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Synthesis of Neurotensin(9–13) Analogues Exhibiting Enhanced Human Neurotensin Receptor Binding Affinities

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Abstract—Recent evidence is consistent with neurotensin (NT)(8–13) adopting a Type I β-turn conformation while binding the NT receptor, which would place the cationic side-chains of Arg(8) and Arg(9) in close proximity. This was the basis for the design, synthesis and analysis of truncated NT(9–13) analogues **1**–**5** with dicationic position 9 side-chains to emulate the functions of the 8 and 9 side-chains of NT(8–13).

Low levels of the brain tridecapeptide neurotensin (NT) have been implicated in the pathogenesis of schizophrenia,[1–4] through an altered modulatory effect on dopamine activity.[5,6] The C-terminal NT hexapeptide fragment, NT(8–13), Arg(8)-Arg(9)-Pro(10)-Tyr(11)-Ile(12)-Leu(13), contains the necessary structural requirements to bind and elicit subsequent biological effects at human NT receptors (hNTRs).[7] The truncated fragment NT(9–13) binds about two orders of magnitude less efficiently than NT(8–13) due to the loss of the critical Arg(8) cationic residue. There is substantial interest in producing analogues of NT(8–13) possessing suitable properties (stability, high receptor binding affinity, blood brain barrier access, etc.) to enable use in vivo as a new class of antipsychotics devoid of classical neuroleptic side-effects.[8] We now report the synthesis and hNTR-1 (the 'high affinity' NTR) binding analysis of a series of novel NT(9–13) analogues (Fig. 1; **1**–**5**) featuring incorporation of an extra cationic charged group to mimic the requisite Arg(8) residue in a simpler molecular configuration. The design of these analogues is based on the structural model of Pang et al.[9] for NT receptor binding while the synthetic method appears to have general applicability for synthesis of peptide derivatives containing a wide variety of non-natural functionalities in the side-chain.

Strategies toward improving the stability of the N-terminal region of NT(8-13) have included modification of the Arg(8)-Arg(9) peptide bond[10] and substitution of Arg(8), Arg(9) and Pro(10) with indole- or pyrrole-based templates containing guanidinium substituents.[11] The synthetic routes to these analogues are lengthy and limited in the types of cationic groups that can be incorporated. We sought to eliminate the Arg(8)-Arg(9) peptide bond entirely by combining the two cationic groups in a single N-terminal position 9 side-chain. Analyses of binding affinities of many different NT(8–13) analogues,[12] and the use of heuristic conformational modeling, were employed by Pang et al.[9] to postulate a receptor binding site in which peptides adopt a Type I β-turn. This model indicated that the guanidinium groups of Arg(8) and Arg(9) are in close proximity. We recreated the Pang et al.[9] model using the Arg-Pro-Tyr reverse turn of serine protease (1hf1.pdb) using the molecular modeling program Sybyl. This was used to demonstrate that the δ-amines of [L-Orn(8), L-Orn(9)]NT(8–13) (**6**, Fig. 1), a compound which exhibits nanomolar potency without large guanidinium groups, can be superimposed without unfavorable energy constraints on the template model.

This simplified model led to the design of NT(9–13) analogues **1**–**3** in which the side-chain of residue 9 would contain two amino groups separated by a fixed distance over a range of several angstroms to mimic the binding pharmacophore of the two cationic side-chains of **6**. The alkyl attachment could then flexibly place the

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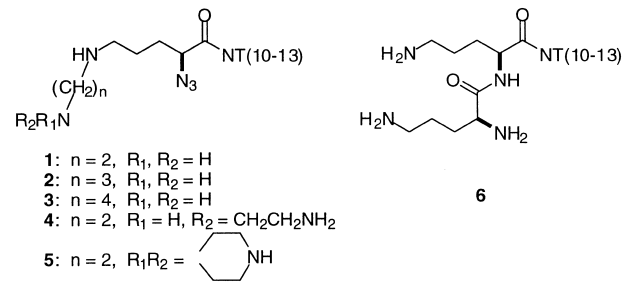
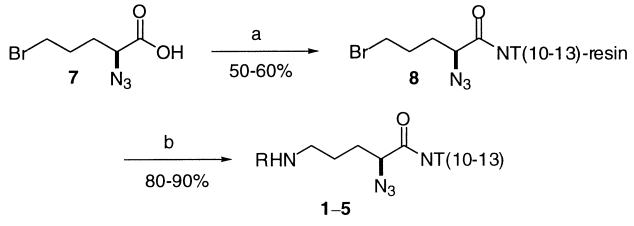


Figure 1. Structures of peptides prepared for this work, with template model.

ω-nitrogen in a position to function as the δ-amino group of L-Orn(8), thereby removing the labile amide bond. Since chiral placement of the position 8 δ-amino group is not essential,[12] optimization of the distance between the two amino groups can be probed by employing different alkyldiamines. Analogues **4** and **5** also were prepared to further explore steric and electrostatic requirements at the receptor interface.

Synthesis of the novel NT(9–13) analogues is presented in Scheme 1. After routine construction of the resin-attached tetrapeptide NT(10–13),[13] 2(*S*)-azido-5-bromovaleric acid (**7**)[14] was incorporated at position 9 by utilizing the neutral bromo functionality and azido group for solubilization and protection, respectively.

After cleavage from the resin and purification by reversed phase- (RP-)HPLC, the brominated pentapeptide (**8**) was dissolved in EtOH, treated with 100 equiv of the requisite amine nucleophile (40% in H_2O), and stirred overnight at ambient temperature and pressure in a closed container.[15] After acidification to neutralize excess amine, purification by RP-HPLC afforded the desired NT(9–13) analogues **1**–**5**. This method is efficient in that many products can be produced from one precursor peptide without the need for protecting groups per se. Additionally, groups which are not amenable to protection are incorporated easily (e.g. tertiary nitrogens, as present in analogue **5**). The α-azido functionality can be retained in the peptides to hinder aminopeptidase action and enhance overall lipophilicity by removal of a nonessential cationic charge, or be reduced to the amine if desired. NT(9–13) was prepared routinely[13] and **6** purchased for use as a standard.



Scheme 1. (a) HOBt, DCC, DMF, Wang-resin attached, side-chain protected NT(10–13), TFA:H_2O; (b) EtOH, 100 equiv of the requisite amines (40%) in H_2O; TFA.

The structures of peptide analogues **1**–**5** and NT(9–13) were characterized by mass spectrometry, exhibiting correct molecular weights (Table 1) and appropriate sequential breakdown patterns. The peptides were assayed by RP-HPLC to assess purity and to demonstrate that the azido-containing peptides were more lipophilic than the standard NT(9–13). As indicated in Table 1, all new peptides are more lipophilic than NT(9–13), based on increased retention times. For all analogues, the azido group enhances lipophilicity as do the addition of alkyl groups around the position 9 cationic side-chain.

The hNTR binding affinities of the analogues, in comparison to NT(9–13) ($K_i = 19$ nM[12]) and **6**, are provided in Table 1. Analogue **2**, containing a propylene spacer, improved the potency of NT(9–13) by five times, while analogues **1**, **3** and **4** also were more potent. The binding affinities of **2**–**4** compared favorably with **6** for these smaller, more lipophilic, and potentially less biodegradable peptides. The relative affinities of these compounds, especially analogue **2**, also provide further support of the Pang et al.[9] model for the NT(8–13) binding conformation. Although **2** can adequately position two amino groups in a spatial arrangement recognizable by the receptor, there still exists some loss in potency in comparison to **6**. This could result from a lack of optimization of the length of the alkyl spacer, entropic costs of freezing the dialkyl group in its binding conformation, or loss of additional peptide–protein stabilizing interactions with the removal of the position 8–9 amide group and the α-ammonium ion. For the triamino side-chain containing analogues **4** and **5**, the piperazine ring of analogue **5** did not improve interaction with the NT receptor when compared to **4**, indicating a steric conflict and/or preference for an ω-primary amino group.

The new analogues offer several potential improvements applicable to in vivo studies. The azido group at the N-terminus offers a more lipophilic, less sterically

Table 1. Binding affinity comparison and analytical data for neurotensin(9–13) analogues **1**–**5**

Peptide[a]	hNTR-1 affinity (nM)[b]	Retention time[c] (min)	M.W. found[d]: (calculated)
NT(9–13)	19.0 ± 2.0	22.4	660.8: (660.8)
1	14.7 ± 0.2	24.6	687.8: (687.8)
2	3.9 ± 0.4	24.7	702.0: (701.9)
3	9.3 ± 1.4	24.6	715.8: (715.9)
4	8.8 ± 2.0	24.4	731.0: (731.0)
5	28 ± 5.0	24.9	757.0: (756.9)
6	1.3	N.D.	N.D.

[a]The relative potency of **6** compared to NT(9–13) was calculated using assay data for both peptides from Cusack et al.[12] N.D., not determined in our laboratory.
[b]Binding data were obtained using hNTR (Leu194) expressed in CHO cells from New England Nuclear (Boston, MA) as described previously.[16]
[c]RP–HPLC retention times were determined as described previously.[16]
[d]ES–MS analysis on a Finnigan LCQ instrument. The tandem mass spectrum of the $[M+2H]^{2+}$ ion for each analogue gave correct sequence data.

hindered, alternative to the α-ammonium ion or α-*N*-acetylation, which may result in enhanced bio-distribution into lipophilic compartments.[17] Since size and number of hydrogen bonding amide groups are important for crossing the blood-brain barrier[18] we note up to a 16% decrease in molecular weight and loss of an amide bond for the analogues in comparison to NT(8–13). The general synthetic route can be used to produce many unique substitutions at the N-terminal region of peptides. Analogues **1**–**5**, or derivations thereof, have the potential for use to further define the role of NT in neurological disorders or for development as a new class of antipsychotics.

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