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Bioorganic & Medicinal Chemistry Letters 18 (2008) 2013–2018
Bioorganic & Medicinal Chemistry Letters

Peptide backbone modifications on the C-terminal hexapeptide of neurotensin

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Received 11 December 2007; revised 28 January 2008; accepted 29 January 2008
Available online 2 February 2008

Abstract—To compare backbone-induced susceptibilities with affinity changes that are caused by side-chain modifications in the respective positions, structure activity relationship studies on a series of NT(8-13) analogues were performed providing valuable insights into the major requirement for neurotensin receptor recognition and activation. The data led us to highly potent NTR1 ligands and the generation of a pharmacophore model that will be helpful for the discovery of therapeutically relevant non-peptidic NTR1 agonists.
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The neuropeptide neurotensin (NT, pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu) acts as a neuromodulator and is associated with various physiological effects. Besides its location in the periphery, NT is found in the central nervous system especially regulating dopaminergic transmission of the mesocorticolimbic pathways.[1] Based on behavioural observations, neurotensin produces preclinical effects similar to antipsychotics, which is likely due to a modulation of dopaminergic activity by stimulation of the G-protein coupled receptor NTR1 being co-localized with the dopamine receptor subtype D2.[2,3] SAR studies including a gradual truncation of the peptide sequence demonstrated that the *C*-terminal hexapeptide NT(8-13) is sufficient for NTR1 binding and ligand efficacy.[4] Probing the contributions of the ligand's side chains employing Ala- and D-amino acid species exchange led to the conclusion that all residues appear to be important for the receptor binding process.[5–10] Structural properties mediated by Pro[10] and Tyr[11] within the core region of NT(8-13) turned out highly crucial for receptor recognition.[11] Very recently, M. Baldus and co-workers were able to gain experimental data on the bioactive conformation of NT(8-13) bound to NTR1 when 2D solid-state NMR spectroscopy was utilized.[12] According to these results and our investigations of conformational constraints on NTR1 binding,[13] a linear rearrangement of the NT(8-13) backbone can be concluded to represent the bioactive conformation. As a complement to previously described SAR studies,[14–17] we herein describe NT(8-13) backbone modifications when we compare the susceptibilities on receptor recognition with affinity changes that are caused by side-chain modifications in the respective positions. Besides systematic homo-β-amino acid exchange and a peptoid scan, our backbone manipulations involve the insertion of lactam bridged scaffolds and the substitution of both amino and the carboxyl functions at the *N*- and *C*-terminal ends, respectively.

Peptide analogues composed exclusively of β-residues (β-peptides) or of a combination of β- and α-amino acid residues have been designed to adopt a variety of conformations that resemble protein secondary structures.[18] Such foldamer classes can serve as rich sources of inhibitors of protein-protein interactions and show remarkable resistance to degradation by proteases,[19] making such oligomers attractive in medicinal chemistry. Employing commercially available chiral building blocks, our initial investigations were directed to the synthesis of the β-homologues **1a**–**c** and **1f**, **g** when straightforward solid phase supported preparations employing Fmoc strategy and HATU as the coupling reagent gave rise to analytically pure oligomers. Proline

Keywords: Neurotensin; GPCR; Backbone modifications; Peptide analogues; Ligand binding; Pharmacophore model; NT(8-13).
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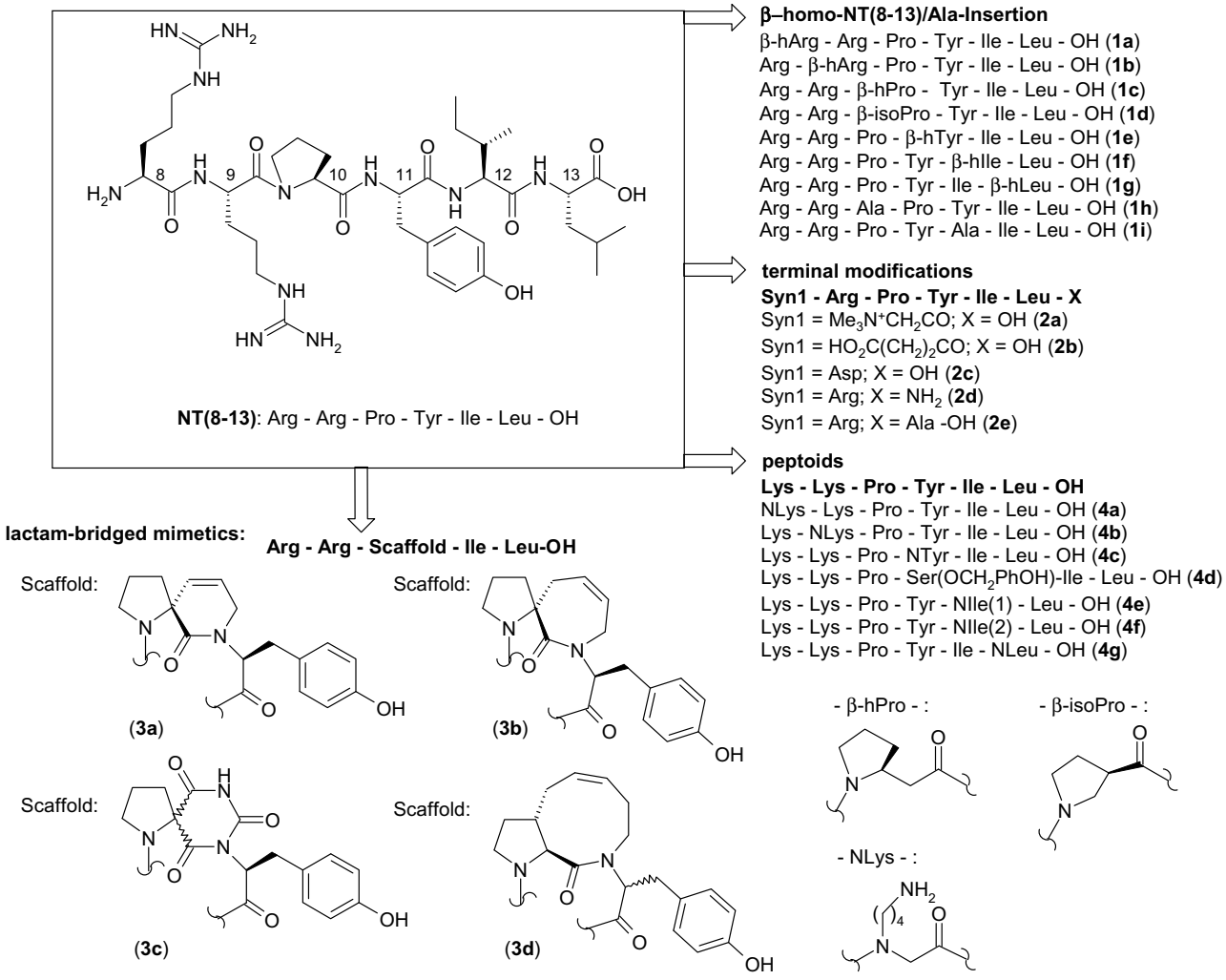
doi:10.1016/j.bmcl.2008.01.110



Scheme 1. Backbone modifications leading to a variety of NT(8-13) analogues.

was replaced by its congeners (*S*)-2-carboxymethylpyrrolidine (β-homoproline) and (*R*)-3-carboxypyrrolidine (β-isoproline)[20] resulting in formation of the peptide analogues **1c** and **1d**, respectively (Scheme 1).

For the synthesis of the β-homotyrosine derived peptide analogue **1e**, we relied on our previously reported homologization protocol giving access to enantiopure *N*,*N*-dibenzyl protected β-homotyrosine[21] that was esterified, debenzylated and *N*-protected by a Boc unit. After alkylation of the aromatic HO-group with 2,6-dichlorobenzylbromide and saponification,[22] the chiral building block was subjected to Boc-SPPS.[23] During the course of this work, our attention was drawn towards the microwave assisted Fmoc deprotection and peptide coupling principle employing PYBOP as the coupling reagent. This fast method is highly useful to achieve excellent purities avoiding the application of cost-intensive HATU.[24] Besides homologization by formally pasting a CH_2 group into the backbone, an Ala moiety as a three-atom spacer was inserted into the peptide bonds connecting the crucial Pro-Tyr unit with the basic *N*-terminal and the lipophilic *C*-terminal fragments to give the heptapeptides **1h** and **1i**, respectively.

To circumvent the perceived major limitations associated with peptides as therapeutic agents, peptoids or peptide–peptoid combinations have been frequently used.[25] The peptoid design strategy also allows to explore the significance of backbone NH functions and to increase the number of energetically relevant conformations for ligand binding.[26] A solid phase supported sub-monomer approach was chosen for the synthesis of the test compounds **4a**, **c**, **e/f**, **g** containing peptoid subunits to simulate the amino acid residues of the reference NTR1 agonist [Lys[8],Lsy[9]]NT(8-13).[27,28] Since we employed 2-butylamine as a racemic building block, the *N*Ile derivative was obtained as a mixture of diastereomers (**4e/f**) that had to be separated by HPLC. To circumvent an acetal-type cleavage of the hydroxybenzylamine substructure of *N*Tyr, we applied a protocol employing 4-allyloxybenzylamine[29] and Fmoc-Lys(Alloc)-OH for the generation of oligomer **4c**. When we utilized 2-chlorotrityl resin, this strategy facilitated a smooth, palladium catalyzed side-chain deprotection and subsequent HFIP promoted cleavage. To further probe the tyrosine binding portion of the receptor, the 4-HO-benzyl function was displaced not only to the amide nitrogen but also onto the primary alcohol func-

tion of [Ser[11]]NT(8-13). In detail, Boc-protected serine was *O*-alkylated by 4-allyloxybenzylbromide[30] using NaH as a base. After Boc-detachment promoted by sulfuric acid in dioxane[31] and subsequent Fmoc-protection, the resulting unnatural amino acid was subjected to solid phase supported peptide synthesis. We took advantage of Alloc protected lysine and 2-chlorotrityl resin to give peptide surrogate **4d**. As the sub-monomer approach did not prove successful for the synthesis of H-Lys-*N*Lys-Pro-Tyr-Ile-Leu-OH (**4b**), we incorporated *N*Lys as a Fmoc-protected monomer.[32]

To compare backbone-induced susceptibilities on receptor recognition with affinity changes that are caused by side-chain modifications in the respective positions, NT(8-13) related hexapeptides obtained upon sequential alanine and D-amino acid replacements were synthesized as reference agents according to Fmoc SPPS protocols.[33,34] Using PYBOP or HATU as activating reagents, spirocyclic and fused Pro-Tyr surrogates could be introduced into the sequence giving access to the lactam bridged NT(8-13) mimics **3a**–**d**.[35,36] For both oligomers **3c** and **3d**, diastereomers were separated by HPLC and further investigated in isomerically pure form. To formally replace the *C*-terminal carboxy function by a carboxamide isostere, Rink amide resin was used to give rise to the test compound H-Arg-Arg-Pro-Tyr-Ile-Leu-NH_2 (**2d**)[8] after sequential coupling of the respective Fmoc-protected amino acids and TFA cleavage. To further explore the *C*-terminus-binding portion of the neurotensin receptor, the heptapeptide NT(8-13)-Ala-OH (**2e**) was synthesized. Finally, the *N*-terminal moiety was modified to evaluate if a cationic element is necessary for receptor recognition. Thus, carboxymethyl(trimethyl)ammonium (betaine), succinic acid and aspartic acid were attached to resin-bound NT(9-13) to afford oligomers **2a**–**c**.

Our initial biological investigations were directed to an exchange of the amino acid residues 8-13 by β-amino acid homologues **1a**–**c** and **1e**–**g**. Since we anticipated the Pro-Tyr fragment to be especially crucial, the β-isoproline derived congener **1d** and the peptide homologues **1h**, **i** that display an insertion of Ala departing the Pro-Tyr moiety from the *N*- and *C*-terminal sequence, respectively, were also investigated. In detail, susceptibilities on NT(8-13) backbone modifications were compared to affinity changes that are caused by side-chain modifications when employing Ala- and D-amino acid species exchange in the positions 8-13. Susceptibility data were derived from specific radioactivity ratios of test compounds and reference agents. An attenuation of affinity compared to NT(8-13) is displayed in Figure 1. The data clearly indicate the low susceptibility for both backbone and side-chain modifications in position 8 when the amount of bound radioligands after competition with the test compounds in 300 nM concentration was comparably low (data in detail, see supporting information). An exchange of arginine in position 9 by homo-arginine led to an approximately five-fold higher amount of bound radioligand indicating a significantly lower but still substantial ligand affinity. These screening data could be corroborated by the measurement of K_i values when the 8-β-homo-NT(8-13) showed a K_i of 0.13 nM indicating an even higher affinity than the reference NT(8-13) (K_i = 0.23 nM). For the 9-β-homo-NT(8-13) (**1b**), a K_i value of 2.3 nM was observed.

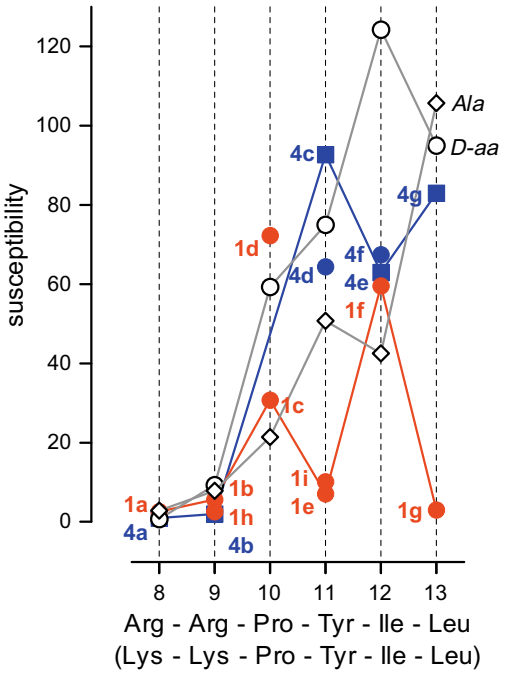


Figure 1. Impact of peptide backbone modifications on the binding properties of the C-terminal hexapeptide of neurotensin. Susceptibility is derived from bound radioactivity determined in a heterologous binding experiment using porcine NTR1 receptors, [^{3}H]neurotensin and the test compounds at 300 nM and is expressed as the ratio of specific radioactivity of test compound over that of reference. Specific radioactivity was calculated using the equation: ((radioactivity − UB)/(TB − UB)) × 100%. The β-homo derivatives **1a**–**i** (full circles (•)), the hexapeptides of the Ala scan (open hash (◇)) and the hexapeptides of the D-amino acid scan (open circles (○)) are compared to the effect of NT(8-13). The peptoid derivatives **4a**–**g** (full squares (■)) are compared to [Lys[8],Lys[9]]NT(8-13).

As expected, β-amino acid exchange in position 10 was more crucial, when we lost a factor of approximately 30 for the ability of **1c** to displace [^{3}H]neurotensin. An alternative evaluation of the β-proline derivative **1d** bearing a carboxylate function in position 3 of the pyrrolidine ring induced an even stronger loss of affinity, which was indicated by a factor of about 70. On the other hand, investigation of the [β-Tyr]NT(8-13) (**1e**) showed a minor susceptibility when repeatedly performed heterologous displacement experiments at eight different concentrations (Table 1) showed an average K_i value in the single digit nanomolar range (K_i = 8.1 nM). Since we inferred from these experiments a certain flexibility of the distance between the Pro-Tyr fragment and both the *N*- and the *C*-terminal portions, alanine was inserted as a three-atom spacer instead of the one-carbon homologation performed before. In fact, in vitro displacement studies corroborated our hypothesis displaying K_i values of 9.6 nM and 6.8 nM for the heptapeptides **1h** and **1i**, respectively. On the other hand, our heterologous competition experiments clearly indicate that chemical manipulation of Ile[12] led to a

Table 1. Receptor binding data for the oligomeres **1a–i**, **2a–e**, **3a–d** and **4a–d** in comparison to the reference hexapeptides NT(8-13) and [Lys^{8}Lys^9]NT(8-13) employing porcine NTR1 receptors

Oligomer	K_i[a] (nM)	Oligomer	K_i[a] (nM)
1a	0.13 ± 0.0050	**3a**	7400 ± 1200
1b	2.3 ± 0.95	**3b**	18,000 ± 5000
1c	28 ± 5.5	**3c**[b]	1400 ± 50
1d	260 ± 35	**3c**[c]	10,000 ± 0
1e	8.1 ± 0.72	**3d**[d]	8600 ± 1,500
1f	100 ± 26	**3d**[e]	2400 ± 200
1g	3.2 ± 0.75	**4a**	0.45 ± 0.14
1h	9.6 ± 4.4	**4b**	5.7 ± 2.0
1i	6.8 ± 2.3	**4c**	11,000 ± 0
2a	0.65 ± 0.19	**4d**	530 ± 80
2b	2300 ± 0		
2c	150 ± 25		
2d	0.18 ± 0.046		
2e	0.27 ± 0.056	NT[f]	1.2 ± 0.21
NT(8-13)	0.23 ± 0.042	[Lys^{8}Lys^9]NT(8-13)	2.6 ± 0.55

[a] K_i values in nM ± SEM are based on the means of 2–7 experiments each done in triplicate.
[b] K_i value of diastereomer 1 of **3c**.
[c] K_i value of diastereomer 2 of **3c**.
[d] K_i value of diastereomer 1 of **3d**.
[e] K_i value of diastereomer 2 of **3d**.
[f] K_d value from 17 homologous competition experiments.

substantial loss of affinity for both side-chain and backbone modifications. Displacement of the leucine residue in position 13 resulted in a surprising biological behaviour of the β-amino acid homologue **1g** since it showed comparable affinity to the reference peptide NT(8-13) (K_i = 3.2 nM). This is different to the observation from our Ala- and D-amino acid scan that indicated an approximately 100-fold loss of affinity.

For the peptoid scan, [Lys^8,Lsy^9]NT(8-13) was employed as a reference. The blue curve depicted in Figure 1 indicates susceptibilities that are comparable to the results of the alanine and D-amino acid exchange for the respective residue positions. Interestingly, the substantial loss of affinity for the *N*Tyr-derivative (**4c**) could be partially compensated by an elongation of the side-chain leading to a higher affinity of the oligomer **4d**. As expected, modifications in position 12 were again highly crucial when both diastereomers **4e** and **4f** showed very poor ability to displace [^{3}H]neurotensin. The strong susceptibility of the peptoid analogue in position 13 compared to the highly potent β-amino acid analogue **1g** led us to conclude that a NH-backbone position close to the C-terminal end might be of primary importance for the receptor recognition process. As a complement to our previous investigations of lactam bridged NT(8-13) mimetics conformationally constraining the backbone ψ-angle in position 11, the spirocyclic NT(8-13) analogues **3a–c** and their fused congener **3d** were also investigated for their ability to compete with neurotensin at the NTR1 target receptor. Although our previously reported spirocyclic γ-lactam has substantial NTR1 affinity,[13] the enlarged congeners **3a–d** incorporating 6-, 7- and 9-membered rings showed only poor receptor binding with K_i values in the micromolar range (K_i = 1.4–18 μM). This indicates that the exchange of the 5-membered lactam by a sterically more demanding surrogate either leads to repulsive interactions or to an increase of the proline ψ-angle resulting in a backbone structure that is significantly different to the bioactive conformation.[37] As a consequence of all data discussed above, we concluded that both *N*- and *C*-terminal groups might be directed to the surface of the receptor whereas the central portion obviously interacts with a tight binding site crevice. To learn if the *C*-terminal carboxyl function and *N*-terminal amino group specifically interact with the neurotensin receptor by an ion-ion interaction or if their hydrophilicity is beneficial to ligand binding, we exchanged the carboxyl function by a carboxamide (oligomer **2d**) and the arginine residue in position 8 by the anionic residues aspartic acid and succinic acid. Additionally, betaine, a permanently positively charged quaternary ammonium salt, was coupled instead of arginine 8. Actually, we were surprised to see that NT(8-13)-NH_2 (**2d**) showed an affinity that even exceeded the binding properties of NT(8-13) (K_i = 0.18 nM). This is in disagreement with previous studies suggesting the C-terminal carboxy function to be of primary importance for NTR1 binding and activation.[38] Interestingly, the acidic terminal OH function can be displaced by an Ala residue without significant reduction of affinity for the heptapeptide (**2e**). On the other hand, exchange of the basic arginine residue in position 8 by aspartate or succinate led to a strong decrease of NTR1 recognition indicating that not only the hydrophilicity but also the basicity and, thus, the formation of a cationic species is crucial for specific binding. This could be confirmed by investigation of the quarternary ammonium salt **2a** revealing a K_i value in the sub-nanomolar range (K_i = 0.65 nM).

To evaluate the effect of our structural modifications on G_q transactivation, the most promising NT(8-13) analogues exhibiting subnanomolar affinities were tested for their ability to increase the intracellular calcium concentration. Comparison of calcium release at concentrations of 100 nM with the ligand efficacy of the standard agents NT, NT(8-13) and [Lys^8,Lsy^9]NT(8-13) indicated full agonist properties and receptor response for the 8-β-homo-NT(8-13) **1a**, the quarternary ammonium salt **2a**, NT(8-13)-NH_2 (**2d**), the *C*-terminally modified heptapeptide **2e** and *N*-Lys derived oligomer **4a** (Table 2).

Using data from previous efforts[9] and the above described SAR analysis, one major aim of this study is to create a pharmacophore model that guides us to discover non-peptidic neurotensin receptor agonists. The conceptional drawing depicted in Figure 2 displays the

Table 2. Ligand potency in the funtional calcium assay

Oligomer	Rel. potency[a]	Oligomer	Rel. potency[a]
NT	100% ± 16%	**2d**	387% ± 14%
NT(8-13)	382% ± 24%	**2e**	220% ± 25%
1a	293% ± 25%	[Lys^{8}Lys^9]NT(8-13)	184% ± 19%
2a	101% ± 35%	**4a**	309% ± 33%

Relative potency of derivatives showing subnanomolar affinities as compared to the native ligand NT.
[a] Values ± SEM are based on the means of 6–12 experiments.

major requirements that we suggest to be essential for NTR1 recognition and activation. Two positive charges attached to the C_α in position 9 within distances of 3–6 and 5 Å, respectively, are suggested to be required since substitution of Arg[8] by betaine (**2a**) retained ligand affinity whereas the introduction of a negatively charged carboxylate (**2b**, **2c**) had an adverse effect on ligand binding. The *N*-terminal portion is connected by a variable linker to the functional unit of Pro[10] and Tyr[11] since the introduction of β-homo-Arg (**1a**), D-Arg or a peptoid (**4a**) had no effect on ligand binding. Obviously, the linker between the cationic centre and the backbone is not fully extended at the native receptor-ligand complex. Proline works as a unique 'kink generating element' specifically redirecting Tyr[11] towards an aromatic pocket and an H-bond acceptor provided by NTR1 residues that has to be addressed by Tyr[11]. This explains the loss of affinity observed for our lactam-bridged analogues (**3a–d**). Separated by a 1–4 Å spacer element, two bulky, hydrophobic moieties, which are provided by Ile[12] and Leu[13] within the natural ligand, are necessary. Additionally, H-bonding backbone-backbone interactions are crucial at this portion of the ligand receptor complex, which was indicated by the above mentioned SAR analysis. A polar, H-bonding *C*-terminal portion and, not necessarily, a negatively charged carboxylate is needed for high affinity ligand binding.

In conclusion, structure activity relationship studies on a series of NT(8-13) analogues provided valuable insights into the major requirement for neurotensin receptor recognition and activation. The data facilitated the generation of a pharmacophore model. In combination with our homology-based protein modelling and site directed mutagenesis studies, this approach will guide us to non-peptidic neurotensin receptor agonists that are suggested to be of special interest for the treatment of schizophrenia.

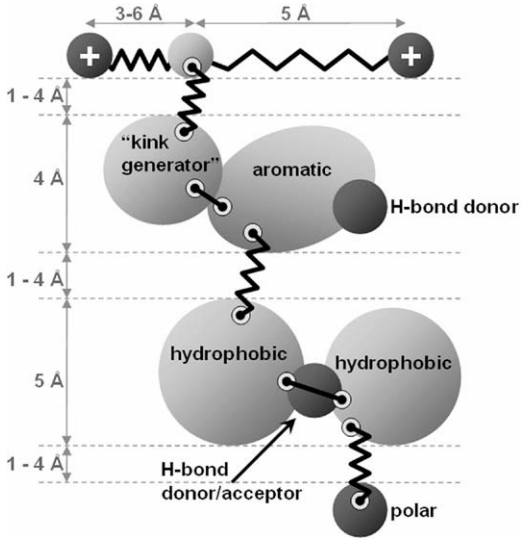


Figure 2. Pharmacophore model for NT(8-13) agonists. Functional units are depicted as gray spheres. Straight black lines indicate fixed distances and zigzag-lines indicate variable distances. Available space is given for rough guidance on the left hand side.

Acknowledgments

The authors thank A. Pinsker, S. Hemmers, I. Torres-Berger and M. Kettler for skillful technical assistance and Dr. H. Bittermann for helpful discussions. This work was supported by the Deutsche Forschungsgemeinschaft (DFG Gm 13/7).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.01.110.

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37. Our NTR1 homology model, containing NT(8-13) as a docked ligand, indicated that steric hindrance in the region of Ile^{329} could be a reason for the loss of affinity observed for the bulky bridged NT(8-13) analogues. However, the human NTR1 triple mutant YIS328-330AAA, constructed by an overlap PCR methodology and expressed in HEK-293 cells, did not regain affinity for the sterically most demanding peptide mimetic **3d** (K_i > 1000 nM). Thus, the increase of the proline ψ-angle, which is a consequence of the lactam-ring enlargement obviously leads to an inactive backbone conformation.
38. NT(8-13) and NT(8-13)-NH_2 affinities have also been investigated in Ref. 9 (K_i values: 0.018 and 10 nM, respectively) when NT receptors in neonatal mouse whole brain preparations have been used, and in Ref.10.