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Synthesis and Pharmacological Properties of the N-Terminal Decapeptide of the Vasoactive Intestinal Peptide (VIP)

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The decapeptide derivative, L-histidyl-L-seryl-L-aspartyl-L-alanyl-L-valyl-L-phenylalanyl-L-threonyl-L-aspartyl-L-asparaginyl-L-tyrosine methyl ester, corresponding to the N-terminal sequence of both porcine and chicken VIP was synthesized in solution, by the stepwise strategy. Its pharmacological properties resemble those of VIP itself, but with a much lower potency, comparable to that of peptides with C-terminal sequences. The presence of *two* independent sequences carrying similar instructions was recognized in VIP.

The vasoactive intestinal peptide (VIP) discovered by Said and Mutt[1] was first isolated from porcine tissues.[2] The sequence (Figure 1) of the 28 amino acids constituting a single chain was determined by the same authors.[3] Subsequently, a compound with similar pharmacological properties and with a closely homologous sequence (Figure 1) was obtained from chicken intestine.[4] In the course of the synthesis,[5] carried out mainly to prove the correctness of the sequence determined by degradation of the porcine peptide, not only the final octacosapeptide but also shorter peptides corresponding to partial sequences of VIP were tested for biological activities.[6] The N-terminal hexapeptide VIP_{1-6} exhibited only slight activity on different smooth muscle preparations and on peripheral (or systemic) blood flow of dogs. Similarly weak potency was detected in the C-terminal sequences, the hendecapeptide VIP_{18-28} and the tetradecapeptide VIP_{15-28}. The C-terminal pentadecapeptide VIP_{14-28} was markedly more effective than the tetradecapeptide in the blood flow experiments, while the docosapeptide VIP_{7-28} exhibited closer similarity to the parent octacosapeptide both in biological activity (on peripheral blood flow and smooth-muscle organs)[6] and in chiroptical properties.[7]

The finding of slight but significant biological activities, resembling those of VIP, both in N-terminal and C-terminal sequences is reminiscent of the recognition of two independent "command sequences" in melanotropin by Eberle and Schwyzer.[8] It seems that similar dual-receptor mechanisms might be operative also in other peptide hormones. Preliminary studies[9] on peptides corresponding to partial sequences of secretin gave support to this view.

The recently determined sequence[10] of chicken VIP closely resembles that of the porcine peptide (Figure 1). Only positions 11, 13, 26, and 28 are occupied by different amino acid residues in the two species and the differences are rather conservative. The complete identity of sequence 1–10 in chicken and porcine VIP suggests that it may have functional significance and remained, therefore, unaltered during evolution. This part of the VIP molecule, VIP_{1-10}, has now been synthesized, in solution, by the stepwise strategy[11] (cf. Chart I). The protected decapeptide intermediate can serve as a larger building block in the synthesis of both chicken and porcine VIP. For the present study it was partially deprotected to afford L-histidyl-L-seryl-L-aspartyl-L-alanyl-L-valyl-L-phenylalanyl-L-threonyl-L-aspartyl-L-asparaginyl-L-tyrosine methyl ester (I) and tested in this form for VIP-like activities.

The potency of compound I in relaxing smooth muscle preparations (rat stomach and guinea pig lung) was approximately half that of the C-terminal peptide of comparable length, VIP_{18-28}. Yet, in its effect on blood flow it surpassed VIP_{18-28} and even VIP_{15-28} (but not VIP_{14-28}). Thus, while the physiological role of VIP remains to be determined, the VIP-like activities found in nonoverlapping N-terminal and C-terminal areas of the chain demonstrate the presence in VIP of two sequences carrying similar instructions. In themselves these parts of the molecule have only weak activity, and no potentiation could be detected when VIP_{1-10} and VIP_{18-28} were applied together. A more exact determination of the role of different portions of VIP through the examination of further partial sequences and their analogues is in progress.

Experimental Section

Synthesis. Capillary melting points were determined and are reported uncorrected. DMF was dried over the molecular sieve, Linde Type 4A. Spots on thin-layer chromatograms (TLC) were revealed by a combination of UV, ninhydrin spray (0.4 g of ninhydrin, 1 mL of collidine, 100 mL of acetone), fluorescamine spray (20 mg of Floram, 100 mL of acetone), *tert*-butyl hypochlorite–KI–starch reagent [1% *tert*-butyl hypochlorite in cyclohexane, followed by a 1:1 (v/v) solution of 1% starch and 1% KI], and charring after spraying with a solution of $(NH_4)HSO_4$ [20 g of $(NH_4)_2SO_4$, 4 mL of H_2SO_4, 100 mL of H_2O]. The solvent systems for TLC were A, $CHCl_3$–MeOH (9:1); B, 1-butanol–AcOH–H_2O (4:1:1); C, EtOAc–AcOH–pyridine–H_2O (60:6:20:11); D, 1-butanol–AcOH–pyridine–H_2O (45:6:24:20). For amino acid analysis, samples were hydrolyzed with constant boiling hydrochloric acid in evacuated, sealed ampules at 110 °C for 16 h and analyzed by the Spackman–Stein–Moore method[12] on a Beckman Spinco 120C amino acid analyzer. The following abbreviations were used: DIEA, diisopropylethylamine; DMF, dimethylformamide; TFA, trifluoroacetic acid.

Boc-Asn-Tyr(Bzl)-OMe (II). *O*-Benzyl-L-tyrosine methyl ester hydrochloride (Bachem, 3.54 g, 11 mmol) was dissolved in DMF (40 mL). DIEA (1.76 mL, 11 mmol) and Boc-Asn-ONo[13] (3.53 g, 10 mmol) were added to the stirred solution. The next day the solvent was removed in vacuo, and the residue was dissolved in $CHCl_3$ (200 mL) and washed with water (2 × 100 mL), dilute $KHSO_4$ solution (pH 2, 2 × 100 mL), water (2 × 100 mL), 0.5 M $NaHCO_3$ (2 × 100 mL), and water (2 × 100 mL). Each aqueous phase was reextracted with $CHCl_3$ (25 mL). The $CHCl_3$ layers were combined, the solvent was evaporated, and the residue was triturated with ether. The product was collected on a filter, washed with H_2O, air-dried, washed with ether, and dried again to yield 4.67 g (93.5%): mp 169–171 °C; TLC R_f (A) 0.44.

For analysis a sample (240 mg) was recrystallized from 95% EtOH (5 mL) and dried at 100 °C and 0.05 mmHg for 4 h: recovery 193 mg; mp 171.5–172 °C; $[\alpha]^{25}_D$ +16° (*c* 2, DMF); Asp 1.00, Tyr 0.72. Anal. ($C_{26}H_{33}N_3O_7$) C, H, N.

Boc-Asp(OBzl)-Asn-Tyr(Bzl)-OMe (III). A sample of compound II (4.99 g, 10 mmol) was dissolved in distilled trifluoroacetic acid (15 mL) and allowed to stand at room temperature for 15 min. The solvent was removed in vacuo, the residue triturated with ether, and the trifluoroacetate salt collected on a filter. It was washed with ether, dried in vacuo in a desiccator

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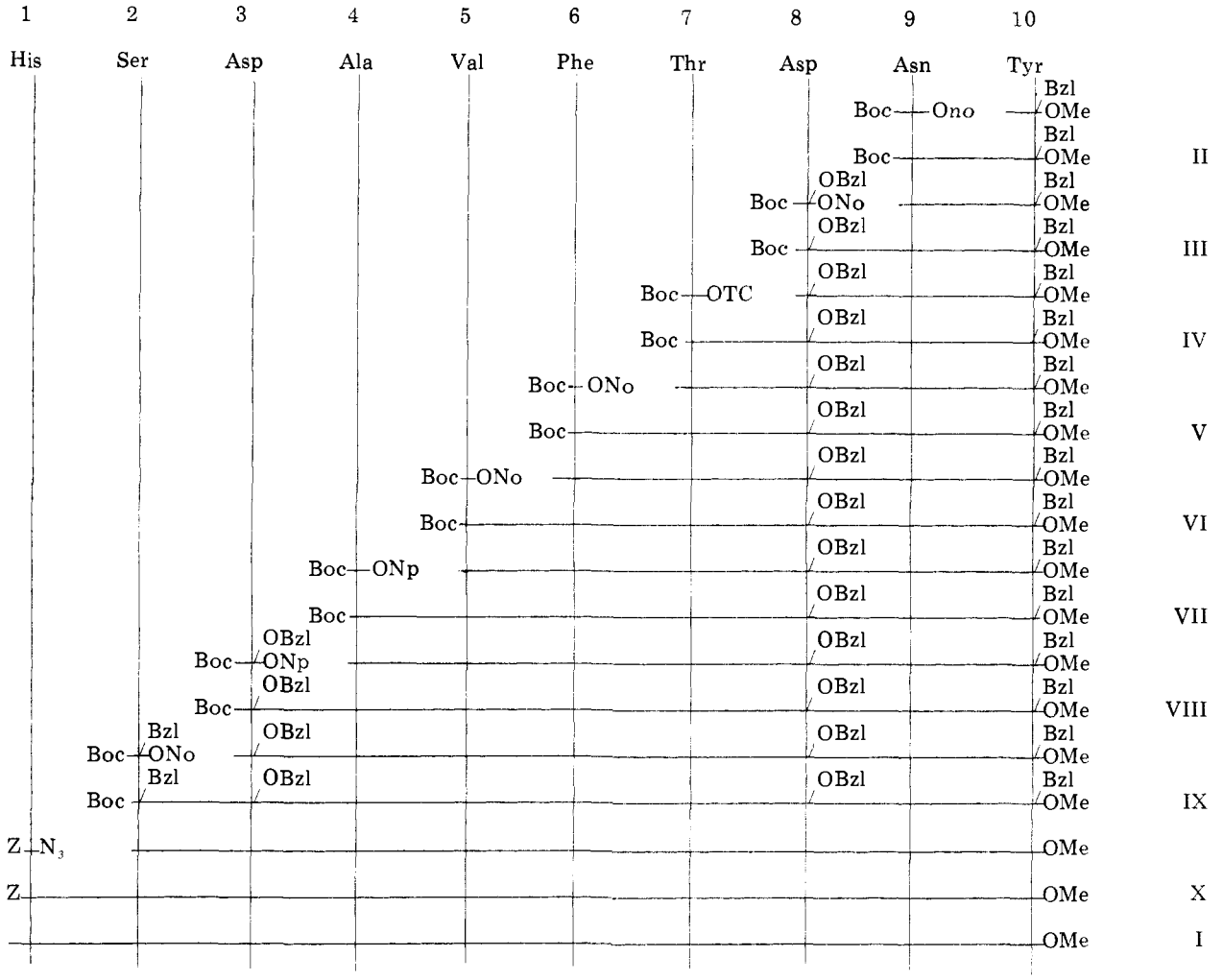
P His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH_2
C His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Ser-Arg-Phe-Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Val-Leu-Thr-NH_2
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28

Figure 1. Sequences of porcine (P) and chicken (C) VIP.

Chart I



over NaOH, and dissolved in dry DMF (30 mL). DIEA (1.6 mL, 10 mmol) and Boc-Asp(OBzl)-ONo[13] (5.33 g, 12 mmol) were added to the stirred solution. The following day, the solution was ninhydrin negative. The solvent was evaporated and the residue triturated with ether, filtered, dried, washed with 95% ethanol, and dried again. The protected tripeptide methyl ester weighed 5.9 g (84%): mp 183–184 °C; R_f (A) 0.45.

For analysis, a sample (100 mg) was recrystallized from 95% EtOH and dried: 87 mg; mp 183.5–185 °C; $[\alpha]^{25}_D$ −2° (*c* 1, DMF with 1% AcOH). Anal. ($C_{37}H_{44}N_4O_{10}$) C, H, N.

Boc-Thr-OTC. A solution of *tert*-butyloxycarbonyl-*O*-benzylthreonine (9.58 g, 31 mmol) and 2,4,5-trichlorophenol (7.9 g, 36 mmol) in EtOAc (100 mL) was cooled in an ice–water bath, and DCC (6.2 g, 30 mmol) was added. After 0.5 h, the mixture was allowed to come to room temperature. The next day, the separated *N,N'*-dicyclohexylurea was removed by filtration and washed with EtOAc, and from the combined filtrate and washings the solvent was evaporated. The residue was dissolved in $CHCl_3$ (50 mL), the solution washed with 0.05 N NaOH (3 × 50 mL) and water (2 × 50 mL), and each aqueous layer reextracted with $CHCl_3$ (15 mL). The combined $CHCl_3$ layers were dried with $MgSO_4$ and evaporated. The residue was dissolved in 95% EtOH containing 1% AcOH (25 mL); the product crystallized overnight. The active ester was collected on a filter, washed with 95% EtOH, and dried: yield 8.0 g (55%); mp 80–81 °C; R_f (A) 0.8; $[\alpha]^{25}_D$ −20° (*c* 1, DMF with 1% AcOH).

A sample of the fully protected amino acid active ester (4.5 g, 912 mmol) was dissolved in a mixture of EtOAc (100 mL) and AcOH (1 mL) and hydrogenated in the presence of a 10% palladium-on-charcoal catalyst (1.3 g) for 16 h. After removal of the catalyst and the solvent the residue was crystallized under hexane, filtered, and washed with hexane. After drying 2.9 g (79%) was obtained: mp 85–88 °C; $[\alpha]^{25}_D$ −46.2° (*c* 2, MeOH); R_f (A) 0.8. Anal. ($C_{15}H_{18}NO_5Cl_3$) C, H, N.

Boc-Thr-Asp(OBzl)-Asn-Tyr(Bzl)-OMe (IV). A sample of the protected tripeptide ester III (4.2 g, 6 mmol) was dissolved in TFA (8 mL). After 15 min at room temperature the TFA was removed in vacuo; the trifluoroacetate salt was precipitated with ether, filtered, washed with ether, and dried. It was dissolved in DMF (35 mL) and DIEA (0.96 mL, 6 mmol), and *tert*-butyloxycarbonyl-L-threonine 2,4,5-trichlorophenyl ester (2.63 g, 6.6 mmol) and 1-hydroxybenzotriazole[14] (0.92 g, 6 mmol) were added. The mixture was kept alkaline with the periodic addition of small amounts of DIEA. Three hours later the solvent was removed in vacuo and the residue treated with EtOAc. The precipitate was filtered, washed with EtOAc, and dried. The crude product (4.0 g) was recrystallized from 95% EtOH to give 3.5 g of IV (73%): mp 179–181 °C; $[\alpha]^{25}_D$ −6.3° (*c* 2, DMF with 1% AcOH); R_f (A) 0.44, R_f (B) 0.82, R_f (D) 0.94; Asp 1.95, Thr 1.0, Tyr 0.90. Anal. ($C_{41}H_{51}N_5O_{12}$) C, H, N.

Boc-Phe-Thr-Asp(OBzl)-Asn-Tyr(Bzl)-OMe (V).[15] The amino-protecting group was removed from a sample (0.806 g, 1 mmol) of compound IV with TFA (2 mL); the trifluoroacetate salt was dissolved in DMF (5 mL) and treated with DIEA (0.16 mL, 1 mmol), Boc-Phe-ONp[16] (0.424 g, 1.1 mmol), and 1-hydroxybenzotriazole (0.15 g, 1 mmol). After 1 h at room temperature, during which time the mixture was kept alkaline with small amounts of DIEA, the solvent was removed in vacuo

and the product isolated by precipitation and washing with EtOAc. After drying 0.86 g (90%) was obtained: mp 180–185 °C; $[\alpha]^{25}_D$ −5° (c 2, DMF with 1% AcOH); R_f (B) 0.81, R_f (D) 0.87; Asp 2.0, Thr 1.0, Tyr 1.0, Phe 1.06. For elemental analysis a sample was reprecipitated from DMF with H_2O; melting point unchanged. Anal. ($C_{50}H_{60}N_6O_{13}\cdot H_2O$) C, H, N.

Boc-Val-Phe-Thr-Asp(OBzl)-Asn-Tyr(Bzl)-OMe (VI). Partial deprotection of compound V (0.75 g, 0.79 mmol) with TFA (2 mL) was carried out as described above. To the solution of the trifluoroacetate salt in DMF (4 mL) DIEA (0.126 mL, 0.79 mmol), Boc-Val-ONo[13] (0.29 g, 0.87 mmol), and 1-hydroxybenzotriazole (0.12 g, 0.79 mmol) were added and the mixture was kept alkaline with addition of small amounts of DIEA. After 2.5 h at room temperature the solvent was removed and the residue triturated with EtOAc. The washed (EtOAc) and dried product weighed 0.75 g (90%): mp 231–233 °C dec; $[\alpha]^{25}_D$ −7.8° (c 2, DMF with 1% AcOH); TLC was carried out after deprotection with TFA, R_f (B) 0.61, R_f (D) 0.76; Asp 1.9, Thr 1.0, Val 1.0, Tyr 0.90, Phe 0.90. Anal. ($C_{55}H_{69}N_7O_{14}$) C, H, N.

Boc-Ala-Val-Phe-Thr-Asp(OBzl)-Asn-Tyr(Bzl)-OMe (VII). Removal of the Boc group from compound VI (0.72 g, 0.68 mmol) yielded the trifluoroacetate salt that was acylated in DMF (3.5 mL) with Boc-Ala-ONp[16] (232 mg, 0.75 mmol) in the presence of DIEA (0.11 mL, 0.68 mmol) and 1-hydroxybenzotriazole (0.10 g, 0.68 mmol) as described in earlier paragraphs. After a 1-h reaction time the protected heptapeptide derivative was isolated by trituration and washing with EtOAc. The product, 0.72 g (94%), melted (with decomposition) at 237–239 °C; $[\alpha]^{25}_D$ −14° (c 2, DMF with 1% AcOH); R_f (B) (after deprotonation with TFA) 0.49; Asp 2.05, Thr 1.0, Ala 1.08, Val 0.94, Tyr 0.87, Phe 0.90. Anal. ($C_{58}H_{74}N_8O_{15}$) C, H, N.

Boc-Asp(OBzl)-Ala-Val-Phe-Thr-Asp(OBzl)-Asn-Tyr(Bzl)-OMe (VIII). Partial deprotection of compound VII (0.69 g, 0.61 mmol) with TFA (3 mL), isolation of the trifluoroacetate salt, and its acylation with Boc-Asp(OBzl)-ONp[17] (297 mg, 0.67 mmol) in DMF (3.8 mL), in the presence of DIEA (97 μL, 0.61 mmol) and 1-hydroxybenzotriazole (91 mg, 0.61 mmol), were carried out as described in previous paragraphs. After 20-min reaction time the solvent was removed and the product precipitated and washed with EtOAc. The dry material (0.77 g, 95%) melted with decomposition at 241–242 °C: $[\alpha]^{25}_D$ −12.1° (c 2, DMF with 1% AcOH). It was too insoluble for TLC; Asp 3.1, Thr 1.10, Ala 1.15, Val 1.0, Tyr 0.93, Phe 0.93. For analysis a sample was reprecipitated from DMF with H_2O; melting point unchanged. Anal. ($C_{69}H_{85}N_9O_{18}$) C, H, N.

Boc-Ser(Bzl)-Asp(OBzl)-Ala-Val-Phe-Thr-Asp(OBzl)-Asn-Tyr(Bzl)-OMe (IX). This intermediate was prepared from compound VIII (0.72 g, 0.54 mmol). For deprotection TFA (2 mL) was used; for chain lengthening in DMF (6 mL) Boc-Ser-(Bzl)-ONo[13] (249 mg, 0.6 mmol) and DIEA (86 μL, 0.54 mmol) were applied. The acylation reaction, catalyzed with 1-hydroxybenzotriazole[14] (81 mg, 0.54 mmol), was complete in less than 1 h. After removal of the solvent, trituration, and washing with EtOAc and with ether, the fully protected intermediate IX was dried to afford 0.75 g (92%): mp 242–244 °C dec; $[\alpha]^{25}_D$ −13° (c 2, DMF with 1% AcOH); too insoluble for TLC; Asp 3.1, Thr 1.0, Ser 0.97, Ala 1.03, Val 0.93, Tyr 0.87, Phe 0.93. For analysis a sample (50 mg) was reprecipitated from DMF with H_2O. The recovered material (40 mg), perhaps because of retention of a trace of water, had a lower melting point, 236–238 °C dec. Anal. ($C_{79}H_{96}N_{10}O_{20}$) C, H, N.

Z-His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-OMe (X). A sample (0.15 g, 0.1 mmol) of the protected nonapeptide derivative IX was suspended in 80% AcOH (25 mL) and hydrogenated in the presence of a 10% palladium-on-charcoal catalyst (43 mg) for 24 h. After removal of the catalyst and the solvent the *tert*-butyloxycarbonyl nonapeptide methyl ester was secured by trituration with ether. It was treated with TFA (0.5 mL) to produce, after trituration with ether, the nonapeptide methyl ester IXa as the trifluoroacetate salt: R_f (D) 0.22 with traces of impurities; Asp 3.1, Thr 1.05, Ser 1.03, Ala 1.10, Val 1.0, Tyr 0.91, Phe 0.93.

A larger preparation of IXa (1.0 g, 0.86 mmol) was dissolved in DMF (8 mL) and DIEA (0.41 mL, 2.58 mmol) was added. The mixture was cooled in an ice–water bath and treated with DMF (7 mL) solution of N^{α}-benzyloxycarbonyl-L-histidine azide, prepared[18] from the hydrazide (0.91 g, 3 mmol). After 3 days at 4 °C and about 16 h at room temperature the solvent was removed in vacuo and the residue triturated with MeOH (20 mL), filtered, washed with MeOH, EtOAc, and ether, and dried. The partially protected decapeptide ester X (0.85 g, 72%) was obtained: mp 217–200 °C dec; R_f (D) 0.35. Anal. ($C_{60}H_{77}N_{13}O_{21}\cdot 3H_2O$) C, N; H: calcd, 6.1; found, 5.6.

A sample of the methyl ester X (131 mg, 0.1 mmol) was converted to the hydrazide by treatment with anhydrous hydrazine (0.8 mL) in MeOH (5 mL) at room temperature overnight. Evaporation in vacuo and trituration of the residue with ether was followed by the addition of 95% EtOH, under which the hydrazonium salt of the partially protected decapeptide hydrazide Xa solidified. It was washed with 95% EtOH and dried: 100 mg (76%); mp 212–215 °C dec; R_f (D) 0.29. Anal. ($C_{59}H_{77}N_{15}O_{20}\cdot H_2NNH_2\cdot C_2H_5OH\cdot 3H_2O$) C, N; H: calcd, 6.5; found, 5.8.

His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-OMe (I). The partially protected decapeptide methyl ester X (105 mg, 0.077 mmol) was dissolved in 80% AcOH (10 mL) and hydrogenated, in the presence of a 10% palladium-on-charcoal catalyst (30 mg), for 4.5 h. The catalyst was filtered, the solvent removed in vacuo, and the residue triturated with EtOAc, filtered, and washed with EtOAc. The crude product (83 mg) was extracted with MeOH and then with EtOAc. The purified material was dissolved in 98% AcOH and lyophilized: 54 mg; R_f (D) 0.2; Asp 3.1, Thr 1.04, Ser 1.08, Ala 1.10, Val 1.0, Tyr 0.94, Phe 0.97, His 1.12.

Biological Activities. VIP-like biological activity was measured on (1) isolated, superfused smooth muscle organs[19] (stomach strip and colon of the rat; trachea and gall bladder of the guinea pig) and (2) peripheral blood flow and arterial blood pressure in anesthetized dogs.[1] Relative potency was assessed by comparing doses eliciting equal responses at two points on the dose–response curve. VIP-like immunoreactivity was assayed in a radioimmunoassay[20,21] using ^{125}I-labeled porcine VIP and antibodies raised in rabbits against a conjugate of the peptide with bovine serum albumin, prepared by the carbodiimide method. Two dilutions of the synthetic fragment were prepared, and their ability to displace bound VIP was related to that of standard VIP solutions.

The vasodilator potency of VIP_{1-10} was approximately 1:500 that of VIP, but its tracheal-relaxant and gastric muscle relaxant potencies were only about 1:5000 that of the whole molecule. Immunoreactivity was even weaker, being about 1:40 000 that of VIP.

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Proteinase Inhibitors. 1. Inhibitors of Elastase

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A series of peptides and depsipeptides containing 2-methylcarbazic acid (H-Mec-OH), the 2-aza analogue of alanine, was prepared and tested as inhibitors of pancreatic and human granulocyte elastases. A requirement for a minimum chain length as well as specific amino acid sequence was observed which correlates well with both substrate and inhibitor studies by others in this field. The most active inhibitors have the structure Ac-Ala-Ala-Pro-Mec-Lac-R. When Lac-R is an ester, only the pancreatic enzyme is inhibited. When Lac-R is an amide or hydrazide, then both enzymes are inhibited. The inhibitory activity is reversible; the inhibitors are not hydrolyzed by the enzyme and the inhibition is noncompetitive with synthetic substrates of similar structure, suggesting that binding at the sites adjacent to the carboxyl group of the amino acid analogue, 2-methylcarbazic acid, is important for this inhibition. The data further demonstrate the differences between pancreatic and granulocyte elastases.

Our interest in the synthesis of elastase inhibitors arises from the probable involvement of this enzyme in tissue destruction associated with arthritis,[1] inflammation,[1] emphysema,[1] and pancreatitis.[2] Effective inhibition of elastase would, therefore, appear to be a worthwhile goal in the amelioration of these disease states.

Several types of elastase inhibitors have been reported in the literature. These include certain di- and tripeptides,[3] peptide chloromethyl ketones,[4-7] and peptide aldehydes,[8] including elastatinal.[9] More recently, Powers has described a new type of elastase inhibitor, namely, peptide carbazate *p*-nitrophenyl esters such as Cbz-Ala-Ala-Pro-Mec-*p*-NP (**2**).[10] In these compounds, the alanine moiety at position P_1[11] of the substrate–inhibitor has been replaced by its nitrogen isostere 2-methylcarbazic acid (Mec). Such compounds acylate the hydroxy group of the active site serine.[12] The resulting carbazoyl enzyme deacylates slowly because of the influence of the nitrogen atom adjacent to the bond which is cleaved.

Because the reactivity of the *p*-nitrophenyl ester group makes its in vivo usefulness doubtful, we prepared a series of peptide carbazates incorporating at the P_1 position the alanine isostere 2-methylcarbazic acid but lacking a terminal activated ester bond such as Ac-Ala-Ala-Pro-Mec-OEt (**3**). The compounds were designed primarily as inhibitors of pancreatic elastase since this enzyme has been well studied and the structural features of its substrates and inhibitors are well known. Human granulocyte elastase was previously believed to be similar to the pancreatic enzyme, but differences between the two with respect to both substrate specificity and sensitivity to inhibitors are now becoming apparent[12-15] and, indeed, were observed in our studies.

Our first objective was to determine whether or not a peptide carbazate terminated by a simple ester instead of the activated *p*-nitrophenyl group was still capable of acylating the enzyme and, if not, could such a compound function as a competitive inhibitor. Compounds **3–10** served to answer this question and also to verify the structural requirements of the peptide backbone as reported by Thompson and Blout[6,8,16] for porcine pancreatic elastase.

Another objective was to make use of the $S_{1'}$ and $S_{2'}$ subsites on the enzyme ($P_{1'}$ and $P_{2'}$ on the inhibitor) to increase the strength of binding between enzyme and inhibitor. The terminology S and P is that of Schechter and Berger.[11]

$\cdots S_2\text{–}S_1\text{–}S_{1'}\text{–}S_{2'}\cdots$ refers to subsites on both sides of the catalytic site of the enzyme. Each subsite may be composed of several amino acid residues arranged in a particular geometric pattern which allows for interaction with an amino acid residue of the substrate–inhibitor. The notation P on the substrate (or inhibitor) denotes the amino acids which bind to these enzyme subsites such that $P_1\text{–}P_{1'}$ represents the bond which is cleaved. Moreover, the utilization of these additional binding sites might confer increased selectivity for inhibiting elastase but not other serine proteases. At the time we initiated our efforts, no study had been reported which characterized the nature of the amino acids preferred at the $P_{1'}$ and $P_{2'}$ subsites of an elastase substrate or inhibitor. During the course of our study, however, Atlas published the finding that lysine and phenylalanine are favored at the $P_{1'}$ and $P_{2'}$ sites, respectively,[17] of synthetic substrates for the porcine pancreatic enzyme.

Our exploration of the $P_{1'}$ subsite was guided by the observation of Thompson[16] that the tetraalanine amide, Ac-Ala-Ala-Ala-Ala-NH_2, could bind to pancreatic elastase in the S_{54321} or $S_{43211'}$ mode, giving rise to ammonia or alaninamide, respectively. This suggested to us the synthesis of a carbazate ester resembling alanine at the $P_{1'}$ site. Accordingly, we prepared compounds **11–13** which contain the alanine isostere lactic acid (Lac) at the $P_{1'}$ site. Compounds **14–19** interchange alanine and its oxygen and nitrogen isosteres, lactic acid, and 2-methylcarbazic acid at the P_1 and $P_{1'}$ sites. Compounds **20–23** verify both length and amino acid requirements of the peptide backbone in the carbazate lactate series. Compounds **24–36** were prepared to evaluate the effect of various substituents on the lactate moiety.

Compounds **2–36** were evaluated for their ability to inhibit the cleavage of the synthetic substrate Ac-Ala-Ala-Pro-Ala-*p*-nitroanilide (**1**) by the enzyme under the conditions specified in the Experimental Section. Results are shown in Table I. K_i values for the most active compounds are shown in Table II.

The majority of the compounds listed in Table I were prepared according to one of the following routes shown