Neurotensin Analogues. Structure-Activity Relationships

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A series of neurotensin (NT) analogues in which each amino acid has been successively replaced by its D isomer, as well as analogues involving modifications at positions 3 and 11 and a cyclic compound [Cys^{2,13}]-NT, has been synthesized by solid-phase methodology. After purification by conventional techniques the compounds were characterized by thin-layer chromatography, amino acid analysis, and optical rotation. Further characterization of the analogues by high-pressure liquid chromatography demonstrates the high resolving power of this new method. Each analogue was studied for its ability to induce hypothermia in cold-exposed rats (4 °C) in vivo and to bind to mast cells in vitro. Although close correlation in potencies was not found for all the analogues tested in both assay systems, they substantiate the basic observation that substitutions in positions 1–9 produced active peptides whereas modification of residues 10–13 considerably decreased biological response in vitro and in vivo. One exception is the higher potency of [D-Phe^{11}]-NT and [D-Tyr^{11}]-NT in vivo. The differences between the efficacies of these analogues in vivo and in vitro are discussed.

Neurotensin (NT)[1] is a tridecapeptide whose sequence is pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH. It was originally isolated and characterized from bovine hypothalamus.[2] Recent studies have demonstrated NT or NT-like activity in canine, bovine, porcine, and human gut.[3-6] NT has a wide spectrum of pharmacologic actions including production of hypotension, gut contraction, increased vascular permeability, hemoconcentration, hyperglycemia, and hyperglucagonemia.[2a,5,7-9] NT also stimulates the secretion of growth hormone and prolactin by a brain-dependent mechanism.[10] Of considerable interest has been the recent observation that NT induces hypothermia after its intracisternal administration to cold-exposed mice or rats.[11,12] Since NT has been demonstrated to be distributed throughout the central nervous system with high levels in the hypothalamus,[13-15] the hypothermic actions of this peptide may suggest that it plays some role in thermoregulation. To elucidate the specificity of NT and to exploit its use to study the various physiologic parameters that it affects, it has been of interest to develop structural analogues of NT. A number of them have already been reported by Carraway and Leeman[16] and Folkers et al.[17] Those are (a) short chain analogues which demonstrated that the hexapeptide C-terminal contained all the necessary information to trigger a biological response and (b) amidated analogues at position 4 (γ-Glu-COOH) and 13 (Leu-OH) which indicated that replacement of Glu^4 by Gln^4 did not alter the biological potency of the analogues, whereas amidation of the C terminus yielded an analogue with low biological potency.

It is known that introduction of D-amino acids in a sequence can lead to longer acting ([D-Trp^6]-LRF[18]) and/or more potent analogues ([D-Arg^8]vasopressin,[19] [D-Trp^8]-somatostatin[20]), perhaps either through resistance to enzymatic degradation and/or better binding. Partial agonists have also been developed by introduction of a D-amino acid in LRF ([D-Phe^2]-LRF).[21] We were thus interested in systematically substituting each amino acid of the NT molecule by its corresponding D isomer and testing the peptides for their hypothermic action. The binding properties of these analogues to mast cells have been described in detail elsewhere.[22] Potency values in those systems are included in Table I. The observation by N. Marks (private communication) that NT might be enzymatically degraded by a carboxypeptidase led us to synthesize NT *N*-methylamide. A model presented by Carraway and Leeman[16] suggesting that the tertiary structure of NT might have a U-shape led us to synthesize [Cys^{2,13}]-NT. During the synthesis of these analogues we were particularly aware of the fact that any contamination by the L isomer of any D-amino acid substituents would yield NT itself as a contaminant. Our newly developed systems[23] for high-pressure liquid chromatography (HPLC) allowed us to quantitate, in most instances, the amount of NT present, if any, in the different preparations.

Table I. Relative Potency Values of NT and NT Analogues to Lower Body Temperature of Cold-Exposed (4 °C) Rats or to Bind Specifically to Mast Cells

No.	Compd	Potency rel to NT[a]	Binding affinity to mast cells rel to NT[b]
1	Neurotensin (NT)	100	100
2	[D-pGlu^1]-NT	100	120
3	[D-Leu^2]-NT	250	120
4	[D-Tyr^3]-NT	200	60
5	[D-Glu^4]-NT	300	100
6	[D-Asn^5]-NT	100	100
7	[D-Lys^6]-NT	110	100
8	[D-Pro^7]-NT	100	100
9	[D-Arg^8]-NT	100	500
10	[D-Arg^9]-NT	50	640
11	[D-Pro^{10}]-NT	<1	12
12	[D-Tyr^{11}]-NT	1000	9.5
13	[D-Ileu^{12}]-NT	20	2
14	[D-Leu^{13}]-NT	<1	30
15	NT-(8-13) hexapeptide	25	67
16	NT-(9-13) pentapeptide	20	76
17	[Phe^3]-NT	100	102
18	[Phe^{11}]-NT	100	100
19	NT-NHMe	<1	0.26
20	[D-Lys^{11}]-NT	<1	3.4
21	[D-Leu^{11}]-NT	30	
22	[D-Phe^{11}]-NT	1000	0.64
23	[Cys^{2,13}]-NT	100	3.4

[a] Relative potency values of neurotensin (NT) and analogues of NT to lower body temperature of cold-exposed rats 60 min following intracisternal administration of peptide. Potency values were calculated using the four- or six-point parallel line bioassay method. 95% confidence limits are of the order of ±10%. [b] See ref 22.

Synthesis, Purification, and Characterization. The NT analogues reported in Table I were synthesized by the solid-phase method previously described for somatostatin[24] and its analogues.[25] Boc-Leu or Boc-D-Leu was esterified to the chloromethylated resin by a modification of Monahan and Gilon's procedure.[20,26] The stepwise buildup of the peptide on the resin was done using standard procedures previously reported, with the exception that all couplings but those of Boc-Arg(Tos), Boc-Gln-PNP, and

Table II. Physical Constants, Yield, and HPLC Results of NT Analogues

No.	TLC system[a] BAW	BPyA	2-BA	2-BW	$[\alpha]^{22}_D$,[b] deg	Yield,[c] %	HPLC,[d] RT (min)	HPLC,[e] % impurities (RT)	Remarks[f]
1	0.37	0.37	0.25	0.20	−92.5	25	8.9	<2	
2	0.39	0.46	0.25	0.23	−89.5	10	6.3	<2	No NT
3	0.38	0.41	0.26	0.25	−73.0	21	12.3	<2	No NT
4	0.38	0.41	0.25	0.24	−85.5	28	11.0	<2	No NT
5	0.38	0.38	0.24	0.18	−79.5	18	9.6	<2	Uncertain
6	0.37	0.35	0.24	0.15	−78.5	25	9.1	<2	Uncertain
7	0.36	0.36	0.24	0.16	−71.0	10	8.9	<2	No NT
8	0.36	0.36	0.25	0.17	−50.5	18	9.9	<5	Possibly 1%
9	0.37	0.36	0.25	0.19	−63.0	21	10.0	<2	No NT
10	0.39	0.41	0.26	0.23	−70.0	22	10.5	<2	Uncertain
11	0.38	0.46	0.27	0.25	−54.5	27	10.6	4 (12.1)	No NT
12	0.37	0.47	0.29	0.26	−84.5	30	11.7	<2	No NT
13	0.40	0.47	0.29	0.27	−82.0	30	10.9	<2	No NT
14	0.38	0.45	0.27	0.26	−84.0	27	15.7	7 (7.2)	Possibly 1%
15	0.40	0.29	0.23	0.17	−57.0	25	8.2	<4	20% CH_3CN
16	0.50	0.44	0.31	0.27	−60.0	25	10.4	<2	20% CH_3CN
17	0.40	0.49	0.46	0.27	−90.0	24	4.9	3 (3.8)	33% CH_3CN
18	0.38	0.41	0.27	0.25	−95.0	22	9.7	<2	28% CH_3CN
19	0.38	0.45	0.28	0.27	−87.0	20	12.7	20 (10.6)	25% CH_3CN
20	0.22	0.19	0.08	0.04	−75.0	25	6.4	<2	20% CH_3CN
21	0.41	0.48	0.31	0.30	−74.0	15	5.2	<4	35% CH_3CN
22	0.40	0.47	0.30	0.30	−82.5	27	5.3	4 (4.4)	36% CH_3CN
23	0.22	0.26	<0.10	<0.10	−63.5	21	5.3	<2	20% CH_3CN

[a] BAW, 1-butanol-acetic acid-water (4:1:5, upper phase): BPyA, 1-butanol-pyridine-0.1 M acetic acid (5:3:11, upper phase); 2-BA, 2-butanol-0.1 M acetic acid (1:1, upper phase); 2-BW, 2-butanol-water (1:1, upper phase); I_2, ninhydrin spray, and Pauly reagent were successively used. Loads varied from 20 to 40 μg per spot. Under those conditions, very closely related impurities in the amount of up to 5% would not be detected. [b] Concentration in 1% AcOH = 1. [c] Peptide yields are calculated on the basis of millimoles of peptides isolated after final purification relative to the total millimoles of starting *tert*-butyloxycarbonylamino acid, viz., as resin ester. Yields were not optimized. [d] HPLC were run using 25% acetonitrile in 0.01 M NH_4OAc, buffer pH 4.5, in an isocratic manner unless indicated otherwise in the column headed "Remarks". [e] In most cases the impurities could be shown to be different from NT; their retention time is indicated in parentheses. [f] No NT indicates that the mixture of NT plus the reported analogue was run together in an equimolar amount and could be separated well enough so that NT if present ≥1% could have been detected in the analogue's preparation when run by itself under the same conditions. Uncertain indicates that even though retention times of NT and analogues were different (generally less than 1 min), when run by themselves equimolar amounts would not resolve. Possibly *x*% indicates that a peak with the retention time of NT could be detected in the proximate area.

Boc-Asn(xanthyl),[27] for which dimethylformamide (DMF) was used, were carried out in CH_2Cl_2. Glutamic acid at the 4 position was introduced as the protected Boc-Glu-(γ-Bzl). Boc-D- or -L-lysine (ε-2ClZ)[28] was coupled at the 6 position. For tyrosine, the 2,6-Cl_2Bzl protecting group[29] was used. D-pGlu[1] was produced by cyclization of the corresponding D-glutamine derivative,[30] itself introduced as the *tert*-butyloxycarbonylglutamine PNP derivative in DMF. pGlu was otherwise introduced as the Z-pGlu derivative since this analogue is more soluble in organic solvents. Neurotensin *N*-methylamide (NT-NHMe) was made on an *N*-methylbenzhydrylamine resin, the synthesis of which will be described elsewhere (J. Rivier, in preparation). [$Cys^{2,13}$]-NT was synthesized using a methodology developed for somatostatin analogues.[25]

After cleavage and deprotection, the peptides were purified by ion-exchange chromatography on carboxymethylcellulose (CMC) and partition chromatography on Sephadex G-25F. R_f values on TLC in four different solvent systems, optical rotation, yields, and percent contamination as measured by HPLC[23] are reported in Table II. Amino acid analysis of peptide hydrolysates is consistent with the desired sequences.

Biological Test and Discussion. The biological test used to evaluate the relative potency of each of the NT analogues reported in Table I gives an indication of the ability of the peptides to lower the body temperature of cold-exposed rats (4 °C). Potency values were calculated by four- and six-point bioassays. A typical graphical representation is shown in Figure 1. Affinities of NT and NT analogues to mast cells are also included in Table I. The high specificity of this test[31] as well as a discussion

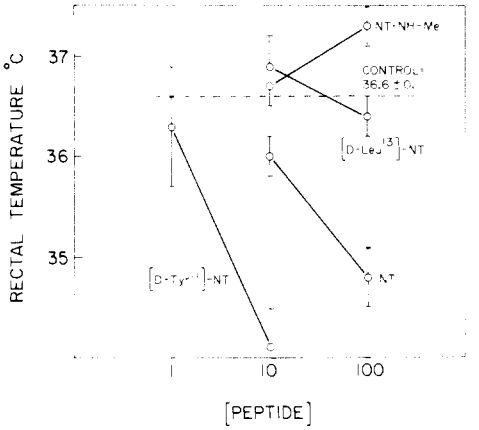

Figure 1. Dose–response of neurotensin (NT, μg) and NT analogues to lower body temperature of cold-exposed rats 60 min following intracisternal administration of peptide. Each point represents four animals.

of structure–activity relationships based on these data has been reported separately.[22,36]

The high biological potency in vivo of analogues **2**–**10**, the lower but significant potency of **13**, **15**, and **16**, and relative inactivity of **11** and **14** are consistent with Carraway's observations[16] that, whereas the NH_2-terminal partial sequences of NT as large as the decapeptide (NT_{1-10}) were found to be ineffectual, COOH-terminal partial sequences of five or more amino acids in length induced hypotension, hyperglycemia, increased cutaneous vascular permeability, and contracted the isolated guinea pig ileum.

The much higher biological potency of [D-Tyr[11]]-NT, however, was unexpected and could be explained in terms of stabilization of its tertiary structure and/or resistance to enzymatic degradation. The requirement for an aromatic amino acid in position 11 for full biological activity of NT is supported by the observation that [D-Leu[11]]-NT (aliphatic) and [D-Lys[11]]-NT (basic) are inactive while [Phe[11]]-NT and [D-Phe[11]]-NT are as potent and ten times as potent as NT, respectively.

In agreement with Folkers et al.,[17] who amidated the NT COOH terminal, we found that N-methylamidation of Leu[13] rendered the molecule almost inactive.

To test Carraway's hypothesis[16] that NT could interact with the receptor while its backbone was U-shaped, allowing the possible hydrogen bonding between amino acids of the N and C termini, we synthesized [Cys[2,13]]-NT which was found as potent as NT in the induction of hypothermia. The fact that this molecule was found inactive in the mast cell binding assay when cyclized but fully active after treatment with dithiothreitol[37] would indicate that the linear form is the active form. It is easily conceivable that **23** could be reduced in vivo.

Similarly, [Cys[1,8]]angiotensin II (J. Rivier and T. Paiva, unpublished results) and several analogues of LRF ([Cys[4,7]]-, [Cys[4,8]]-, [Cys[4,9]]-, and [Cys[4,10]]-LRF, J. Rivier and W. Vale, unpublished results) have been found to be inactive in the proper respective biological tests probably because of drastic alterations of the peptide side chain. None of the inactive analogues reported in Table I was tested for possible competitive antagonism in vivo.

If one compares the biological potencies obtained in vivo and in vitro, one sees a fairly good correlation for the modifications at most positions. Alterations at position 8, 9, and 11, however, give drastically different values for which we have no good explanation. One should realize, however, that the mechanism by which body temperature is controlled is not known and most probably does not involve interaction of a substrate with mast cells.

Experimental Section

Synthesis. Chloromethylated polystyrene resin cross-linked with 1% divinylbenzene was obtained from Lab. Systems, Inc. (LS 601, 0.90 mM/g as Cl). Esterification of Boc-Leu·H_2O or Boc-D-Leu·H_2O to the resin was performed as for α-Boc-Cys(SpOMe-Bzl) already described in detail.[20,25,26] Substitution varied from 0.35 to 0.50 mequiv/g of resin. For the synthesis of NT *N*-methylamide, an N-methylated derivative of the benzyhydrylamine was obtained by using *N*-methylammonium formate instead of ammonium formate during the Leuckart's reductive ammonolysis step.[32] This resin was developed for the making of peptide methylamides which are widely used as model compounds for the study of physicochemical properties of peptides. Boc-Leu·H_2O was coupled to that resin by using DCC in CH_2Cl_2 (substitution, 0.37 mequiv of NH_2/g of resin). Deblocking of the Boc group was achieved in 20 min by TFA–CH_2Cl_2 (50:50) containing 5% ethanedithiol, followed by neutralization with 12.5% Et_3N in CH_2Cl_2. Successive coupling of each amino acid (3 M excess) was mediated by DCC (3 M excess) for periods varying from 45 to 120 min in CH_2Cl_2 with the exception of Boc-Arg(Tos), Boc-Asn(xanthyl), and Boc-D-glutamine-PNP which were coupled in DMF. Completion of some selected coupling reactions was checked by the ninhydrin test of Kaiser et al.[33] in only a few instances (after the last coupling reaction). Most couplings were, in fact, made in an automated synthesizer (Beckman Model 990) using a program developed in this laboratory (Rivier, Kaiser, and Galyean, unpublished results).

The protected amino acids [α-Boc-D-Gln(PNP), Z-pGlu, α-Boc-D- or -L-Leu·H_2O, α-Boc-Tyr(2,6-Cl_2Bzl), α-Boc-D- or -L-Glu(γ-Bzl), α-Boc-D- or -L-Asn(xanthyl), α-Boc-D- or -L-Lys(ε-2ClZ), α-Boc-D- or -L-Pro, α-Boc-D- or -L-Arg(Tos), α-Boc-D- or -L-Ile·0.5H_2O, and α-Boc-D- or -L-Phe] were bought from Bachem. They were checked for optical purity by determination of their optical rotation.

Cleavage, Deprotection, and Purification. The protected peptide resin (3–6 g) was treated with HF[34] (~100 mL) for 40 min at −20 °C and for 20 min at 0 °C in the presence of anisole (6–12 mL). Under those conditions, both the chloromethylated resin and the N-methylated benzhydrylamine resin gave good yields.

After rapid removal of HF and drying under vacuum (the temperature was kept at ≤0 °C) the light brown colored resins were washed with ether (4 × 50 mL). The dried resin was immediately extracted with 25% AcOH (50 mL) and water (250 mL) and lyophilized. The crude products thus obtained (500–1500 mg) were then applied onto a CMC column (2 × 12 cm); a gradient of NH_4OAc (0.01–0.4 M, pH 6.5) was applied. The peptides, depending on their composition, were eluted at different concentrations of buffer. After lyophilization and elimination of the NH_4OAc the peptides (250–1000 mg) were applied on a partition column[35] [maximum 400 mg/column, 3 × 100 cm, and eluted in the upper phase of 1-butanol–acetic acid–H_2O (4:1:5)]. Retention time on the column was also dependent on the composition of the peptide. Elution patterns were recorded at A_{280nm}. Cuts in the peak fraction were made to favor purity rather than quantity.

Characterization. Homogeneity of peptides 1–**23** was demonstrated by thin-layer chromatography on Eastman chromatogram sheets (6061 silica gel with fluorescent indicator) in four solvent systems and by HPLC (see Table II).

The HPLC apparatus consisted of a Waters Associates Model 204 liquid chromatograph equipped with a Model UK6 injector, two Model 6000A pumps, and a Model 660 programmer for gradient elution. Detection of separated components was carried out using a Schoeffel Model 770 absorbance detector. Tryptophan or tyrosine containing peptides were followed by their absorbance at 280 nm, but since readings at 280 and 210 nm gave similar profiles for all such peptides, we now routinely work at 210 nm. An Infotronics Model 110 integrator was used to determine peak areas. Chromatography was performed using two 4 mm × 30 cm columns of μ Bondapak/C_{18} (Waters Associates) which consisted of octadecyltrichlorosilane chemically bonded to 10-μ porous silica particles; the 30-cm columns were connected by 0.51 mm i.d. × 5 cm stainless steel tubing. Solvents were mixtures of CH_3CN (Burdick and Jackson, glass distilled) in 0.01 M NH_4OAc, pH 4.5 buffer, which had been filtered through a 0.5-μ Millipore filter and degassed in vacuo. Gradients were used to determine proper solvent systems for individual compounds but isocratic solvent mixtures gave larger separation factors with closely related compounds.[23] Each analogue (40 μg) was injected in solution in the solvent mixture used for developing (see legend of Table II for further details).

Amino acid analyses were performed on peptide hydrolysates (6 N HCl containing 2.5% thioglycolic acid in evacuated sealed tubes at 110 °C for 20 h) using a Beckman/Spinco Model 119 amino acid analyzer. Peak areas were determined by an Infotronics Model CRS-100A electronic integrator. All amino acid analyses gave the expected ratios ± 5% with the exception of Ile which was consistently low (0.90–1.00), Lys (0.93) in 4, Lys (1.07) in **8**, and Tyr (0.91) in **15**. Cysteic acid value in **23** (1.85) was obtained after performic oxidation.[25]

Male Sprague–Dawley rats weighing 200–300 g were used in the biological studies. Rats were fed Purina Rat Chow and tap water ad libitum and were housed in temperature and humidity controlled quarters with 14 h of light (0600–2000) and 10 h of dark. Substances were dissolved in artificial CSF (pH 7.2, 126 mM NaCl, 6 mM KCl, 1 mM NaH_2PO_4, 0.88 mM $MgSO_4$, 1.45 mM $CaCl_2$, 25 mM Hepes) and administered in a 10-μL volume via the cisterna magna into rats lightly anesthetized with ether. Animals were then placed in a cold room (4 °C) and rectal temperatures were recorded using a Yellowsprings Instrument Thermoprobe at 30-min time intervals following the injections. Experiments were carried out in randomized block design with four animals per treatment. Data were subjected to analysis of variance and differences between control and treatment groups were determined by the multiple range tests of Dunnett and Duncan. Binding studies were performed as described in detail by Lazarus et al.[22,31,37] NT was iodinated by chloramine T (CT) at an equal molar ratio of peptide, iodide (Amersham/Searle, carrier free $Na^{125}I$), and

CT for 30 s at room temperature and the iodination was terminated by a 4 M excess of sodium metabisulfite.[31] Iodinated NT was isolated from a Sephadex LH-20 (Pharmacia) column (10 × 0.7 cm) preequilibrated in and eluted with butanol–acetic acid–water (10:2:1, BAW).[31] The peak tubes of [^{125}I]-NT were stored at either 0 or −90 °C. The preparation of iodinated NT was found to be biologically active; it produces hypotension in rats and exhibits tachyphalaxis (Brown and Lazarus, unpublished observations).

Mast cells were collected from both the peritoneal and thoracic cavities of decapitated, exsanguinated mature male rats according to Johnson and Moran.[38] The cells were washed three times each in saline and 0.32 M sucrose and stored at 4 °C in sucrose containing 0.02% NaN_3. The proportion of mast cells was found to be between 20 and 70% as determined by staining with methylene blue followed by eosin and hematoxylin.[31] Neither erythrocytes, the main cellular contaminant in the mast cell preparation, nor lymphocytes (1M-9 cell line, donated by Jesse Roth) specifically bind NT.

Specific binding was determined in triplicate 100-μL reaction assays containing 0.25–2 × 10^5 mast cells, 1 mg of BSA, 80 mM sucrose, 10 mM Tris–acetate buffer, pH 7.0, and 40–180 fmol of [^{125}I]-NT in the presence or absence of 6 nmol (10 μg) of unlabeled NT. After incubation for 15 min at 0 °C, the cell–[^{125}I]-NT complex was rapidly filtered with 0.5 mL of 2% BSA through glass fiber filters (Whatman GF/C) presoaked in 2% BSA and washed once in 0.5 mL of cold 2% BSA.[31] All peptides to be analyzed were diluted in 10 mg/mL of BSA and assayed at concentrations from 10 μg to 1 ng.

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References and Notes

(1) Symbols and abbreviations are in accordance with the recommendations of the IUPAC–IUB Commission on Biochemical Nomenclature, *J. Biol. Chem.*, **247**, 977 (1971). Other abbreviations used are NT, neurotensin; LRF, luteinizing hormone releasing factor; HPLC, high-pressure liquid chromatography; TFA, trifluoroacetic acid; CMC, carboxymethylcellulose; SpOMe-Bzl, *S*-*p*-methoxybenzyl; PNP, *p*-nitrophenyl ester; DMF, dimethylformamide; Z, benzyloxycarbonyl; ε-2ClZ, 2-chlorobenzyloxycarbonyl.
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