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IDENTIFICATION OF SIMPLER ANALOGS OF NEUROTENSIN(9-13) WHICH RETAIN ANTINOCICEPTIVE ACTIVITY

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Abstract. The neurotensin C-terminal pentapeptide has been systematically simplified to identify a minimal fragment with *in vivo* analgesic activity and neurotensin receptor binding ability. Di-, tri-, and tetrapeptide fragments were inactive. Pentapeptide simplifications established that only the arginine could be replaced with simpler amino acids and retain activity in each assay.

Neurotensin (NT) is a thirteen amino acid, biologically active peptide originally isolated from bovine hypothalamus,[1] with sequence of pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu. Neurotensin has been found to possess a host of pharmacological effects,[2] including analgesic,[3-6] hypotensive,[1] hypothermic,[7] and psychotropic actions.[2] A short time after its isolation, Carraway and Leeman[8] determined that the full thirteen amino acid sequence is not required for biological activity. By the stepwise removal of N-terminal amino acid residues, they found that the smallest peptide which retained significant activity in a number of assays was the carboxy terminal pentapeptide Arg-Pro-Tyr-Ile-Leu.

Neurotensin is an extremely potent analgesic, surpassing even morphine when compared by direct injection into the brains of mice.[9] It is known that the full NT(1-13) sequence is not required for analgesic activity. Nicolaides[5] *et al.* found that the C-terminal hexapeptide NT(8-13) was three to ten times less potent than NT itself upon intracerebroventricular (i.c.v.) administration in mice in the acetic acid induced writhing assay. In the mouse tail pressure assay, Furuta[6] *et al.* found that the NT(8-13) hexapeptide maintained full i.c.v. potency and the NT(9-13) pentapeptide maintained 58% relative potency compared to NT(1-13).

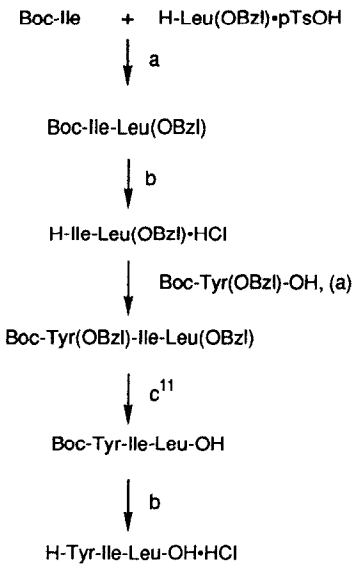
Given the extreme potency and relatively small size of the known antinociceptive fragments of neurotensin, we began a study with the ultimate goal of finding an orally active nonpeptide analgesic acting at the neurotensin receptor. The first objective, as described in this Communication, was to ascertain the minimum neurotensin fragment which retained antinociceptive activity. We expected to identify the minimum active structure by first testing smaller di-, tri-, and tetrapeptides within the NT(9-13) pentapeptide. Having determined the smallest active peptide fragment, we then planned to systematically simplify the functionality of that fragment to arrive at a core active structure.

Chemistry

Each of the dipeptide fragment subunits of NT(9-13) was available commercially. Two of the possible tripeptide fragment subunits, **9** and **10** (Table I), were prepared by custom synthesis at Peninsula Laboratories (Belmont, CA). Literature standard compounds **1**, NT(1-13), and **2**, NT(8-13), were purchased from Peninsula

Labs or Bachem Bioscience (Philadelphia, PA). The pentapeptide standard **3**, NT(9-13), was obtained from Bachem. Other peptides were synthesized within Du Pont Merck using standard solution phase methodology.[10] A representative example, the synthesis of tripeptide **12**, is shown in Scheme I.

Scheme I[a]



[a] (a) isobutylchloroformate, N-methylmorpholine, THF, DMF, -15 °C to RT; (b) HCl in dioxane (4 M), RT; (c) 20% $Pd(OH)_2/C$, cyclohexene, ethanol, reflux

Examples **13** and **16** required synthetic procedures beyond those illustrated in Scheme I. The Arg residue in tetrapeptide **13** was introduced by coupling with N^{α}-Boc-N^{g}-(Tos)Arg. After standard Boc removal, the tetrapeptide was further deprotected with HF/anisole, worked up, and purified by reverse phase HPLC. Tetrapeptide isoamyl ester **16** was prepared by acid catalyzed exchange of the carboxylic acid in the presence of a large excess of isoamyl alcohol.

Biology

Two biological assays were used to monitor the activity of the test compounds. Initially, the affinity of the test compound for the neurotensin receptor was determined by its ability to displace [^{3}H]neurotensin binding *in vitro*.[12] Secondly, the compounds were tested for antinociceptive activity using the mouse phenylquinone writhing (PQW) assay[13] with i.c.v. administration. For the analgesia assay, compounds were dissolved in either saline or DMSO, depending on solubility. It should be noted that while DMSO proved to be a non-toxic solvent for insoluble compounds, it diminished the analgesic activity of the saline-soluble compound, NT(1-13), by sixteen-fold (example **1a** and **1b**).

As shown in Table I, we have confirmed the activities of intact neurotensin as well as the two smaller C-terminal fragments, NT(8-13) and NT(9-13), in both neurotensin receptor binding and the *in vivo* mouse PQW antinociceptive assay. All of the di-, tri-, and tetrapeptide subunit fragments, examples **4** to **14**, were found, however, to have extremely poor to no biological activity in both of the assays. Apparently, the neurotensin receptor does not recognize tetrapeptides or smaller fragments.

Table I. Biological activity of neurotensin and smaller neurotensin fragments

			Mouse PQW	
		NT binding	ED_{50} (95% Conf. Lim.)	
no.	Compound	K_i, nM	μg/mouse, i.c.v.	
1a	NT(1-13) / Saline [a]	5	0.090	(0.036 - 0.22)
1b	NT(1-13) / DMSO [b]		1.4	(0.52 - 3.8)
2	NT(8-13), Arg-Arg-Pro-Tyr-Ile-Leu [a]	4	0.092	(0.023 - 0.38)
3	NT(9-13), Arg-Pro-Tyr-Ile-Leu [a]	73	0.021	(0.0058 - 0.075)
4	Arg-Arg [a]	>100,000	>50	
5	Arg-Pro [a]	>10,000	>50	
6	Pro-Tyr [a]	13,000	>50	
7	Tyr-Ile [a]	>1000	>50	
8	Ile-Leu [b]	>1000	>50	
9	Arg-Arg-Pro [a]	ND	≥500	
10	Arg-Pro-Tyr [a]	>10,000	55	(30 - 100)
11	Pro-Tyr-Ile [b]	>1000	>50	
12	Tyr-Ile-Leu [b]	>10,000	>50	
13	Arg-Pro-Tyr-Ile [a]	>1000	≥50	
14	Pro-Tyr-Ile-Leu [b]	>1000	>50	

PQW = Mouse phenylquinone writhing test (analgesia; 5 or 10 min peak-effect values shown)

[a] Sample dissolved in saline for i.c.v. dosing ND = Not Determined

[b] Sample dissolved in DMSO for i.c.v. dosing

Having determined that at least five residues are required for receptor activity, the C-terminal pentapeptide was then simplified in a number of ways (Table II), so as to learn the importance of each side chain and functional group in maintaining activity. The first simplification was to replace the residue Arg-9 with a Lys, example **15**, because this replacement has been successfully made in the C-terminal hexapeptide,[14] and because Lys is easier to routinely work with using solution phase chemistry. This modification of the basic side chain led to a ten-fold loss of binding activity, and a three hundred eighty-fold loss of mouse PQW activity. The importance of the C-terminal carboxylic acid function is revealed in **16**, which lacks the carboxyl group but retains the leucine side chain linkage as an ester. Removal of the -COOH destroyed all activity. In examples **17** to **21**, each residue in the [Lys^9]NT(9-13) pentapeptide was sequentially substituted with an Ala, thereby removing the functionality of each side chain, leaving only a methyl group, yet retaining the asymmetric center. While side chain replacement with methyl in residues 9 and 12, examples **17** and **20**, did not abolish the binding ability of these compounds, their activities decreased by an additional order of magnitude. Methyl group substitution of the side chains in the other residues did destroy all binding affinity. Shortening the [Lys^9] basic side chain by one carbon, example **22**, had no significant effect on receptor binding affinity or antinociceptive activity. When the N$^{\varepsilon}$-amino group of [Lys^9]NT(9-13) was removed to give **23**, the binding ability decreased by four-fold. Antinociceptive activity, however, was abolished. Conversely, when the N$^{\alpha}$-amino group was removed from [Lys^9]NT(9-13) to give **24**, binding decreased two-fold (not significant), yet the analgesic potency increased six-fold ($P<0.05$). The presence of the N$^{\alpha}$-amino group of residue 9, therefore, appears not to be critical.

Table II. Biological activity of simplified pentapeptides

			Mouse PQW	
		NT binding	ED_{50} (95% Conf. Lim.)	
no.	Compound	K_i, nM	μg/mouse, i.c.v.	
3	NT(9-13), Arg-Pro-Tyr-Ile-Leu [a]	73	0.021	(0.0058 - 0.075)
15	Lys-Pro-Tyr-Ile-Leu [b]	690	8.0	(2.7 - 24)
16	Lys-Pro-Tyr-Ile(O-isoC$_5$H$_{11}$)	>10,000	ND	
17	Ala-Pro-Tyr-Ile-Leu [b]	5900	<50	
18	Lys-Ala-Tyr-Ile-Leu [b]	>10,000	>50	
19	Lys-Pro-Ala-Ile-Leu [b]	>10,000	>50	
20	Lys-Pro-Tyr-Ala-Leu [b]	4200	>50	
21	Lys-Pro-Tyr-Ile-Ala [b]	>10,000	>50	
22	Orn-Pro-Tyr-Ile-Leu [b]	520	20	(12 - 32)
23	Nle-Pro-Tyr-Ile-Leu [b]	3000	>50	
24	Aca-Pro-Tyr-Ile-Leu [b]	1300	1.3	(0.30 - 5.4) *

[a] Sample dissolved in saline for i.c.v. dosing
[b] Sample dissolved in DMSO for i.c.v. dosing
ND = Not Determined
* $P < 0.05$ vs. compound 16

Among the simplified pentapeptides (Table II) which were active in each assay (examples **3**, **15**, **22**, and **24**), there is not a direct correlation between NT receptor binding ability and antinociceptive activity. We believe this lack of correlation arises due to the many factors which impinge on activity in the mouse PQW assay, as in any *in vivo* environment: differing rates of metabolism, diffusion, membrane permeability, etc.

In summary, the C-terminal neurotensin pentapeptide NT(9-13) is the smallest fragment which retains *significant* activity in both binding affinity at the NT receptor and *in vivo* in the mouse analgesia assay. Each of the tested di-, tri-, and tetrapeptide subunits of NT(9-13) is devoid of activity in either assay. Removal of the C-terminal carboxylic acid group, or simplification of the side chains in residues 10-13 also leads to inactive compounds. Only the residue 9 arginine could be replaced with simpler amino acids and retain notable, albeit diminished, activity in each assay.

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