Design and Structure–Activity Relationships of C-Terminal Cyclic Neurotensin Fragment Analogues

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Neurotensin (NT) is a linear tridecapeptide with a broad range of central and peripheral pharmacological effects. The C-terminal hexapeptide of NT (NT_{8-13}) has been shown to possess similar properties to NT itself, and in fact, an analogue of NT_{8-13} ($N^{\alpha}MeArg^{8}$-Lys-Pro-Trp-Tle-Leu^{13}, Tle = *tert*-leucine) has been reported to possess central activity after peripheral administration. Cyclic derivatives of this hexapeptide were synthesized by a combination of solution and solid-phase peptide synthetic methodologies, and several analogues had low nanomolar binding affinity for the NT receptor. In particular, cyclo[Arg-Lys-Pro-Trp-Glu]-Leu (cyclized between the α amine of Arg and the γ carboxylate of Glu) possessed 16 nM NT receptor affinity and was determined to be an agonist *in vitro*. ^{1}H-NMR and ^{13}C-edited ^{1}H-NMR spectroscopy were performed on this and related cyclic analogues to help identify structural properties which may be important for receptor recognition. These cyclic peptides represent novel molecular probes to further investigate NT receptor pharmacology, as well as to advance our understanding of the structure–conformation relationships of NT and to help establish a working basis for additional pharmacophore mapping studies.

Introduction[1]

Neurotensin (NT, $pGlu^{1}$-Leu^{2}-Tyr^{3}-Glu^{4}-Asn^{5}-Lys^{6}-Pro^{7}-Arg^{8}-Arg^{9}-Pro^{10}-Tyr^{11}-Ile^{12}-Leu^{13}) is a linear tridecapeptide that was originally isolated from the bovine hypothalamus.[2] NT is found in high concentrations in the ileum and the hypothalamus and has a broad range of biological effects including hypotension, analgesia, gut contraction, and increase of vascular permeability.[3-5] In addition, NT has been shown to possess pharmacological properties similar to dopamine antagonists that are effective antipsychotics.[6]

NT is one member of a family of biologically active peptides that includes xenopsin (pGlu-Gly-Lys-Arg-Pro-Trp-Ile-Leu, isolated from the skin of *Xenopus laevis*)[7] and neuromedin N (Lys-Ile-Pro-Tyr-Ile-Leu, isolated from the porcine spinal cord).[8] Both of these peptides interact with the NT receptor[9,10] and are homologous with the C-terminal hexapeptide of NT (NT_{8-13}, Arg^{8}-Arg-Pro-Tyr-Ile-Leu^{13}).

Historically, the design and syntheses of NT analogues, including fragment derivatives and prototypic cyclic congeners, dates to the very important work of a number of research groups, as exemplified by Carraway and Leeman,[11] Folkers *et al.*,[12] Rivier *et al.*,[13,14] St-Pierre *et al.*,[15] Bayer *et al.*,[16] Quirion *et al.*,[17] Kitabgi *et al.*,[18] Jolicoeur *et al.*,[19] and Clineschmidt *et al.*[20] In particular, these pioneering studies provided the first systematic analysis of NT in terms of the structure–activity relationships (SAR) of the critical NT_{9-13} message sequence and provided insight to the structure–conformation properties of both the native peptide and a NT_{6-13} analogue. More recently, NT research has focused primarily on the C-terminal pentapeptide or hexapeptide sequence to further advance structure–activity studies and NT-based drug discovery, *vide infra*.

Both NT and NT_{8-13} fail to cross the blood–brain barrier when administered intravenously (iv) or orally,[5] and their relatively short *in vivo* half-lives have prompted a search for metabolically stable analogues for further evaluation. Reduced peptide bond analogues (Ψ[CH_2-NH]) of NT_{8-13} have shown increased stability, especially when the Arg^{8}–Arg^{9} bond was modified.[21-24] In addition, it has been reported that a modified NT_{8-13} hexapeptide (**1**, $N^{\alpha}MeArg^{8}$-Lys-Pro-Trp-Tle-Leu^{13}, Tle = *tert*-leucine)[25] binds to the NT receptor with low nanomolar affinity. Interestingly, this compound possessed central nervous system (CNS) activity after peripheral administration, suggesting that it may cross the blood–brain barrier and be metabolically stable.[25-27]

NT has been suggested to have a role in the effects of antipsychotic drug action. Enhanced NT levels have been found in the cerebrospinal fluid of a subpopulation of schizophrenic patients undergoing antipsychotic drug therapy versus a control group.[28] Antipsychotic drugs have also been shown to increase NT immunoreactivity in the brain and levels of NT messenger RNA in the rat.[29] Thus, NT agonists could potentially be a novel class of antipsychotics. Such compounds could replace current anti-dopamine therapies, since they may not possess the extrapyramidal side effects and tardive dyskinesia.[30] Also, the antinociceptive effects of NT are of interest, since they are not associated with the respiratory depression and addictive properties of the opiates.[31]

The three-dimensional solution conformation of NT has been studied by two-dimensional proton NMR spectroscopy in water and methanol, but due to its inherent flexibility little structural information was obtained.[32] In NT_{8-13}, the replacement of the Pro^{10}-

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Table 1. N-Terminal to Side Chain Cyclized Neurotensin and Related Analogues

no.	compound	constraint	NT binding (± SEM)[a] (nM, K_i)
1[b]	N$^{\alpha}$MeArg^{8}-Lys-Pro-Trp-Tle-Leu^{13}	none	0.67 ± 0.03
2	cyclo[Arg^{8}-Lys-Pro-Trp-Glu]-Leu^{13}	α-NH_2(Arg)→γ-CO_2H(Glu)	16 ± 4
3	cyclo[Arg^{8}-Lys-Pro-Trp-Asp]-Leu^{13}	α-NH_2(Arg)→β-CO_2H(Asp)	910 ± 25
4	cyclo[Arg^{8}-Lys-Pro-Trp-Glu]-Gly^{13}	α-NH_2(Arg)→γ-CO_2H(Glu)	250 ± 13
5	cyclo[Arg^{8}-Ala-Pro-Trp-Glu]-Leu^{13}	α-NH_2(Arg)→γ-CO_2H(Glu)	>10000
6	cyclo[Arg^{8}-Lys-Pro-Trp-Glu^{12}]	α-NH_2(Arg)→γ-CO_2H(Glu)	>10000
7	cyclo[Arg^{8}-Pro-Trp-Glu]-Leu^{12}	α-NH_2(Arg)→γ-CO_2H(Glu)	340 ± 8
8	N$^{\alpha}$MeArg^{8}-cyclo[Lys-Pro-Trp-Tle-Asp^{13}]	ε-NH_2(Lys)→β-CO_2H(Asp)	>10000
9	cyclo[Gly-N$^{\alpha}$MeArg^{8}-Lys-Pro-Trp-Asp]-Leu^{13}	α-NH_2(Gly)→β-CO_2H(Asp)	560 ± 44
10	cyclo[Gly-N$^{\alpha}$MeArg^{8}-Lys-Pro-Trp-Glu]-Leu^{13}	α-NH_2(Gly)→γ-CO_2H(Glu)	28 ± 11
11	cyclo[N$^{\alpha}$MeArg^{8}-Lys-Pro-Trp-Glu]-Leu^{13}	α-NH(MeArg)→γ-CO_2H(Glu)	700 ± 20
12	cyclo[D-Arg^{8}-Lys-Pro-Trp-D-Glu]-Leu^{13}	α-NH_2(D-Arg)→γ-CO_2H(D-Glu)	>10000[c]
13	cyclo[Arg^{8}-Lys-Pro-Trp-Hgl[d]]-Leu^{13}	α-NH_2(Arg)→δ-CO_2H(Hgl[d])	830[c] ± 175

[a] $n = 6$ (number of replicate determinations) unless otherwise noted. [b] See refs 25, 26, 27, and 39. [c] $n = 3$. [d] Hgl = homoglutamic acid.

Table 2. N-Terminal to Side Chain Cyclized Neurotensin Analogues and Corresponding Linear Analogues

no.	compound	constraint	NT binding (± SEM)[a] (nM, K_i)
2	cyclo[Arg^{8}-Lys-Pro-Trp-Glu]-Leu^{13}	α-NH_2(Arg)→γ-CO_2H(Glu)	16 ± 4
14	N$^{\alpha}$AcArg^{8}-Lys-Pro-Trp-Gln-Leu^{13}	none	260 ± 80
11	cyclo[N$^{\alpha}$MeArg^{8}-Lys-Pro-Trp-Glu]-Leu^{13}	α-NH(MeArg)→γ-CO_2H(Glu)	700[b] ± 20
15	N$^{\alpha}$MeArg^{8}-Lys-Pro-Trp-Glu-Leu^{13}	none	>10000[b]

[a] $n = 6$ (number of replicate determinations) unless otherwise noted. [b] $n = 3$.

Tyr^{11} dipeptide with β-turn mimetics has suggested the presence of a β-turn-like structure, but overall there is a paucity of information about the biologically active conformation of NT or its C-terminal hexapeptide.[33-35] Recently, we have systematically investigated the Pro^{10}-Tyr^{11} sites of the NT_{8-13} analogue **1** by incorporating a series of backbone and/or side chain modified amino acids which induced striking biological effects *in vitro* and *in vivo*.[27] In order to prepare conformationally rigid analogues of NT_{8-13} and further understand the three-dimensional pharmacophore, we have synthesized a series of cyclic derivatives of the linear hexapeptide **1**.

Several approaches were undertaken to obtain a cyclic NT_{8-13} analogue that possessed high binding affinity for the NT receptor. Previous NT_{8-13} structure–activity relationships have indicated residues that are critical for receptor recognition. For example, an alanine scan of NT_{8-13} suggested[36] the relative importance of the individual amino acid side chains to be as follows: Leu^{13} > Tyr^{11} ≫ Ile^{12} > Arg^{9} > Pro^{10} > Arg^{8}. Fragment studies also suggested[13-16] the requirement of at least one basic residue, since NT_{8-13} and NT_{9-13} have similar binding affinities, while the NT_{10-13} fragment has much lower affinity. Finally, a free C-terminal carboxylate also appears to be essential for high receptor affinity since the NT_{8-13} C-terminal amide has 2 orders of magnitude less binding.[36]

Given this information, we replaced the Tle^{12} of compound **1** with either a glutamic acid or aspartic acid residue. These substitutions provided a free carboxylate for cyclization via a lactam bridge, to either the Lys^{9} side chain or the N-terminal amine, without removal of the important C-terminal carboxylic acid moiety. The preferred stereochemistry of the side chains forming the lactam bridge was also explored. Furthermore, alanine and glycine substitutions were incorporated to probe the importance of individual side chains. In several peptides glycine was added to the N-terminal Arg^{9} and cyclized through the side chain carboxylate of position 12 to increase the flexibility of the lactam bridge. The Leu^{13} residue was replaced with aspartic acid such that the cyclic head-to-tail compound could be prepared to probe the relative importance of the Leu^{13} side chain and/or the free C-terminal carboxylate. Finally, cyclic disulfide-containing peptides were prepared for comparison with the corresponding cyclic lactams.

Results and Discussion

The side chain to N-terminus and side chain to side chain cyclics were prepared and cyclized on a solid support. Utilizing an N^{α}-*t*-Boc (*tert*-butyloxycarbonyl) protection strategy for the solid phase synthesis, the side chain carboxylates of Asp^{12} or Glu^{12} were protected as Fmo (9-fluorenylmethyl) esters. For the side chain to side chain cyclic analogues, the Lys^{9} side chain was also Fmoc protected and was simultaneously deprotected along with the Asp^{12} or Glu^{12} side chain using piperidine. Cyclization on the resin takes advantage of the self-diluting effects of the resin.[37,38] Each peptide chain is isolated by the polymeric matrix such that the cyclization can occur rapidly and with less oligomeric byproducts. In general, for the cyclic neurotensin analogues prepared in this study on resin cyclization led to crude products that were relatively pure and easily isolated by subsequent RP-HPLC.

Neurotensin Receptor Binding Studies. Table 1 illustrates the NT binding affinity for compounds cyclized from the N-terminus to a side chain carboxylate. Compound **2** is the most potent analogue with 16 nM binding for the NT receptor. This is only 20-fold less potent than the linear NT_{8-13} analogue (compound **1**), from which these compounds were designed. In the preparation of compound **2**, the α amine of Arg^{8} of compound **1** was incorporated in the lactam reducing the overall basicity of the molecule. Previously, it has been reported that Ac-Arg^{8}-Lys-Pro-Trp-Tle-Leu^{13} exhibited similar affinity for the NT receptor (1.4 nM) as compound **1**, suggesting that the loss of the N-terminal backbone basic group does not alone account for the differences in NT binding affinity of compounds **1** and **2**.[39] In addition, compound **14**, was prepared as an acyclic control of compound **2** (Table 2) is 20-fold less potent than compound **2**. This suggests that cyclization provides peptide **2** with an entropic advantage for binding. The linear analogue of compound **11**, compound **15**, also possesses less binding affinity (see Table

Table 3. N-Terminal to C-Terminal Cyclized Neurotensin Analogues

no.	compound	constraint	NT binding (± SEM)[a] (nM, K_i)
16	cyclo[Arg[8]-Lys-Pro-Trp-Tle-Leu[13]]	α-NH_2(Arg)→α-CO_2H(Leu)	410 ± 106
17	cyclo[Arg[8]-Lys-Pro-Tyr-Tle-Leu[13]]	α-NH_2(Arg)→α-CO_2H(Leu)	440[b] ± 18
18	cyclo[Lys[9]-Pro-Trp-Tle-Leu[13]]	α-NH(Lys)→α-CO_2H(Leu)	350 ± 158

[a] $n = 6$ (number of replicate determinations) unless otherwise noted. [b] $n = 9$.

Table 4. Side Chain to Side Chain Cyclized Neurotensin Analogues

no.	compound	constraint	NT binding (± SEM)[a] (nM, K_i)
19	N[α]MeArg[8]-cyclo[Lys-Pro-Trp-Glu-Leu[13]]	ε-NH_2(Lys)→γ-CO_2H(Glu)	770 ± 26
20	N[α]MeArg[8]-cyclo[D-Lys-Pro-Trp-Glu-Leu[13]]	ε-NH_2(Lys)→γ-CO_2H(Glu)	8500 ± 500
21	N[α]MeArg[8]-cyclo[Lys-Pro-Trp-D-Glu-Leu[13]]	ε-NH_2(Lys)→γ-CO_2H(Glu)	1600 ± 152
22	N[α]MeArg[8]-cyclo[D-Lys-Pro-Trp-D-Glu-Leu[13]]	ε-NH_2(Lys)→γ-CO_2H(Glu)	2500[b] ± 200

[a] $n = 6$ (number of replicate determinations) unless otherwise noted. [b] $n = 9$.

Table 5. Disulfide Side Chain to Side Chain Cyclized Neurotensin Analogues

no.	compound	constraint	NT binding (± SEM)[a] (nM, K_i)
23	N[α]MeArg[8]-cyclo[Cys-Pro-Trp-Pen[b]]-Leu[13]	Cys→Pen[b]	83 ± 19
24	N[α]MeArg[8]-cyclo[Cys-Pro-Trp-Cys]-Leu[13]	Cys→Cys	810[c] ± 200
25	N[α]MeArg[8]-cyclo[Pen-Pro-Trp-Pen]-Leu[13]	Pen[b]→Pen[b]	4400[c] ± 19
26	cyclo[Cys-Arg[8]-Lys-Pro-Trp-Pen]-Leu[13]	Cys→Pen[b]	640[c] ± 10

[a] $n = 6$ (number of replicate determinations) unless otherwise noted. [b] Pen = penicillamine (β,β-dimethylcysteine). [c] $n = 3$.

2), providing further evidence that cyclization can be beneficial to ligand-receptor recognition. Here, the differences in binding affinities between cyclic and acyclic compounds is greater than that for compounds **2** and **14**, but it should be noted that compound **15** has an extra charge on the free Glu[12] side chain and the amine terminus that are not present in acyclic compound **11**. Compound **11**, however, has nearly 50-fold less binding affinity than compound **2**. It is possible that N-methylation of the Arg[8] residue may remove an essential hydrogen bonding interaction, or, perhaps, the N-Me amide bond leads to a conformational change of the ring via *cis/trans* amide isomerization.

Compounds **9** and **13**, which have larger ring sizes than compound **2**, have lower binding affinities, perhaps due to their flexibility. (Compound **10**, which also has a larger ring size, exhibits a similar trend in binding affinity, although it is not statistically significant.) Interestingly, replacement of the Glu[12] in compound **2** with Asp (compound **3**), which should constrain the molecule further, decreased the binding affinity by more than 50-fold. This suggests that the smaller ring may have changed the orientation of key side chains such that the molecule can no longer adopt a favorable conformation for NT binding.

Table 3 summarizes the NT binding results for compounds cyclized from the N- to C-terminus (head-to-tail). All compounds possessed less binding affinity than compound **2**, underlining the importance of the free C-terminal carboxylate. The reduced binding affinities of compounds **4**, **6**, and **8** (Table 1), however, may indicate that the side chain of the C-terminal Leu[13] is important, as well. Compound **5** did not bind to the NT receptor, suggesting an important role for the Lys[9] side chain in this series of compounds. In contrast, compound **7**, in which one of the two basic residues is deleted, displayed only 20-fold lower binding affinity with respect to compound **2**. Also, compound **18**, which has only one basic side chain, had virtually the same if not better binding when compared to the cyclic hexapeptides **16** and **17**, which contain both the Arg[8] and the Lys[9] side chains. This suggests that the position next to the Pro is more important than the Arg[8] position in this series, which is consistent with the previous data from the Ala scan performed on the C-terminal hexapeptide.[36]

Table 4 provides binding data for the side chain to side chain cyclic analogues, all of which have weak affinity for the NT receptor (compounds **19** to **22**). This may be due to the fact that two key side chains are involved in the cyclization and not available for receptor interaction, or that the peptide is constrained in a conformation that is unfavorable for receptor recognition. In any case, the orientation of the amino acids in position 9 (Lys) or 12 (Glu) seemed to have little effect on NT binding affinity.

Comparison of these data to the data in Table 5 for the disulfide cyclized peptides shows that the Lys[9] side chain can be removed while retaining moderate affinity for the NT receptor (83 nM) (compound **23**). Therefore, the disulfide bridge may constrain the molecule differently than a lactam (Table 4) and orient the peptide in a favorable conformation for binding. These data contradict the data for the N-terminus to side chain cyclics, which suggests that the Lys[9] side chain is critical for binding, but it is possible that the smaller ring of the disulfide has moved the Arg[8] side chain to a position where it is able to mimic the Lys[9] side chain. Nevertheless, the binding affinity of compound **23** is still 5-fold less than that of compound **2**, so both basic side chains may be contributing to the binding.

Table 5 also illustrates the effect of structural modifications proximate to the disulfide (i.e., cysteine versus penicillamine). Specifically, removal of the β,β-dimethyls from Pen[12] in compound **23** by replacement with a cysteine residue, as in compound **24**, resulted in a 10-fold loss in binding affinity. It could be postulated that the penicillamine β,β-dimethyl groups may be binding in the same pocket as the methyl groups of Tle[12] (compound **1**). Interestingly, compound **25** exhibited a 50-fold decrease in NT binding affinity relative to compound **23** and suggested the possibility that the β,β-dimethyls of Pen[9] effected steric hinderance to ligand-receptor recognition and/or intramolecular effects to the conformation of the cyclic NT_{8-13} analogue which compromised binding to the NT receptor, *vide infra*. Also,

compound **26** exhibited approximately an 8-fold loss of affinity with respect to compound **23**. This may simply be an effect of the excess entropy resulting from the larger ring size.

Intracellular Calcium Mobilization Studies. The relatively low binding affinity of the cyclic analogues versus neurotensin, itself or compound **1**, precluded the evaluation of functional activity in an *in vivo* system. In lieu of this, several compounds were evaluated for their ability to stimulate calcium mobilization in HT-29 cells. NT and compound **1** were shown to be agonists with EC_{50}'s of 12 and 120 nM, respectively. Likewise, preliminary studies for compounds **2**, **18**, and **23** revealed that these were able to stimulate intracellular calcium mobilization with low micromolar EC_{50}'s. In fact, compounds **1** and **2** were full agonists, using NT as a control for a full agonist, while compounds **18** and **23** were only partial agonists. Clearly, the receptor binding affinities observed with 10-day-old postnatal mouse brain do not correlate directly to the functional activity observed in HT-29 cells making a concise evaluation of weaker binding compounds very difficult. Additional studies with these and other cyclic analogues, including an evaluation of their ability to stimulate intracellular calcium mobilization, are in progress and will be published elsewhere.

^{1}H-NMR Studies. While NOE measurements provide a rich source of structural constraints for small proteins, their utility for the structural analysis of peptides is limited, due to the inherent flexibility and relatively large surface area of these compounds. Therefore, in order to assess the structural characteristics of some of these cyclic/constrained NT analogues, coupling constants and amide proton temperature coefficients were measured for compounds **2**, **3**, **23**, and **25** (Table 6). Typically, limited conformational averaging can be inferred if the $^3J_{NH,H\alpha}$ value is less than 6 Hz or greater than 8 Hz. However, even when this condition is met $^3J_{NH,H\alpha}$ values may be consistent with more than one distinct dihedral angle, but such ambiguity can be resolved by determining heteronuclear coupling constants. For compounds **2** and **3**, a ^{13}C-edited TOCSY experiment[40,41] was used to determine $^3J_{NH,C\beta}$ values. Also, reduced temperature coefficients for exchangeable protons can be employed to suggest reduced contact with the solvent. In water, a fully exposed backbone amide proton is expected to possess a temperature coefficient of about −10 ppb/K. A smaller temperature coefficient (closer to zero) indicates reduced exchange with the bulk solvent and possible involvement in hydrogen bonding especially when it ($\Delta\delta/\Delta T$) is less than or equal to ~3 ppb/K. Unfortunately, for these compounds the $^3J_{NH,H\alpha}$ values are in the motionally averaged range. Also, the distinct lack of long-range crosspeaks in the NOESY and ROESY spectra suggest that all of these compounds are somewhat flexible. These factors have precluded the determination of highly refined structures, although some interesting trends do exist and deserve comment.

Compounds **2** and **3** have 17 and 16 membered rings, respectively, and as expected for such large cyclic systems both are somewhat flexible. As might be anticipated from a comparison of the two peptides, the smaller of the two, compound **3**, has some spectral features that are consistent with a greater degree of conformational rigidity. The $^3J_{NH,H\alpha}$ values for Lys^9 (2.6 Hz) and Asp^{12} (9.4 Hz) lie outside the range expected for motional averaging. Furthermore, for compound **3**, the Trp^{11} and Leu^{13} residues have relatively small amide proton temperature coefficients of −1.2 and −3.8 ppb/K, respectively. In compound **2**, all of the ^{1}H-NMR parameters are indicative of a more flexible peptide. However, it should be noted that, with the exception of the Leu^{13} amide proton temperature coefficient, the same trend is observed for compounds **2** and **3** (e.g., for both compounds the residues Lys^9 and Glu/Asp^{12} possesses the smallest and largest $^3J_{NH,H\alpha}$ values, respectively, and Trp^{11} possesses the smallest amide temperature coefficient). This suggests that while compound **2** is more flexible than compound **3**, they may both share one or more common three-dimensional conformation(s). Interestingly, both peptides show strong Lys^{9}Hα to Pro^{10}Hα NOE's indicating a *cis* amide bond between Lys^9 and Pro^{10}. Linear proline-containing peptides commonly exhibit *cis* and *trans* isomers that are in slow exchange (relative to the chemical shift time scale), but for compounds **2** and **3**, these peptides exist exclusively in the *cis*-proline conformation.

Compounds **23** and **25** represent two extremes in terms of NT receptor binding affinity (Table 5) for the 14-membered ring disulfide-cyclized NT analogues, yet they only differ by two methyl groups. A critical examination of the ^{1}H-NMR data in Table 6 reveals that several interesting features exist. First, both compounds **23** and **25** exist as a major (~80%) and minor (~20%) isomer on the ^{1}H-NMR time scale. In the case of compound **25**, the major isomer has a *trans*-proline and the minor has a *cis*-proline amide bond. This is based upon the strong NOE's observed between Pen^{9}Hα–Pro^{10}Hδ and Pen^{9}Hα–Pro^{10}Hα, respectively. (The equivalent NOEs could not be identified for compound **23** due to poor signal-to-noise and spectral overlap.) For both

Table 6. Proton NMR Data for Compounds **2**, **3**, **23** and **25**

residue		$^3J_{NH,H\alpha}$ (Hz)	$^3J_{NH,C\beta}$ (Hz)	$\Delta\delta/\Delta T$ (ppb/K)	δNH (ppm) 288 K	293 K	298 K	308 K
Compound 2								
Arg^8		8.0	1.0	−8.0	8.20		8.12	
Lys^9		5.9		−8.0	8.27		8.19	
Trp^{11}		7.0	1.5	−4.0	7.88		7.84	
Glu^{12}		8.7	0.6	−6.0	7.92		7.86	
Leu^{13}		8.3	1.5	−9.0	8.03		7.94	
Compound 3								
Arg^8		6.4		−8.3	7.52		7.43	
Lys^9		2.6		−7.7	8.66		8.58	
Trp^{11}		8.1	1.8	−1.2	7.44		7.43	
Asp^{12}		9.4	1.6	−5.2	8.38		8.33	
Leu^{13}		7.7	2.9	−3.8	7.31		7.27	
Compound 23								
Cys^9	major:	6.3		−7.0	9.18		9.05	8.98
	minor:	8.2		−8.0	8.98		8.90	
Trp^{11}	major:	6.7		−12.0	8.01		7.88	7.77
	minor:	6.5		−8.0	8.77		8.69	
Pen^{12}	major:	9.1		−4.0	7.77		7.73	7.69
	minor:	8.1		−3.0	7.65		7.68	
Leu^{13}	major:	6.7		−6.5	8.41		8.35	8.28
	minor:	7.5		−9.5	8.68		8.58	
Compound 25								
Pen^9	major:	7.4		−7.0	8.68	8.71		
	minor:	10.0		−8.0	8.62	8.66		
Trp^{11}	major:	8.3		−8.0	6.80	6.84		
	minor:	11.0		−7.8	7.68	7.72		
Pen^{12}	major:	7.0		−4.4	7.65	7.68		
	minor:	10.3		−2.0	8.72	8.73		
Leu^{13}	major:	7.6		−9.2	8.58	8.62		
	minor:	6.2		−5.8	8.37	8.40		

of the peptides the major isomer has medium sized $^3J_{NH,H\alpha}$ values (except for Pen^{12} in **23** and Trp^{11} in **25**) and large amide proton temperature coefficients. Both of these observations are consistent with motional averaging. Two features distinguish the major isomers of compounds **23** and **25** and suggest that their preferred conformations are not identical. First, the only residues that display $^3J_{NH,H\alpha}$ values greater than 8 Hz are Pen^{12} in compound **23** and Trp^{11} in compound **25**, *vide supra*. Secondly, a 1 ppm chemical shift difference exists between the NH resonances of Trp^{11} in the major isomers of compounds **23** and **25**.

In contrast to the major isomers, several differences in chemical shifts and coupling constants are even more obvious in the minor isomers. For example, the minor (*cis*) isomer of compound **25** has large $^3J_{NH,H\alpha}$ values for all of the amino acids in the cyclic portion of the molecule (Table 6). This limits the allowed conformational space for the ϕ angle from approximately $-170°$ to $-80°$. This further suggests that the backbone conformation of the minor isomer of compound **25** is well defined, at least with respect to the other cyclic NT peptides discussed here. Also, the Pen^{12} amide proton in the minor isomer has the smallest temperature coefficient (-2.0 ppb/K) of any of the these analogues. For all of the peptides presently investigated, the minor conformer of compound **25** is clearly the most conformationally constrained. Due to the relatively low abundance (15−20%) of this conformer, we could not determine meaningful NOE's for structural analysis. Currently, we are optimizing conditions for the structural analysis of these peptides.

Conclusions

We report the synthesis of several conformationally constrained cyclic neurotensin fragment (NT_{8-13}) analogues, several of which possess nanomolar binding affinities at the NT receptor. Specifically, compounds **2**, **10**, and **23** are the only cyclic peptides of the C-terminal hexapeptide that have been shown to possess low nanomolar affinity for the NT receptor. These analogues provide further evidence that a reverse-turn in the C-terminal hexapeptide in NT may be important for NT binding.[27] It is interesting that compounds **2** and **23**, which are structurally very different, have similar binding affinities. These compounds may provide novel molecular probes to further investigate NT receptor pharmacology, as well as further advance our understanding of the structure−conformation relationships of NT and establish a working basis for future pharmacophore mapping studies. To this extent, future molecular modeling and ^{1}H-NMR studies of these compounds may provide some insight toward the identification of the three-dimensional structural requirements for NT binding. Such work will extend previous studies[27] which implicated reverse-turn type conformational preferences within a series of linear NT_{8-13} analogues as a result of modifications of the Pro^{10}-Tyr^{11} dipeptide. Future studies will be directed at the design of NT_{8-13} pseudopeptides and peptidomimetics that may exploit such structure−conformation relationships in the discovery of yet more effective NT agonists.

Experimental Section

General. All reagents and solvents were purchased from commercial sources unless otherwise specified. Solvents were of reagent or HPLC grade as indicated. All amino acids and substituted peptide resins were purchased from commercial sources, used without further purification, and amino acids were of the L configuration unless indicated. Routine ^{1}H-NMR spectra were obtained on a Bruker AM-250 spectrometer, and all samples were dissolved in D_2O or DMSO-d_6, as indicated. Mass spectra were recorded on a VG Analytical 7070E/HF, a VG Masslab Trio-2A, or a Fisons VG Trio200 mass spectrometer. Amino acid analyses were performed on an Applied Biosystems International (ABI) 420H derivatizer with a 130A separation system.

Peptide Synthesis Method A (PAM Resin). The peptides were prepared by standard solid phase peptide methodology[42] on an ABI 430A peptide synthesizer using the N^{α}-*t*-Boc *N*-hydroxybenzotriazole (HOBt), *N*-methylpyrrolidone (NMP) strategy (Version 1.40). Single couplings were performed with 4 equiv of the suitably protected amino acid activated ester formed by reaction with *N,N'*-dicyclohexylcarbodiimide (DCC) and HOBt. A typical cycle for the coupling of an individual amino acid was as follows: (1) activation of the next amino acid to be coupled with DCC and HOBt; (2) deprotection of the amino acid on the resin with 50% trifluoroacetic acid (TFA)/dichloromethane (DCM) + indole (1 mg/mL, used as a scavenger); (3) washes with DCM and neutralization with 10% diisopropylethylamine (DIEA)/DCM; (4) coupling of the HOBt activated ester in NMP; (5) five washes with NMP and capping with 10% acetic anhydride (Ac_2O)/DCM. The N-terminal Boc group was removed with 50% TFA/DCM, and the resin was neutralized with 10% DIEA/DCM (except for peptides to be cyclized by cyclization method A). The resin was then washed with DCM and dried under reduced pressure.

Peptide Synthesis Method B (Sasrin Resin[43]). Sasrin resin (1 g, 1 mmol) was placed in a shaker vessel and washed with 3 × 25 mL of DCM. The first coupling was performed using 3 equiv of the Fmoc-protected amino acid, 3 equiv of *N,N'*-diisopropylcarbodiimide (DIC), and 0.3 equiv of 4-(dimethylamino)pyridine (DMAP), in 25 mL of DCM overnight. The resin was then rinsed with *N,N*-dimethylformamide (DMF) (3 × 25 mL). The Fmoc group was removed by treatment with 20% piperidine/DMF for 1 and 10 min (2 × 25 mL). Amino acids were successively coupled using 3 equiv of 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU),[44] 3 equiv of DIEA, and 3 equiv of the amino acid (N^{α}-Fmoc-protected, Arg and Lys side chains protected as the tosyl and *t*-Boc derivatives, respectively) in DMF for 60 min, followed by Fmoc deprotection. After the final Fmoc deprotection, the resin was washed with DMF (3 × 25 mL) and DCM (3 × 25 mL) and was dried under reduced pressure.

Resin Cyclization, Method A (Side Chain to Side Chain, PAM Resin). To deprotect the Fmo-ester-protected side chains of Glu and Lys, 25 mL of 20% piperidine/DCM was added to a shaker vessel containing the resin, and the mixture was shaken for 15 min and drained. Another 25 mL of 20% piperidine/DCM was added to the shaker vessel, and the mixture was shaken for 30 min. The vessel was drained and washed with DCM (3 × 25 mL) and DMF (3 × 25 mL). For side chain to side chain cyclizations, the resin was suspended in 25 mL of DMF, and 400 μL of DIEA was added followed by 270 mg of HOBt and 300 μL of DIC. The mixture was shaken continuously, and the solvent/reagents were changed every 24 h until the resin yielded a negative Kaiser test.[45] The vessel was then drained, and the resin was washed with DMF (3 × 25 mL) and DCM (3 × 25 mL). To deprotect the N-terminal *t*-Boc group, 25 mL of 50% TFA/DCM was added, and the mixture was shaken for 15 min. The vessel was drained, another 25 mL of 50% TFA/DCM was added, and the mixture was shaken for an additional 30 min. The vessel was drained, and the resin was washed with DCM (3 × 25 mL), 10% DIEA/DCM (3 x 25 mL), and DCM (3 × 25 mL), and the resin was dried under reduced pressure.

Resin Cyclization, Method B (Side Chain to N-Terminus, PAM Resin). To deprotect the Fmo-ester-protected side chains of Glu or Asp, 25 mL of 20% piperidine/DCM was added to a shaker vessel containing the resin, and the mixture was shaken for 15 min and drained. Another 25 mL of 20% piperidine/DCM was added to the shaker vessel, and the

mixture was shaken for 30 min. The vessel was drained and washed with DCM (3 × 25 mL) and DMF (3 × 25 mL). The resin was suspended in 25 mL of DMF, and 400 μL of DIEA was added followed by 270 mg of HOBt and 300 μL of DIC. The mixture was shaken continuously, and the solvent/reagents were changed every 24 h until the resin yielded a negative Kaiser test.[45] The vessel was then drained, and the resin was washed with DMF (3 × 25 mL) and DCM (3 × 25 mL) and dried under reduced pressure.

Peptide Cleavage Method A (PAM Resin). The peptides were removed from the resin and the side chains deprotected by treatment with 90% anhydrous hydrogen fluoride (HF) and 10% scavengers (3-methylindole and anisole) at 0 °C for 1 h. Nitrogen was bubbled through the mixture to evaporate the HF, and the residue was triturated with 75 mL of Et_2O and filtered. The crude precipitate was dissolved in 70% CH_3CN/H_2O + 0.1% TFA and filtered, and the solvent was removed under reduced pressure. The residue was resuspended in ~100 mL of H_2O and lyophilized.

Peptide Cleavage Method B (Sasrin Resin[43]). A solution of 1% TFA/DCM (20 mL) was added to the resin in a shaker vessel, and the mixture was agitated for 10 min and drained. This procedure was repeated three times, and the combined eluants were evaporated under reduced pressure. The residue was resuspended in ~100 mL of H_2O and lyophilized.

Solution Cyclization, Method C (Disulfides). The crude lyophilized peptide after HF cleavage (peptide cleavage method A) (~300 mg) was dissolved in 2.5 L of H_2O, and the pH was adjusted to 8.5 with 1 N potassium hydroxide (KOH). Dithiothreitol (DTT, ~200 mg) was added, and the mixture was stirred for 1 h. Potassium ferricyanide ($K_3Fe(CN)_6$, 500 mg in 100 mL of H_2O) was then added dropwise to the solution while the pH was maintained at ~8–9 with 1 N KOH until a pale yellow color was maintained for 60 min. At this point, the pH was then adjusted to 4.5 with glacial acetic acid, and the solution was stirred for 10 min with an excess of BioRad AG4-X4 anion exchange resin (~10-fold). The solution was filtered and passed through a C18 cartridge preequilibrated with H_2O. The absorbed cyclic peptide was eluted with 70% CH_3CN/H_2O + 0.1% TFA, concentrated under reduced pressure, resuspended in H_2O (~100 mL), and lyophilized.

Solution Cyclization, Method D (C-Terminus to N-Terminus). A solution of the linear peptide (240 mg) in 30 mL of DMF was added dropwise with stirring over 60 min to a solution of (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP)[38,46] or HBTU[44] (0.6 mmol, 3 equiv) and DIEA (0.6 mmol, 3 equiv) in 200 mL of DMF at 0 °C. The reaction was followed by analytical HPLC, which showed the disappearance of starting material after 60 min. The reaction mixture was allowed to slowly warm to room temperature, the solvent was removed under reduced pressure, and the peptide was lyophilized from H_2O (~100 mL). The side chain protecting groups were then removed with HF as described above (peptide cleavage method A).

Peptide Purification. The crude peptide was dissolved in approximately 3–5 mL of H_2O + 20% CH_3CN (with increasing amounts of CH_3CN as needed for dissolution) and chromatographed on a Vydac 218TP1022 (2.2 × 25.0 cm) preparative HPLC column. A linear gradient of 0.1% TFA/water to 0.1% TFA/CH_3CN was used to elute the peptide from the column. A flow rate of 15 mL/min was maintained on a Waters 600E System Controller, and the absorbance of the eluant at 214 and 280 nm was recorded on a Waters 490E programmable multiwavelength detector. Individual fractions were collected and analyzed by analytical HPLC. Appropriate fractions were combined and concentrated under reduced pressure, diluted with H_2O, and lyophilized to give the purified peptide.

Peptide Characterization. The peptides were assayed for purity by analytical HPLC on a Vydac 218TP54 (0.46 × 25.0 cm) analytical HPLC column connected to a Waters 600E system controller with a Waters 490E programmable multiwavelength detector. A gradient of 20–86% B over 22 min (A = 0.1% TFA/H_2O, B = 0.1% TFA/CH_3CN) was used with a flow rate of 1.5 mL/min. The purity of the peptides was confirmed by capillary zone electrophoresis (CZE) using an ABI 270A capillary electrophoresis system with a 72 cm capillary at +20 kV and a buffer of 20 mM sodium citrate, pH 2.5. The peptides were also analyzed by fast-atom bombardment (FAB) or electrospray (ES) mass spectrometry, amino acid analysis (AAA), and ^{1}H-NMR. Values found were in good agreement with the predicted values.

Compound 2 was synthesized using peptide synthesis method A, cyclization method B, followed by peptide cleavage method A. Analytical; RP-HPLC, t_R 8.07 min; purity, 91% at 214 nm. CZE: t_R 17.54 min; purity, >99% at 214 nm. MS (ES, m/z): 809.8 $[MH]^+$. AAA and ^{1}H-NMR (D_2O) were consistent with the proposed structure.

Compound 3 was synthesized using peptide synthesis method A, cyclization method B, followed by peptide cleavage method A. Analytical; RP-HPLC, t_R 9.16 min; purity, 90% at 214 nm. CZE: t_R 9.44 min; purity, 89% at 214 nm. MS (FAB, m/z): 796.4 $[MH]^+$. AAA and ^{1}H-NMR (D_2O) were consistent with the proposed structure.

Compound 4 was synthesized using peptide synthesis method A, cyclization method B, followed by peptide cleavage method A. Analytical; RP-HPLC, t_R 5.18 min; purity, 97% at 214 nm. CZE: t_R 9.18 min; purity, 95% at 214 nm. MS (FAB, m/z): 754.3 $[MH]^+$. AAA and ^{1}H-NMR (DMSO-d_6) were consistent with the proposed structure.

Compound 5 was synthesized using peptide synthesis method A, cyclization method B, followed by peptide cleavage method A. Analytical; RP-HPLC, t_R 10.18 min; purity, 95% at 214 nm. CZE: t_R 12.68 min; purity, >99% at 214 nm. MS (FAB, m/z): 753.8 $[MH]^+$. AAA and ^{1}H-NMR (DMSO-d_6) were consistent with the proposed structure.

Compound 6 was synthesized using peptide synthesis method A, cyclization method B, followed by peptide cleavage method A. Analytical; RP-HPLC, t_R 13.33 min; purity, 90% at 214 nm. CZE: t_R 6.06 min; purity, 95% at 214 nm. MS (FAB, m/z): 697.6 $[MH]^+$. AAA and ^{1}H-NMR (DMSO-d_6) were consistent with the proposed structure.

Compound 7 was synthesized using peptide synthesis method A, cyclization method B, followed by peptide cleavage method A. Analytical; RP-HPLC, t_R 10.63 min; purity, >99% at 214 nm. CZE: t_R 17.32 min; purity, >99% at 214 nm. MS (ES, m/z): 682.6 $[MH]^+$. AAA and ^{1}H-NMR (DMSO-d_6) were consistent with the proposed structure.

Compound 8 was synthesized using peptide synthesis method A, cyclization method B, followed by peptide cleavage method A. Analytical; RP-HPLC, t_R 6.77 min; purity, >99% at 214 nm. CZE: t_R 10.10 min; purity, >99% at 214 nm. MS (FAB, m/z): 810.7 $[MH]^+$. AAA and ^{1}H-NMR (DMSO-d_6) were consistent with the proposed structure.

Compound 9 was synthesized using peptide synthesis method A, cyclization method B, followed by peptide cleavage method A. Analytical; RP-HPLC, t_R 7.38 min; purity, 91% at 214 nm. CZE: t_R 13.83 min; purity, >99% at 214 nm. MS (ES, m/z): 867.4 $[MH]^+$. AAA and ^{1}H-NMR (DMSO-d_6) were consistent with the proposed structure.

Compound 10 was synthesized using peptide synthesis method A, cyclization method B, followed by peptide cleavage method A. Analytical; RP-HPLC, t_R 9.07 min; purity, 95% at 214 nm. CZE: t_R 10.58 min; purity, 93% at 214 nm. MS (FAB, m/z): 881.4 $[MH]^+$. AAA and ^{1}H-NMR (DMSO-d_6) were consistent with the proposed structure.

Compound 11 was synthesized using peptide synthesis method A, cyclization method B, followed by peptide cleavage method A. Analytical; RP-HPLC, t_R 7.88 min; purity, 90% at 214 nm. CZE: t_R 14.78 min; purity, 93% at 214 nm. MS (FAB, m/z): 824.8 $[MH]^+$. AAA and ^{1}H-NMR (DMSO-d_6) were consistent with the proposed structure.

Compound 12 was synthesized using peptide synthesis method A, cyclization method B, followed by peptide cleavage method A. Analytical; RP-HPLC, t_R 9.42 min; purity, 94% at 214 nm. CZE: t_R 9.41 min; purity, 90% at 214 nm. MS (ES, m/z): 405.7 $[M + 2H]^{2+}$. AAA and ^{1}H-NMR (DMSO-d_6) were consistent with the proposed structure.

Compound 13. N^{α}-Boc-homoglutamic acid γ-Fmo ester (homoglutamic acid (Hgl) = 2-aminoadipic acid) was prepared according to the procedure of Al-Obeidi *et al.*[47] Compound **13**

was synthesized using peptide synthesis method A, cyclization method B, followed by peptide cleavage method A. Analytical; RP-HPLC, t_R 10.47 min; purity, 93% at 214 nm. CZE: t_R 6.04 min; purity, >99% at 214 nm. MS (ES, m/z): 823.8 $[MH]^+$. AAA and ^{1}H-NMR (DMSO-d_6) were consistent with the proposed structure.

Compound 14 was synthesized using peptide synthesis method A, followed by peptide cleavage method A. Analytical; RP-HPLC, t_R 6.78 min; purity, 95% at 214 nm. CZE: t_R 10.03 min; purity, 96% at 214 nm. MS (FAB, m/z): 869.5 $[MH]^+$. AAA and ^{1}H-NMR (DMSO-d_6) were consistent with the proposed structure.

Compound 15 was synthesized using peptide synthesis method A, followed by peptide cleavage method A. Analytical; RP-HPLC, t_R 6.13 min; purity, >99% at 214 nm. CZE: t_R 12.27 min; purity, >99% at 214 nm. MS (FAB, m/z): 842.9 $[MH]^+$. AAA and ^{1}H-NMR (DMSO-d_6) were consistent with the proposed structure.

Compound 16 was synthesized using peptide synthesis method B, peptide cleavage method B, followed by cyclization method D. Analytical; RP-HPLC, t_R 9.73 min; purity, 98% at 214 nm. CZE: t_R 9.46 min; purity, 98% at 214 nm. MS (FAB, m/z): 794.7 $[MH]^+$. AAA and ^{1}H-NMR (DMSO-d_6) were consistent with the proposed structure.

Compound 17 was synthesized using peptide synthesis method B, peptide cleavage method B, followed by cyclization method D. Analytical; RP-HPLC, t_R 9.95 min; purity, 96% at 214 nm. CZE: t_R 13.06 min; purity, 97% at 214 nm. MS (FAB, m/z): 799.7 $[MH]^+$. AAA and ^{1}H-NMR (DMSO-d_6) were consistent with the proposed structure.

Compound 18 was synthesized using peptide synthesis method B, peptide cleavage method B, followed by cyclization method D. Analytical; RP-HPLC, t_R 13.04 min; purity, >95% at 214 nm. CZE: t_R 13.96 min; purity, 95% at 214 nm. MS (ES, m/z): 638.4 $[MH]^+$. AAA was consistent with the proposed structure.

Compound 19 was synthesized using peptide synthesis method A, cyclization method A, followed by peptide cleavage method A. Analytical; RP-HPLC, t_R 9.38 min; purity, 98% at 214 nm. CZE: t_R 7.34 min; purity, 93% at 214 nm. MS (ES, m/z): 823.5 $[MH]^+$. AAA and ^{1}H-NMR (DMSO-d_6) were consistent with the proposed structure.

Compound 20 was synthesized using peptide synthesis method A, cyclization method A, followed by peptide cleavage method A. Analytical; RP-HPLC, t_R 9.43 min; purity, 92% at 214 nm. CZE: t_R 10.30 min; purity, 95% at 214 nm. MS (FAB, m/z): 824.5 $[MH]^+$. AAA and ^{1}H-NMR (DMSO-d_6) were consistent with the proposed structure.

Compound 21 was synthesized using peptide synthesis method A, cyclization method A, followed by peptide cleavage method A. Analytical; RP-HPLC, t_R 9.28 min; purity, >99% at 214 nm. CZE: t_R 9.99 min; purity, 99% at 214 nm. MS (FAB, m/z): 824.5 $[MH]^+$. AAA and ^{1}H-NMR (DMSO-d_6) were consistent with the proposed structure.

Compound 22 was synthesized using peptide synthesis method A, cyclization method A, followed by peptide cleavage method A. Analytical; RP-HPLC, t_R 9.26 min; purity, 95% at 214 nm. CZE: t_R 10.71 min; purity, 93% at 214 nm. MS (FAB, m/z): 824.6 $[MH]^+$. AAA and ^{1}H-NMR (DMSO-d_6) were consistent with the proposed structure.

Compound 23 was synthesized using peptide synthesis method A, peptide cleavage method A, followed by cyclization method C. Analytical; RP-HPLC, t_R 10.69 min; purity, 99% at 214 nm. CZE: t_R 7.10 min; purity, 98% at 214 nm. MS (FAB, m/z): 818.5 $[MH]^+$. AAA and ^{1}H-NMR (D_2O) were consistent with the proposed structure.

Compound 24 was synthesized using peptide synthesis method A, peptide cleavage method A, followed by cyclization method C. Analytical; RP-HPLC, t_R 9.28 min; purity, 99% at 214 nm. CZE: t_R 14.95 min; purity, 98% at 214 nm. MS (ES, m/z): 789.3 $[MH]^+$. AAA and ^{1}H-NMR (DMSO-d_6) were consistent with the proposed structure.

Compound 25 was synthesized using peptide synthesis method A, peptide cleavage method A, followed by cyclization method C. Analytical; RP-HPLC, t_R 9.33 min; purity, 98% at 214 nm. CZE: t_R 6.03 min; purity, 98% at 214 nm. MS (FAB, m/z): 845.6 $[MH]^+$. AAA and ^{1}H-NMR (DMSO-d_6) were consistent with the proposed structure.

Compound 26 was synthesized using peptide synthesis method A, peptide cleavage method A, followed by cyclization method C. Analytical; RP-HPLC, t_R 11.95 min; purity, 98% at 214 nm. CZE: t_R 6.70 min; purity, 99% at 214 nm. MS (FAB, m/z): 931.4 $[MH]^+$. AAA and ^{1}H-NMR (DMSO-d_6) were consistent with the proposed structure.

NMR Measurements for Structural Studies. The NMR spectra were acquired on a Bruker AMX500 spectrometer using 2 mg of peptide dissolved in 0.5 mL of H_2O/5% D_2O. The proton chemical shifts were referenced to tetramethylsilane (TMS) at 0.00 ppm, and the water signal was presaturated during acquisition. Proton assignments were based on the TOCSY[40] experiment acquired into 512 t1 blocks of 2048 t2 data points using a 7 kHz MLEV-17[48] spin lock field applied at the transmitter frequency (ca. 4.76 ppm) for 65 ms. Sequential assignments were based on the rotating frame nuclear Overhauser spectroscopy (ROESY)[49] experiment (512 t1 blocks of 2048 t2 data points). A 4 kHz continuous wave spin-lock field was applied during the 250 ms mixing time at the transmitter frequency. The temperature dependence of the amide protons was determined by acquiring proton spectra at 5° increments between 288 and 308 K. Homonuclear coupling constants were measured directly from the resolution-enhanced proton spectra (Gaussian broadening (GB) = 0.6, line broadening (LB) = −6). Heteronuclear coupling constants were measured from the ^{13}C-edited TOCSY[41] spectra at natural abundance of the ^{13}C nuclei. The pulse sequence includes BIRD type heteronuclear editing to select protons attached to ^{13}C nuclei followed by a conventional TOCSY pulse sequence.

Neurotensin Binding Assay. [^{3}H]NT (107.2 Ci/mmol) or [^{125}I-Tyr^3]NT (2200 Ci/mmol) was obtained from Dupont-New England Nuclear, Boston, MA. Membranes were prepared according to the methods of Mazella *et al.*[50] with minor modifications. Briefly, seven frozen 10-day-old postnatal mouse brains were thawed and the cerebellum removed. The membranes were homogenized in 20 volumes of ice cold 5 mM tris(hydroxymethyl)aminomethane (TRIZMA) buffer, pH 7.5 (buffer A). The homogenates were centrifuged at 100000*g* for 15 min at 4 °C. Resulting pellets were resuspended in the same volume of buffer and recentrifuged as above, twice repeated. Final pellets were resuspended in 1 g wet weight in each milliliter of buffer B (50 mM TRIZMA, pH 7.4, 0.1% bovine serum albumin (BSA), 0.2 mM bacitracin, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenanthroline, 0.3 mM toluenesulfonyl fluoride (PMSF)) and used for the assay.

To label the NT receptor, [^{3}H]NT or [^{125}I-Tyr^3]NT (0.20 nM or 0.21 nM final concentration, respectively) was used. (In general, [^{3}H]NT was used for all the analyses, with the exception of compounds **2**, **20**, and **21** for which [^{125}I-Tyr^3]NT was the radioligand.) The binding assay was slightly modified from that of Kitabgi *et al.*[51] Briefly, 25 μL of [^{3}H]ligand, 25 μL of either drug or buffer, and 200 μL of brain membranes (all diluted with ice cold buffer B) were added to polypropylene microtubes (96-well microplate format; total volume 250 μL). Incubation proceeded for 30 min at 25 °C and was terminated by rapid filtration through Whatman GF/B glass fiber filters which had been presoaked for 1 h in 0.5% polyethylenimine. Radioactivity on the filters was determined on a γ or β counter following the addition of liquid scintillation cocktail. Under these conditions 96% specific binding was observed for [^{125}I-Tyr^3]NT and 90% specific binding for [^{3}H]NT. Nonspecific binding was determined using 1 μM NT. Binding affinities for NT standards were determined from competition curves.

Cellular Calcium Measurements. Changes in $[Ca^{2+}]_i$ were determined with the Ca^{2+}-sensitive dye fura-2/AM and a Proton Technology International Photoscan-2 dual excitation spectrofluorometer. The protocol followed was essentially that of Turner *et al.*[52] Briefly, a suspension of HT-29 cells pelleted by centrifugation at 1000*g* was washed in a balanced salt solution consisting of 130 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 15 mM glucose, 10 mM HEPES, 0.1% BSA, 1.0 mM $MgCl_2$, and 0.2 mM $CaCl_2$ at pH 7.4 (solution 1). The pellet obtained was suspended in a different balanced salt solution

containing 1.0 mM $MgCl_2$ 0.2 mM $CaCl_2$, and 0.1% BSA (solution 2). To load cells, cell suspensions (3×10^6 cells/mL) were incubated with 2 μM fura-2/AM for 40 min at 37 °C in a shaking incubator. After washing and resuspension in solution 2 at the original density, the cell suspensions were further incubated for 40 min to appropriately allow for the maximal de-esterification process. Measurement of autofluorescence was conducted in an equal aliquot of cell suspension taken through the same steps as in the fura-2/AM-treated suspension, but without the dye added. After centrifugation, cells were washed twice in solution 2. The final pellet was resuspended in a balanced salt solution containing 1.0 mM $MgCl_2$ 1.0 mM $CaCl_2$, and 0.1% BSA. Samples were temporarily stored in a water bath maintained at 37 °C which was placed on top of a stir plate. For $[Ca^{2+}]$ measurements, 1 mL aliquots of fura-2/AM-loaded cells were transferred to quartz cuvettes and the latter placed in the spectrophotometer to establish a stable baseline. Compounds were then added to the cell suspension. A magnetic stir bar was always placed in each cuvette to allow proper mixing of compounds and to prevent cells form settling to the bottom.

The $[Ca^{2+}]_i$ concentration was determined using the Proton Technology International Deltascan II spectrofluorometer by measuring the ratio of fluorescence excited by 340 nm to that excited by 380 nm (emission was at 505–510 nm). This was calibrated using the equation developed by Grynkiewicz *et al.*[53]: $[Ca^{2+}]_i = K_D Sf_2/Sb_2(R - R_{min}/R_{max} - R)$, where K_D = 224 nM, Sf_2 is the signal of the free fura-2/AM at 380 nm, Sb_2 is the signal of the bound fura-2/AM at 380 nm, and R is the ratio of the fluorescence. Values for R_{min} were obtained from measurements with a final concentration of 500 nM EGTA (pH balanced to 7.4 with TRIZMA base) added to the cuvette containing a 2 mL sample of cells; while R_{max} was determined in a different sample of cells after addition of 0.1% Triton-X. The 340 and 380 nm values for a sample of unloaded cells were determined to correct for tissue or background autofluorescence. Percent of maximal calcium mobilized for each compound was measured and the EC_{50} values determined.

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Supplementary Material Available: A table of the amino acid analyses and ^{1}H-NMR spectra (except for compound **18**) and mass spectra for compounds **2–26** (50 pages). Ordering information is given on any current masthead page.

References

(1) Symbols and abbreviations are in accordance with the recommendation of the IUPAC–IUB Commission on Biochemical Nomenclature (*Eur. J. Biochem.* **1984**, *138*, 9–37). The optically active amino acids are of the L-stereochemistry unless otherwise noted. Other abbreviations included: AAA, amino acid analysis; ABI, Applied Biosystems Incorporated; Ac_2O, acetic anhydride; *t*-Boc, *tert*-butyloxycarbonyl; BOP, (benzotriazol-1-yloxy)tris-(dimethylamino)phosphonium hexafluorophosphate; BSA, bovine serum albumin; CZE, capillary zone electrophoresis; DCC, *N,N'*-dicyclohexylcarbodiimide; DCM, dichloromethane; DIC, *N,N'*-diisopropylcarbodiimide; DIEA, diisopropylethylamine; DMAP, 4-(dimethylamino)pyridine; DMF, *N,N*-dimethylformamide; DMSO, dimethyl sulfoxide; D_2O, deuterium oxide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether); ES, electrospray; FAB, fast atom bombardment; Fmo, 9-fluorenylmethyl; Fmoc, 9-fluorenylmethoxycarbonyl; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid); HF, hydrogen fluoride; Hgl, homoglutamic acid or 2-aminoadipic acid; ^{1}H-NMR, proton nuclear magnetic resonance; HOBt, *N*-hydroxybenzotriazole; N$^{\alpha}$MeArg, *N* $^{\alpha}$-methylarginine; MS, mass spectrometry; NMP, *N*-methylpyrrolidone; NOE, nuclear Overhauser effect, NT, neurotensin (pGlu^1-Leu-Tyr-Glu^4-Asn-Lys-Pro-Arg^8-Arg-Pro-Tyr-Ile-Leu^{13}); NT_{8-13}, Arg^8-Arg-Pro-Tyr-Ile-Leu^{13}; PAM, [(phenylacetyl)amino]methyl; Pen, penicillamine or β,β-dimethylcysteine; pGlu, pyroglutamic acid or 2-pyrrolidone-5-carboxylic acid; PMSF, phenylmethylsulfonyl fluoride; Sasrin resin, super acid sensitive resin or 2-methoxy-4-alkoxybenzyl alcohol resin; ROESY, rotating frame nuclear Overhauser spectroscopy; RP-HPLC, reversed-phase high-performance liquid chromatography; SAR, structure–activity relationships; TFA, trifluoroacetic acid; Tle, *tert*-leucine; TMS, tetramethylsilane; TOCSY, total correlation spectroscopy; TRIZMA, tris(hydroxymethyl)aminomethane.

(2) Carraway, R.; Leeman, S. E. The isolation of a new hypotensive peptide, neurotensin, from bovine hypothalami. *J. Biol. Chem.* **1973**, *248*, 6854–6861.

(3) Clineschmidt, B. V.; McGuffin, J. C.; Bunting, P. B. Neurotensin: Antinociceptive action in rodents. *Eur. J. Pharmacol.* **1979**, *54*, 129–139.

(4) St-Pierre, S. A.; Kérouac, R.; Quirion, R.; Jolicouer, F. B.; Rioux, F. Neurotensin. In *Peptide Protein Review*; Hearn, M. T. W., Ed.; Marcel Dekker Inc.: New York, 1984; Vol. 2, pp 83–171.

(5) Nemeroff, C. B.; Cain, S. T. Neurotensin-dopamine interactions in the CNS. *Trends Pharmacol. Sci.* **1985**, *6*, 201–205.

(6) Davis, M. D.; Nemeroff, C. B. Neurotensin, dopamine and schizophrenia. In *Receptors and Ligands in Psychiatry*; Sen, A. K., Lee, T., Eds.; Cambridge University Press: Cambridge, 1988; pp 167–186.

(7) Araki, K.; Tachibana, S.; Uchiyama, M.; Nakajima, T.; Yasuhara, T. Isolation and structure of a new active peptide "xenopsin" on the smooth muscle, especially on a strip of fundus from a rat stomach, from the skin of *Xenopus Laevis*. *Chem. Pharm. Bull.* **1973**, *21*, 2801–2804.

(8) Minamino, N.; Kangawa, K.; Matsuo, H. Neuromedin N: A novel neurotensin-like peptide identified in porcine spinal cord. *Biochem. Biophys. Res. Commun.* **1984**, *122*, 542–549.

(9) Checler, F.; Vincent, J. P.; Kitabgi, P. Neuromedin N: High affinity interaction with brain neurotensin receptors and rapid inactivation by brain synaptic peptidases. *Eur. J. Pharmacol.* **1986**, *126*, 239–244.

(10) Checler, F.; Labbé, C.; Granier, C.; van Rietschoten, J.; Kitabgi, P.; Vincent, J. P. [Trp^{11}]-Neurotensin and xenopsin discriminate between rat and guinea-pig neurotensin receptors. *Life Sci.* **1982**, *31*, 1145–1150.

(11) Carraway, R. E.; Leeman, S. E. The synthesis of neurotensin. *J. Biol. Chem.* **1975**, *250*, 1912–1918.

(12) Folkers, K.; Chang, D.; Humphries, J.; Carraway, R. E.; Leeman, S. E.; Bowers, C. Y. Synthesis and activities of neurotensin and its acid and amide analogues: Possible natural occurance of [Gln^4]-neurotensin. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 3833–3837.

(13) Rivier, J. E.; Lazarus, L. H.; Perrin, M. H.; Brown, M. R. Neurotensin analogues. stucture-activity relationships. *J. Med. Chem.* **1977**, *20*, 1409–1413.

(14) Lazarus, L. H.; Perrin, M. H.; Brown, M. R.; Rivier, J. E. Verification of both the sequence and conformational specificity of neurotensin in binding to mast cells. *Biochem. Biophys. Res. Commun.* **1977**, *76*, 1079–1085. (b) Lazarus, L. H.; Perrin, M. H.; Brown, M. R.; Rivier, J. E. Mast cell binding of neurotensin. II. Molecular conformation of neurotensin involved in the stereospecific binding to mast cell receptor sites. *J. Biol. Chem.* **1977**, *252*, 7180–7183.

(15) St-Pierre, S.; Lalonde, J. M.; Gendreau, M.; Quirion, R.; Regoli, D.; Rioux, F. Synthesis of peptides by the solid-phase method. 6. Neurotensin, fragments, and analogues. *J. Med. Chem.* **1981**, *24*, 370–376.

(16) Bayer, V. V.; Miroshnikov, A. I.; Klusa, V. I. Misina, I. P. Synthesis and biological activity of neurotensin and its analogues. *Bioorg. Khim.* **1980**, *6*, 379–385.

(17) Quirion, R.; Rioux, F.; Regoli, D.; St-Pierre, S. Pharmacological studies of neurotensin, several fragments and analogues in the isolated perfused rat heart. *Eur. J. Pharmacol.* **1980**, *66*, 257–266.

(18) Kitabgi, P.; Poustis, C.; Granier, C.; van Rietschoten, J.; Rivier, J.; Morgat, J. L.; Freychet, P. Neurotensin binding to extraneural and neural receptors: Comparison with biological activity and structure–actvity relationships. *Mol. Pharmacol.* **1980**, *18*, 11–19.

(19) (a) Jolicoeur, F. B.; Rioux, F.; St-Pierre, S.; Barbeau, A. Structure–activity studies of neurotensin's neurobehavioral effects. *Neuroendocrinol. Lett.* **1981**, *3*, 77–84. (b) Jolicoeur, F. B.; St-Pierre, S.: Aubé, Rivest, R.; Gagné, M. A. Relationships between structure and duration of neurotensin's central action: Emergence of long acting analogues. *Neuropeptides* **1984**, *4*, 467–476.

(20) Clineschmidt, B. V.; Martin, E. E.; Veber, D. F. Antinocisponsive effects of neurotensin and neurotensin-related peptides. *Ann. N. Y. Acad. Sci.* **1982**, *400*, 283–306.

(21) Lugrin, D.; Vecchini, F.; Doulut, S.; Rodriguez, M.; Martinez, J.; Kitabgi, P. Reduced peptide bond pseudopeptide analogues of neurotensin: binding and biological activities, and in vitro metabolic stability. *Eur. J. Pharmacol.* **1991**, *205*, 191–198.

(22) Dubuc, I.; Costentin, J.; Doulut, S.; Rodriguez, M.; Martinez, J.; Kitabgi, P. JMV 449: A pseudopeptide analogue of neurotensin-(8–13) with highly potent and long-lasting hypothermic and analgesic effects in the mouse. *Eur. J. Pharmacol.* **1992**, *219*, 327–329.

(23) Doulut, S.; Rodriguez, M.; Lugrin, D.; Vecchini, F.; Kitabgi, P.; Aumelas, A.; Martinez, J. Reduced peptide bond pseudopeptide analogues of neurotensin. *Pept. Res.* **1992**, *5*, 30–38.
(24) Couder, J.; Tourwé D.; van Binst, G.; Schuurkens, J.; Leysen, J. E. Synthesis and biological activities of Ψ(CH_2NH) pseudopeptide analogues of the C-terminal hexapeptide of neurotensin. *Int. J. Peptide Res.* **1993**, *41*, 181–184.
(25) (a) Tsuchiya, Y.; Sasaki, A.; Yoshino, H.; Karibe, N.; Sugimoto, H.; Kubota, A.; Kosasa, M.; Araki, S.; Ikeda, M.; Yamanishi, Y.; Machida, R.; Yamatsu, K. New hexapeptide neurotensin analogues useful as cerebral medicaments, antipsychotic agents, analgesics, and methamphetamine antagonists. European Patent Application 89104302.8, 1989. (b) Machida, R.; Tokumura, T.; Sasaki, A.; Tsuchiya, Y.; Abe, K. High-performance liquid chromatographic determination of (Me)Arg-Lys-Pro-Trp-*tert*.-Leu-Leu in plasma. *J. Chromatogr.* **1990**, *534*, 190–195. (c) Tokumura, T.; Tanaka, T.; Sasaki, A.; Tsuchiya, Y.; Abe, K.; Machida, R. Stability of a novel hexapeptide, (Me)Arg-Lys-Pro-Trp-*tert*-Leu-Leu-OEt, with neurotensin activity, in aqueous solution and in the solid state. *Chem. Pharm. Bull.* **1990**, *38*, 3094–3098.
(26) Cooke, L. W.; Wustrow, D. J.; Pugsley, T. A.; Akunne, H. C.; Heffner, T. G.; Cody, W. L.; Davis, M. D. Effect of systemic and central administration of natural and novel neurotensin analogues on behavior and neurotransmitter turnover in the rodent brain. *Soc. Neurosci. Abstr.* **1992**, *18*, 273.
(27) Heyl, D. L.; Sefler, A. M.; He, J. X.; Sawyer, T. K.; Wustrow, D. J.; Akunne, H. C.; Davis, M. D.; Pugsley, T. A.; Heffner, T. G.; Corbin, A. E.; Cody, W. L. Structure–activity and conformational studies of a series of modified C-terminal hexapeptide neurotensin analogues. *Int. J. Pept. Protein Res.* **1994**, *44*, 233–238.
(28) Widerlöv, E.; Lindström, L. H.; Besev, G.; Manberg, P. J.; Nemeroff, C. B. Breese, G. R.; Kizer, J. S.; Prange, A. J., Jr. Subnormal CSF levels of neurotensin in a subgroup of schizophrenic patients: Normalization after neuroleptic treatment. *Am. J. Psychiatry* **1982**, *139*, 1122–1126.
(29) Nemeroff, C. B.; Luttinger, D.; Hernandez, D. E.; Mailman, R. B.; Mason, G. A.; Davis, S. D.; Widerlöv, E.; Frye, G. D.; Kilts, C. A.; Beaumont, K.; Breese, G. R.; Prange, A. J. Jr. Interactions of neurotensin with brain dopamine systems: Biochemical and behavioral studies. *J. Pharmacol. Exp. Ther.* **1983**, *225*, 337–345.
(30) Garver, D. L.; Bissette, G.; Yao, J. K.; Nemeroff, C. B. Relation of CSF neurotensin concentrations to symptoms and drug response of psychotic patients. *Am. J. Psychiatry* **1991**, *148*, 484–488.
(31) al-Rodhan, N. R.; Richelson, E.; Gilbert, J. A.; McCormick, D. J.; Kanba, K. S.; Pfenning, M. A.; Nelson, A.; Larson, E. W.; Yaksh, T. L. Structure-antinociceptive activity of neurotensin and some novel analogues in the periaqueductal gray region of the brainstem. *Brain Res.* **1991**, *557*, 227–235.
(32) Xu, G. Y.; Deber, C. M. Conformations of neurotensin in solution and in membrane environments studied by 2-D NMR spectroscopy. *Int. J. Pept. Protein Res.* **1991**, *37*, 528–535.
(33) (a) García-López, M. T.; Domínguez, M. J.; González-Muñiz, R.; Herranz, R.; Johansen, N. L.; Madsen, K.; Suzdak, P.; Thøgersen, H. β-Turn constrained analogues of neurotensin(8–13) containing a 3-oxoindolizidine skeleton. In *Peptides 1992: Proceedings of the Twenty-Second European Peptide Symposium*; Schneider, C. H., Eberle, A. N., Eds.; ESCOM Science Publishers B.V.: Leiden, The Netherlands, 1993; pp 623–624. (b) García-López, M. T.; Domínguez, M. J.; González-Muñiz, R.; Herranz, R.; Johansen, N. L.; Madsen, K.; Suzdak, P.; Thøgersen, H. Novel 3-oxoindolizidine containing neurotensin (8–13) analogues. In *Program and Abstracts: The Thirteenth American Peptide Symposium*, Edmonton, Canada, 1993; p 212.
(34) Maduskuie, T. P., Jr.; Bleicher, L. S.; Cacciola, J.; Cheatham, W.; Fevig, J. M.; Johnson, A. L.; McComb, S. A.; Nugiel, D. A.; Schimdt, W. K.; Spellmeyer, D. A.; Voss, M. E.; Wagner, R. M. The design and synthesis of a peptidomimetic for neurotensin. *J. Cell. Biochem.* **1993**, *51* (Suppl. 17C), 232.
(35) Johansen, N. L.; Thøgersen H.; Madsen, K.; Suzdak, P.; Hansen, K. T.; Weis, J. U.; García-López; M. T.; Gomez-Monterrey, I.; González-Muniz, R.; Herranz, R. Receptor specific neurotensin peptide mimics. In *Program and Abstracts: The Thirteenth American Peptide Symposium*, Edmonton, Canada, 1993; p 216.
(36) Henry, J. A.; Horwell, D. C.; Meecham, K. G.; Rees, D. C. A structure-affinity study of the amino acid side-chains in neurotensin: N and C terminal deletions and Ala-scan. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 949–952.
(37) (a) Schiller, P. W.; Nguyen, T. M.; Miller, J. Synthesis of side-chain to side-chain cyclized peptide analogues on solid supports. *Int. J. Pept. Protein Res.* **1985**, *25*, 171–177. (b) Al-Obeidi, F.; Castrucci, A. M. de L.; Hadley, M. E.; Hruby, V. J. Potent and prolonged acting cyclic lactam analogues of α-melanotropin: Design based upon molecular dynamics. *J. Med. Chem.* **1989**, *32*, 2555–2561.
(38) Felix, A. M.; Wang, C. T.; Heimer, E. P.; Fournier, A. Applications of BOP reagent in solid phase synthesis. II. Solid phase side-chain to side-chain cyclizations using BOP reagent. *Int. J. Pept. Protein Res.* **1988**, *31*, 231–238.
(39) Cody, W. L.; He, J. X.; Heyl, D. L.; Thieme-Sefler, A. M.; Wustrow, D. J.; Sawyer, T. K.; Akunne, H.; Pugsley T. A.; Corbin, A. E.; Davis, M. D. Neurotensin: Structure–activity relationships of the C-terminal hexapeptide (Arg-Arg-Pro-Tyr-Ile-Leu). In *Peptides 1992: Proceedings of the Twenty-Second European Peptide Symposium*; Schneider, C. H., Eberle, A. N., Eds.; ESCOM Science Publishers B.V.: Leiden, The Netherlands, 1993; pp 677–678.
(40) Braunschweiler, L.; Ernst, R. R. Coherence transfer by isotropic mixing: Application to proton correlation spectroscopy. *J. Magn. Reson.* **1983**, *53*, 521–528.
(41) (a) Wagner, G. NMR investigations of protein structures. *Prog. Nucl. Magn. Reson. Spectrosc.* **1991**, *22*, 101–140. (b) Reily, M. D.; Thanabal, V.; Lunney, E. A.; Repine, J. T.; Humblet, C. C.; Wagner, G. Design, synthesis and solution structure of a renin inhibitor. *FEBS Lett.* **1992**, *302*, 97–103.
(42) (a) Stewart, J. M.; Young, J. D. *Solid Phase Peptide Synthesis, 2nd ed.*; Pierce Chemical Co.: Rockford, IL, 1984. (b) Fields, G. B.; Noble, R. L. Solid phase peptide synthesis utilizing 9-fluorenylmethoxycarbonyl amino acids. *Int. J. Pept. Protein Res.* **1990**, *35*, 161–214.
(43) (a) Mergler, M.; Tanner, R.; Gosteli, J. Grogg, P. Peptide synthesis by a combination of solid-phase and solution methods I: A new very acid-labile anchor group for the solid phase synthesis of fully protected fragments. *Tetrahedron Lett.* **1988**, *29*, 4005–4008. (b) Mergler, M.; Nyfeler, R.; Tanner, R.; Gosteli, J. Grogg, P. Peptide synthesis by a combination of solid-phase and solution methods II: Synthesis of fully protected peptide fragments of 2-methoxy-4-alkoxy-benzyl alcohol resin. *Tetrahedron Lett.* **1988**, *29*, 4009–4012.
(44) (a) Knorr, R.; Trzeciak, A.; Bannwarth, W.; Gillessen, D. New coupling reagents in peptide chemistry. *Tetrahedron Lett.* **1989**, *30*, 1927–1930. (b) Beck-Sickinger, A. G.; Dürr, H.; Jung, G. Semiautomated T-bag peptide synthesis using 9-fluorenylmethoxycarbonyl strategy and benzotriazol-1-yl-tetramethyluronium tetrafluoroborate activation. *Pept. Res.* **1991**, *4*, 88–94. (c) Fields, C. G.; Lloyd, D. H.; Macdonald, R. L.; Otteson, K. M.; Noble, R. L. HBTU activation for automated Fmoc solid-phase peptide synthesis. *Pept. Res.* **1991**, *4*, 95–101. (d) Reid, G. E.; Simpson, R. J. Automated solid-phase peptide synthesis: Use of 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate for coupling of *tert*-butyloxycarbonyl amino acids. *Anal. Biochem.* **1992**, *200*, 301–309.
(45) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. *Anal. Biochem.* **1970**, *34*, 595–598.
(46) (a) Castro, B.; Dormoy, J. R.; Evin, G.; Selve, C. Reactifs de couplage peptidique IV. (1)-L' hexafluorophosphate de benzotriazolyl N-oxytrisdimethylamino phosphonium (B. O. P.). (Peptide coupling reagents. IV. N-[Oxytris(dimethylamino)phosphonium]benzotriazole hexafluorophosate.) *Tetrahedron Lett.* **1975**, *14*, 1219–1222. (b) Hudson, D. Methodological implications of simultaneous solid-phase peptide synthesis. 1. Comparison of different coupling procedures. *J. Org. Chem.* **1988**, 617–624. (c) Fournier, A.; Wang, C. T.; Felix, A. M. Applications of BOP reagent in solid phase synthesis. Advantages of BOP reagent for difficult couplings exemplified by a synthesis of [Ala^{15}]-GRF-(1–29)-NH_2. *Int. J. Pept. Protein Res.* **1988**, *31*, 86–97.
(47) Al-Obeidi, F.; Sanderson, D. G.; Hruby, V. J. Synthesis of β- and γ-fluorenylmethyl esters of respectively N^{α}-Boc-L-aspartic acid and N^{α}-Boc-L-glutamic acid. *Int. J. Pept. Protein Res.* **1990**, *35*, 215–218.
(48) Bax, A.; Davis, D. G. MLEV-17-based two-dimensional homonuclear magnetization transfer spectroscopy. *J. Magn. Reson.* **1985**, *65*, 355–360.
(49) Bothner-By, A. A.; Stephens, R. L.; Lee, J.; Warren, C. D.; Jeanloz, R. W. Structure determination of a tetrasaccharide: Transient nuclear Overhauser effects in the rotating frame. *J. Am. Chem. Soc.* **1984**, *106*, 811–813.
(50) Mazella, J.; Chabry, J.; Kitabgi, P.; Vincent, J. Solubilization and characterization of active neurotensin receptors from mouse brain. *J. Biol. Chem.* **1988**, *263*, 144–149.
(51) Kitabgi, P.; Rostene, W.; Dussaillant, M.; Schotte, A.; Laduron, P.; Vincent, J. Two populations of neurotensin binding sites in murine brain: Discrimination by the antihistamine levocabastine reveals markedly different radioautographic distribution. *Eur. J. Pharmacol.* **1987**, *140*, 285–293.
(52) Turner, J. T.; James-Krcake, M. R.; Camden, J. M. Regulation of the neurotensin receptor and intracellular calcium mobilization in HT29 cells. *J. Pharmacol. Exp. Ther.* **1989**, *253*, 1049–1056.
(53) Grynkiewicz, G.; Poenie, M.; Tsien, R. Y. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **1985**, *260*, 3440–3450.

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