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Impact of the Proline Residue on Ligand Binding of Neurotensin Receptor 2 (NTS2)-Selective Peptide–Peptoid Hybrids

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To investigate the binding mode and structure–activity relationships (SARs) of selective neurotensin receptor 2 (NTS2) ligands, novel peptide–peptoid hybrids that simulate the function of the endogenous ligand were developed. Starting from our recently described NTS2 ligands of type **1**, structural variants of type **2** and the metabolically stable analogues **3a**,**b** were developed. Replacement of the proline unit by a collection of structural surrogates and evaluation of the respective molecular probes for NTS2 affinity and selectivity indicated similar SARs as described for NT(8–13) derivatives bound to the subtype NTS1. Peptide–peptoid hybrids **2d**, **3a**,**b** showed substantial NTS2 binding affinity (K_i = 8.1–16 nM) and 2400–8600-fold selectivity over NTS1. The thiazolidine derivative **3b** showed metabolic stability over 32 h in a serum degradation assay. In an inositol phosphate accumulation assay, the neurotensin mimetics **3a** and **3b** displayed an inhibition of constitutive activity exceeding 1.7–2.0 times the activity of NT(8–13). The fluorinated derivative **3a** could afford attractive opportunities to detect NTS2 by ^{19}F magnetic resonance imaging.

Introduction

NT(8–13) (H-Arg-Arg-Pro-Tyr-Ile-Leu-OH) is the fully active C-terminal hexapeptide of the neuropeptide neurotensin,[1–4] which exerts its function through activation of the G-protein-coupled receptors (GPCRs) NTS1[5,6] and NTS2[7,8] or through subtype 3 (NTS3), which belongs to the Vps10p family of sorting receptors.[9] Grisshammer and co-workers recently determined the crystal structure of neurotensin receptor 1 (NTS1) bound to the activating peptide NT(8–13).[10] This excellent work provides valuable insight into the shape and electrostatic properties of the binding pocket and the bioactive conformation of the endogenous ligand. Moreover, the crystal structure will serve as a guideline for the rational design of subtype-selective NT(8–13) analogues. Neurotensin receptor 2 (NTS2), which is structurally related to NTS1, has attracted growing interest because its physiological effects range from hypothermia to antipsychotic properties.[11] Promoting μ-opioid-independent antinociception,[12] NTS2 is mainly involved in the modulation of tonic pain sensitivity.[13] Thus, NTS2-selective ligands could serve as valuable therapeutic agents.

In the course of our recent studies, identification of screening hits and the chemical synthesis of structural variants led us to the highly potent and NTS2-selective peptide–peptoid hybrids of type **1** (Figure 1).[14] Except for an *N*-2-(4-hydroxyphenyl)ethylglycine peptoid moiety[15] (*N*-homotyrosine, Nhtyr) in place of the tyrosine unit of the parent peptide, the sequence of **1a** is identical to that of the hexapeptide NT(8–13). Further replacements of the basic amino acids with *N*-methylarginine and lysine resulted in the discovery of the metabolically stable derivative **1b**.[16]

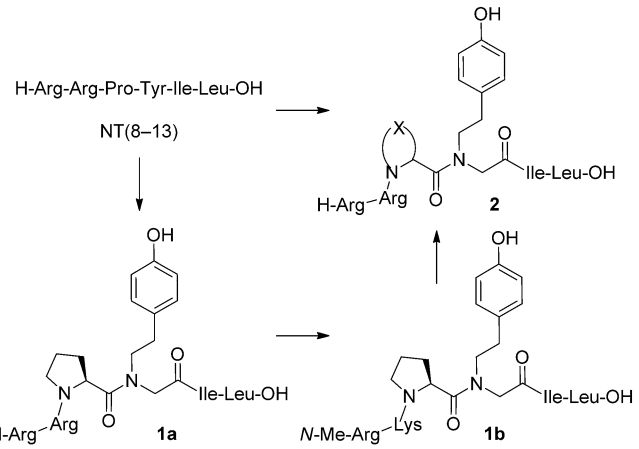

Figure 1. Structural modification leading to target compounds of type **2**.

SAR studies involving alanine,[17] D-amino acid,[18,19] and homo-β-amino acid scans[20] on NT(8–13), as well as the introduction of conformational constraints,[21] indicated a crucial role for the proline residue of neurotensin and NT(8–13) in

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NTS1 binding. This was corroborated by the crystal structure of the GPCR–peptide complex, which shows a tight hydrophobic binding site for the pyrrolidine ring of the proline residue (Figure 2).

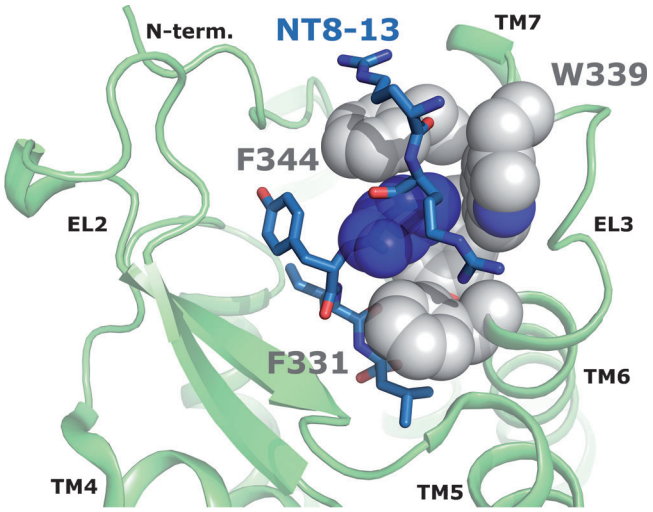


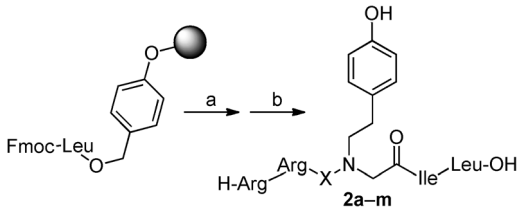
Figure 2. Close view of the crystal structure of the NTS1–NT(8–13) complex: NTS1 is represented by green ribbons, the ligand NT(8–13) is shown in blue sticks. The proline residue of NT(8–13) and the residues of the receptor's hydrophobic binding site are highlighted as blue and grey spheres, respectively. The image was prepared with PyMOL.

To learn more about the binding mode and the bioactive conformation of NTS2-selective NT(8–13) analogues, we envisioned the synthesis of target compounds of type **2** incorporating a collection of proline surrogates and to study these molecular probes for NTS2 affinity and selectivity over NTS1. Using alternative cyclic systems and substituents at position 4 of the pyrrolidine ring that can control ring pucker, we sought to investigate the impact of shape and conformational properties, respectively, on receptor–ligand recognition.

Results and Discussion

Chemistry

Several proline surrogates were incorporated into NT(8–13) and investigated for NTS1 receptor binding.[22,23] A significant decrease in affinity was observed with the use of 3,4-dehydroproline, azetidine-2-carboxylic acid, and pipecolic acid. Thiazolidine-2-carboxylic acid proved to be a potent bioisostere, leading to a slight increase in NTS1 binding affinity. To determine if the pharmacophore of the peptide–peptoid hybrids **1** causes analogous SARs at NTS2, the commercially available proline surrogates mentioned above were used to synthesize target compounds **2a**–**d**. Solid-phase peptide synthesis (SPPS) was started from Fmoc-leucinyl-loaded Wang resin. The peptide–peptoid hybrids **2a**–**d** were prepared by microwave irradiation to accelerate Fmoc deprotection, PyBOP-promoted acylation, and HATU-induced proline surrogate incorporation (Scheme 1).



Scheme 1. *Reagents and conditions*: a) 1. Fmoc-AA-OH, PyBOP, DIPEA, HOBt, DMF, μ-wave, except for proline surrogates: Fmoc-Xaa-OH, HATU, DIPEA, μ-wave, 2. piperidine/DMF, μ-wave; b) TFA/phenol/H_2O/TIS, RT, 2 h.

The ring pucker of proline can be strongly influenced by substituents at position C^{γ}.[24–28] This conformational control, which is caused by stereoelectronic,[25,26] steric,[27] and/or hydrogen bonding effects,[28] can be used as a SAR investigation tool. For example, with electron-withdrawing groups at C^{γ} (e.g., fluorine as the most electronegative element), the substituents of the C^{δ}–C^{γ} bond are not arranged "anti-periplanar" (as expected based on steric considerations) but "gauche". Thus, an electron-withdrawing substituent at a 4*R*-configured center stabilizes a C^{γ} *exo* pucker, and a 4*S*-configured substituent induces a C^{γ} *endo* pucker[25,26] (Figure 3).

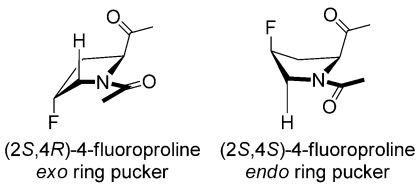
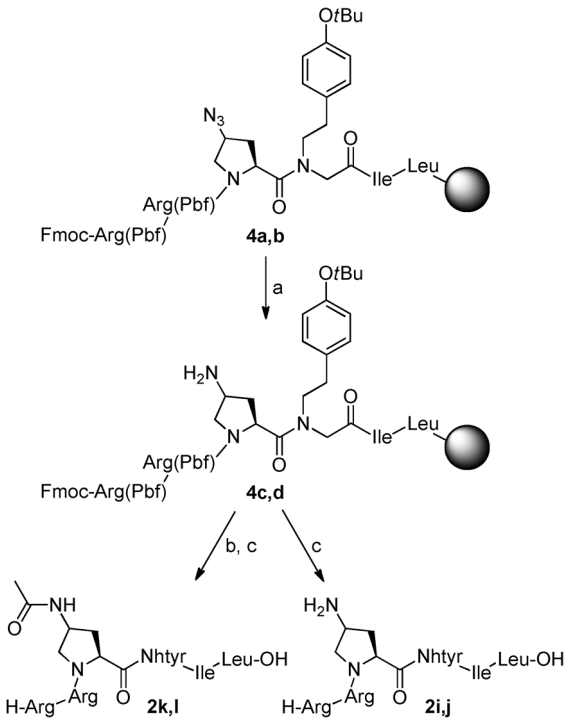


Figure 3. Stereo projection of 4-substituted prolines.

These properties have also been described for azido groups.[26,29] Using SPPS, the chiral building blocks Fmoc-protected (2*S*,4*S*)-4-azidoproline, (2*S*,4*R*)-4-azidoproline,[26] (2*S*,4*R*)-4-fluoroproline, and (2*S*,4*R*)-4-fluoroproline[25,30] were incorporated to afford the peptide–peptoid hybrids **2e**–**h**.

To investigate the effect of charge and hydrogen bonding, amino- and acetamido-substituted analogues were also synthesized.[28] The peptide–peptoid hybrids containing (2*S*,4*S*)-4-aminoproline, (2*S*,4*R*)-4-aminoproline, (2*S*,4*S*)-4-acetamidoproline and (2*S*,4*R*)-4-acetamidoproline were prepared from the fully protected solid-phase-bound (2*S*,4*S*)-4-azidoproline and (2*S*,4*R*)-4-azidoproline derivatives **4a**,**b**, respectively. Using trimethylphosphine, intermediates **4a**,**b** were reduced to the primary amines **4c**,**d**, which could be N-deprotected and cleaved from the resin to obtain aminoprolines **2i**,**j**.[31,32] N-Acetylation of **4c**,**d** by acidic anhydride and subsequent N-deprotection and cleavage resulted in the formation of acetamidoproline derivatives **2k**,**l** (Scheme 2).

To examine the effect of fluorine substituents in greater detail, difluoroproline[33] was used to synthesize peptide–peptoid **2m** (Scheme 1). Representatively, (2*S*,4*R*)-4-fluoroproline and thiazolidine-2-carboxylic acid were converted into the peptide–peptoids **3a**,**b**, which contain the metabolically stable N-terminal motif *N*-methylarginine-lysine instead of Arg-Arg (see Supporting Information).



Scheme 2. *Reagents and conditions*: a) H_2O, Me_3P, THF, RT, 2 h; b) DIPEA, Ac_2O, CH_2Cl_2, RT, 30 min; c) 1. piperidine/DMF, μ-wave, 2. TFA/phenol/H_2O/TIS, RT, 4 h.

Biological investigations

Radioligand binding studies were conducted to evaluate the peptide–peptoids **2a**–**m** and **3a**,**b** for NTS1 and NTS2 affinity (Table 1). Binding data were determined using the radioligand [^{3}H]neurotensin and stably transfected Chinese hamster ovary (CHO) cells expressing human NTS1. [^{3}H]NT(8–13) was used for binding assays investigating human NTS2, which was transiently transfected in human embryonic kidney (HEK293) cells.

The binding data of dehydropyrrolidine and piperidine derivatives **2a** and **2c** show that slight modifications to the geometry of the proline-mimicking ring system made by introduction of a double bond or an additional methylene unit lead to slightly decreased NTS2 affinity (K_i = 23 and 24 nM) relative to the lead structure **1a**. This was also observed for the respective NT(8–13) derivatives at NTS1.[22] Employing azetidine-2-carboxylic acid instead of proline resulted in a 10-fold decrease in NTS2 binding affinity for **2b** which is similar to the 50-fold loss of affinity in NTS1 binding described for the respective NT(8–13) analogue.[22] Formal exchange of the pyrrolidine moiety of **1a** by a thiazolidine unit resulted in preservation of NTS2 binding and an increase in selectivity; the thia-analogue **2d** showed K_i values of 10 and 66000 nM for NTS2 and NTS1, respectively.

Stereospecific substitution of the proline C^γ position of **1a** for fluoro, azido, and acetamido groups resulted in respective K_i values of K_i = 74, 83, and 52 nM for the 4*R* isomers **2f**, **2h**, and **2l**. In contrast, the affinities of the 4*S* isomers **2e**, **2g**, and **2k** were two- to fivefold lower. This indicates that the adoption of an *exo* ring pucker, which is stabilized by the 4*R* configuration, is favored. Introduction of a primary amino group at position 4, facilitating formation of a cationic center upon protonation, led to a drastic loss of NTS2 affinity for **2i** and **2j**. Interestingly, the geminally substituted difluoride **2m** (K_i = 30 nM) showed higher NTS2 binding affinity than the 4*R* fluoro analogue **2f**, indicating that the *exo* ring pucker might be more favorable; however, a fluorine substituent will be bound better by the receptor if located at the 4*S* position of the ligand. To take advantage of the metabolic stability that results from a formal exchange of the arginine residues at the N terminus by the sequence *N*-Me-Arg-Lys, the peptide–peptoid hybrids **3a** and **3b** were included into the collection of test compounds. Interestingly, the NTS2 ligands showed high binding affinity (8.1 nM for **3a**, 16 nM for **3b**) and selectivity over NTS1 (> 2000). The thiazolidine derivative **3b** was subjected to a serum degradation assay that indicated metabolic stability over 32 h (see Supporting Information).

To investigate the intrinsic activity of the peptide–peptoid hybrids **2d**, **2f**, **2m**, **3a**, and **3b** in comparison with the lead compound **1a** and NT(8–13),[34] we used a previously established inositol phosphate (IP) accumulation assay (Table 2).[16,35] $G\alpha_q$-promoted modulation of IP_3 production in HEK293 cells transiently expressing NTS2 was recorded. Constitutive activity could be attenuated by the reference agents neurotensin, NT(8–13), and **1a**. Remarkably, all peptide–peptoid hybrids **2d**, **2f**, **2m**, **3a**, and **3b** displayed strong intrinsic activity, substantially exceeding the maximal efficacy of the endogenous ligand neurotensin. The strongest effect was exerted by the thiazolidine derivative **3b**, showing a maximum effect that is twofold stronger than that of NT(8–13).

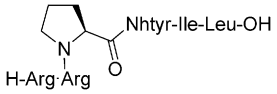
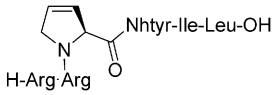
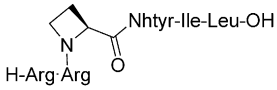
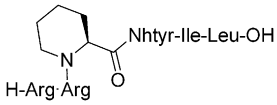
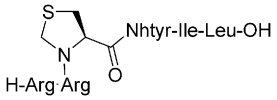
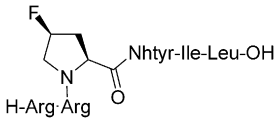
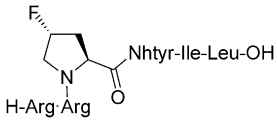
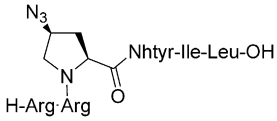
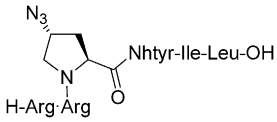
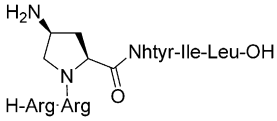
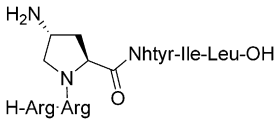
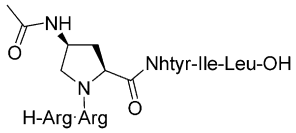
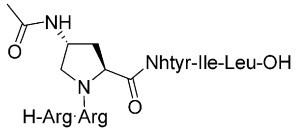
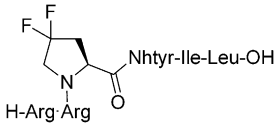
Conclusions

Using the NTS2 ligands of type **1** as lead compounds, structural variants of type **2** were synthesized. Replacement of the proline unit with a collection of structural surrogates and evaluation of the respective molecular probes for NTS2 affinity and selectivity indicated a related binding mode and similar SARs as described for NT(8–13) derivatives bound to the subtype NTS1. The peptide–peptoid hybrids of type **2e**–**m** suggest that NTS2 has a preference for an *exo*-puckered conformation of the pyrrolidine ring. The thiazolidine-2-carboxylic acid derivative **2d** and the N-terminally modified (2*S*,4*R*)-4-fluoroproline NT(8–13) analogue **3a** show excellent receptor binding affinity and subtype selectivity. These SAR investigations will be of particular interest in drug discovery because selective NTS2 ligands could serve as useful molecular probes and analgesic agents without the side effects of opioid-mediated antinociception. Furthermore, the fluorinated derivative **3a** might provide attractive opportunities to develop NTS2-selective ^{19}F magnetic resonance imaging agents.

Experimental Section

Chemistry: Reagents and dry solvents were obtained from commercial sources and were used as received. Reactions were conducted under dry N_2. Evaporations of product solutions were car-

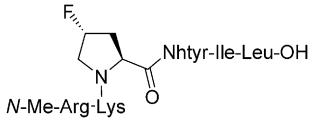
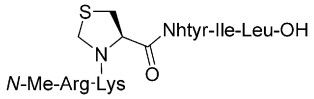
Table 1. Human NTS1 and NTS2 receptor binding data for the peptide–peptoids **1a**, **2a–m**, and **3a,b** in comparison with the reference agent NT(8–13).

Compd	Sequence	K_i [nM][a] NTS1[b]	NTS2[c]	SR[f]
NT(8–13)		0.24 ± 0.024	1.2 ± 0.17[d]	0.20
1a		31 000 ± 7000	8.0 ± 0.94	3900
2a		60 000 ± 8700	23 ± 5.4	2600
2b		59 000 ± 15 000	91 ± 12	650
2c		23 000 ± 7800	24 ± 5.6	930
2d		66 000 ± 21 000	10 ± 1.7	6600
2e		25 000 ± 5500	340 ± 100	67
2f		44 000 ± 9000	74 ± 10	590
2g		59 000 ± 4900[e]	190 ± 0[e]	310
2h		57 000 ± 13 000	83 ± 19	690
2i		> 100 000	1800 ± 150	55
2j		59 000 ± 8700	880 ± 62	67
2k		20 000 ± 3500	200 ± 20	100
2l		55 000 ± 23 000	52 ± 11	1100
2m		67 000 ± 17 000	30 ± 11	2200

ried out in vacuo with a rotatory evaporator. Column chromatography was performed using 60 μm silica gel. For thin-layer chromatography (TLC) silica gel 60 μm plates were used (UV, I_2, or ninhydrin detection). Melting temperatures are uncorrected. IR spectra were registered from a thin film on a NaCl crystal. NMR data were acquired with 360 or 600 MHz spectrometers, if not stated otherwise in $CDCl_3$; ^{13}C NMR spectra were recorded at 90 MHz if not stated otherwise in $CDCl_3$. Chemical shifts (δ) are given in ppm relative to tetramethylsilane (TMS), with TMS = 0. MS data were acquired using electronic ionization (EI), atmospheric pressure chemical ionization (APCI), or electrospray ionization (ESI) techniques. HPLC analysis revealed a purity of > 95% for all SAR compounds.

Synthesis of peptide–peptoid hybrids: Peptide synthesis was performed according to standard protocols as described below. The synthesis was performed by starting from commercially available Fmoc-Leu Wang resin. α-Amino acids were incorporated as their commercially available derivatives: Fmoc-Ile-OH, Fmoc-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-*N*-Me-Arg(Mtr)-OH, Fmoc-(2*S*)-thiazolidine-2-carboxylic acid, Fmoc-(2*S*)-azetidine-2-carboxylic acid, Fmoc-(2*S*)-3,4-dehydroproline, Fmoc-(2*S*)-pipecolic acid. Elongation of the peptide chain was done by repetitive cycles of Fmoc deprotection with the help of microwave irradiation and subsequent coupling of the amino acid derivative. After the final acylation step, the N-terminal Fmoc residue was deprotected, the resin was rinsed with CH_2Cl_2 (10×) and dried in vacuo. Cleavage from the resin was performed with a mixture of trifluoroacetic acid (TFA)/phenol/H_2O/triisopropylsilane (TIS) 88:4:6:2 for 4 h, followed by filtration of the resin. After evaporation of the solvent in vacuo and precipitation in *tert*-butylmethyl ether, the crude peptides were purified by preparative RP-HPLC: Agilent 1100 preparative series, column Zorbax Eclipse XDB-C8, 21.2 mm × 150 mm, 5 μm particles [C_8], employing solvent systems,

Table 1. (Continued)

Compd	Sequence	K_i [nM][a] NTS1[b]	NTS2[c]	SR[f]
3a		70000 ± 15000	8.1 ± 2.3	8600
3b		39000 ± 21000	16 ± 5.1	2400

[a] Values are the means ± SEM of 3–9 individual experiments, each done in triplicate. [b] Determined with [^{3}H]neurotensin and membranes from CHO cells stably expressing human NTS1. [c] Determined with [^{3}H]NT(8–13) and homogenates from HEK293 cells transiently expressing human NTS2. [d] K_D value. [e] Values are the means ± SD of two individual experiments, each done in triplicate. [f] Selectivity ratio: K_i(NTS1)/K_i(NTS2).

Table 2. Determination of the functional activity of selected test compounds, reference agent NT(8–13), and neurotensin obtained from an IP accumulation assay in NTS2-expressing cells.

Compd	IP accumulation[a]
1a	1.5 ± 0.21
2d	2.0 ± 0.11
2f	1.4 ± 0.18
2m	1.6 ± 0.14
3a	1.7 ± 0.13
3b	2.0 ± 0.11
NT(8–13)	1.0 ± 0.21

[a] Fold effect relative to NT(8–13): ligand efficacy at 1 μM [NT(8–13)] and 10 μM [test compounds] measured in an IP accumulation assay; values are the means ± SEM of five individual experiments, each done in triplicate.

linear gradient and flow rate [FR] as specified below or VP 250/32 Nucleodur C18 HTec, 5 μm particles [C_{18}] employing solvent systems, linear gradient, and flow rate [FR] as specified below.

After separation, the peptides were lyophilized, and peptide purity and identity were assessed by analytical HPLC (Agilent 1100 analytical series equipped with QuatPump and VWD detector; column Zorbax Eclipse XDB-C8 analytical column, 4.6 mm × 150 mm, 5 μm, FR: 0.5 mL min^{-1}) coupled to a Bruker Esquire 2000 mass detector equipped with an ESI-trap. System 1 (S1): *x*–*y*% CH_3OH in H_2O + 0.1% HCO_2H in 18 min (S1A: 10–55% in 18 min, 55–95% in 2 min, 95–95% in 2 min; S1B: 10–65%; S1C: 10–40%). System 2 (S2): *x*–*y*% CH_3CN in H_2O + 0.1% HCO_2H in 26 min (S2A: 3–40%).

Elongation method (EM): Microwave-assisted (Discover microwave oven, CEM Corp.) peptide synthesis was carried out in silanized glass tubes loosely sealed with a silicon septum. The development of overpressure was avoided by using DMF as the solvent along with intermittent cooling. Fmoc deprotection: the resin was treated 1× with 25% piperidine in DMF (microwave irradiation: 7 × 5 s, 100 W), followed by washings with DMF (5×). Peptide coupling was done by using 5 equiv each of Fmoc-AA/PyBOP/DIPEA and 7.5 equiv 1-hydroxybenzotriazole (HOBt) dissolved in a minimum amount of DMF (irradiation: 20 × 10 s, 50 W). Between each irradiation step, cooling of the reaction mixture to −10 °C was achieved by sufficient agitation in an EtOH–ice bath. The peptoid (3 equiv) was coupled with 3 equiv PyBOP/DIPEA and 4.5 equiv HOBt in DMF. Fmoc-Pro-OH (5 equiv) was routinely coupled 2× with HATU (5 equiv) and DIPEA (10 equiv) in DMF.

H-Arg-Arg-3,4-dehydroproline-Nhtyr-Ile-Leu-OH (2a): Synthesized according to EM. Purification [C_{18}]: CH_3CN (A) and 0.1% HCO_2H in H_2O (B) applying a linear gradient starting from 3% A in 97% B to 32% A in 68% B in 10.9 min; FR: 32.0 mL min^{-1}; t_R: 8.6 min; purity: S1B 97% (t_R: 12.0 min); S2A >97% (t_R: 14.8 min); $[M+H]^+$: calcd 829.5, found 829.6.

H-Arg-Arg-azetidine-2-carboxylic acid-Nhtyr-Ile-Leu-OH (2b): Synthesized according to EM. Purification [C_8]: CH_3CN (A) and 0.1% HCO_2H in H_2O (B) applying a linear gradient starting from 3% A in 97% B to 37% A in 63% B in 18.0 min; FR: 10.0 mL min^{-1}; t_R: 10.1 min; purity: S1A >99% (t_R: 14.8 min); S2A >99% (t_R: 15.6 min); $[M+H]^+$: calcd 817.5, found 817.5.

H-Arg-Arg-pipecolic acid-Nhtyr-Ile-Leu-OH (2c): Synthesized according to EM. Purification [C_{18}]: CH_3CN (A) and 0.1% HCO_2H in H_2O (B) applying a linear gradient starting from 3% A in 97% B to 29.6% A in 70.4% B 10.0 min; FR: 32.0 mL min^{-1}; t_R: 9.0 min; purity: S1B 99% (t_R: 12.7 min); S2A >99% (t_R: 15.3 min); $[M+H]^+$: calcd 845.5, found 845.7.

H-Arg-Arg-thiazolidine-2-carboxylic acid-Nhtyr-Ile-Leu-OH (2d): Synthesized according to EM. Purification [C_8]: CH_3CN (A) and 0.1% HCO_2H in H_2O (B) applying a linear gradient starting from 3% A in 97% B to 37% A in 63% B in 18.0 min; FR: 10.0 mL min^{-1}; t_R: 10.4 min; purity: S1A >99% (t_R: 15.5 min); S2A >99% (t_R: 16.0 min); $[M+H]^+$: calcd 849.5, found 849.6.

H-Arg-Arg-(2*S*,4*S*)-4-fluoroproline-Nhtyr-Ile-Leu-OH (2e): Synthesized according to EM. Purification [C_8]: CH_3OH (A) and 0.1% HCO_2H in H_2O (B) applying a linear gradient starting from 10% A in 90% B to 50% A in 50% B in 18.0 min; FR: 10.0 mL min^{-1}; t_R: 9.5 min; purity: S1A >99% (t_R: 14.4 min); S2A >99% (t_R: 16.3 min); $[M+H]^+$: calcd 849.5, found 849.7.

H-Arg-Arg-(2*S*,4*R*)-4-fluoroproline-Nhtyr-Ile-Leu-OH (2f): Synthesized according to EM. Purification [C_8]: CH_3OH (A) and 0.1% HCO_2H in H_2O (B) applying a linear gradient starting from 10% A in 90% B to 50% A in 50% B in 18.0 min; FR: 10.0 mL min^{-1}; t_R: 9.0 min; purity: S1A >99% (t_R: 13.8 min); S2A >99% (t_R: 14.6 min); $[M+H]^+$: calcd 849.5, found 849.6.

H-Arg-Arg-(2*S*,4*S*)-4-azidoproline-Nhtyr-Ile-Leu-OH (2g): Synthesized according to EM. Purification [C_8]: CH_3OH (A) and 0.1% HCO_2H in H_2O (B) applying a linear gradient starting from 10% A in 90% B to 50% A in 50% B in 18.0 min; FR: 10.0 mL min^{-1}; t_R: 9.6 min; purity: S1A >99% (t_R: 13.7 min); S2A 96% (t_R: 14.9 min); $[M+H]^+$: calcd 872.5, found 872.6.

H-Arg-Arg-(2*S*,4*R*)-4-azidoproline-Nhtyr-Ile-Leu-OH (2h): Synthesized according to EM. Purification [C_8]: CH_3OH (A) and 0.1% HCO_2H in H_2O (B) applying a linear gradient starting from 10% A in 90% B to 50% A in 50% B in 18.0 min; FR: 10.0 mL min^{-1}; t_R: 9.6 min; purity: S1A 95% (t_R: 13.6 min); S2A 95% (t_R: 14.8 min); $[M+H]^+$: calcd 872.5, found 872.6.

H-Arg-Arg-(2*S*,4*S*)-4-aminoproline-Nhtyr-Ile-Leu-OH (2i): Peptide **2g** was synthesized according to EM. After completed peptide coupling, the resin-bound peptide–peptoid [H-Arg(Pbf)-Arg(Pbf)-(2*S*,4*S*)-4-azidoproline-Nhtyr(*t*Bu)-Ile-Leu Wang resin (**4a**)] was washed with CH_2Cl_2 (5×2 mL), dried, and swollen with THF (2 mL) for 30 min. After solvent removal, a mixture of H_2O (130 μL), Me_3P (1 M in THF, 30 equiv), and THF (2 mL) was added, and the reaction vessel was shaken for 2 h at RT (reaction monitored by Kaiser test). After complete reaction, the resin was filtered and washed with THF (5×2 mL), DMF (5×2 mL), and CH_2Cl_2 (3×2 mL) and dried afterward. Then, the resin **4c** was divided into two parts: one part was processed to **2k**, the other part was swollen in DMF for 30 min to deprotect the Fmoc group via standard protocol and cleave the peptide from the resin. Purification [C_8]: CH_3CN (A) and 0.1% HCO_2H in H_2O (B) applying a linear gradient starting from 3% A in 97% B to 35% A in 65% B in 18.0 min; FR: 10.0 mL min^{-1}; t_R: 7.8 min; purity: S1 C 99% (t_R: 10.4 min); S2 A 99% (t_R: 12.0 min); $[M+H]^+$: calcd 846.5, found 846.5.

H-Arg-Arg-(2*S*,4*R*)-4-aminoproline-Nhtyr-Ile-Leu-OH (2j): Synthesized analogously to **2i** using (2*S*,4*R*)-4-azidoproline instead of (2*S*,4*S*)-4-azidoproline. One part of the resin-bound intermediate [Fmoc-Arg(Pbf)-Arg(Pbf)-(2*S*,4*R*)-4-aminoproline-Nhtyr(*t*Bu)-Ile-Leu Wang resin (**4d**)] was used for the synthesis of **2l**, the other part was N-terminally cleaved from the Fmoc protecting group. Afterward, resin cleavage was performed. Purification [C_8]: CH_3CN (A) and 0.1% HCO_2H in H_2O (B) applying a linear gradient starting from 3% A in 97% B to 35% A in 65% B in 18.0 min; FR: 10.0 mL min^{-1}; t_R: 8.1 min; purity: S1 C 99% (t_R: 12.3 min); S2 A 99% (t_R: 12.2 min); $[M+H]^+$: calcd 846.5, found 846.6.

H-Arg-Arg-(2*S*,4*S*)-4-acetamidoproline-Nhtyr-Ile-Leu-OH (2k): The resin-bound intermediate from the synthesis of **2i** [Fmoc-Arg(Pbf)-Arg(Pbf)-(2*S*,4*S*)-4-aminoproline-Nhtyr(*t*Bu)-Ile-Leu Wang resin (**4c**)] was swollen in CH_2Cl_2 for 30 min. Afterward, the solvent was removed and a mixture of DIPEA (30 equiv) and acetic anhydride (30 equiv) in CH_2Cl_2 (2 mL) was added, and the reaction vessel was shaken for 30 min at RT (reaction monitored by Kaiser test). After solvent removal the N-terminal Fmoc protecting group was cleaved before the resin cleavage was performed. Purification [C_8]: CH_3CN (A) and 0.1% HCO_2H in H_2O (B) applying a linear gradient starting from 3% A in 97% B to 35% A in 65% B in 18.0 min; FR: 10.0 mL min^{-1}; t_R: 9.6 min; purity: S1 A >99% (t_R: 13.1 min); S2 A >99% (t_R: 14.5 min); $[M+H]^+$: calcd 888.5, found 888.6.

H-Arg-Arg-(2*S*,4*R*)-4-acetamidoproline-Nhtyr-Ile-Leu-OH (2l): Synthesized analogously to **2k** by using the intermediate **4d** instead of **4c**. Purification [C_8]: CH_3CN (A) and 0.1% HCO_2H in H_2O (B) applying a linear gradient starting from 3% A in 97% B to 35% A in 65% B in 18.0 min; FR: 10.0 mL min^{-1}; t_R: 10.3 min; purity: S1 A 99% (t_R: 13.7 min); S2 A 99% (t_R: 14.8 min); $[M+H]^+$: calcd 888.5, found 888.6.

H-Arg-Arg-4,4-difluoroproline-Nhtyr-Ile-Leu-OH (2m): Synthesized according to EM. Purification [C_8]: CH_3OH (A) and 0.1% HCO_2H in H_2O (B) applying a linear gradient starting from 10% A in 90% B to 50% A in 50% B in 18.0 min; FR: 10.0 mL min^{-1}; t_R: 11.0 min; purity: S1 A >99% (t_R: 14.7 min); S2 A >99% (t_R: 16.3 min); $[M+H]^+$: calcd 867.5, found 867.6.

Receptor binding experiments: Receptor binding data were determined according to protocols as described previously.[14,36] In detail, NTS1 binding was measured using homogenates of membranes from CHO cells stably expressing human NTS1 at a final concentration of 1–2 μg per well, and the radioligand [^{3}H]neurotensin (specific activity: 116 Ci mmol^{-1}; PerkinElmer, Rodgau, Germany) at a concentration of 0.50 nM. Specific binding of the radioligand was determined at K_D values of 0.37–0.96 nM and a B_{max} of 6170–9300 fmol (mg protein)$^{-1}$. Nonspecific binding was determined in the presence of 10 μM neurotensin. NTS2 binding assays were carried out by the calcium phosphate method, using homogenates of membranes from HEK293 cells, which were transiently transfected with the pcDNA3.1 vector containing the human NTS2 gene (Missouri S&T cDNA Resource Center (UMR), Rolla, MO, USA).[37] Membranes were incubated at a final concentration of 6–20 μg per well together with 0.50 nM [^{3}H]NT(8–13) (specific activity: 136 Ci mmol^{-1}; custom synthesis of [leucine-^{3}H]NT(8–13) by GE Healthcare, Freiburg, Germany) at K_D values in the range of 0.67–2.02 nM and a B_{max} value of 310–930 fmol (mg protein)$^{-1}$. Nonspecific binding was determined in the presence of 10 μM NT(8–13), and the protein concentration was generally determined by the method of Lowry using bovine serum albumin as standard.[38]

Data analysis: Data analysis of the competition curves from the radioligand binding experiments was accomplished by nonlinear regression analysis using the algorithms in Prism 5.0 (GraphPad Software, San Diego, CA, USA). EC_{50} values derived from the resulting dose–response curves were transformed into the corresponding K_i values using the equation of Cheng and Prusoff.[39]

Determination of IP accumulation: The functional test on NTS2 receptor-mediated IP accumulation was performed according to published procedures.[14,35a] Briefly, HEK293 cells were transiently transfected and transferred into 24-well plates at a density of 1×10^5 cells per well at a volume of 270 μL. After the addition of 30 μL *myo*-[^{3}H]inositol (specific activity: 22.5 Ci mmol^{-1}, PerkinElmer, Rodgau, Germany), cells were incubated for 15 h. The medium was then aspirated, cells were washed with medium supplemented with 10 mM LiCl, and test compounds were added for 60 min at 37 °C. To stop incubation, cells were lysed by adding 150 μL ice-cold 0.1 M NaOH. The cell extract was diluted in buffer (5 mM sodium tetraborate, 0.5 mM Na·EDTA) and was separated by anion-exchange chromatography using an AG1-X8 resin (BioRad, Munich, Germany). After washing with water and elution buffer I (5 mM sodium tetraborate, 60 mM sodium formate) and again with water, total IP was eluted with 2.5 mL elution buffer II (1.0 M ammonium formate) and directly collected into scintillation counting vials. Radioactivity was measured by scintillation counting after adding 2.5 mL of Emulsifier-Safe (PerkinElmer, Rodgau, Germany). Data were analyzed by normalizing the dpm values; this was done by setting the data for non-stimulated receptor (buffer) equal to zero and the effect for NT(8–13) equal to one.

Glossary: NTS1, neurotensin receptor 1; NTS2, neurotensin receptor 2; NTS3, neurotensin receptor 3; NT, neurotensin; GPCR, G-protein-coupled receptor; AA, amino acid; Nhtyr, *N*-homotyrosine; CHO, Chinese hamster ovary; HEK, human embryonic kidney; SPPS: solid-phase peptide synthesis; Fmoc, 9-fluorenylmethyloxycarbonyl; HATU, 2-(1*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; PyBOP, (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; Boc, *tert*-butyloxycarbonyl; Mtr; 4-Methoxy-2,3,6-trimethylbenzenesulfonyl; HOBt, 1-hydroxybenzotriazole; DMF, *N*,*N*-dimethylformamide; DIPEA, diisopropylethylamine; TFA, trifluoroacetic acid; TIS, triisopropylsilane; MALDI-TOF-MS, Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; IS, internal standard; IP inositol phosphate.

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Keywords: GPCRs · neurotensin · NTS2 · peptide–peptoid hybrids · proline analogues · structure–activity relationships

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