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PREPARATION AND RECEPTOR BINDING AFFINITIES OF CYCLIC C-TERMINAL NEUROTENSIN (8–13) AND (9–13) ANALOGUES

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Abstract: Cyclic analogues of neurotensin (NT) C-terminal fragments NT(8–13) and NT(9–13) were produced via intramolecular nucleophilic substitution of the Tyr(11) phenoxide anion on a 6-bromohexanoyl side chain substituted at position 8 or 9 and tested for NT receptor binding affinity.

Neurotensin (NT) is an endogenous tridecapeptide that has been implicated in the pathogenesis of schizophrenia through an altered modulatory effect on dopamine activity. In a population of schizophrenic patients, CSF levels of the peptide were correlated with responses to neuroleptic treatment.[1] An electrophysiological animal model indicated that NT possesses an atypical response comparable to clozapine,[2] which suggests that NT analogues designed for use in vivo could serve as an alternative neuroleptic treatment without extrapyramidal side effects and tardive dyskinesea.[3] Structure–activity studies of NT and truncated fragments indicated that the C-terminal fragment NT(8–13) (Arg8-Arg9-Pro10-Tyr11-Ile12-Leu13) elicits comparable or enhanced biological effects to NT by binding at the human NT receptor (hNTR) while NT(9–13) (Arg9-Pro10-Tyr11-Ile12-Leu13) also exhibits significant receptor binding.[4] Since a crystal structure is not available for NT binding to the hNTR, structural alterations and binding analyses of these ligands may provide useful data to aid in the design of improved analogues. Cyclization of peptide domains containing the necessary elements to trigger biological responses of interest has proven a rational approach to gain information about the active binding conformation of the pharmacophore at the receptor interface and has successfully produced improved peptide-based drugs.[5] Previous examples of cyclic NT(8–13) derivatives were prepared through amide cyclization to link the N-terminal α-amino group or the side chain nitrogen of Lys(8) to Glu(12) or by disulfide formation between different thiol residues at the 8 and 12 position.[6] Recently, Pang et al.[7] implicated the adoption of a β-turn conformation when NT(8–13) is bound to hNTR, which features the cationic side chain of Arg(9) hydrogen bonding to the Tyr(11) hydroxyl functionality. It also was suggested that the Arg(8) side chain curls back to interact with the Arg(9) side chain. The design, synthesis, and evaluation of NT(8–13) and (9–13) fragment analogues cyclized between either position 9 or 8 and the Tyr(11) side chain (**1** and **2**, Figure 1) are described in this manuscript. These compounds mimic the proposed conformation of NT fragments bound to the hNTR.

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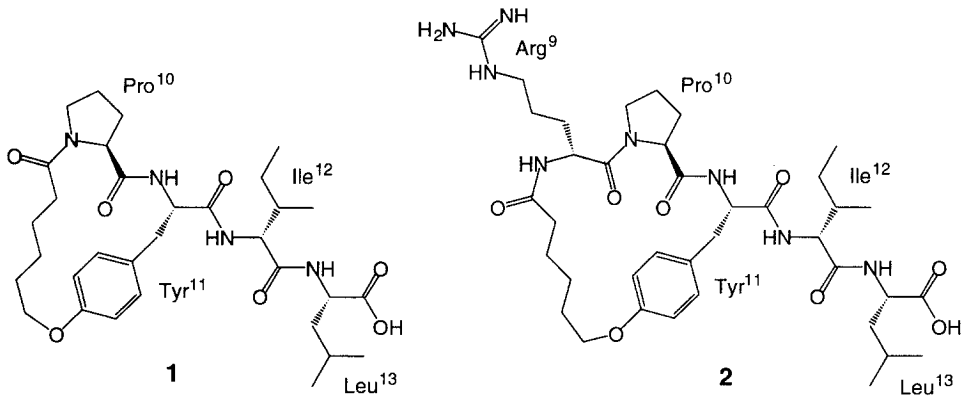
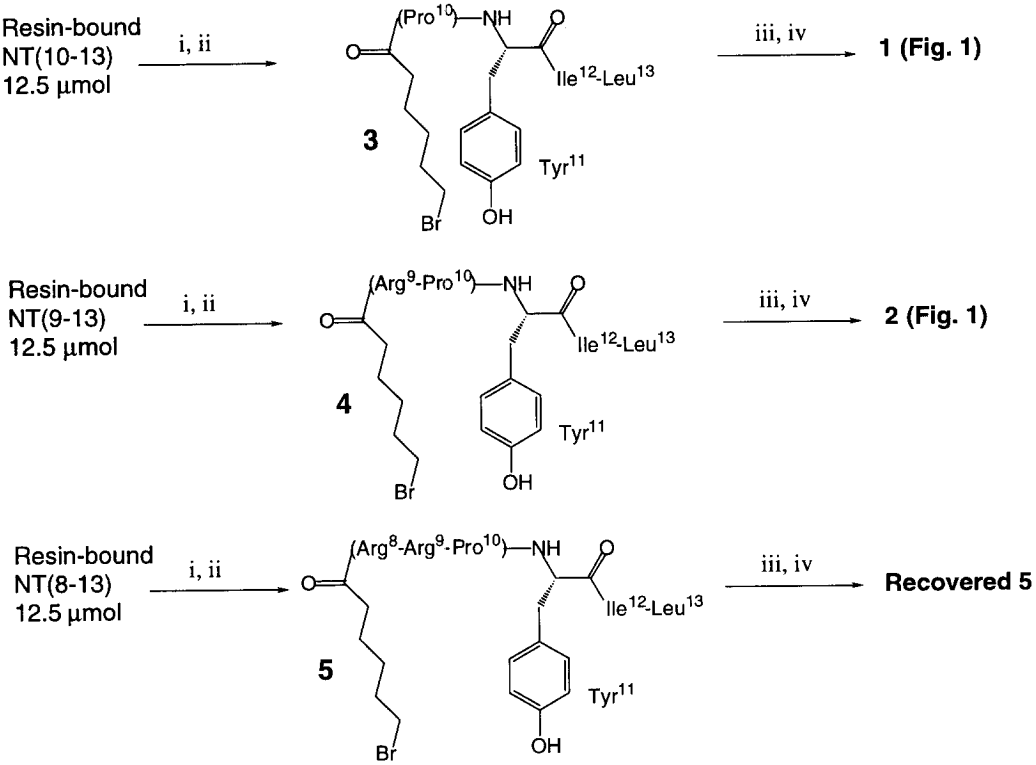


Figure 1. Cyclic Neurotensin Binding Fragment Analogues

It was hypothesized that a halogenated side chain in the N-terminal 9 position (**3**, Scheme 1) or 8 position (**4**, Scheme 1) could be used to covalently cross-link the Tyr(11) hydroxyl group if these reactive groups were placed in the positions to be occupied in a reverse-turn conformation. The achiral 6-bromohexanoic acid side chain was chosen as the linker, so that steric bias of an α-substituent would not adversely hinder binding of the conformationally defined products. A linker consisting of five methylene units was selected to bridge the distance covered by the putative Arg-Tyr electrostatic bond in the binding conformation of NT(8–13) and (9–13), which also would allow some conformational flexibility after cyclization. It also was envisioned that the seven-residue peptide (**5**, Scheme 1) could be converted into a cyclic peptide containing the two guanidinium groups of NT(8–13) that promote optimal receptor interaction.

The synthesis of these analogues is shown in Scheme 1. The N-terminal α-amine of the appropriate acid-labile Wang resin-bound side chain initially was deprotected for coupling with 6-bromohexanoic acid.[8] After coupling, the resin-bound halogenated intermediates **3**–**5** were cleaved from the resin with TFA/H_2O (without scavengers), concentrated, and purified (overall yields 50%). For cyclization, **3**–**5** were dissolved in dilute cesium carbonate solution and EtOH to form the Tyr(11) phenoxide anion. After solvent removal, each peptide fragment was redissolved in dry DMF and allowed to react under ambient conditions for 24 h. The reaction mixtures were neutralized with TFA and purified by RP-HPLC. Quantitative conversion of **3** to cyclic product **1** was achieved. Intermediate **4** was converted to several minor products (possibly different Arg(9) salt forms) with the largest peak isolated as cyclized product **2** (ca. 50% yield). Intermediate **5** was recovered unchanged from reaction mixtures. This appears to be a function of sterics inhibiting access of the nucleophilic phenolic hydroxyl to the electrophilic site, as molecular modeling demonstrated a high energy barrier for bringing these groups within reacting distance. This was not the case with synthetic intermediates **3** and **4**, both of which could access a reacting conformation with little energy penalty (data not shown).



Scheme 1. (i) 6-Bromohexanoic acid (4 equiv), HOBt (4 equiv), DCCI (4 equiv) (ii) TFA:H_2O (80:20), RP-HPLC (iii) Cesium carbonate (10 equiv,100 μL H_2O, 100 μL EtOH), evaporate to dryness (iv) DMF (100 μL), 24 h, TFA, RP-HPLC

Production of analogues **1** and **2** demonstrated the feasibility of cyclizing side chains in small peptides separated by one or two amino acid monomers using this method. The distance between the reactive functionalities is important and the intramolecular reaction may depend on a reverse-turn conformation around the Pro(10) residue for positioning. While the possibility of intramolecular ester formation with the carboxyl of Leu(13) also exists for these molecules, a tandem electrospray mass spectrum of each analogue demonstrated that cyclization had occurred incorporating the Tyr(11) hydroxyl, as the $[M+2H]^{2+}$ ion for each analogue showed the correct cyclized fragment with the loss of the Leu(13) residue.

Physiochemical properties and binding affinity data at the hNTR of cyclic peptides **1** and **2** are provided in Table 1. Both analogues are significantly more lipophilic in comparison to the NT(9–13) standard as assessed by RP-HPLC. This can be attributed primarily to loss of the ionized α-amino and guanidinium functionalities. The enhanced lipophilicity of these analogues, which can promote passive diffusion across the blood brain barrier, could result in an intravenously-deliverable drug.[9] Losses in binding affinity, however,

Table 1

Peptide	Binding Affinity Comparison and Analytical Data		
	K_i (nM)[a]	Ret. Time[b] (min)	M.W. (Calcd) : Found[c]
NT(9–13)	28 ± 2	22.4	(660.8) : 660.8
1	> 2000	36.5	(600.7) : 600.3
2	450 ± 77	32.0	(756.9) : 756.5

[a] Binding data were obtained by assaying the ability of an analogue to compete with [^{125}I]Tyr^3-NT (0.15 nM) for human NT (Leu^{194}) receptors cloned in CHO cells (New England Nuclear) following manufacturers instructions. Assays were performed in triplicate and are the geometric means reported with ± S.E. given. Nonspecific binding was determined using 20 μM of NT(9–13).

[b] Reversed-phase HPLC: The mobile phase consisted of 0.1% trifluoroacetic acid in water (solvent A) and 0.1% trifluoroacetic acid in 80% acetonitrile (solvent B). Analytical HPLC was performed on a Waters dual pump HPLC system in combination with a Bakerbond (C18, wide bored) column. About 10 to 50 μg of peptide was automatically injected and separated at a flow rate of 1 mL/min. Linear gradients from 5% to 50% B over 30 min were used and the effluent detected by UV absorbance at 220 nm. All peptides were of greater than 95% purity with enhanced hydrophobic character evidenced by increased retention time in comparison to NT(9-13).

[c] An electrospray mass spectrum for each analogue was obtained using Finnigan LCQ instrument. The tandem mass spectrum of the $[M+2H]^{2+}$ ion for each analogue gave correct sequential data.

are noted with replacement of the ionizable groups in cyclization. Analogue **1** was envisioned to lock in a β-turn–like structure as proposed by Pang et al.[7] This strategy could give rise to antagonistic activity by blocking the Tyr(11) hydroxyl group and removal of the cationic functionalities. The decreased binding affinity of analogue **2** compared to NT(9–13) can be attributed to either masking of the Tyr(11) hydroxyl group or an adverse conformational constraint. It might be possible to improve the loss of affinity due to *O*-alkylation by optimizing the length of the N-terminal alkyl chain. Parallel production of many analogues is feasible with this route since common intermediates and inexpensive starting materials are available. In summation, cyclization through ether bond formation offers an alternative chemical means and different bond-angle constraints when compared to amide and disulfide cyclization strategies and may be applicable for exploration of structure activity relationships in other peptide-based leads.

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