Evaluation of Lactam-Bridged Neurotensin Analogues Adjusting ψ(Pro[10]) Close to the Experimentally Derived Bioactive Conformation of NT(8−13)

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Received May 13, 2004

The neurotensin C-terminal hexapeptide, NT(8−13), which has been found to adopt a β-strand-like conformation while bound to the NT1 receptor, was modified by the introduction of conformational constraints. Synthesis of the four stereoisomeric 4.4-spirolactams **1**−**4** and subsequent NT1 receptor binding studies showed that the restriction of ψ(Pro[10]) to approximately 130° leads to a more than 1000-fold increase of binding affinity for **1** (K_i = 12 nM) when compared to the more flexible analogue [*N*MeTyr[11]]NT(8−13).

Neurotensin (NT, pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu) is a neuropeptide found in the periphery and in the central nervous system especially regulating dopaminergic transmission of the mesocorticolimbic pathways.[1] Because of several behavioral effects when administrated into the CNS, neurotensin has been considered as an endogenous antipsychotic, which is due to a modulation of dopaminergic activity by stimulation of the G-protein-coupled receptor NT1 being colocalized with the dopamine D2 receptor.[2,3] SAR studies demonstrated that the C-terminal hexapeptide NT(8−13) is sufficient for NT1 binding and ligand efficacy when the structural properties mediated by Pro[10] and Tyr[11] within the core region of NT(8−13) turned out highly crucial for receptor recognition.[4−8] Very recently, M. Baldus and co-workers were able to determine the bioactive conformation of NT(8−13) bound to NT1 when 2D solid-state NMR spectroscopy was employed.[9] Following preceding discussions suggesting a linear rearrangement as well as a type I β-turn conformation indicating a key dihedral angle of −30°,[10,11] the direct structural methods revealed a β-strand-like orientation of the peptide, including a diagnostic ψ(Pro[10]) dihedral angle of 146 ± 15°. As a complement to these structural investigations, we tried to investigate the importance of the secondary structure to the binding process by bridging the α-C of Pro[10] with the amino group of Tyr[11] and, thus, constraining ψ(Pro[10]) of NT(8−13). To observe the consequence of the conformational restriction, NT1 binding affinities of the peptide mimetics should be compared to those of the more flexible analogue [*N*MeTyr[11]]NT(8−13).[12] According to recent X-ray analyses in the field of neuropeptide research,[13−15] 4.4-spiro-lactams adopting a ψ-angle of approximately 130° should enable us to adjust a torsion angle ψ(Pro[10]) approximately matching to the NMR-derived data when starting from natural proline. Inverting also Φ(Pro[10]), the opposite ψ-angle (approximately −130°) can be adjusted based on the respective unnatural (*R*)-amino acid enantiomer. Backbone representations of NT(8−13) and the spirocyclic analogues **1** and **2**, presuming consistent backbone dihedral angles for Arg[8], Arg[9], Ile[12], and Leu[13] as well as for the Tyr moiety of the inserted templates, are displayed in Figure 1 and indicate the structural similarity of the β-strand-like bioactive conformation of NT 8−13 and the (*S*)-proline derived spiro-lactam **1** as well as the distinct shape and geometry for the diastereomer based on unnatural proline (**2**). To learn more about the side chain geometry of the bioactive core scaffold and the conformational space available to the aromatic system

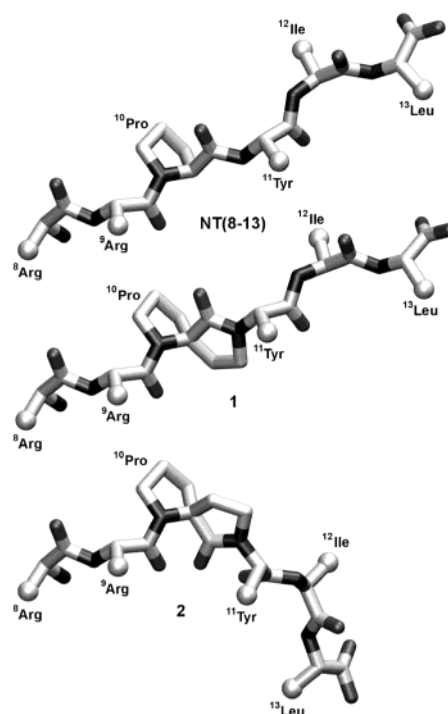

Figure 1. Backbone representations of NT(8−13) (top) and spirocyclic analogues **1** (center) and **2** (bottom). The conformation of NT(8−13) was obtained by applying the dihedral angles found by Baldus et al.[9] Conformations shown for **1** and **2** represent supposable alignments presuming consistent backbone dihedral angles for Arg[8], Arg[9], Ile[12], Leu[13], as well as for the Tyr moiety of the inserted templates, and +128.7° (**1**) or −128.7° (**2**) for ψ(Pro).

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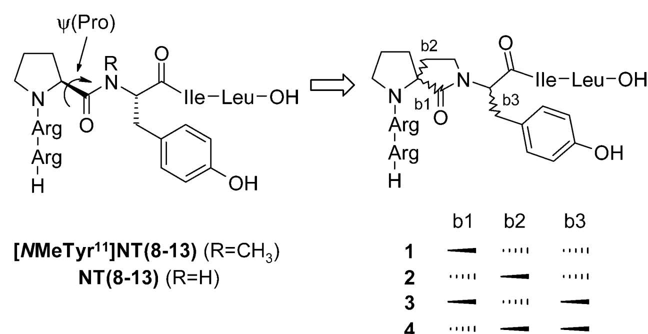
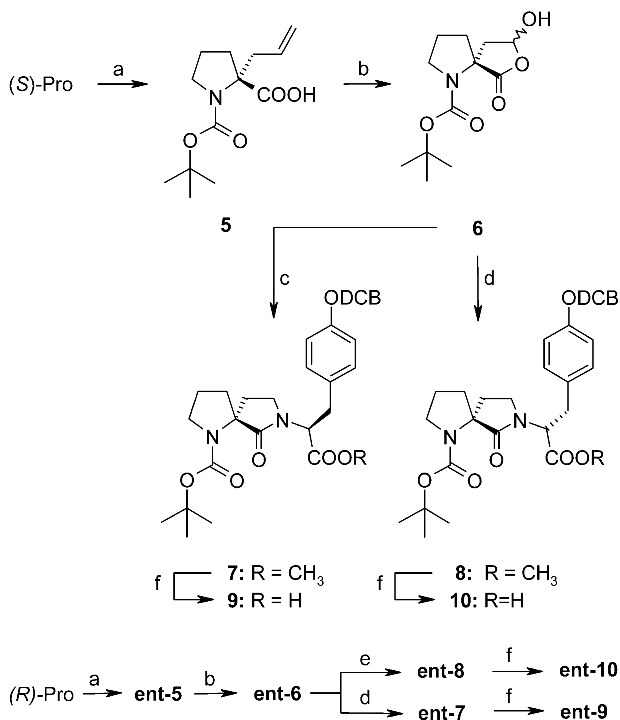
10.1021/jm049644y CCC: $27.50 © 2004 American Chemical Society
Published on Web 09/29/2004

Chart 1



Scheme 1[a]



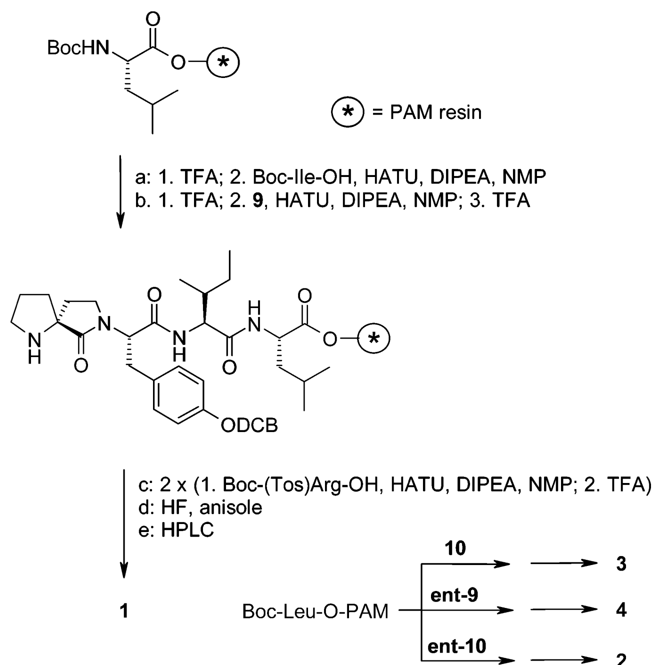
[a] Reagents and conditions: (a) refs 16–19; (b) 1. O_3, CH_2Cl_2, −78 °C, 2. PPh_3, RT, 2.5 h (75%) (c) 1. (*S*)-*O*-(2,6-dichlorobenzyl)tyrosine methyl ester, CH_2Cl_2, RT, 30 min, 2. Na[BH(OAc)$_3$], 2.5 h, 3. $CHCl_3$, RT, 40 h (82%); (d) (*R*)-*O*-(2,6-dichlorobenzyl)-tyrosine methyl ester hydrochloride, Na[BH(OAc)$_3$], CH_2Cl_2, RT, 15–24 h (43–52%); (e) (*S*)-*O*-(2,6-dichlorobenzyl)tyrosine methyl ester hydrochloride, Na[BH(OAc)$_3$], CH_2Cl_2, RT, 5 h (81%); (f) NaOH, MeOH, THF, H_2O, 0 °C, 25 min–3.5 h (55–100%).

of Tyr[11], both enantiomers of tyrosine should be inserted to give the peptide mimetics **1**–**4** after solid-phase-supported peptide synthesis.

Chemistry

For the synthesis of the spirocyclic dipeptide mimetics **9** and **10** (Scheme 1), *N*-Boc-protected (*R*)-2-allylproline (**5**)[16] was employed as a key starting material, when we applied Seebach's concept of self-reproduction of chirality which was recently reinvestigated by Germanas and co-workers.[17,18] Thus, natural proline was transformed into the α-allylproline methyl ester[19] and, subsequently, Boc-protected and saponified to afford the building block **5** in 32% overall yield. For the construction of the 4.4-spirocylic system, activation was envisioned by ozonolysis when subsequent reductive coupling with a protected tyrosine derivative should result in lactam formation.[20]

Scheme 2



Thus, treatment of the terminal alkene **5** with ozone, followed by a reductive workup procedure, gave the hydroxylactone **6** as a stable central intermediate. According to the NMR spectra, **6** exists as a 4:1 mixture of diastereomers when the chemical shift values of the OH protons indicate an intramolecular H-bond to the Boc C=O oxygen for the major isomer and thus (*R*)-configuration. By employing sodium triacetoxyborohydride as a reducing agent, the carbaldehyde equivalent **6** could be readily reacted with *O*-2,6-dichlorobenzyl-protected (*S*)- and (*R*)-Tyr methyl ester,[21,22] giving direct and efficient access to the spirocyclic lactams **7** and **8**, respectively. Saponification of the ester functionalities using sodium hydroxide yielded the Boc-protected dipeptide mimetics **9** and **10**. Employing the identical reaction sequence, we synthesized the optical antipodes *ent*-**9** and *ent*-**10**, starting from (*R*)-proline.

Solid-phase-supported synthesis of the conformationally constrained peptide mimetics **1**–**4** was carried out following a Boc strategy on a PAM (4-(hydroxymethyl)-phenylacetamide) resin preloaded with Boc-Leu when the Boc-protected amino acids isoleucine, N_ω-tosylarginine and the dipeptide mimetics **9**, **10**, *ent*-**9**, and *ent*-**10** were attached following standard HATU (*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate)[23] coupling and TFA deprotection protocols (Scheme 2). For the synthesis of [*N*MeTyr[11]]NT-(8–13), proline was incorporated by a BTC (bis-(trichloromethyl)carbonate)[24]-mediated coupling of Fmoc-Pro and subsequent piperidine/DBU deprotection. Cleavage and deprotection of the peptides were accomplished using neat HF. Finally, purification by HPLC gave rise to the test compounds **1**–**4**.

Biological Results and Discussion

The lactam-bridged neurotensin analogues **1**–**4** and the more flexible peptide mimetic [*N*MeTyr[11]]NT(8–13) were evaluated in vitro for their ability to compete with [^{3}H]neurotensin in binding to porcine striatal NT1

Table 1. Receptor Binding Data for the Lactam-Bridged Test Compounds **1**−**4** in Comparison to the Natural Transmitter Neurotensin (NT) and [*N*MeTyr[11]]NT(8−13) Employing Porcine NT1 Receptors[a]

compd	[^{3}H]NT: K_i (nM)
1	12 ± 0.73
2	17000 ± 1200
3	>100 000
4	14000 ± 1500
NT	1.3 ± 0.28[b]
[*N*MeTyr[11]]NT(8−13)	1400 ± 150

[a] K_i values in nM ± SEM are based on the means of two to six experiments each done in triplicate. [b] K_D value for the porcine NT1 receptor as average value derived from eight different homogenates.

receptors (Table 1) when competition experiments employing NT (K_D = 1.3 nM) and NT(8−13) (K_i = 0.27 nM) showed strong analogy to the values found for human NT1 (K_D = 1.8 nM and 0.16 nM, respectively[25]) indicating the suitability of our test system as a model for hNT1. Our initial investigations were directed to the binding properties of [*N*MeTyr[11]]NT(8−13) being structurally very close to the test compounds **1**−**4** since the sequence of residues and the number and position of backbone NH functionalities are identical. The difference is the conformational constraint exerted by the 4.4-spirolactam system. When ψ(Pro[10]) is adjusted properly, the reduction of conformational freedom and the loss of entropy should be reduced resulting in a significant increase of binding affinity. The K_i values of the heterologous competition assay reflect substantial NT1 affinity of [*N*MeTyr[11]]NT(8−13) (K_i = 1400 nM). Interestingly, albeit **1** is missing the hydrogen-bond donating properties of NT and NT(8−13) at the NH of Tyr[11], the affinity was improved by approximately 3 orders of magnitude when ψ(Pro[10]) was adjusted to approximately 130° indicated by a K_i value of 12 nM for the spirolactam **1**. Obviously, the similarity to the experimentally investigated ψ(Pro[10]) enables **1** to simulate the natural neurotransmitter neurotensin (K_D = 1.3 nM) in its bioactive conformation. The fact that the affinity of **1** is still lower than the receptor binding of NT and NT(8−13) is obviously due to the hydrogen bridge donor functionality of the NH. Configurational switch of the tyrosine residue resulting in a different disposition of the 4-hydroxybenzyl side chain revealed a dramatic loss of NT1 recognition (K_i > 100 000 nM) for the 11(*R*)-isomer **3** indicating that the aromatic residue needs to be located in an area inaccessible to the side chain of (*R*)-Tyr. Interestingly, an analogous chiral switch at the tyrosine position of NT(8−13) gave only a 10-fold reduction of affinity.[5] Obviously, mismatching structural properties can be better compensated by more flexible receptor ligands, which is an effect that is generally known to be responsible for selectivity problems of highly flexible drug candidates. Investigation of the (*R*)-Pro derived spirolactams **2** and **4** displayed only moderate binding affinities indicated by K_i values of 17 000 and 14 000 nM, respectively, clearly demonstrating the importance of ψ(Pro[10]) and Φ(Pro[10]).

In conclusion, we could demonstrate that the loss of entropy as a consequence of the conformational restriction of ψ(Pro[10]) leads to a substantial increase of NT1 binding affinity when the lactam-bridged peptide mimetic **1** is compared to to [*N*MeTyr[11]]NT(8−13) as the more flexible analogue. The study corroborates the NMR-derived bioactive conformation of NT(8−13) discovered by M. Baldus and co-workers. With the goal of developing a nonpeptidic neurotensin NT1 receptor agonist, further structural modifications are in progress.

Experimental Section

(5*R*,8*RS*)-8-Hydroxy-6-oxo-7-oxa-1-azaspiro[4.4]nonane-1-carboxylic Acid *tert*-Butyl Ester (6). A solution of (*R*)-N-Boc-2-allylproline (**5**; 1.00 g, 3.92 mmol) in dry CH_2Cl_2 (50 mL) was cooled to −78 °C, then an ozone enriched stream of oxygen was bubbled through the solution until it turned light blue (19 min). Excess ozone was removed with a stream of oxygen, followed by further flushing with nitrogen. A solution of triphenylphosphine (1.24 g, 4.69 mmol) in CH_2Cl_2 (10 mL) was added dropwise over 5 min, and the mixture was allowed to warm to room temperature. After 2.5 h the solvent was removed in vacuo. Column chromatography (hexane/EtOAc 95:5 increasing to 1:1) provided 752 mg (75%) of **6** as a colorless solid.

(2*S*,5′*R*)-3-[4-(2,6-Dichlorobenzyloxy)phenyl]-2-(1-*tert*-butoxycarbonyl-6-oxo-1,7-diazaspiro[4.4]nonane-7-yl)-propanoic Acid Methyl Ester (7). (*S*)-*O*-(2,6-Dichlorobenzyl)-tyrosine methyl ester hydrochloride (200 mg, 0.512 mmol) was dissolved in water (15 mL) and ethanol (0.5 mL). The pH was adjusted to 10 with saturated $NaHCO_3$, and the mixture was extracted with CH_2Cl_2 (5 × 5 mL). The combined organic layers were dried with $MgSO_4$ and evaporated. The residue (181.1 mg) was dissolved in CH_2Cl_2 (6.02 mL). To 5.00 mL of this solution **6** (100 mg, 0.389 mmol) was added. After 30 min sodium triacetoxyborohydride (413 mg, 1.95 mmol) was added and stirring was continued for 2.5 h. The reaction was quenched with NaOH (2 N, 0.5 mL), dried with $MgSO_4$, and filtered. The solution was concentrated and redissolved in $CHCl_3$ (30 mL). After 40 h, the solvent was removed and the residue was purified by column chromatography (hexane/ethyl acetate 3:2 increasing to 1:1) yielding 184 mg (82%) of **7** as a colorless solid.

(2*S*,5′*R*)-3-[4-(2,6-Dichlorobenzyloxy)phenyl]-2-(1-*tert*-butoxycarbonyl-6-oxo-1,7-diazaspiro[4.4]nonane-7-yl)-propanoic Acid (9). **7** (100 mg, 0.173 mmol) was dissolved in THF (2 mL) and MeOH (1 mL). NaOH (2 N, 1 mL) was added dropwise while stirring on an ice bath. After 25 min at 0 °C, THF and MeOH were removed in vacuo (40 °C, 100 mbar), and the remaining solution was diluted with water. After acidification with citric acid (5%), a precipitation formed. The mixture was extracted with ether (3×), followed by addition of HCl (2 N) and further extraction steps (2×). The combined organic layers were dried with $MgSO_4$, filtered and concentrated. Precipitation from CH_2Cl_2/hexane and drying in vacuo afforded **9** (97.8 mg, 100%) as a colorless solid. NMR and TLC indicate the presence of approximately 10% of **10**.

Peptide Synthesis. Commercially available PAM (4-hydroxymethyl)phenylacetamide) resin preloaded with Boc-Leu was deprotected using TFA/CH_2Cl_2/indole (50/50/0.1; 20 min), followed by neutralization with 10% DIPEA in CH_2Cl_2 followed by several washes with CH_2Cl_2. Boc-Ile, **9**, **10**, *ent*-**9**, and *ent*-**10**, and Boc-(Tos)Arg were coupled according to the following procedure: HATU (3−5 equiv) and the carboxylic acid (3−5 equiv) were dissolved in NMP (least volume possible). After addition of DIPEA (6−10 equiv) the mixture was added to the resin and agitated for 8−16 h, followed by several CH_2Cl_2 washes. If possible, complete acylation was monitored with the Kaiser Test. When the test indicated incomplete coupling or when Boc-(Tos)Arg was coupled to the secondary amino group of **9** and **10** or *ent*-**9/10**, respectively, the procedure was repeated. After deprotection with TFA (20 min), the next coupling cycle was started. In case of [*N*MeTyr[11]]NT(8−13), Boc-*N*MeTyr(2,6-Cl_2Bn)-OH was incorporated as described above using HATU followed by TFA cleavage of the Boc group. Introduction of proline was accomplished by the activation of Fmoc-Pro (5 equiv) with BTC (1.67 equiv) in the presence of 2,6-lutidine (12.5 equiv) and dioxane as the solvent. After 1 h, the resin was washed with CH_2Cl_2 and the Fmoc group was

removed by the treatment with piperidine/DBU (2%/2% in DMF). Subsequently, Boc-Tos(Arg) was attached two times as described above. Upon completion, the N-termini were deblocked (TFA) and the HF-cleavage from the resin using anisole as the scavenger (HF/anisole 9/1, 2 h, 0 °C) was performed. HF was evaporated, and the resin was washed with *tert*-butyl methyl ether. The pure peptides were obtained by extraction of the resin with acetic acid, followed by lyophilization and purification via preparative HPLC (gradient elution: 5−35% CH_3CN + 0.1% TFA/H_2O + 0.1% TFA) on a ZORBAX 300SB−C18 PrepHT (21.2 × 250 mm, 7 μm) column.

Receptor Binding Experiments. Receptor binding data were determined utilizing homogenates of membranes from porcine striatum which were prepared from fresh brains obtained from the local slaughterhouse. Dissection of the striatum and preparation of membrane homogenates were performed as described previously.[26]

The neurotensin receptor binding assay was run at a final volume of 1.5 mL on 24-well plates. The experiment was started by adding membranes which were diluted with binding buffer (50 mM TrisHCl, 1 mM EDTA, 0.2 mM bacitracin, 0.1% BSA; pH 7.4) to a final concentration of 660 μg/tube to a mixture of 0.2 nM [^{3}H]neurotensin (specific activity 91 Ci/mmol; Perkin-Elmer, Boston, MA) and the test compound at eight different concentrations (in the range from 0.001 nM to 100 μM). Incubation was continued for 30 min. at 37 °C and stopped by rapid filtration through GF/B filters precoated with 0.3% polyethyleneimine. Filters were washed five times with ice-cold washing buffer (50 mM TrisHCl, 1 mM EDTA; pH 7.4) and dried, and the radioactivity was counted in a Microbeta Trilux (Perkin-Elmer, Freiburg, Germany). Unspecific binding was determined in the presence of 2 μM neurotensin (Sigma-Aldrich). Protein concentration was established by the method of Lowry using bovine serum albumin as standard.[27]

Data analysis of the resulting competition curves was accomplished by nonlinear regression analysis using the algorithms in PRISM (GraphPad Software, San Diego, CA). K_i values were derived from the corresponding EC_{50} data utilizing the equation of Cheng and Prusoff.[28]

Supporting Information Available: Methods and materials, synthetic procedures, and analytical data of **5**, *ent*-**6**, *ent*-**7**, **8**, *ent*-**8**, *ent*-**9**, **10**, and *ent*-**10**, analytical data of **1**−**4**, **6**, **7**, **9**, and [*N*MeTyr^{11}]NT(8−13), and elemental analysis data. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM049644Y