preparation of 10. Chromatographic purification was required on a chloroform-packed silica gel column [35 g (27.5 × 2.5 cm)], eluted with chloroform (200 mL), 10% ethyl acetate in chloroform (100 mL), 20% ethyl acetate in chloroform (100 mL), 40% ethyl acetate in chloroform (100 mL), 80% ethyl acetate in chloroform (100 mL), and ethyl acetate (700 mL). Crude **15a** was collected in fractions (4 mL) 190–229 and crystallized from ethyl acetate–petroleum ether: yield 0.20 g (42%); mp 135–138 °C; $[\alpha]^{28}_D$ −15.2 (c 0.983, $CHCl_3$); one spot on TLC (B), UV and Cl-tolidine positive, ninhydrin negative. The NMR spectrum supported the assigned structure. Anal. ($C_{25}H_{28}ClN_3O_4$) C, H, Cl, N.

***N*-[4-(ClAc)Bz]-L-Ala-L-Phe-Pyrr (15b).** To a solution of **13a** (0.41 g, 1.24 mmol) in methylene chloride (10 mL) and *N*-methylmorpholine (0.12 mL) was added **5b** (0.20 g), followed by additional *N*-methylmorpholine (0.12 mL). The reaction was stirred for 3 h at room temperature and then worked up as described above. Chromatographic purification of **15b** on a silica gel column [40 g (2.5 × 30 cm)] and elution with chloroform (200 mL), 10% ethyl acetate in chloroform (100 mL), 20% ethyl acetate in chloroform (100 mL), 40% ethyl acetate in chloroform (100 mL), 80% ethyl acetate in chloroform (100 mL), and ethyl acetate (700 mL) yielded **15b** in fractions (4 mL) 203 to 238. Crystallization from ethyl acetate gave pure crystalline **15b** with the expected NMR spectrum: yield 0.22 g (46%); mp 194–197 °C; $[\alpha]^{28}_D$ +12.2 (c 0.975, $CHCl_3$); one spot on TLC (B), UV and Cl-tolidine positive, ninhydrin negative. Anal. ($C_{25}H_{28}ClN_3O_4$) C, H, Cl, N.

Conclusion

Seven analogues of TRH are reported, six of which have replacements of the pyroglutamic acid moiety. The agonist activity observed for several of these analogues indicated that the pyroglutamyl moiety is not essential for intrinsic activity. It is possible that the carbonyl of the chloroacetyl moiety is fitting the binding site normally occupied by the lactam carbonyl of the pyroglutamic acid. It is interesting to note that in those analogues where the chloroacetyl is part of an amide the agonist activity is not observed. However, when the chloroacetyl group is present as a ketone the agonist activity is apparent. No significant antagonist activity was observed up to a dose of 30 μg.

Synthesis of Peptides by the Solid-Phase Method. 6. Neurotensin, Fragments, and Analogues[1a]

S. St-Pierre,*[1b] J.-M. Lalonde, M. Gendreau, R. Quirion,[1c] D. Regoli,[1d] and F. Rioux[1e]

Department of Physiology and Pharmacology, University of Sherbrooke Medical School, Sherbrooke, Canada J1H 5N4.
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Neurotensin (NT) and 24 related compounds, including fragments or analogues modified at the C-terminal end of the parent molecule, have been prepared by the solid-phase method. After purification by cation-exchange chromatography, the compounds were characterized by thin-layer chromatography, amino acid analysis, elemental analysis, and high-pressure liquid chromatography. The stimulating effects of the peptides were evaluated in rat stomach strips, in isolated spontaneously beating atria of guinea pigs, and in the coronaries of perfused rat hearts. The differences between the biological activities of these compounds are discussed.

Neurotensin (NT) is a recently discovered peptide hormone[2a] which is largely distributed in the central nervous system[2b,3] and in some regions of the digestive tract[2b,4,5] in various mammals. The peptide exhibits a large spectrum of biological activities, which have been discussed by several authors during the last few years.[6,7] The recent demonstration of the presence of immunoreactive NT in the blood of humans,[8] as well as of the elevated blood concentration of NT following the ingestion of food,[9] more strikingly fat-rich food,[10] seriously raised the possibility that this peptide behaves as a circulating hormone. A likely hypothesis to explain the above results would be that NT is released from endocrine cells located in the wall of the small intestines into the blood and/or the lumen from where it can diffuse into the blood. Recent reports from Swedish[9] and British[11] investigators support these hypotheses. The presence of NT or NT metabolites in the circulating blood raised also the possibility that these peptides influence the function of several organs, including the vasculature and the heart.

At the time we became interested in NT in 1978, this peptide was already known to exert an hypotensive effect in rats[2a] and dogs.[12] Other related actions of NT were an increased vascular permeability in rats[2a] as well as vasodilation in the small intestine and vasoconstriction in subcutaneous adipose tissues in dogs.[12] NT had been reported to be without effect on the heart rate in an-

(1) (a) 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; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; DEA, diisopropylethylamine; DMF, dimethylformamide; 2-BrZ and 2-ClZ, 2-bromo- and 2-chlorocarbobenzoxy; Xan, xanthyl; Me, methyl ether. (b) Chercheur Boursier, Conseil de la Recherche en Santé du Québec. (c) Owner of a Studentship from le Conseil de la Recherche en Santé du Québec. (d) Associate of the Medical Research Council of Canada. (e) Scholar from the Quebec Heart Foundation.

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esthetized dogs[12] and to be completely inactive in isolated, perfused hearts of rabbits and spontaneously beating auricles of guinea pigs.[12] Since the latter results were based upon a rather limited number of experiments, they were repeated in our laboratories as part of a research program on the cardiovascular actions of NT. The powerful positive, chronotropic and inotropic effect of NT in isolated, spontaneously beating auricles of rats and guinea pigs was subsequently published by Quirion et al.[13] Thereafter we provided evidence, for the first time, that NT is a potent constrictor of the coronary vessels in perfused rat hearts[14] and that starvation selectively increased the sensitivity of the coronary vessels of rat hearts to NT.[15] More recently, Rioux et al. described the contractile effect of neurotensin in the rat portal vein.[16]

Several structure–activity studies of NT have been published in recent years[17,20] since the first synthesis of the natural peptide was reported.[21] The order of potency of NT, fragments, and analogues was evaluated in in vitro tests involving biological preparations of smooth-muscle tissues[20,22] or mast cells[18,19] and in vivo by measuring the variations in the rat blood pressure,[20] the hyperglycemic activity,[20] and the body temperature.[18] A good agreement exists between the results obtained by the various groups concerning the activities of fragments and analogues of NT. Convincing evidence was provided suggesting that the major determinants of biological activities reside primarily in its C-terminal region[20,22] and that a free C-terminal carboxyl group is required for full potency in several tests.[18] No specific NT antagonist was developed as a consequence of these studies.

In the present article, we describe the preparation of (a) the complete series of N- and C-terminal fragments of NT, destined to determine the biologically active portion of this peptide in the cardiovascular system, and (b) a set of monosubstituted analogues of NT, in order to pinpoint biologically essential residues in the 8–13 C-terminal fragment and to search for specific antagonists of NT in the cardiovascular system. We also report the biological activities of the fragments and analogues in vitro (guinea pig atria, perfused rat heart, and rat stomach strips).

Synthesis of the Fragments and Analogues. All the peptides were synthesized by the solid-phase method,[23] using a Beckman Model 990 B synthesizer, according to procedures previously described for des-Arg^9-bradykinin[24] and substance P.[25] The C-terminal amino acids (Boc-Leu-OH, Boc-Ile-OH, Boc-Tyr(2-BrZ)-OH, Boc-Pro-OH) were esterified to the chloromethylated polystyrene–divinylbenzene copolymer by the cesium salt procedure.[26] All subsequent amino acid derivatives were added to the peptide via the symmetrical anhydride coupling method:[27] couplings of Boc-Arg(Tos)-OH, Boc-Asn(Xan)-OH, Boc-D-Trp-OH, and Z-pGlu-OH were carried out in DMF–CH_2Cl_2 (9:2, v/v); otherwise, CH_2Cl_2–DMF (9:1, v/v) was used as the coupling solvent. Side-chain protection for other trifunctional Boc amino acids used in this work was the following: Glu(OBzl)-OH, Tyr(2-BrZ)-OH, and Lys(2-ClZ)-OH. The L- or D-Trp side chain was left unprotected, but D,L-methionine (1%, w/v) was added to the deprotection reagent (TFA–CH_2Cl_2, 4:6, v/v) after the addition of this residue to the peptide, which prevented most of the alkylation of the indole moiety. HF cleavage of the completed peptides from the resin was carried out for 0.5 h at −25 °C and for an additional 0.5 h at 0 °C, in a mixture of double-distilled HF (CoF_3)–anisole (80:20, v/v). This procedure was shown effective to prevent the side reaction on the Glu side chain in the presence of anisole.[28] When Trp was present, D,L-methionine (1%, w/v) was added to the HF–anisole mixture. After extraction from the resin and lyophilization, all fragments and analogues, except NT_{10-13}, were purified to homogeneity (≥99%) in a single operation of ion-exchange chromatography operation on Whatman CM-52 microgranular resin. NT_{10-13} was found homogeneous after a further step of purification using a column of Sephadex LH-20 equilibrated with methanol–H_2O (8:2, v/v). All peptides were characterized by TLC, elemental analysis, and analytical HPLC. Amino acid analyses of acid hydrolysates were consistent with the desired sequences. These data are reported in Table I. The percentage of contaminating L isomer in analogues containing D residues in positions 9, 10, and 11 was shown to be less than 1% by HPLC.

Structure–Activity Studies and Discussion

The relative affinities of NT fragments and analogues in the three pharmacological preparations are listed in Table II. The portion of NT that is necessary for its action on cardiac muscle and stomach smooth muscle was determined by the fragment study. It is shown in Table II that NT_{9-13} is the smallest C-terminal fragment possessing full intrinsic activity in the three assays. NT_{8-13} possessed the full potency of NT in vivo.[20] Moreover, the importance of residues Leu^{13} and Ile^{12} for affinity and Tyr^{11} for activity was demonstrated by preparing fragments NT_{1-12}, NT_{1-11}, and NT_{1-10}. Consequently, the sequence Arg^9-Pro^{10}-Tyr^{11} contains all the essential information for the activation of NT receptors in the three in vitro preparations and is required for intrinsic activity. However, this fragment study did not allow us to determine whether the substance that is responsible for NT peripheral activity is the sequence 1–13 of NT, a metabolite, or a closely related molecule that could cross-react with NT antibodies in radioimmunoassays. Assuming that this substance is a metabolite, one can suggest that the sequence 1–13 containing the basic doublet Arg^8-Arg^9 determined by Leeman corresponds to a prohormone. Basic doublets are

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Table I. Characterization of the Fragments and Analogues of NT by TLC, Amino Acid Analysis, Elemental Analysis, and High-Pressure Liquid Chromatography

no.	yield,[a] %	TLC (R_f) BAPW[b]	TLC (R_f) BAW[c]	HPLC[d] T_R	HPLC[d] flow rate	HPLC[d] CH_3CN, %	formula	(acetate)$_x$	$(H_2O)_y$	M_r
1	45	0.65	0.28	10.3	1.0	25	$C_{80}H_{124}N_{22}O_{20}$	1	12	1951
2	51	0.56	0.23	10.5	1.5	20	$C_{77}H_{123}N_{21}O_{20}$	2	9	1846
3	46	0.47	0.20	6.6	1.5	20	$C_{71}H_{112}N_{20}O_{19}$	2	9.5	1742
4	48	0.29		5.6	1.5	20	$C_{62}H_{103}N_{19}O_{17}$	2	9.5	1579
5	33	0.43	0.11	5.0	1.5	20	$C_{55}H_{94}N_{16}O_{14}$	3	7.5	1360
6	40	0.51	0.21	7.1	1.5	20	$C_{47}H_{78}N_{14}O_{11}$	2	5.5	1143
7	45	0.39	0.20	3.2	1.5	25	$C_{42}H_{71}N_{13}O_8$	2	5	1028
8	34	0.47	0.31	11.8	1.5	20	$C_{34}H_{55}N_9O_7$	1	3.5	785
9	28	0.60		3.8	1.5	25	$C_{26}H_{39}N_5O_4$			
10	25	0.42	0.22	9.0	1.5	20	$C_{74}H_{113}N_{21}O_{19}$	1	10	1802
11	46	0.38					$C_{66}H_{98}N_{20}O_{16}$			
12	48	0.35	0.13	3.3	1.5	20	$C_{57}H_{89}N_{19}O_{14}$	1	9	1491
13	52	0.66	0.20	7.6	1.0	25	$C_{80}H_{124}N_{22}O_{20}$	1	11.5	1942
14	50	0.71	0.21	6.0	1.0	28	$C_{75}H_{113}N_{19}O_{10}$	0	12	1806
15	50	0.64	0.19	9.0	1.0	25	$C_{80}H_{124}N_{22}O_{20}$	1	12.5	1960
16	37	0.63	0.24	6.7	1.0	25	$C_{77}H_{120}N_{22}O_{20}$	1	12	1909
17	48	0.67	0.24	9.0	1.0	28	$C_{80}H_{124}N_{22}O_{20}$	1	10.5	1924
18	45	0.68	0.26	7.2	1.0	28	$C_{80}H_{124}N_{22}O_{19}$	1	11	1917
19	45	0.71	0.30	5.1	1.0	36	$C_{80}N_{124}N_{22}O_{19}$	1	11	1917
20	40	0.64	0.25	3.6	1.0	25	$C_{72}H_{120}N_{22}O_{19}$	1	9	1805
21	38	0.72	0.28	14.2	1.0	25	$C_{77}H_{126}N_{22}O_{19}$	1	11.5	1892
22	25	0.63	0.21	6.8	1.0	30	$C_{84}H_{129}N_{23}O_{21}$	2	12	2033
23	24	0.64	0.23	19.8	1.0	30	$C_{84}H_{129}N_{23}O_{21}$	2	12	2033
24	30	0.66	0.23	20.2	1.0	30	$C_{81}H_{126}N_{22}O_{20}$	1	10	1929
25	49	0.63	0.22	5.6	1.5	25	$C_{80}H_{124}N_{22}O_{20}$	1	10.5	1924

amino acid analysis[e]											
Glu	Leu	Tyr	Asp	Lys	Pro	Arg	Ile	Trp[f]	Ala	Gly	Phe
2.09	2.00	1.98	0.94	1.02	1.89	2.11	0.99				
1.10	2.00	1.97	0.95	1.04	1.97	2.03	0.95				
1.04	1.00	1.98	0.95	1.00	1.89	2.09	1.01				
1.04	1.00	0.95	0.94	1.01	1.96	1.97	0.96				
	1.00	0.97		0.97	1.92	2.15	0.96				
	1.00	0.95			2.01	2.00	0.98				
	1.00	0.93			1.03	2.11	1.03				
	1.00	0.98			0.97	1.06	0.99				
	1.00				0.91		0.93				
2.08	1.00	2.03	0.98	1.02	1.94	2.11	1.00				
2.04	1.00	1.95	0.91	1.00	1.89	2.04					
1.91	1.00	0.95	0.93	1.04	1.91	2.06					
2.04	2.00	1.97	1.02	0.97	1.97	2.00	1.01				
2.07	2.00	1.93	0.91	0.95	1.89	1.07	0.96		1.01		
2.04	2.00	1.95	0.98	1.05	2.06	1.94	0.94				
2.10	2.00	2.00	0.93	1.08	0.93	2.08	0.99				
2.06	2.00	1.97	0.99	0.99	1.91	2.05	0.97			1.05	
2.08	2.00	1.03	0.92	1.04	1.86	2.00	0.95				0.95
2.04	2.00	0.96	1.03	1.01	1.94	2.05	0.96				0.98
2.03	2.00	0.95	1.00	0.97	1.91	1.99	0.95		0.96		
1.99	2.00	0.91	0.94	1.03	2.09	2.02	1.02				
2.07	2.00	0.88	0.99	1.05	1.88	2.10	1.03	0.88			
2.00	2.00	0.94	1.02	0.99	1.92	2.05	0.98	0.83			
2.04	2.00	1.88	1.00	1.06	1.86	2.06	1.00				
2.06	2.00	1.90	0.93	0.98	2.02	2.04	1.03				

[a] Yield of final product obtained after chromatography, based on the total quantity of C-terminal amino acid attached on the resin before the synthesis. [b] 1-Butanol–acetic acid–water–pyridine (30:6:24:20, v/v). [c] Top phase of 1-butanol–acetic acid–water (4:1:5, v/v). [d] Ioscratic solvent conditions, using mixtures of 0.01 M ammonium acetate buffer with acetonitrile as indicated. Solvent was run through two 0.4 × 30 cm columns of μBondapak/C_{18} (Waters) in series, connected by a 0.51 mm i.d. × 50 mm stainless-steel tubing, using Waters Model 6000A pumps and a Model UK-6 injector. Detection at 210 nm (Schoeffel Model SF770). T_R = retention time. [e] Amino acid analysis from acid hydrolysates (distilled 6 N HCl from Pierce Chemical at 110 °C in evacuated sealed tubes for 20 h). [f] 4 N methanesulfonic acid (Pierce Chemicals), same conditions as in footnote *e*.

known to be located, among other examples, between the portions of β-MSH and β-endorphin in lipotropin,[29] at the C-terminal end of the Leu-enkephalin sequence in dynorphin,[30] and between the C-peptide and A chain of insulin.[31] The existence of a proteolytic process that specifically cleaves peptide chains at a basic doublet remains to be demonstrated.

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Table II. Primary Structures and Biological Activities of NT, Fragments, and Analogues

no.	compd	primary structure													relative potency,[a] %		
		1	2	3	4	5	6	7	8	9	10	11	12	13	guinea pig atria	rat stomach strips	perfused rat heart
1	NT	pGlu	Leu	Tyr	Glu	Asn	Lys	Pro	Arg	Arg	Pro	Tyr	Ile	Leu	100 (65)	100 (68)	100
2	NT_{2-13}		Leu	Tyr	Glu	Asn	Lys	Pro	Arg	Arg	Pro	Tyr	Ile	Leu	94 (8)	90 (7)	87 (6)
3	NT_{3-13}			Tyr	Glu	Asn	Lys	Pro	Arg	Arg	Pro	Tyr	Ile	Leu	84 (7)	90 (7)	85 (6)
4	NT_{4-13}				Glu	Asn	Lys	Pro	Arg	Arg	Pro	Tyr	Ile	Leu	99 (7)	85 (7)	92 (6)
5	NT_{6-13}						Lys	Pro	Arg	Arg	Pro	Tyr	Ile	Leu	73 (7)	78 (7)	84 (6)
6	NT_{7-13}							Pro	Arg	Arg	Pro	Tyr	Ile	Leu	49 (6)	65 (7)	59 (6)
7	NT_{8-13}								Arg	Arg	Pro	Tyr	Ile	Leu	52 (6)	69 (7)	102 (8)
8	NT_{9-13}									Arg	Pro	Tyr	Ile	Leu	1.3 (7)	11 (7)	1.6 (8)
9	NT_{10-13}										Pro	Tyr	Ile	Leu	0 (7)	0 (7)	0 (6)
10	NT_{1-12}	pGlu	Leu	Tyr	Glu	Asn	Lys	Pro	Arg	Arg	Pro	Tyr	Ile		0.09 (6)	0.65 (8)	0.03 (6)
11	NT_{1-11}	pGlu	Leu	Tyr	Glu	Asn	Lys	Pro	Arg	Arg	Pro	Tyr			<0.01 (3)	<0.01 (3)	<0.01 (6)
12	NT_{1-10}	pGlu	Leu	Tyr	Glu	Asn	Lys	Pro	Arg	Arg	Pro				0 (4)	0 (4)	0 (6)
13	[D-Arg[9]]-NT	pGlu	Leu	Tyr	Glu	Asn	Lys	Pro	Arg	*D-Arg*	Pro	Tyr	Ile	Leu	4.1 (7)	0.65 (7)	0.01 (6)
14	[Ala[9]]-NT	pGlu	Leu	Tyr	Glu	Asn	Lys	Pro	Arg	*Ala*	Pro	Tyr	Ile	Leu	2.6 (7)	1.5 (7)	0.3 (6)
15	[D-Pro[10]]-NT	pGlu	Leu	Tyr	Glu	Asn	Lys	Pro	Arg	Arg	*D-Pro*	Tyr	Ile	Leu	0.4 (7)	0.1 (8)	0.02 (6)
16	[Gly[10]]-NT	pGlu	Leu	Tyr	Glu	Asn	Lys	Pro	Arg	Arg	*Gly*	Tyr	Ile	Leu	~0.01 (7)	~0.01 (8)	<0.01 (6)
17	[D-Tyr[11]]-NT	pGlu	Leu	Tyr	Glu	Asn	Lys	Pro	Arg	Arg	Pro	*P-Tyr*	Ile	Leu	~0.01 (4)	~0.05 (6)	<0.01 (6)
18	[Phe[11]]-NT	pGlu	Leu	Tyr	Glu	Asn	Lys	Pro	Arg	Arg	Pro	*Phe*	Ile	Leu	15 (8)	16 (8)	1.4 (6)
19	[D-Phe[11]]-NT	pGlu	Leu	Tyr	Glu	Asn	Lys	Pro	Arg	Arg	Pro	*D-Phe*	Ile	Leu	~0.01 (4)	~0.02 (6)	<0.01 (6)
20	[Ala[11]]-NT	pGlu	Leu	Tyr	Glu	Asn	Lys	Pro	Arg	Arg	Pro	*Ala*	Ile	Leu	<0.01 (6)	0.01 (6)	0.01 (6)
21	[Leu[11]]-NT	pGlu	Leu	Tyr	Glu	Asn	Lys	Pro	Arg	Arg	Pro	*Leu*	Ile	Leu	~0.01 (6)	~0.03 (6)	0.01 (6)
22	[Trp[11]]-NT	pGlu	Leu	Tyr	Glu	Asn	Lys	Pro	Arg	Arg	Pro	*Trp*	Ile	Leu	3.2 (6)	130 (7)	102 (8)
23	[D-Trp[11]]-NT[b]	pGlu	Leu	Tyr	Glu	Asn	Lys	Pro	Arg	Arg	Pro	*D-Trp*	Ile	Leu	0.01 (7)	0.64 (7)	0.1 (8)
24	[Tyr(Me)[11]]-NT[b]	pGlu	Leu	Tyr	Glu	Asn	Lys	Pro	Arg	Arg	Pro	*Tyr(Me)*	Ile	Leu	0.03 (8)	0.5 (6)	<0.01 (8)
25	[D-Glu[4]]-NT	pGlu	Leu	Tyr	*D-Glu*	Asn	Lys	Pro	Arg	Arg	Pro	Tyr	Ile	Leu	100	100	100

[a] Relative potency expressed in percent of that of NT. The number in parentheses represents the number of replicate experiments. [b] These compounds also behave as antagonists in the perfused rat heart preparation. As agonists, they possess between 60 and 70% of the intrinsic activity of NT.

The results of fragment studies are consistent with the important biological role of the Tyr[11] residue. Therefore, it was expected that chemical modifications of this side chain and of its spatial orientation would lead to important variations of biological activity. Low potencies obtained with [D-Pro[10]]- and [Gly[10]]-NT are consistent with the important role of Pro[10] in properly orienting the Tyr[11] side chain for recognition by the receptor. The important loss of affinity of analogues [D-Arg[9]]- and [Ala[9]]-NT could be explained in terms of the stabilizing role of the positive charge of residue 9 for maximum interaction of NT with the receptor. Moreover, eliminating hydrophobic residues 12 and 13 drastically decreases potency, which tends to show the important function of hydrophobic interactions for full receptor activation.

Substitution of Tyr[11] by Leu, Ala, Phe, Trp, Tyr(Me), D-Tyr, D-Phe, and D-Trp showed that an aromatic residue, with the L configuration, bearing an heteroatom capable of hydrogen bonding with the receptor, is essential in that position for maximum potency in our three preparations. [Leu[11]]- and [Ala[11]]-NT, on the one hand, and [D-Tyr[11]]-, [D-Phe[11]]-, and [D-Trp[11]]-NT, on the other hand, are weak agonists. However, [D-Trp[11]]- and [Tyr(Me)[11]]-NT are selective antagonists of the vasoconstrictor action of NT in the coronaries of perfused rat hearts.[32,33] The pA_2 values of [D-Trp[11]]- and [Tyr(Me)[11]]-NT were, respectively, 6.8 and 6.6. The inhibition was found to be reversible and presumably of the competitive type. [L-Phe[11]]-NT is a good agonist in rat stomach strips and guinea pig atria but a weaker one in the coronaries. [L-Trp[11]]-NT is slightly more potent than NT in rat stomach strips and coronaries but is weak in guinea pig atria.

Different orders of potencies exist for several analogues in the three aforementioned preparations. Furthermore, two analogues ([D-Trp[11]]- and [Tyr(Me)[11]]-NT) are specific NT antagonists in only one of the preparations (rat coronaries). These data suggest that various types of NT receptors could exist in peripheral systems such as rat stomach strips, guinea pig atria, and rat coronaries. Moreover, good evidence in favor of the existence of different receptors of NT in periphery and in the brain has been recently provided by Barbeau et al. (in preparation). It was shown that some analogues with low potencies in periphery ([D-Phe[11]]-, [D-Tyr[11]]-, and [D-Trp[11]]-NT) are more potent than NT, when injected intracerebrally, to induce hypothermia or to act on motor activity. The higher biological potency of analogues [D-Tyr[11]]- and [D-Phe[11]]-NT had already been observed by Rivier et al.[18] in the hypothermia test.

Recent results from several research groups, including our own, strongly support the hypothesis for an important physiological role of NT in the central nervous system and in periphery. The increase in circulating concentrations of immunoreactive NT following the ingestion of lipid-rich food,[10] as well as the discovery of its presence in[2a,4] and action on gastrointestinal tissues[20,34] and intestinal blood flow[12] seriously raised the possibility that NT is a circulating hormone whose role may be to participate in postprandial cardiovascular homeostasis. The sensitization of the cardiovascular system of the rat to the action of NT after a prolonged period of fasting also supports the above hypothesis. The development of specific and competitive antagonists of the various biological effects of NT may greatly contribute to the elucidation of its physiological role.

Table III. Program for Automatic Solid-Phase Synthesis of Neurotensin

	synthesis step	no. of repetitions of step	approx vol of solvent, mL/g of resin	mix time, min
(1)	CH_2Cl_2 wash	3	10	1
(2)	TFA[a] prewash	2	6	1
(3)	TFA deblocking		6	20
(4)	CH_2Cl_2 wash	3	10	1
(5)	CH_2Cl_2–dioxane[b]	3	6	1
(6)	CH_2Cl_2 wash	6	10	1
(7)	DEA prewash[c]	2	6	1
(8)	DEA neutralization		6	5
(9)	CH_2Cl_2 wash	6	10	1
(10)	Boc amino acid SA delivery[d]		5	
(11)	amino acid check and transfer			
(12)	column rinse and fill (CH_2Cl_2); mix for coupling			30-60
(13)	CH_2Cl_2 transfer and wash	4	10	1
(14)	2-propanol shrink and wash	2	6	1
(15)	CH_2Cl_2 swell and wash	3	10	1
(16)	repeat line 14	2	6	1
(17)	repeat line 15	3	10	1
(18)	search next program			

[a] 40%, v/v, in CH_2Cl_2 + 5% thioanisole. [b] 50%, v/v, in CH_2Cl_2. [c] 5%, v/v, in CH_2Cl_2. [d] 0.3–0.5 mmol of Boc amino acid SA/mL of CH_2Cl_2.

Experimental Section

Reagents and Solvents. Boc amino acids and Z-pGlu were purchased from Bachem Fine Chemicals Inc. Their purity was carefully checked in two TLC systems and their optical rotation measured before use. The Merrifield resin (chloromethylated copolystyrene–divinylbenzene, 1%, 0.75 mequiv of Cl/g) was obtained from Bachem or Lab Systems Inc. Reagent grade solvents were bought from Anachemica and Fisher Scientific and purified prior to use: methylene chloride was distilled from sodium carbonate, dioxane from sodium, and DMF from ninhydrin, in vacuo, after shaking with KOH pellets. Methylene chloride and dioxane were stored over Linde type 4A molecular sieves. TFA (Halocarbons) was distilled from calcium sulfate and DEA (Aldrich) from ninhydrin. DCC was obtained from Chemical Dynamics and used without further purification, after testing its solubility in ethyl ether.

Peptide Synthesis. (i) Protected Peptide-Resin. C-Terminal Boc-Leu, Boc-Ile, and Boc-Tyr(Br-Z) were coupled to the resin via the cesium salt procedure.[26] Substitution varied between 0.3 and 0.5 mmol/g, as determined by the picric acid method.[35]

(ii) Solid-Phase Peptide Synthesis. Peptide synthesis was carried out with a Beckman Model 990B peptide synthesizer operated in the automatic mode. The instrument was equipped with jacketed amino acid reservoirs in order to keep the temperature of the solutions of Boc amino acid symmetrical anhydrides at −20 °C. A 1:1 mixture of water–ethylene glycol was circulated in the reservoirs by means of a Lauda Model RC-3 circulating bath.

For a typical synthesis of NT (compound 1), 2.2 g (1 mmol) of Boc-Leu resin was placed in the Teflon reaction vessel, and the operations were carried out according to the schedule shown in Table III. The deprotection was performed for 20 min with a solution of 40% trifluoroacetic acid–CH_2Cl_2. For the synthesis

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of Trp-containing NT analogues, subsequent deprotections following the introduction of Boc-Trp were made with 40% TFA in CH_2Cl_2 containing D,L-Met (1%, w/v). After the resin was copiously washed with CH_2Cl_2 and 1:1 dioxane–CH_2Cl_2, the neutralization was carried out with a 5% solution of DEA in CH_2Cl_2. *N*-α-Boc protection was used for the following residues, with the side-chain protecting group shown in parentheses: L-Arg(Tos), L-Tyr(2-BrZ), D-Tyr(2-BrZ), Lys(2-ClZ), L-Glu(OBzl), D-Glu(OBzl), Asn(Xan), Leu, L-Pro, D-Pro, Ile, Trp, Ala, Phe, D-Phe, Gly and Tyr(Me); L-pGlu was introduced as the Z-amino acid. Solutions of symmetrical anhydrides were prepared as previously described,[24] poured into their respective reservoirs on the synthesizer, and transferred into the reaction vessel, via the metering tower, at the appropriate step of the synthesis. DMF was used as a solvent for coupling of derivatives Boc-Arg(Tos), Boc-Asn(Xan), Boc-Trp, and Z-pGlu. Other Boc derivatives were coupled in CH_2Cl_2–DMF (10:2, v/v). After 30 min of coupling, 1 equiv of DEA (5% solution) was added and the reaction allowed to proceed for another 10 min. Every coupling step was checked using the ninhydrin test.[36] After proper washing of the resin (shrinking and swelling by the alternate use of CH_2Cl_2 and 2-propanol) the program reached the last line and the machine proceeded to the addition of the next amino acid.

After the last amino acid was introduced, the synthesizer stopped and the peptide-resin was collected and washed with methanol on a fritted funnel. It was dried overnight in vacuo and weighed 4.5 g.

(iii) Cleavage of the Peptides from the Support. The dried, protected NT-resin (2 g) was placed in the reaction vessel of a liquid HF apparatus (Protein Research Foundation), 5 mL of anisole was added, and 20 mL of HF was distilled from CoF_3 in the vessel. When Trp was present in NT analogues, D,L-methionine (1%, w/v) was added to the reaction mixture. The cleavage reaction proceeded for 30 min at −25 °C ($CaCl_2$–H_2O–CO_2 bath) and at 0 °C for another 30 min. HF was rapidly evaporated in vacuo and the resin was copiously washed with ether and ethyl acetate. The peptide was extracted from the resin with ca. 200 mL of 30% acetic acid, and the solution was lyophilized to give 670 mg of crude material.

(iv) Purification of the Peptides. The white powder (200 mg) was dissolved into 10 mL of 0.005 M ammonium acetate buffer, pH 4.5, and the solution was applied to a 1.5 × 10 cm column (Pharmacia) of Whatman microgranular CM-52 cation-exchange resin equilibrated with the same buffer. The buffer was pushed through the column at a 750 mL/h flow rate, using a Buchler peristaltic pump. A linear gradient of salt (0.005 to 0.2 M) and pH (4.5 to 6.0) was performed, using a 350-mL mixing chamber. The elution was monitored at 280 nm by means of a double-beam Schoeffel Model SF770 flow spectrophotometer, recorded on a Picker dual-pen recorder, and 12-mL fractions were collected with a LKB fraction collector. The peptide was eluted as a symmetrical peak at 3.5 mmho, as measured with a Radiometer conductivity meter. The peptide was separated from three minor peaks at 0.5 (injection), 2.9, and 4.5 mmho, respectively. The solution was lyophilized three times and 117 mg of material was obtained. The overall yield of peptide was 45%, based on the quantity of Boc-Leu originally attached to the resin. Overall yields of other peptides varied between 24 and 52%.

Characterization of the Peptides. (i) Thin-Layer Chromatography. Homogeneity of the peptides was demonstrated by TLC on Brinkmann chromatogram plates (F-254 with fluorescent indicator) in the solvent systems BAPW and BAW; ca. 25 μg of peptide was applied, and the spots were revealed on the plates by spraying with a solution of ninhydrin in ethanol (1%), with the Reindel–Hoppe reagent,[37] or the Pauly reagent. The R_f values of the peptides in the two TLC systems are given in Table I.

(ii) High-Pressure Liquid Chromatography. All peptides were also analyzed for homogeneity by high-performance liquid chromatography (HPLC). The Waters HPLC apparatus consisted of the two Model 6000A pumps, a Model UK6 injector, and a Model 660 programmer for gradient elution. Detection of components was carried out using a Schoeffel Model SF770 variable-wavelength flow spectrophotometer, operated at 210 nm. Chromatography was performed using two 0.4 × 30 cm columns of μBondapak/C_{18} (Waters associates) in series, connected by a 0.51 mm i.d. × 50 mm stainless-steel tubing. Solvents were mixtures of acetonitrile (Fisher Scientific) in 0.01 M ammonium acetate, pH 4.5, buffer, filtered through Millipore (0.5 μm), and degassed in vacuo.[18] Proper solvent systems for each analogue were determined by means of gradients. Thereafter, isocratic solvent conditions were used to separate closely releated compounds. Each analogue (ca. 30 μg) was injected in solution in the solvent mixture used for developing. Retention times are given in Table I.

(iii) Analyses. Amino acid analyses were performed on peptide hydrolysates [distilled 6 N HCl (Pierce Chemicals) at 110 °C in evacuated sealed tubes for 20 h or 4 N methanesulfonic acid, under the same conditions, for Trp-containing peptides], using a JEOL Model JLC 6AH or a Beckman Model 120 automatic amino acid analyzer. Elemental analyses were performed by Galbraith Laboratories (Knoxville, Tenn.), and the results corresponded to the theoretical compositions (see Table I).

Determination of Biological Activity. The biological experiments were performed using Albino Wistar rats of either sex (Canadian Breeding Laboratories, St-Constant, Quebec), weighing between 250 and 350 g, or male and female guinea pigs (450–550 g) purchased from a local breeder. The animals were killed by a blow on the neck and exsanguinated by cutting the carotid arteries. The assay of peptides in rat stomach strips and guinea pig atria was done essentially according to procedures described previously.[38]

(i) Rat Stomach Strips. The stomach was taken out. The fundus was separated from the whole organ, emptied of its content, washed with a cold (4 °C) Krebs solution, and cut longitudinally into strips averaging 3.0-cm long and 1.5-mm wide. The strips were mounted under a resting tension of 2 g in 15-mL organ baths containing an oxygenated (95% O_2, 5% CO_2) Krebs solution maintained at 37 °C with a thermostated circulator (Haake Model FJ). The composition of the Krebs solution has been described previously.[38] The tissues were equilibrated for 45 to 60 min before being exposed to the peptides. The contractions of the tissues were recorded by means of force-displacement transducers (Grass FTO3) coupled to a Grass polygraph (Model 79).

(ii) Guinea Pig Atria. The preparation of guinea pig atria was as follows: both atria were dissected out from the ventricles, cleaned of fat and blood, and placed under a resting tension of 0.5 g in 15-mL organ baths containing an oxygenated Krebs solution at 30 °C. Under these conditions, the atria started beating spontaneously. The force of contraction was recorded as described above for the rat stomach strip. The period of equilibration of the tissues was 45 to 60 min.

(iii) Perfused Rat Hearts. The experiments performed with perfused rat hearts were done as described previously.[14] Following an intraperitoneal injection of heparin sodium (2.5 mg/rat), male albino Wistar rats (see above) were killed as mentioned above. The heart was quickly excised, put in a small Petri dish containing oxygenated Krebs solution at room temperature, cleaned of fat, and transferred into a Plexiglas-enclosed chamber thermoregulated at 37 °C. The aortic end of the organ was fixed to a plastic cannula positioned close to the coronary ostia. The heart was perfused with a warmed (37 °C), oxygenated, carefully filtered Krebs solution using a peristaltic pump (Sage). The perfusion rate was increased gradully during the first 5 min to 8 mL/min to reach a stable perfusion pressure of 70–90 mmHg. The perfusion pressure was measured using pressure transducer (Statham P23 Db) attached to the side arm of the aortic cannula. Changes of perfusion pressure were recorded on a Grass Polygraph (Model 79). The organs were allowed to stabilize for 30 min before starting the infusions of peptides.

For further technical details concerning the use of the above preparations for testing the peptides described in this paper, the reader is referred to recent publication from our team.[14,38]

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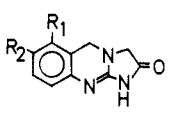
Cyclic Guanidines. 14.[1] Imidazo[1,2-*a*]thienopyrimidin-2-one Derivatives as Blood Platelet Aggregation Inhibitors

Fumiyoshi Ishikawa,* Akira Kosasayama, Hitoshi Yamaguchi, Yoshifumi Watanabe, Junji Saegusa, Seiichi Shibamura, Kyoko Sakuma, Shin-ichiro Ashida, and Yasushi Abiko

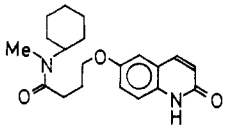
Research Institute, Daiichi Seiyaku Company, Ltd., 1-16-13 Kitakasai, Edogawa-ku, Tokyo 132, Japan. *Received July 2, 1980*

A series of novel 1,2,3,5-tetrahydroimidazo[1,2-*a*]thieno[2,3-*d*]-, -[3,2-*d*]-, and -[3,4-*d*]pyrimidin-2-one derivatives has been prepared and tested for the activity of inhibiting platelet aggregation in rats in vitro and ex vivo. These compounds were synthesized through the following reactions: sodium borohydride reduction of 2,4-dichlorothienopyrimidines, followed by ethoxycarbonylmethylation and successive amination. Most of the compounds were found to be potent inhibitors of blood platelet aggregation. Structure–activity relationships have indicated the essential contribution of the lactam structure and lipophilic substituents on the thiophene ring to the effective interaction of the compounds with a receptor site on the platelet. Among the compounds studied, 1,2,3,5,6,7,8,9-octahydro[1]benzothieno[2,3-*d*]imidazo[1,2-*a*]pyrimidin-2-one (**9m**) exhibited the most favorable activity.

In view of the important contribution of platelet functions to thrombus formation, considerable effort has been devoted in a search for inhibitors of platelet aggregation. Among the structures of reported inhibitors, we were interested in a lipophilic lactam structure as a common, essential prerequisite for the active compounds, e.g., 1,2,3,5-tetrahydroimidazo[2,1-*b*]quinazolin-2-ones[2] (I),



Ia R_1 = Me, R_2 = H
Ib R_1 = R_2 = Cl

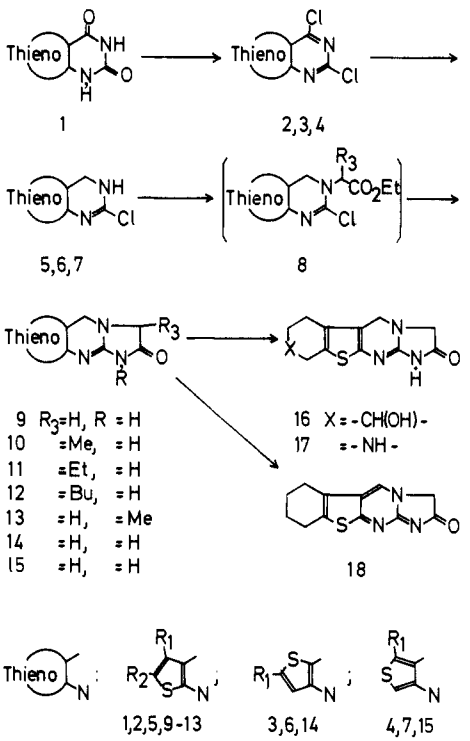


II

3,4-dihydroimidazo[1,2-*a*]benzimidazol-2(1*H*)-one derivatives,[3] 4-(3,4-disubstituted-benzyl)imidazolidin-2-one derivatives,[4] and 4-(6-carbostyryloxy)butyramide derivatives[5] (II). The amide structure may be essentially required for interaction of the compounds with an enzyme protein through hydrogen bondings, and the lipophilic moiety in these compounds may increase the effectiveness of the interaction. On the basis of this assumption, the present study was undertaken to prepare potent inhibitors of platelet aggregation and to confirm the structure–activity relationships along this line.

Our desired compounds were 1,2,3,5-tetrahydroimidazo[1,2-*a*]thienopyrimidin-2-ones (**9**–**17**) which have

Scheme I



the thieno moiety, in place of the benzo group of quinazoline compounds[1] (I), containing a 3,4-dihydrothienopyrimidine ring as a partial structure in the molecule. To our knowledge, the above 3,4-dihydro ring system has been found only in 4-alkyl-3,4-dihydrothieno[2,3-*d*]pyrimidines prepared by the reaction of thieno[2,3-*d*]pyrimidine with alkyllithiums.[6] The desired compounds having this ring system were easily obtained, however, by a novel synthetic route and were found to be highly potent inhibitors of platelet aggregation. This paper describes the synthesis and biological activities of a series of 1,2,3,5-tetrahydroimidazo[1,2-*a*]thieno[2,3-*d*]-, -[3,2-*d*]-, and -[3,4-*d*]pyrimidin-2-ones.

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