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Racemic synthesis and solid phase peptide synthesis application of the chimeric valine/leucine derivative 2-amino-3,3,4-trimethyl-pentanoic acid

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The synthesis of non natural amino acid 2-amino-3,3,4-trimethyl-pentanoic acid (Ipv) ready for solid phase peptide synthesis has been developed. Copper (I) chloride Michael addition, followed by a Curtius rearrangement are the key steps for the Ipv synthesis. The racemic valine/leucine chimeric amino acid was then successfully inserted in position 5 of neuropeptide S (NPS) and the diastereomeric mixture separated by reverse phase HPLC. The two diastereomeric NPS derivatives were tested for intracellular calcium mobilization using HEK293 cells stably expressing the mouse NPS receptor where they behaved as partial agonist and pure antagonist.

1. Introduction

Non natural amino acids are important tools for investigating the structure-activity relationship (SAR) of bioactive peptides. In the case of peptide/receptor systems, the amino acid side chain replacement can give important information for understanding the receptor recognition mechanisms. In the recent past we performed a series of SAR studies on neuropeptide S (NPS) (primary sequence of human NPS: SFRNGVGTGMKK-TSFQRAKS) (Guerrini et al. 2010) and we found that the replacement of Gly⁵ with D-amino acids leads to the generation of NPS receptor (NPSR) antagonists. In particular, we replaced Gly⁵ with hydrophobic residues and we identified [D-Cys(^tBu)⁵]NPS, [D-Val⁵]NPS, [^tBu-D-Gly⁵]NPS and [D-Pen⁵]NPS as NPSR pure antagonists with pK_b value in the range 6.5–7.0 (Guerrini et al. 2009a, b). Interestingly, subtle changes of the amino acid side chain produce important modifications of peptide efficacy. For example, the isopropyl moiety is the common structural feature of both Val and Leu side chains, but in the latter case the chiral carbon and the featuring substituent are separated by the presence of a methylene group. Interestingly, such a subtle chemical modification produced an important increase in efficacy without affecting the potency of the ligands (Guerrini et al. 2009b). Similar results have been obtained with the replacement ^tBu-D-Gly/^tBu-D-Ala. In fact [^tBu-D-Gly⁵]NPS behaved as pure antagonist and [^tBu-D-Ala⁵]NPS as partial agonist (Guerrini et al. 2009a) (see Fig. 1). Thus, a branched aliphatic side chain directly linked to the D-amino acid chiral carbon of NPS position 5 seems to be an important chemical requirement for generating pure NPSR antagonism. As far as ligand and potency is concerned, amino acids with similar side chain shape (Val/Leu; ^tBu-D-Gly/ ^tBu-D-Ala) but shifted from the peptide backbone of one carbon atom (Val versus Leu and ^tBu-D-Gly versus ^tBu-D-Ala) generated [D-Xaa⁵]NPS analogues

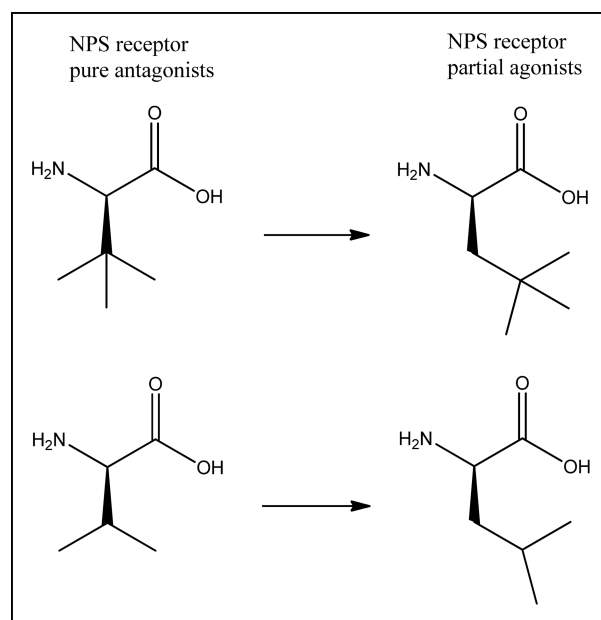
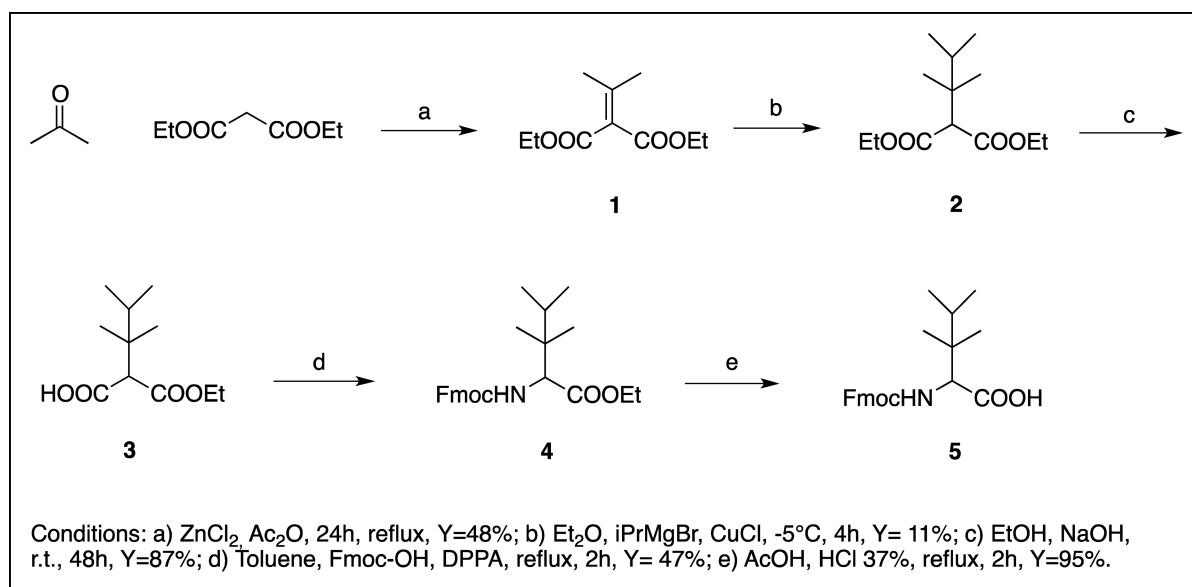


Fig. 1: Increase in the side chain length of the D-amino acid in NPS position 5 enhances ligand efficacy.

with similar potencies (Guerrini et al. 2009a, b). On these basis, to further investigate [Xaa⁵]NPS chemical requirements for NPSR interaction, we designed and synthesized a novel unnatural amino acid with a bulky side chain group, a rather unusual chimeric valine/leucine amino acid (Ipv). We describe in this paper a successful route for the preparation in racemic form of this unusual amino acid, its insertion in position 5 of the NPS sequence, and the biological activity of the two [Ipv⁵]NPS diastereomers.



Scheme: Synthetic approach adopted for the synthesis of racemic 2-amino-3,3,4-trimethyl-pentanoic acid.

2. Investigations, results and discussion

In the past many methods for the synthesis of both chiral and racemic amino acids have been reported (Williams et al. 1991; Bøgevig et al. 2002). Firstly, we adopted the synthetic strategy developed by Porzi et al. (Orena et al. 1992) aimed to the asymmetric synthesis of chiral Ipv (isopropylvaline) through the enantioselective alkylation of a chiral monolactim ether with the selected amino acid side chain. However, attempts to prepare the required 3-(1,1,2 trimethyl-propyl)-piperazine-2,5-dione using the readily available 2-bromo-2,3-dimethylbutane (Bunnett et al. 1971) were frustrating by the easy elimination occurring to the alkyl halide under the strongly basic conditions required for the diketopiperazine alkylation. This failure led us to modify our approach for generating the desired Ipv amino acid; the synthetic approach start from the olefinic diester **1**, easily obtained through anhydrous zinc dichloride-catalyzed Knoevenagel condensation between acetone and diethyl malonate (Eliel et al. 1970) (Scheme).

Compound **1** underwent copper (I) chloride-catalyzed (Eliel et al. 1970) conjugated addition of *i*-propyl magnesium bromide affording the desired Michael adduct **2**. The low yield (11%) could be ascribed to the steric hindrance of both Michael acceptor and donor involved in this reaction. Mono-saponification of the diester **2** generated the monoacid derivative **3** that reacted with DPPA followed by Curtius rearrangement (Shioiri et al. 1972) using the rather uncommon nucleophile fluorene-methanol with good yield, allowing to obtain the Fmoc protected aminoester **4** in one pot reaction. The desired Fmoc aminoacid **5** was then generated in good yield by acid hydrolysis of the corresponding ester. Its incorporation in NPS position 5 was successfully accomplished using classical solid phase peptide synthesis procedures previously applied for the synthesis of NPS analogues (Camarda et al. 2008). In this case, double coupling was performed for the acylation of the sterically hindered Ipv. The use of racemic Fmoc-Ipv-OH generated a diastereomeric mixture of [L-Ipv⁵]NPS and [D-Ipv⁵]NPS that was successfully separated in preparative HPLC. Using experimental HPLC conditions previously reported (Camarda et al. 2008) we obtained two peaks completely baseline resolved. The absolute configuration at position 5 of the two final peptides was not assigned and [Ip⁵]NPS **A** and [Ip⁵]NPS **B** correspond to the product with the shorter and longer HPLC elution time, respectively.

The novel peptides were tested in calcium mobilization studies using HEK293 cells stably expressing the mouse NPSR (Camarda et al. 2008). In these experiments NPS produced a concentration dependent stimulatory effect showing maximal effects equal to $404 \pm 24\%$ over the basal levels and a pEC_{50} value of 8.86 ($\text{CL}_{95\%}$ 8.55 – 9.17) (Fig. 2, left panel). These values are in line with previous results (Camarda et al. 2008; Guerrini et al. 2009a, b). Under the same experimental conditions [Ip⁵]NPS **A** displayed weak stimulatory effects showing low potency (6.22 ($5.90 - 6.54$)) and maximal effects ($165 \pm 20\%$). [Ip⁵]NPS **B** was found inactive up to $10 \mu\text{M}$ (Figure 2, left panel). In separate experiments the antagonistic properties of [Ip⁵]NPS **B** were investigated and [³H-Bu-D-Gly⁵]NPS was used as positive control (Guerrini et al. 2009a). $10 \mu\text{M}$ [Ip⁵]NPS **B** elicited a rightward shift of the concentration response curve to NPS without significantly modify its maximal effects. A pA_2 value of 5.97 ($5.58 - 6.36$) has been derived from these experiments. [³H-Bu-D-Gly⁵]NPS produced superimposable results at tenfold lower concentrations ($1 \mu\text{M}$). A pA_2 value of 7.01 ($6.71 - 7.31$) has been obtained; this value is closed to what previously reported at mouse (6.78, (Guerrini et al. 2009a)) and rat (7.17, Ruzza et al. 2012) NPSR. Collectively these studies demonstrated that [Ip⁵]NPS **A** behaves as a NPSR partial agonist while [Ip⁵]NPS **B** as a pure antagonist; both peptides displayed low potency at the mouse NPSR.

Collectively the results we obtained with [Ip⁵]NPS corroborate and extend our previous findings demonstrating that Xaa⁵ chirality of NPS derivatives is crucial for pharmacological activity while the size of the aliphatic side chain is important for both ligand potency and efficacy. In particular regarding the relationship between potency and size of the side chain, the present results suggest that the aliphatic highly branched side chain of Ipv is less tolerated for NPSR binding compared to the less bulky moieties of Val and Leu.

In conclusion, we have described a method for the preparation of a chimeric valine/leucine amino acid in racemic form the key step being the copper (I) chloride-catalyzed conjugated addition of *i*-propyl magnesium bromide to 2-isopropylidene-malonic acid diethyl ester. Despite the low yield, the practical ease of reaction, amenable in multigram scale, the common and cheap reagents involved, the variety of Grignard reagents available make this route suitable for further synthetic applications

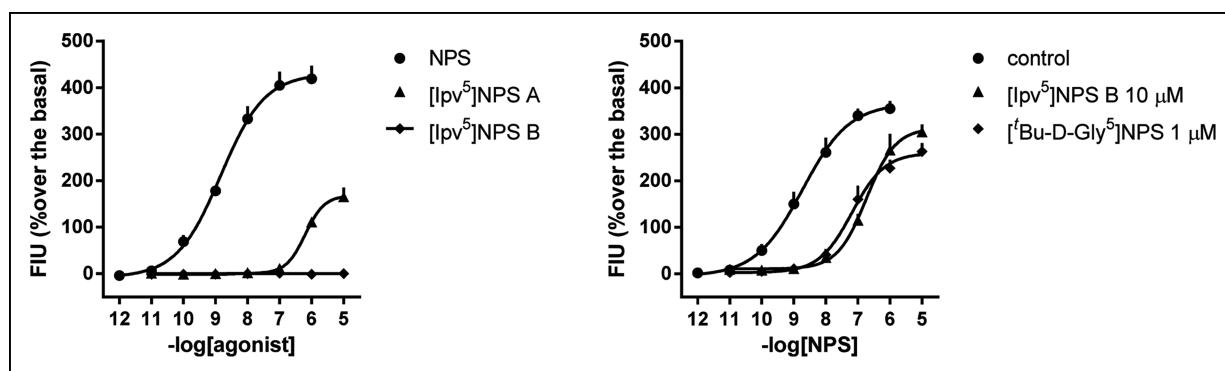


Fig. 2: Calcium mobilization studies in HEK293_{MNSR} cells. Left panel: agonist effects of NPS, [I^{pv}]⁵NPS A and B. Right panel: antagonist effects of [I^{pv}]⁵NPS B and [t^{Bu}-D-Gly⁵]⁵NPS vs NPS. Data are the mean $\pm$ sem of 4 separate experiments performed in duplicate.

including the synthesis of novel non natural Val derivatives. The incorporation of Ipv in position 5 of NPS was successfully performed using classical solid phase peptide synthesis procedures and the diastereomeric mixture of [L-Ipv⁵]NPS and [D-Ipv⁵]NPS obtained was successfully separated in preparative HPLC. In calcium mobilization assays [I^{pv}]⁵NPS A behaves as a NPSR partial agonist while [I^{pv}]⁵NPS B as a pure antagonist and both peptides displayed low potency at the mouse NPSR. Our results indicated that the unnatural amino acid described in this study could be used as a novel building block for combinatorial libraries of peptides, as well as for structure-activity relationship studies of bioactive peptides.

3. Experimental

3.1. Synthesis

3.1.1. General procedures

HPLC grade solvents were purchased from Sigma Aldrich (Steinheim, Germany). The purity of the tested compounds 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.

3.1.2. 2-Isopropylidene-malonic acid diethyl ester (1)

To a stirred solution of diethyl malonate (20 mL, 131.87 mmol), acetone (14.5 mL 197.81 mmol) and acetic anhydride (15.57 mL, 164.84 mmol) anhydrous zinc dichloride (2.68 g, 19.78 mmol) was added. The reaction mixture was heated at reflux for 24 h and checked by TLC using EtOAc/light petroleum 0.5/9.5 as eluent. After cooling at room temperature, toluene (50 mL) was added and the organic phase was washed twice with water (20 mL each), the organic phase was concentrated *in vacuo* and the crude product purified by flash chromatography (EtOAc/Light petroleum 0.5/9.5) to yield compound **1**, Yield 12.63 g (48%). ¹H NMR (400 MHz, CDCl₃): δ 4.27-4.17 (4H, q, J = 7 Hz, OCH₂CH₃), 2.05 (6H, s, 2CH₃), 1.31-1.24 (6H, t, J = 7 Hz, 2(OCH₂CH₃)). MS (ESI): [MH]⁺ = 201.

3.1.3. 2-(1,1,2-Trimethyl-propyl)-malonic acid diethyl ester (2)

In a two neck round bottom flask, under argon atmosphere, diethyl ether (20 mL), magnesium (82.4 mg, 3.4 mmol) and isopropylbromide (0.38 mL, 4.06 mmol) were added. When all the metallic magnesium was consumed, the solution was cooled at -5 °C and a catalytically amount of copper (I) chloride (4.5 mg, 0.045 mmol) was added. After 15 min compound **1** (451.5 mg, 2.26 mmol) dissolved in diethyl ether were added. After 4 h, 10 mL of 10% sulphuric acid were added and the organic phase was separated, dried over sodium sulphate and purified by flash chromatography (EtOAc/ light petroleum 0.5/9.5) to give compound **2**, Yield 60.66 mg (11%). ¹H NMR (400 MHz, CDCl₃): δ 4.19-4.11 (4H, q, J = 7.2 Hz, OCH₂CH₃), 3.50 (1H, s, CH(COOEt)₂), 1.85-1.79 (1H, m, CH), 1.28-1.21 (6H, t, J = 7.2 Hz, 2(OCH₂CH₃)), 1.04 (6H, s, -C(CH₃)₂), 0.87-0.84 (6H, d, J = 6.8 Hz, -

CH(CH₃)₂). ¹³C NMR (100 MHz, CDCl₃): δ 168.7, 60.8, 58.6, 39.1, 34.5, 20.9, 17.3, 14.2. MS (ESI): [MH]⁺ = 245.

3.1.4. 2-(1,1,2-Trimethyl-propyl)-malonic acid monoethyl ester (3)

To a stirred solution of **2** (521 mg, 2.135 mmol) in 15 mL of ethanol, a 1N solution of NaOH (5.5 mL, 7.9 mmol) was added dropwise. The reaction mixture was stirred at room temperature for 10 h, monitored by TLC (EtOAc/Light Petroleum/AcOH, 1/5/0.3) and mass spectrometry until to the appearing of diacid molecular weight. The solvent was evaporated to dryness and the aqueous layer extracted twice with ethyl acetate (10 mL each) to remove the di-ester derivative. Then aqueous layer was acidified with 1N HCl and extracted 3 times with ethyl acetate (20 mL each) to recover the desired monoacid, Yield 401 mg (87%), based on the consumed di-ester, enough pure to be used in the next reaction without further purification. ¹H NMR (400 MHz, CDCl₃): δ 4.27-4.16 (2H, q, J = 7.2 Hz, OCH₂CH₃), 3.56 (1H, s, EtOOC-CH-COOH), 1.85-1.78 (1H, m, CH(CH₃)₂), 1.32-1.25 (3H, t, J = 7.2 Hz, OCH₂CH₃), 1.05 (3H, s, CH₃), 1.00 (3H, s, CH₃), 0.92-0.90 (3H, d, J = 3.6 Hz, CH(CH₃)₂), 0.88-0.87 (3H, d, J = 3.6 Hz, CH(CH₃)₂). ¹³C NMR (100 MHz, CDCl₃): δ 171.7, 170.8, 61.7, 58.1, 40.1, 34.4, 21.1, 20.9, 17.4, 17.3, 14.1. MS (ESI): [MH]⁺ = 217.

3.1.5. 2-(9H-Fluoren-9-yl-methoxycarbonylamino)-3,3,4-trimethyl-pentanoic acid ethyl ester (4)

To a stirred solution of **3** (113 mg, 0.52 mmol) in dry toluene, triethylamine (0.18 mL, 1.3 mmol) and diphenylphosphoryl azide (0.17 mL, 0.78 mmol) were added. The reaction mixture was heated at reflux for 2 h. After cooling at room temperature, fluorenyl-methanol (204 mg, 1.04 mmol) was added and the reaction was heated again at reflux overnight. After evaporation of the solvent, the crude material was purified by flash chromatography (EtOAc/Light petroleum, 0.5/9.5) to give compound **4**, Yield 99.96 mg (47%). ¹H NMR (400 MHz, CDCl₃): δ 7.78-7.74 (4H, d, J = 8 Hz, Ar Fmoc), 7.61-7.57 (4H, d, J = 8 Hz, Ar Fmoc), 5.34 (2H, d, J = 8 Hz, CH-CH₂-O), 4.41 (1H, s, EtOOC-CH-NH), 4.20 (2H, q, J = 7.6 Hz, CH₃-CH₂-O), 4.17 (1H, m, (Ar)₂-CH-CH₂-O), 1.58 (1H, m, CH₃-CH-CH₃), 1.28 (3H, t, J = 7.6 Hz, CH₃-CH₂-O), 0.93 (6H, s, (CH₃)₂-C), 0.89 (6H, m, (CH₃)₂-CH).

3.1.6. 2-(9H-Fluoren-9-yl methoxycarbonylamino)-3,3,4-trimethyl-pentanoic acid (5)

Compound **4** (75 mg, 0.183 mmol) was dissolved in glacial acetic acid (2 mL) and HCl 37% (2 mL); reaction mixture was then heated at reflux over night and monitored by TLC (EtOAc/Light petroleum/AcOH, 1/1/0.3) successively, reaction mixture was evaporated *in vacuo* and the crude residue was dissolved in water (5 mL) and extracted three times with EtOAc (15 mL each). The organic phases were dried over Na₂SO₄ and evaporated to dryness to obtain compound **5** in 95% yield as a pure compound. ¹H NMR (400 MHz, CDCl₃): δ 7.78-7.74 (4H, d, J = 8 Hz, Ar Fmoc), 7.61-7.57 (4H, d, J = 8 Hz, Ar Fmoc), 5.34 (2H, d, J = 8 Hz, Fmoc-CH₂-O), 4.41 (1H, s, HOOC-CH-NH), 4.17 (1H, m, (Ar)₂-CH-CH₂-O), 1.58 (1H, m, CH₃-CH-CH₃), 0.93 (6H, s, (CH₃)₂-C), 0.89 (6H, m, (CH₃)₂-CH). HR-MS (ESI): [MH]⁺ = 382.20171, calc: 382.20183.

3.1.7. General procedures for the solid phase peptide synthesis and purification

Fmoc-Ser(tBu)-4-benzyloxybenzyl alcohol resin (Fmoc-Ser(tBu)-Wang resin) (0.62 mmol/g, 0.2 g) was treated with 20% piperine/N, N-dimethylformamide (DMF) and linked with Fmoc-Lys(Boc)-OH

by using [O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate] (HATU) as the coupling reagent. The following *N*^α-Fmoc amino acids were sequentially coupled to the growing peptide chain: Fmoc-Ala-OH, Fmoc-Arg(Pmc)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Phe-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Met-OH, Fmoc-Gly-OH, Fmoc-Thr(tBu)-OH, Fmoc-Gly-OH, Fmoc-Val-OH, Fmoc-Ipv-OH, Fmoc-Asn(Trt)-OH, Fmoc-Arg(Pmc)-OH, Fmoc-Phe-OH, and Fmoc-Ser(tBu)-OH. All of the *N*^α-Fmoc amino acids (4 equiv) were coupled to the growing peptide chain by using HATU (4 equiv) in DMF in the presence of an equimolar concentration of 4-methylmorpholine (NMM); the coupling reaction time was 1 h. Double coupling was performed for the acylation of the sterically hindered Ipv. To improve the analytical profile of the crude peptide, capping with acetic anhydride (0.5 M/DMF) in the presence of NMM (0.25 M/DMF) (3:1 v/v; 2 mL/0.2 g of resin) was performed at any step. To remove the Fmoc group at every step, 20% piperidine/DMF was used. The peptide resin was washed with methanol and dried *in vacuo* to yield the protected [Ipv⁵]NPS resin. The protected peptide resin was treated with trifluoroacetic acid (TFA)/H₂O/phenol/triisopropylsilane 88:5:5:2 v/v; 10 mL/0.2 g of resin) for 1.5 h at room temperature. After filtration of the resin, the solvent was concentrated *in vacuo*, and the residue was triturated with ether.

Crude peptide was purified by preparative reversed-phase HPLC using a Water Delta Prep 3000 system with a Jupiter column C18 (250 mm x 30 mm, 300 Å, 15 µm spherical particle size). The column was perfused at a flow rate of 25 mL/min with a mobile phase containing solvent A (10%, v/v, acetonitrile in 0.1% TFA), and at a linear gradient from 0 to 50% of solvent B (60%, v/v, acetonitrile in 0.1% TFA) over 25 min for the elution of final diastereomers [Ipv⁵]NPS A and [Ipv⁵]NPS B. Analytical HPLC analyses were performed on a Beckman 116 liquid chromatograph equipped with a Beckman 166 diode array detector. Retention time was obtained using a Jupiter C18 column (4.6 mm x 100 mm, 2 µm particle size) with the above solvent system (solvents A and B) programmed at a flow rate of 1 mL/min using a linear gradient from 0% to 70% B over 25 min.

Final peptides showed >95% purity when monitored at 220 nm. Molecular weights of compounds were determined with a mass spectrometer ESI Micromass ZMD-2000; values are expressed as MH⁺.

3.2. *In vitro* pharmacology

3.2.1. Calcium mobilization assay

HEK293_{mNPSR} cells were maintained in DMEM medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, hygromycin (100 mg/l), and cultured at 37 °C in 5% CO₂ humidified air. HEK293_{mNPSR} 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) 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 and NPS analogues were made in bidistilled water and kept at -20 °C. Serial dilutions were carried out in HBSS/HEPES (20 mM) 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. In antagonism experiments [Ipv⁵] B and [tBu-D-Gly⁵]NPS were injected 24 min before NPS.

3.2.2. Data analysis and terminology

The pharmacological terminology adopted in this paper is consistent with IUPHAR recommendations (Neubig et al. 2003). *In vitro* data were expressed as mean ± sem of at least four independent experiments made in

duplicate. Maximum change in fluorescence, expressed as percent over the 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 were given as pEC₅₀ (the negative logarithm to base 10 of the molar concentration of an agonist that produces 50% of the maximal possible effect). Antagonist potencies were derived, assuming a slope of the regression line equal to unity, with the following equation: $pA_2 = -\log(DR - 1)/[A]$ where DR is the ratio between agonist potency in the presence and absence of antagonist and [A] is the antagonist molar concentration (Gaddum et al. 1955).

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References

- Bogevig A, Juhl K, Kumaragubaran N, Zhuang W, Jorgensen KA (2002) Direct organo-catalytic asymmetric alpha amination of aldehydes. A symple approach to optically active alpha amino aldehydes, alpha amino alcohols and alpha amino acids. *Angew Chem Int Ed* 41: 1790–1793.
- Bunnett JF, Eck DL (1971) Reactions of 2-halo-2,3,3-trimethylbutanes in methanol solution. Rates and product ratios in solvolysis and in reactions with anionic bases. *J Org Chem* 36: 897–901.
- Camarda V, Trapella C, Calo G, Guerrini R, Rizzi A, Ruzza C, Fiorini S, Marzola E, Reinscheid RK, Regoli D, Salvadori S (2008) Synthesis and biological activity of human neuropeptide S analogues modified in position 2. *J Med Chem* 51: 655–658.
- Eliel E, Hutchins RO, Knoeber M (1970) Diethyl tert-buthylmalonate. *Org Synth Coll* 6: 38–42.
- Gaddum JH, Hameed KA, Hathway DE, Stephens FF (1955) Quantitative studies of antagonists for 5-hydroxytryptamine. *Q J Exp Physiol Cogn Med Sci* 40: 49–74.
- Guerrini R, Camarda V, Trapella C, Calo G, Rizzi A, Ruzza C, Fiorini S, Marzola E, Reinscheid RK, Regoli D, Salvadori S (2009a) Further studies at neuropeptide S position 5: discovery of novel neuropeptide S receptor antagonists. *J Med Chem* 52: 4068–4071.
- Guerrini R, Camarda V, Trapella C, Calo G, Rizzi A, Ruzza C, Fiorini S, Marzola E, Reinscheid RK, Regoli D, Salvadori S (2009b) Synthesis and biological activity of human neuropeptide S analogues modified in position 5: identification of potent and pure neuropeptide S receptor antagonists. *J Med Chem* 52: 524–529.
- Guerrini R, Salvadori S, Rizzi A, Regoli D, Calo G (2010) Neurobiology, pharmacology, and medicinal chemistry of neuropeptide S and its receptor. *Med Res Rev* 30: 751–777.
- Neubig RR, Spedding M, Kenakin T, Christopoulos A (2003) International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification. XXXVIII. Update on terms and symbols in quantitative pharmacology. *Pharmacol Rev* 55: 597–606.
- Orena M, Porzi G, Sandri S (1992) Diastereoselective alkylation of (3S)- and (3R)-methylpiperazine-2,5-dione derivatives. A convenient approach to both (S)- and (R)-alanine *J Org Chem* 57: 6532–6536.
- Ruzza C, Rizzi A, Camarda V, Pulga A, Marzola G, Filafiero M, Novi C, Ruggieri V, Marzola E, Vitale G, Salvadori S, Guerrini R, Calo G (2012) [tBu-D-Gly⁵]NPS, a pure and potent antagonist of the neuropeptide S receptor: *in vitro* and *in vivo* studies. *Peptides* 34: 404–411.
- Shioiri T, Ninomiya K, Yamada S (1972) Diphenylphosphoryl azide. A new convenient reagent for a modified Curtius reaction and for the peptide synthesis. *J Am Chem Soc* 94: 6203–6205.
- Williams RM, Im MN (1991) Asymmetric synthesis of monosubstituted and alpha, alpha-disubstituted alpha amino acids via diastereoselective glycine enolate alkylations. *J Am Chem Soc* 113: 9276–9286.