK-801-induced neurotoxicity in rats.¹⁰ Finally recent findings indicate that in the rat intracerebroventricular injection of NPS increased conditioned reinstatement of cocaine seeking, whereas peripheral administration of compound **1** reduced it.¹¹ These pharmacological investigations were performed using the racemic compound **1**. Molecular modelling studies investigated non-peptide ligand binding to NPSR.¹² In the frame of these studies, docking analyses were performed and a defined NPSR binding pocket was proposed; of note, only the (S) enantiomer of compound **1** was used in such simulations. The importance of ligand chirality for NPSR interaction has been recently supported by the identification of two novel classes of non-peptide NPSR antagonists: the quinoline¹³ and the tricyclic imidazole¹⁴ based compounds. In both cases, a single bioactive enantiomer was obtained by chiral chromatography separation from the corresponding racemic mixture.

In the present study, we report the synthesis, chiral HPLC analysis, X-ray crystallographic assignment and in vitro pharmacological evaluation of the two compound **1** enantiomers.

Results and Discussion

The reference compound **1** was synthesized following the procedures reported by Okamura et al.⁸ In Scheme 1 is described the synthetic approach adopted for the synthesis of **10** and **10a** starting from (S)-phenyl ethyl amine. As reported in literature^{15, 16} the use of phenyl ethyl amine was expected to induce the stereochemistry of C9 of the tetrahydro-oxazolo[3,4-a]pyrazine nucleus. Unfortunately, we obtained only a slight chiral induction corresponding approximately to a 60 / 40% ratio determined by NMR spectroscopy. Similar results were obtained using (R)-phenyl ethyl amine as chiral auxiliary. Nevertheless, diastereomers **8** and **8a** were successfully separated in good yield by flash chromatography. The removal of the chiral auxiliary to obtain **9** and **9a** and the acylation of N7 with *p*-fluoro-benzylisocyanate to obtain final compounds were achieved using the procedure reported by Okamura et al.⁸ The purity grade and enantiomeric excess of **10** and **10a** were determined by chiral HPLC analysis. The top panel of Figure 1 shows the chromatogram for the single enantiomer **10a** (first eluted component), the middle panel that of **10** (second eluted species) and the bottom panel of the figure displays the chromatogram for the racemate. As it is evident from this figure, there is no trace of **10** in the chromatogram corresponding to the elution of **10a**, nor of **10a** in that of **10**. In order to define the absolute configuration of the C9 chiral centre, we explored different crystallization conditions for compounds **8**, **8a**, **9**, **9a** and for the final products. Only with compound **8** we were able to obtain crystals suitable for further X-ray

investigation. In particular, compound **8** was crystallized from ethanol/ ethyl acetate and its X-ray analysis demonstrated the absolute configuration R at the chiral center C9. The absolute C9 configuration of compound **8** has been assigned by reference to the unchanged chiral centre C10 in configuration S (Figure 2). On the basis of the absolute configuration of **8**, we were able to assign the absolute C9 configuration to compounds **8a**, **9**, **9a** and that to the final products **10** and **10a**.

In parallel, we performed a series of NMR experiments. In Figure 3 the enlarged [¹H]NMR spectra of the C9 proton region of the **10a** and **10** isomers are depicted. The coupling constant analysis between H_x and H_a/H_b C8 protons are very similar (11.3/3.7 Hz for **10a**, panel A and 11.2/3.7 Hz for **10**, panel B) in both compounds. This result together with X-ray data of **8** (see Figure 2), confirms the axial position of C9 proton in both enantiomers.

Next, we evaluated and compared the in vitro NPSR antagonist properties of compound **1**, compound **10**, and compound **10a**. The three samples were tested in calcium mobilization studies performed on HEK293 cells expressing the murine NPSR or the two isoforms of the human receptor (hNPSR^{Asn107} and hNPSR^{Ile107}).¹⁷

The natural peptide NPS was able to induce calcium mobilization in a concentration dependent manner in HEK 293 mNPSR (pEC₅₀ 8.97 ± 0.11; E_{max} 250 ± 11%), hNPSR^{Asn107} (pEC₅₀: 9.07 ± 0.11; E_{max} 316 ± 13%) and hNPSR^{Ile107} (pEC₅₀: 9.17 ± 0.15; E_{max} 333 ± 17%). The three samples were challenged against the stimulatory effect of 10 nM NPS in inhibition response curves (Figure 4). Compound **1**, compound **10**, and compound **10a** did not stimulated per se calcium mobilization up to 10 μM. Compound **1** inhibited in a concentration dependent manner the stimulatory effect of NPS showing similar high values of potency (pK_B ≅ 8). These values of potency are superimposable to those previously published.^{8,9} Compound **10** was also able to antagonize in a concentration dependent manner the stimulatory effect of NPS displaying values of potency similar or slightly higher than the racemic mixture. By contrast, compound **10a** showed a slight inhibitory effect only at micromolar concentrations. The values of potency of the three compounds in the three cells lines are summarized in Table 1. Collectively, these results demonstrated that compound **10** is the active enantiomer while the contribution of compound **10a** to the biological activity of the racemic mixture is negligible. This information can be extremely useful for the refinement of the recently proposed molecular models of NPSR and its binding pocket.¹² As already mentioned in the introduction, the relevance of ligand chirality for NPSR binding is also corroborated by the fact that the biological activity of chemically different molecules, such as the quinoline¹³ and the tricyclic imidazole¹⁴ compounds, could be attributed to a single bioactive enantiomer.

In conclusion, the present study described the synthesis and separation of the two compound **1** enantiomers. The synthetic scheme we used can be easily scaled up to multi-grams. Compound **10** was demonstrated to be the bioactive enantiomer. Nowadays this molecule represents the standard non peptide NPSR antagonist that has been, and surely will be, used to investigate the biological functions controlled by the NPS / NPSR system and to evaluate the therapeutic potential of innovative drugs acting as NPSR selective ligands.

Experimental Section

Materials

HPLC grade solvent were purchased from Sigma Aldrich (Steinheim, Germany). The purity of the tested compound **1**, compound **10** and compound **10a** has been assessed by RP-HPLC. All compounds showed >95% purity. One-dimensional and two dimensional NMR spectra were recorded on a VARIAN 400 MHz instrument. Chemical shifts are given in ppm

(δ) relative to TMS and coupling constants are in Hz. MS analyses were performed on a ESI-Micromass ZMD 2000. Optical rotation data were recorded on a Perkin-Elmer polarimeter 241. Flash chromatography was carried out on a silica gel (Merck, 230–400 Mesh). Silica gel (Polygram SIL G/UV254) was used for thin layer chromatography.

Typical Procedures for the Synthesis of 10 and 10a

[(1-Phenyl-ethylcarbamoyl)-methyl]-carbamic acid *tert*-butyl ester (3)—To a stirred solution of Boc-Gly-OH (5 g, 28.5 mmol) in CH_2Cl_2 (50 mL), WSC (3.64 g, 19 mmol) and (*S*)-phenylethyl amine (3.45 g, 28.5 mmol) were added. After 24 h at room temperature the reaction was monitored by TLC (EtOAc/light petroleum 2:1). The organic layer was washed with 10% citric acid (20 mL), 5% NaHCO_3 (20 mL) and brine (20 mL). The organic phase was dried, concentrated in vacuo and purified by flash chromatography (EtOAc/light petroleum 2:1) to obtain **3** in 60% yield. ^1H NMR (400MHz, CDCl_3): δ 7.31-7.24 (m, 5H, Ar); 6.62 (bs, 1H, *NH*-CO); 5.31 (bs, 1H, *NH*-Boc); 5.11 (m, 1H, *CH*- CH_3); 3.75 (m, 2H, *CH}_2*-*NH*-Boc); 1.47 (d, 3H, *CH}_3*-*CH*-Ar, $J=6.8$ Hz); 1.42 (s, 9H, ^tbu -); ^{13}C NMR (100MHz, CDCl_3): δ 168.6, 156.2, 143.0, 128.7, 127.4, 126.1, 80.2, 48.7, 44.6, 28.3, 21.9; MS (ESI): $[\text{M}+\text{H}]^+ = 279$; $[\alpha]_{\text{D}}^{20} = -41$ ($c = 0.121\text{g}/100\text{ mL}$, chloroform).

[2-(1-Phenyl-ethylamino)-ethyl]-carbamic acid *tert*-butyl ester (4)—To a stirred suspension of LiAlH_4 (0.85g, 22.38mmol) at 0 °C in anhydrous THF, a solution of **3** (3.11 g, 11.19 mmol) was added drop wise. The reaction was monitored by TLC (EtOAc/light petroleum 3:1) and after 24 hours the excess of hydride was quenched with water and the salts were filtered trough a celite pad. The solvent was evaporated in vacuo to yield **4** (2.66 g, 10.07 mmol) in 90% yield. ^1H NMR (400MHz, CDCl_3): δ 7.32-7.27 (m, 5H, Ar); 4.96 (bs, 1H, *NH*-Boc); 3.77-3.73 (q, 1H, *CH}_3*-*CH*-Ar, $J=6.6$ Hz); 3.16-3.13 (m, 2H, *NH*-*CH}_2*-*CH}_2*); 2.59-2.51 (m, 2H, *NH*-*CH}_2*-*CH}_2*); 1.75 (bs, 1H, -*NH*); 1.42 (s, 9H, ^tbu -); 1.36-1.33 (d, 3H, *CH}_3*-*CH*-Ar, $J=6.6$ Hz);. MS (ESI): $[\text{M}+\text{H}]^+ = 265$; $[\alpha]_{\text{D}}^{20} = -29^\circ$ ($c = 0.11\text{ g}/100\text{ mL}$, chloroform).

{2-[(2-Chloro-acetyl)-(1-phenyl-ethyl)-amino]-ethyl}-carbamic acid *tert*-butyl ester (5)—To a stirred solution of **4** (1.94 g, 7.34 mmol) in EtOAc (50 mL) at 0 °C, saturated solution of NaHCO_3 (5 mL) was added. After 10 min, chloroacetyl chloride (1.17 mL, 14.68 mmol) was added drop wise. The reaction was monitored by TLC (EtOAc/light petroleum 3:1) and after 24 h at room temperature, NaHCO_3 (2 mL) was added to the organic phase. The organic layer was separated and the aqueous phase was extracted twice with EtOAc (50 mL). The combined organic phases were concentrate to dryness to obtain **5** in quantitative yield. ^1H NMR (400MHz, CDCl_3): δ 7.22-6.98 (m, 5H, Ar); 5.59-5.43 (q, 1H, *CH}_3*-*CH*-Ar, $J=8$ Hz); 4.85 (bs, 1H, *NH*-Boc); 4.10-3.97 (m, 2H, *NH*-*CH}_2*-*CH}_2*); 3.76 (s, 2H, *C=O*-*CH}_2*-Cl); 3.18-3.15 (m, 2H, *NH*-*CH}_2*-*CH}_2*); 1.40-1.25 (d, 3H, *CH}_3*-*CH*-Ar, $J=8$ Hz); 1.06 (s, 9H, ^tbu). ^{13}C NMR (100MHz, CDCl_3): δ 170.12, 155.85, 139.47, 128.45, 128.20, 128.03, 80.69, 59.44, 42.86, 41.14, 38.24, 27.48, 19.98. MS (ESI): $[\text{M}+\text{H}]^+ = 341$.

3-oxo-4-(1-phenyl-ethyl)-piperazine-1-carboxylic acid *tert*-butyl ester (6)—To a stirred suspension of 60% NaH (1.14 g, 28.61 mmol) in a mixture of THF/DMF 1/1 (20 mL) at 0 °C, a solution of **5** (3.25 g, 9.54 mmol) in THF/DMF 1/1 (10 mL) was added. After 24 h the reaction was quenched by adding NH_4Cl saturated solution (15 mL) and the solvent was removed in vacuo. The residue was dissolved in EtOAc (50 mL) and washed twice with water (20 mL). The organic layer was dried, evaporated under reduced pressure and the crude product purified by flash chromatography (eluent: EtOAc/light petroleum 1:1) to obtain **6** in 45% yield. ^1H NMR (400MHz, CDCl_3): δ 7.36-7.28 (m, 5H, Ar); 6.08 (q, 1H, *CH}_3*-*CH*-Ar, $J=8\text{Hz}$); 4.23-4.18 (d, 1H, *N-CHeHa-C=O*, $J=20\text{Hz}$); 4.10 (d, 1H, *N-CHeHa-*

C=O, $J=20\text{Hz}$); 3.62 (bs, 1H, CH₂ Piperazine); 3.27 (bs, 1H, CH₂ Piperazine); 3.19 (bs, 1H, CH₂ Piperazine); 2.83 (bs, 1H, CH₂ Piperazine); 1.53-1.51 (d, 3H, CH₃-CH-Ar, $J=8\text{Hz}$); 1.45 (s, 3H, ^tbu-). ¹³C NMR (100MHz, CDCl₃): δ 165.44, 153.78, 139.41, 128.63, 127.68, 127.36, 80.69, 50.08, 47.98, 40.24, 28.32, 15.34. MS (ESI): $[M+H]^+ = 305$; $[\alpha]_D^{20} = -116.0^\circ$ ($c = 0.318$ g/100 mL, chloroform).

4-(1-phenyl-ethyl)-piperazine-carboxylic acid *tert*-butyl ester (7)—To a stirred suspension of LiAlH₄ (453 mg, 18.9 mmol) in anhydrous THF (20 mL) at room temperature, a solution of **6** (1.15 g, 3.78 mmol) in THF (10 mL) was added. After 30 minutes the reaction was completed as showed by TLC analysis (EtOAc/light petroleum 1:2). The reaction was quenched by adding 15% NaOH (1 mL) and Et₂O (20 mL). The resulting precipitate was filtered through a Celite pad and the solvent was concentrate to dryness. The crude product was purified by flash chromatography (eluent: EtOAc/light petroleum 1:2) to give **7** (920 mg, 3.17 mmol) in 84% yield. ¹H NMR (400MHz, CDCl₃): δ 7.31-7.25 (m, 5H, Ar); 3.41-3.35 (m, 5H, CH₂-N-Boc, CH-CH₃); 2.41-2.33 (m, 4H, CH₂-N); 1.43 (s, 9H, ^tbu-); 1.36 (d, 3H, CH₃-CH-Ar). ¹³C NMR (100MHz, CDCl₃): δ 146.80, 134.13, 128.38, 127.74, 127.10, 85.27, 50.36, 29.78, 28.49, 27.48. MS (ESI): $[M+H]^+ = 291$; $[\alpha]_D^{20} = -32^\circ$ ($c = 0.0104$ g/100 mL, chloroform).

1,1-Diphenyl-7-(1-phenyl-ethyl)-hexahydro-oxazolo[3,4-*a*] pyrazin-3-one (8 and 8a)—To a stirred solution of **7** (380 mg, 1.31 mmol) in anhydrous THF (5 mL), TMEDA (0.53 mL, 3.54 mmol) was added. The reaction was cooled at -78 °C and *sec*-BuLi 1.4M in exane (2.53 mL, 3.54 mmol) was added. The reaction was heated at -35 °C and after 2 h a solution of benzophenone (480 mg, 2.62 mmol) in anhydrous THF (7 mL) was added drop wise. The reaction became green and was stirred at room temperature for 24 h. After this time the reaction was monitored by TLC (EtOAc/light petroleum 1:2) and quenched by adding NH₄Cl saturated solution (20 mL). The solvent was removed in vacuo and the aqueous phase extracted 3 times with EtOAc (30 mL). The combined organic layer was dried and evaporated to dryness. The crude diastereomers mixture was purified by flash chromatography using EtOAc/light petroleum 1:2 as eluent to obtain the fast running diastereomer **8a** in 40% yield and the low running diastereomer **8** in 45% yield.

8a: ¹H NMR (400 MHz, CDCl₃): δ 7.55 - 7.49 (m, 2H), 7.41 - 7.21 (m, 11H), 7.19 - 7.14 (m, 2H), 4.51 (dd, 1H, $J = 10.9, 3.6$ Hz), 3.74 (ddd, 1H, $J = 13.2, 3.5, 1.3$ Hz), 3.34 (q, 1H, $J = 6.7$ Hz), 3.04 (ddd, 1H, $J = 13.0, 12.1, 3.6$ Hz), 2.70 - 2.61 (m, 2H), 1.86 (td, 1H, $J = 11.9, 3.6$ Hz), 1.50 - 1.41 (m, 1H), 1.22 (d, 3H, $J = 6.7$ Hz). ¹³C NMR (100 MHz, CDCl₃): δ 156.17, 142.85, 142.52, 138.91, 128.69, 128.58, 128.50, 128.35, 128.01, 127.51, 127.37, 126.19, 125.95, 85.50, 64.52, 61.56, 52.66, 49.30, 42.07, 19.34. MS ESI $[M+H]^+ = 399$; $[\alpha]_D^{20} = +216$ ($c = 0.108$ g/100 mL, chloroform).

8: ¹H NMR (400 MHz, CDCl₃): δ 7.50 - 7.45 (m, 2H), 7.39 - 7.20 (m, 11H), 7.18 - 7.14 (m, 2H), 4.44 (dd, 1H, $J = 3.56, 10.93$ Hz), 3.86 - 3.79 (m, 1H), 3.48 (q, 1H, $J = 6.8$ Hz), 3.11 (ddd, 1H, $J = 13.0, 12.0, 3.81$ Hz), 2.80 - 2.73 (m, 1H), 2.44 (ddd, 1H, $J = 11.5, 3.5, 1.6$ Hz), 2.07 - 1.97 (m, 1H), 1.50 (m, 1H), 1.27 (d, 3H, $J = 6.8$ Hz). ¹³C NMR (100 MHz, CDCl₃): δ 156.20, 142.48, 142.28, 138.81, 128.65, 128.51, 128.32, 127.98, 127.59, 127.28, 126.11, 125.92, 125.84, 85.39, 63.86, 61.67, 53.24, 47.58, 42.11, 17.16; MS ESI $[M+H]^+ = 399$; $[\alpha]_D^{20} = -132^\circ$ ($c = 0.11$ g/100 mL, chloroform).

3-Oxo-1,1-diphenyl-tetrahydro-oxazolo[3,4-*a*]pyrazine-7-carboxylic acid 9*H*-fluoren-9-ylmethyl ester (9 and 9a)—To a stirred solution of **8** or **8a** (200 mg, 0.52 mmol) in acetonitrile (10 mL) at reflux, Fmoc-Cl (148 mg, 0.57 mmol) dissolved in acetonitrile (7 mL) was added. The reaction, monitored by TLC (EtOAc/light petroleum

1:2), was completed in 12 h. The desired precipitate was filtered off to obtain **9** or **9a** in about 67% yield and pure enough to be used in the next reaction.

3-Oxo-1,1-diphenyl-tetrahydro-oxazolo[3,4-a]pyrazine-7-carboxylic acid 4-fluoro-benzylamide (10 and 10a)—To a stirred solution of **9** (59 mg, 0.11 mmol) in anhydrous THF (15 mL) *p*-fluoro-benzylisocyanate (34.4 mg, 0.228 mmol) and DBU (19.2 mg, 0.126 mmol) were added. The reaction was monitored by TLC (EtOAc/light petroleum 1:2) and by mass spectrometry. After 24 h the reaction was treated as for **8** and **8a**. The organic phase was dried and evaporate to dryness to give **10** in 76% yield after column chromatography using EtOAc/light petroleum 1/1 as eluent. $^1\text{H NMR}$ (400 MHz, CDCl_3): δ 7.51 - 7.47 (m, 2H), 7.41 - 7.18 (m, 10H), 7.03 - 6.94 (m, 2H), 4.95 (t, 1H, $J = 5.5$ Hz), 4.45 - 4.27 (m, 3H), 4.03 (ddd, 1H, $J = 13.5, 3.5, 1.2$ Hz), 3.81 (dd, 1H, $J = 13.1, 2.7$ Hz), 3.69 - 3.60 (m, 1H), 3.05 (td, 1H, $J = 12.7, 3.7$ Hz), 2.93 - 2.82 (m, 1H), 2.14 (dd, 1H, $J = 13.3, 11.3$ Hz). $^{13}\text{C NMR}$ (100 MHz, CDCl_3): δ 157.20, 156.11, 141.81, 138.30, 134.91, 129.48, 129.41, 129.17, 129.09, 128.82, 128.72, 128.37, 125.99, 125.85, 115.70, 115.48, 85.90, 60.55, 46.58, 44.47, 43.76, 41.37. MS ESI [$\text{M}+\text{H}^+$]= 445.9; $[\alpha]_{\text{D}}^{20} = +92$ ($c = 0.1$ g/100 mL, MeOH). Compound **10a** was obtained in the same manner, starting from **9a**. Analytical data: Yield: 83%; $^1\text{H NMR}$ (400 MHz, CDCl_3): δ 7.51 - 7.47 (m, 2H), 7.41 - 7.18 (m, 10H), 7.03 - 6.94 (m, 2H), 4.95 (t, 1H, $J = 5.5$ Hz), 4.45 - 4.27 (m, 3H), 4.03 (ddd, 1H, $J = 13.3, 3.6, 1.3$ Hz), 3.81 (dd, 1H, $J = 13.1, 2.7$ Hz), 3.69 - 3.60 (m, 1H), 3.05 (td, 1H, $J = 12.7, 3.7$ Hz), 2.93 - 2.82 (m, 1H), 2.14 (dd, 1H, $J = 13.3, 11.3$ Hz). $^{13}\text{C NMR}$ (100 MHz, CDCl_3): δ 157.20, 156.11, 141.81, 138.30, 134.91, 129.48, 129.41, 129.17, 129.09, 128.82, 128.72, 128.37, 125.99, 125.85, 115.70, 115.48, 85.90, 60.55, 46.58, 44.47, 43.76, 41.37. MS ESI [$\text{M}+\text{H}^+$]= 445.9; $[\alpha]_{\text{D}}^{20} = -91$ ($c = 0.12$ g/100 mL, MeOH).

Chiral chromatography analysis—A micro HPLC (Agilent 1100 micro series, Agilent Technologies) equipped with a micro diode array detector was employed. A 150mm \times 2mm stainless steel column packed with Lux Cellulose-1 (cellulose tris 3,5-dimethylphenylcarbamate from Phenomenex) was used for all the measurements. The average size of the packing material was 3 μm . The mobile phase was a binary mixture of hexane/isopropyl alcohol (80/20 v/v). Flow rate was 200 $\mu\text{L}/\text{min}$. Injection volume was 3 μL . Analyte solutions were filtered with PTFE filters (0.45 μm , Supelco, Bellefonte, PA, USA) before injection. All chromatograms were recorded at 230 nm. The retention times for the first (**10a**) and second (**10**) eluted enantiomers were 6.5 and 7.9 min, respectively.

Crystal structure determination of compound **8**

The crystal data of compound **8** were collected at room temperature using a Nonius Kappa CCD diffractometer with graphite monochromated Mo- $K\alpha$ radiation. The data sets were corrected for Lorentz and polarization effects. The structure was solved by direct methods¹⁸ and refined using full-matrix least-squares with all non-hydrogen atoms anisotropically and hydrogens included on calculated positions, riding on their carrier atoms. All calculations were performed using SHELXL-97¹⁹ and PARST²⁰ implemented in WINGX²¹ system of programs.

Crystal Data— $\text{C}_{26}\text{H}_{26}\text{N}_2\text{O}_2$, orthorhombic, space group $P2_12_12_1$, $a = 11.2339(2)$, $b = 11.6808(3)$, $c = 16.4783(5)$ Å, $V = 2162.30(9)$ Å³, $Z = 4$, $D_c = 1.224$ g cm^{-3} , intensity data collected with $\theta \leq 26^\circ$, 4215 independent reflections measured, 3460 observed reflections [$I > 2\sigma(I)$], final R index = 0.0365 (observed reflections), $R_w = 0.0904$ (all reflections), $S = 1.048$. The absolute configuration has not been established by anomalous dispersion effects in diffraction measurements on the crystal. The enantiomer has been assigned by reference to an unchanging chiral centre in the synthetic procedure. ORTEP²² view of compound **8** is shown in Figure 2.

CCDC deposition number: 810351.

Calcium mobilization experiments—HEK293 cells stably expressing the murine NPSR or the human receptor isoforms NPSRIle107 and NPSRAsn107 were generated as previously described.¹⁷ HEK293_{mNPSR} and HEK293_{hNPSRIle107} cells were maintained in DMEM medium supplemented with 10% fetal bovine serum, 2mM l-glutamine, hygromycin B (100 mg/L). HEK293_{hNPSRAsn107} cells were maintained in DMEM medium supplemented with 10% fetal bovine serum, 2 mM glutamine, zeocin (100 mg/L). Cells were cultured at 37 °C in 5% CO₂ humidified air. Cells were seeded at a density of 50,000 cells/well into poly-D-lysine coated 96-well black, clear-bottom plates. The following day, the cells were incubated with medium supplemented with 2.5 mM probenecid, 3 μM of the calcium sensitive fluorescent dye Fluo-4 AM and 0.01% pluronic acid, for 30 min at 37 °C. After that time the loading solution was aspirated and 100 μL/well of assay buffer (Hank's Balanced Salt Solution; HBSS) supplemented with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2.5 mM probenecid and 500 μM Brilliant Black (Aldrich) was added. Concentrated solutions (1 mM) of NPS were made in bidistilled water and kept at -20 °C. Compound **1**, compound **10** and compound **10a** were dissolved DMSO at a final concentration of 10 mM and stock solutions were kept at -20 °C until use. The successive dilutions were carried out in HBSS/HEPES (20mM) buffer (containing 0.02% bovine serum albumin fraction V). After placing both plates (cell culture and master plate) into the fluorometric imaging plate reader FlexStation II (Molecular Devices, Sunnyvale, CA), fluorescence changes were measured. On-line additions were carried out in a volume of 50 μL/well. To facilitate drug diffusion into the wells in antagonist type experiments, the present studies were performed at 37 °C and three cycles of mixing (25 μL from each well moved up and down 3 times) were performed immediately after antagonist injection to the wells. Inhibition response curves were determined against the stimulatory effect of 10 nM NPS. Compound **1**, compound **10** and compound **10a** were injected into the wells 24 min before adding NPS.

Data analysis and terminology—The pharmacological terminology adopted in this paper is consistent with IUPHAR recommendations. Data were expressed as mean ± sem of at least four independent experiments made in duplicate. Maximum change in fluorescence, expressed in percent of baseline fluorescence, was used to determine agonist response. Non-linear regression analysis using GraphPad Prism software (v.4.0) allowed logistic iterative fitting of the resultant responses and the calculation of agonist potencies and maximal effects. Agonist potencies are given as pEC₅₀ (the negative logarithm to base 10 of the molar concentration of an agonist that produces 50% of the maximal possible effect). Compound **1**, compound **10** and compound **10a** antagonist properties were evaluated in inhibition response curve experiments; the antagonist potency, expressed as pK_B, was derived from the following equation:

$$K_B = IC_{50} / ([2 + ([A]/EC_{50})^n]^{1/n} - 1)$$

where IC₅₀ is the concentration of antagonist that produces 50% inhibition of the agonist response, [A] is the concentration of agonist, EC₅₀ is the concentration of agonist producing a 50% maximal response and n is the Hill coefficient of the concentration response curve to the agonist.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DMEM	Dulbecco's modified Eagle's medium
DMF	<i>N,N</i> -Dimethylformamide
Fmoc-Cl	9-Fluorenylmethyl chloroformate
HBSS	Hank's Balanced Salt Solution
HEK	Human Embryonic Kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
MS-ESI	electron spray ionization mass spectrometry
NMDA	<i>N</i> -Methyl-D-aspartic acid
PFTE	Polytetrafluoroethylene
RP-HPLC	reversed-phase high-performance liquid chromatography
THF	<u>tetrahydrofuran</u>
TMEDA	tetramethylethylenediamine
WSC	1-Ethyl-3-(3'-dimethylaminopropyl)carbodiimide

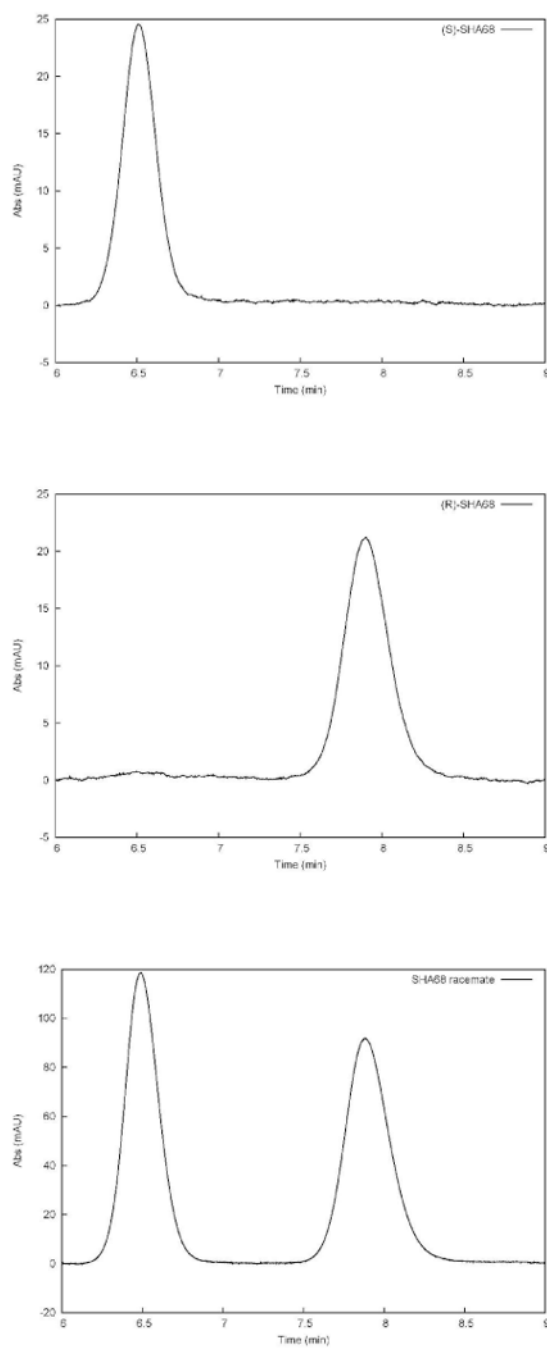


Figure 1. Chromatograms of compound **10a** (top panel) and **10** (middle panel) in comparison with the racemate (bottom panel).

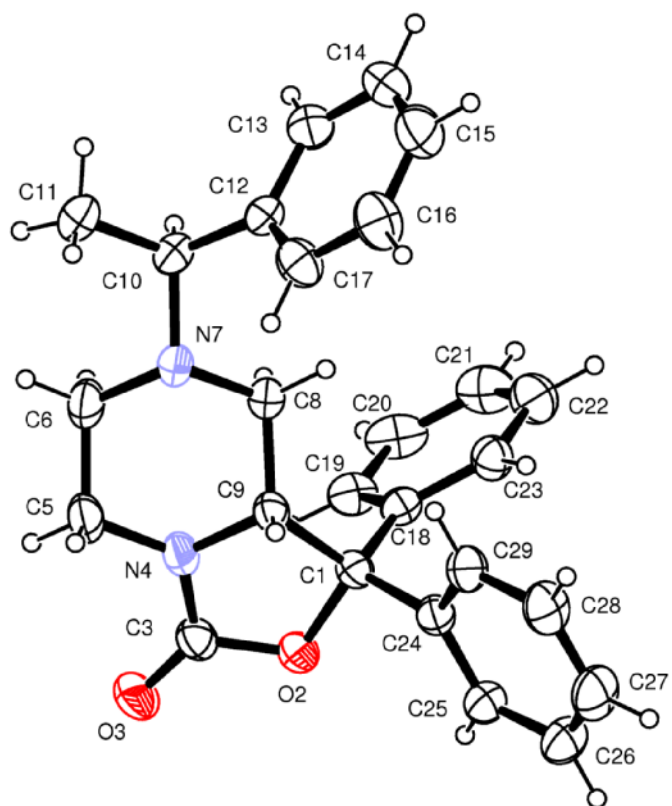
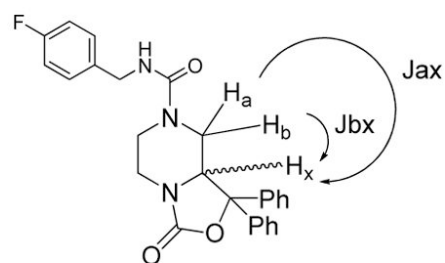
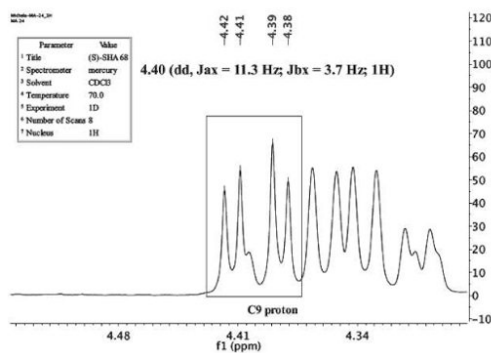


Figure 2. ORTEP view of compound **8**. The thermal ellipsoids are drawn at 30% probability level.



Panel A



Panel B

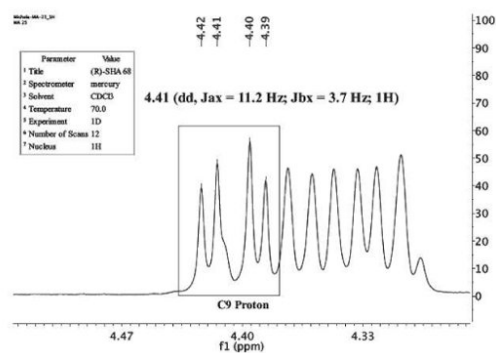


Figure 3. Enlarged ^1H NMR spectra of the C9 proton region of the **10a** (panel A) and **10** (panel B) isomers

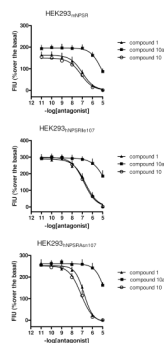
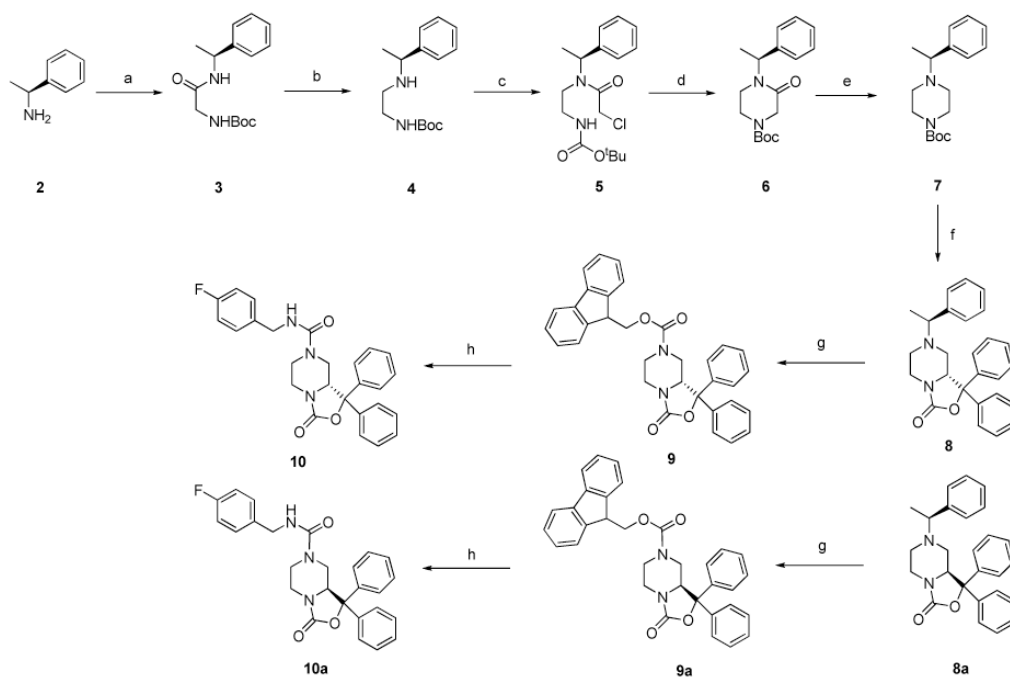


Figure 4. Inhibition response curves to compound **1** (SHA 68), compound **10** and compound **10a** in HEK293 cells expressing the murine NPSR and the human NPSR isoforms. Data are mean \pm s.e.m. of four separate experiments made in duplicate.



Scheme 1. Synthesis of compound 10 and 10a

Reagents and conditions: (a) CH₂Cl₂, WSC, Boc-Gly-OH, room temp, 12h; (b) LiAlH₄, THF, 0°C, 1h; (c) Chloroacetyl-chloride, EtOAc, NaHCO₃, 0°C to room temp., 24h; (d) THF/DMF 1/1, NaH, 0°C to room temp, 24h; (e) THF, LiAlH₄, room temp, 4h; (f) THF, Benzophenone, *sec*-BuLi, TMEDA, -78°C to -30°C to room temp, 24h; (g) CH₃CN, Fmoc-Cl, reflux, 12h; (h) THF, DBU, *p*-fluoro-benzylisocyanate, room temp, 12h.

Table 1
Potencies (pK_B) of compound 1 (SHA 68), compound 10 and compound 10a in HEK293 cells expressing the murine NPSR and the human NPSR isoforms

Compound	mNPSR	hNPSR Ile107	hNPSR Asn107
	pK_B	pK_B	pK_B
1	8.16 (7.79 - 8.53)	8.03 (7.77 - 8.37)	7.99 (7.73 - 8.25)
10	8.29 (7.93 - 8.65)	8.18 (7.90 - 8.46)	8.28 (7.72 - 8.84)
10a	<6	<6	<6