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Synthesis of the Vasoactive Intestinal Peptide (VIP)
II. The *N*-Terminal Hexapeptide[1]

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The protected hexapeptide derivative *t*-butyloxycarbonyl-L-histidyl-*O*-benzyl-L-seryl-β-benzyl-L-aspartyl-L-alanyl-L-valyl-L-phenylalanine methyl ester was prepared from L-phenylalanine methyl ester by stepwise chain lengthening. Hydrogenation of the protected hexapeptide ester followed by hydrazinolysis afforded a derivative that is expected to be useful in the synthesis of VIP. Complete removal of the protecting groups yielded a free hexapeptide, indistinguishable from the *N*-terminal chymotryptic fragment of the natural hormone. Identical products were obtained from side-by-side treatment of the natural and synthetic hexapeptides with thermolysin and with dipeptidyl aminopeptidase I.

A vasoactive peptide (VIP) was isolated from hog intestines by Said and Mutt (*1*). The sequence of the 28 amino acids constituting the single chain of this hormone molecule has not yet been completely elucidated, but information on partial sequences of VIP became available recently (*2*, *3*). In a previous communication (*4*) from these laboratories, the synthesis of a hendecapeptide, the *C*-terminal cyanogen bromide fragment of VIP, was reported. The present paper deals with the preparation of a protected hexapeptide corresponding to the *N*-terminal sequence of VIP, *tert*-butyloxycarbonyl-L-histidyl-L-seryl-L-aspartyl-L-alanyl-L-valyl-L-phenylalanine methyl ester (X). This ester was converted to the hydrazide (XI) which is expected to serve in the total synthesis of the 28-membered chain of the vasoactive peptide.

The synthesis of X was carried out in the stepwise manner (*5*) (cf. Chart 1). Benzyloxycarbonyl-L-valine was coupled to L-phenylalanine methyl ester by the dicyclohexylcarbodiimide (DCC) method (*6*) to give the known (*7*) protected dipeptide, benzyloxycarbonyl-L-valyl-L-phenylalanine methyl ester (I). Hydrogenation of I followed by acylation (DCC) with benzyloxycarbonyl-L-alanine afforded the protected tripeptide benzyloxycarbonyl-L-alanyl-L-valyl-L-phenylalanine methyl ester (III) in good yield. After treatment of III with hydrogen bromide in acetic acid (*8*), the chain was lengthened by incorporation of aspartic acid in the form of *t*-butyloxycarbonyl-β-benzyl-L-aspartic acid *p*-nitrophenyl ester (*9*). The resulting protected tetrapeptide was treated with trifluoracetic acid and then acylated with *t*-amyloxycarbonyl-*O*-benzyl-L-serine *p*-nitrophenyl ester.[3] After partial deprotection with trifluoroacetic acid, the histidine

[1] For the first paper in this series, cf. Ref. 4.

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[3] Prepared according to the general procedure described in *Biochem. Prep.* 9, 110 (1962).

residue was introduced by the Honzl–Rudinger modification of the azide method (*10*). The benzyl groups were removed from the fully protected hexapeptide ester IX by hydrogenolysis and the partially deprotected ester X was converted to the hydrazide XI with a methanolic solution of hydrazine at room temperature.

The amino-protecting group (Boc) was removed from a sample of the protected hexapeptide methyl ester X and the ester was hydrolyzed with chymotrypsin. The free hexapeptide was cleaved by thermolysin (*11*) into two fragments, as had previously been found to be the case for the *N*-terminal chymotryptic fragment of VIP (*3*). When the thermolytic cleavage products obtained from the natural and synthetic hexa-

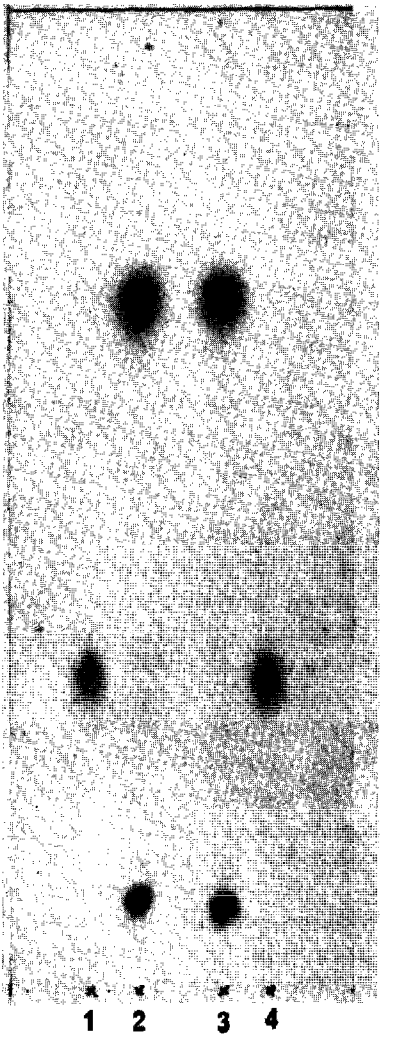

FIG. 1. From left to right: (1) 20 μg natural VIP_{1-6}; (2) thermolytic degradation products of 20 μg natural VIP_{1-6}; (3) thermolytic degradation products of 20 μg synthetic VIP_{1-6}; (4) 20 μg synthetic VIP_{1-6}. Degradation with thermolysine as described in Ref. 11. Paper chromatography on Whatman 42 paper in *n*-butanol/acetic acid/pyridine/water (30:6:20:24), as described in Ref. 21.

peptides were run in parallel on a paper chromatogram, they were found to be indistinguishable, as were the hexapeptides themselves (Fig. 1). The two thermolytic cleavage products were separated on a column of DEAE–cellulose and identified as valylphenylalanine, obviously VIP_{5-6}, and the *N*-terminal tetrapeptide. The synthetic and natural products behaved identically. Both tetrapeptides could then be cleaved, albeit somewhat sluggishly, by dipeptidyl aminopeptidase I (*12*) into the dipeptides aspartylalanine and histidylserine (which were easily separable by chromatography on carboxymethylcellulose at pH 6.0). These degradations and comparisons support the sequence proposed by Mutt and Said (*3*).

SYNTHESIS[4]

Capillary melting points are reported uncorrected. Thin-layer chromatograms (silica gel, Merck) were developed with the solvent systems A: *n*-butanol/acetic acid/water (4:1:1); B: *n*-butanol/pyridine/acetic acid/water (30:24:6:20). Spots were revealed with a modified Rydon–Zahn reagent (*13*) and with Pauly's reagent (*14*). For amino acid analysis, samples were hydrolyzed with constant boiling hydrochloric acid in evacuated, sealed ampoules at 110°C for 16 hr, and analyzed by the method of Spackman, Stein, and Moore (*15*) on a Beckman–Spinco 120C instrument. Countercurrent distribution was carried out with a 60-tube Craig apparatus, 10 ml of each phase in a tube.

Benzyloxycarbonyl-L-Alanyl-L-Valyl-L-Phenylalanine Methyl Ester (III)

Benzyloxycarbonyl-L-valyl-L-phenylalanine methyl ester (I) (*7*) (3.3 g, 8 m*M*) was suspended in 95% ethanol (150 ml) and hydrogenated for 4 hr in the presence of 1.0 *N* HCl (8.8 ml, 8.8 m*M*) and 10% palladium on charcoal (600 mg). The solution was filtered and the filtrate was evaporated to dryness at room temperature. The crystalline residue was dried overnight *in vacuo* to give 2.72 g, tlc R_f A 0.60. The dipeptide hydrochloride II was suspended in dichloromethane (150 ml) and cooled to 0°C. Benzyloxycarbonyl-L-alanine (1.78 g, 8 m*M*) was added, followed by triethylamine (1.12 ml, 8 m*M*) and dicyclohexylcarbodiimide (1.65 g, 8 m*M*). After 15 min the product began to separate. Cooling was continued for 30 min and the reaction was then allowed to proceed at room temperature for 4 hr. THF (100 ml) was added to dissolve the product, followed by 4 *N* HCl (4 ml). After 10 min the precipitate was collected by filtration and washed with THF (3 × 10 ml). The combined filtrates were evaporated, the residue was dissolved in ethylacetate (200 ml) and washed with 2 × 15 ml 1 *N* HCl, 2 × 15 ml 1 *M* $NaHCO_3$, 2 × 15 ml H_2O, and 15 ml of saturated solution of NaCl. The organic layer was dried over Na_2SO_4, filtered, and concentrated to a small volume *in vacuo*. The product was precipitated with petroleum ether (bp 37–50°C), collected on a filter, and washed with petroleum ether. The air-dried product (3.23 g, 84%) melts at 195–196°C. A sample was recrystallized from hot ethanol (mp 204–205°C); $[\alpha]_D^{24} - 5.8°$ (*c* 1, DMF); tlc R_f A 0.68, R_f B 0.73. Amino acid analysis: Ala, 1.0; Val, 0.9; Phe, 1.0.

[4] The following abbreviations are used: DCC (dicyclohexylcarbodiimide), DMF (dimethylformamide), THF (tetrahydrofuran), TFA (trifluoroacetic acid).

Anal. Calcd for $C_{26}H_{33}N_3O_6$ (483.6): C, 64.6; H, 6.9; N, 8.7. Found: C, 64.6; H, 6.8; N, 8.8.

t-Butyloxycarbonyl-β-Benzyl-L-Aspartyl-L-Alanyl-L-Valyl-L-Phenylalanine Methyl Ester (V)

The protected tripeptide III (2.9 g, 6 m*M*) was suspended in acetic acid (18 ml). Hydrobromic acid in acetic acid (4 *N*, 18 ml) was added, followed after 1 hr by dry ether (200 ml). The ether was decanted and hydrobromide was triturated with dry ether (100 ml) and dried for 1 hr *in vacuo* over sodium hydroxide and phosphorous pentoxide. The tripeptide ester hydrobromide IV, *N-t*-butyloxycarbonyl-β-benzyl-L-aspartic acid *p*-nitrophenyl ester (*9*) (3.47 g, 7.8 m*M*), and triethylamine (0.84 ml, 6 m*M*) were dissolved in DMF (30 ml). The reaction mixture was kept slightly basic by the addition of small amounts of the same base. After 2 hr, *uns*-diethylaminopropylamine (*16*) (0.25 ml) was added and 2 hr later ethylacetate (200 ml) was added. The organic phase was washed with 2% citric acid (3 × 20 ml), 0.5 *N* ammonium hydroxide until free of *p*-nitrophenol, 2% citric acid (20 ml), water (2 × 20 ml) and saturated sodium chloride solution. It was dried over sodium sulfate, filtered, and evaporated *in vacuo*. The residue was dissolved in methanol, precipitated with water, collected by filtration and washed with water. The air-dried product (3.8 g, 97%) melts at 143–144°C (softens at 133°C). A sample was recrystallized from methanol–water, without change in mp; $[\alpha]_D^{24} - 14°$ (*c* 1, DMF); tlc R_fA 0.70, R_fB 0.63. Amino acid analysis: Asp, 1.1; Ala, 1.0; Val, 1.0; Phe, 1.0.

Anal. Calcd for $C_{34}H_{46}N_4O_9$ (654.7): C, 62.4; H, 7.1; N, 8.6. Found: C, 62.3; H, 7.3; N, 8.5.

t-Amyloxycarbonyl-O-Benzyl-L-Seryl-β-Benzyl-L-Aspartyl-L-Alanyl-L-Valyl-L-Phenylalanine Methyl Ester (VII)

The protected tetrapeptide V (2.62 g) was dissolved in TFA (20 ml). After 10 min at room temperature, the TFA was evaporated *in vacuo*. Dry ether (200 ml) was added; the product was collected by filtration, washed with dry ether (100 ml) and dried *in vacuo* over sodium hydroxide and phosphorous pentoxide to give 2.62 g (98%); mp 151–153°C; tlc R_fA 0.69, R_fB 0.69.

The tetrapeptide ester trifluoroacetate VI (2.6 g), *N-t*-amyloxycarbonyl-*O*-benzyl-serine *p*-nitrophenyl ester (prepared from 6 m*M* *N-t*-amyloxycarbonyl-*O*-benzyl-L-serine dicyclohexylammonium salt) (*17*) (obtained from the Institute for Protein Research, Peptide Center, Osaka University, Osaka, Japan) and *N*,N^{ϵ}-diisopropylethylamine (0.68 ml, 4 m*M*) were dissolved in DMF (20 ml). The reaction mixture was kept slightly basic by the addition of small amounts of the base. After standing overnight, *uns*-diethylaminopropylamine (0.25 ml) was added and, 2 hr later, ethylacetate (300 ml). The product was isolated as described for compound V. The air-dried material (2.95 g, 87%) melts at 145–146°C (softens at 135°C). A sample was dissolved in a large volume of methanol and the volume was reduced until precipitation began: mp 169–171°C; $[\alpha]_D^{24} - 14°$ (*c* 2, DMF); tlc R_fA 0.83, R_fB 0.76. Amino acid analysis: Ser, 0.9; Asp, 1.0; Ala, 1.0; Val, 1.0; Phe, 1.0.

Anal. Calcd for $C_{45}H_{59}N_5O_{11}$ (846.0): C, 63.9; H, 7.0; N, 8.3. Found: C, 63.7; H, 7.1; N, 8.2.

*t-Butyloxycarbonyl-*L*-histidyl-O-benzyl-*L*-seryl-β-benzyl-*L*-aspartyl-*L*-alanyl-*L*-valyl-*L*-phenylalanine hydrazide (XI)*

The protected pentapeptide (VII) (2.88 g) was dissolved in TFA (20 ml). After 10 min at room temperature, the TFA was evaporated *in vacuo*. Dry ether (250 ml) was added; the product was collected by filtration, washed with dry ether (100 ml) and dried *in vacuo* over NaOH and P_2O_5 to give 2.75 g (96%); mp 211–213°C; tlc R_fA 0.76, R_fB 0.76.

The pentapeptide ester trifluoroacetate VIII (2.75 g) was dissolved in DMF (10 ml) and neutralized with triethylamine (0.46 ml). It was immediately added to a solution of *t*-butyloxycarbonyl-L-histidyl azide prepared as follows: *t*-butyloxycarbonyl-L-histidine hydrazide (1.35 g; obtained from Fluka A. G., Switzerland) was dissolved in

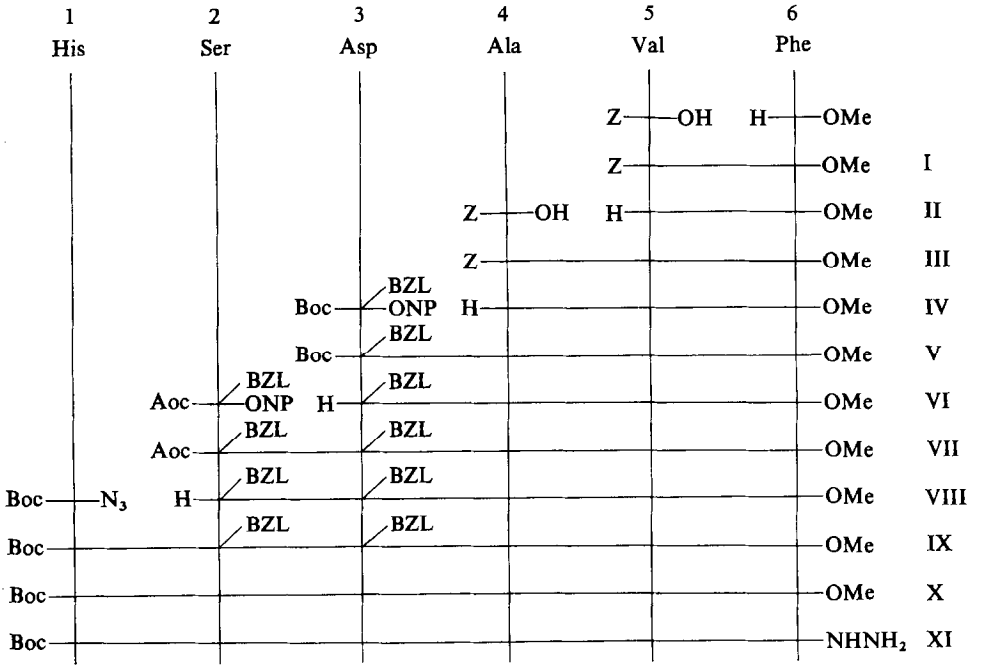

Chart 1

DMF (8 ml). The solution was cooled in acetone–dry ice bath to –25°C, Hydrochloric acid in dioxane (5 ml, 4.78 *N*) was added, followed by *t*-butylnitrite (0.75 ml). After 45 min at –25°C, the reaction mixture was cooled to –50°C and neutralized with triethylamine (3.35 ml). After 3 days of stirring at 4°C, a second portion of azide (2 m*M*) in DMF (2 ml) was added. Next day, the mixture was filtered, the precipitate was washed with DMF (5 ml), and the DMF was concentrated *in vacuo* to small volume. Cold water (200 ml) was added, the product was collected by filtration, washed with water (40 ml), and dried *in vacuo* over P_2O_5 to give 3.07 g. This material was purified by countercurrent distribution in the system chloroform/toluene/methanol/water (5:5:8:2). Two peaks were obtained, with $k = 0.39$ and $k = 3.4$. The slower-moving

band yielded 1.32 g and the faster, 1.06. Both materials melted at 168–170°C; tlc R_fA 0.70, R_fB 0.76, and gave superimposable NMR spectra. Amino acid analysis for the slower-moving material: His, 0.9; Ser, 0.9; Asp, 1.0; Ala, 1.0; Val, 1.1; Phe, 1.1. For the material with $k = 3.4$: His, 1.0; Ser, 0.9; Asp, 0.9; Ala, 1.1; Val, 1.1; Phe, 0.9. As shown below, this is a trifluoroacetate.

Anal. Calcd for $C_{50}H_{64}N_8O_{12}$: C, 62.0; H, 6.7; N, 11.6. Found (for the material with $k = 0.39$): C, 62.2; H, 6.5; N, 10.9. Calcd for $C_{50}H_{64}N_8O_{12} \cdot CF_3COOH$: C, 57.6; H, 6.0; N, 10.3; F, 5.3. Found (for the material with $k = 3.4$): C, 58.0; H, 6.1; N, 10.4; F, 5.9.

The protected hexapeptide methyl ester from the slower-moving fraction IX (1.07 g) was hydrogenated for 24 hr in a mixture of AcOH (8 ml) and 80% methanol (180 ml) in the presence of a 10% Pd on charcoal catalyst (300 mg). The solution was filtered from the catalyst, and evaporated to dryness *in vacuo* to give 790 mg; mp 164–165°C; tlc R_fA 0.47, R_fB 0.59.

The partially protected hexapeptide methyl ester X (790 mg) was suspended in methanol (27 ml). Hydrazine (3 ml, 97%) was added, whereupon a clear solution resulted. After 24 hr, the solvent was removed *in vacuo* and the oil dried overnight over concentrated H_2SO_4. It was triturated with ether (100 ml), filtered, washed with ether (50 ml), and dried; 740 mg; mp 190–194°C (dec.), softens at 186°C; $[\alpha]_D^{25} - 32°$ (*c* 1, AcOH); tlc R_fA 0.41, R_fB 0.36. The NMR spectrum showed absence of the methoxy group. Amino acid analysis: His, 0.9; Ser, 0.9; Asp, 1.1; Ala, 1.1; Val, 1.0; Phe,1.1.

Anal. Calcd for $C_{35}H_{52}N_{10}O_{11} \cdot 3H_2O$: C, 49.8; H, 6.9; N, 16.6; hydrazide (*18*) N, 3.3. Found: C, 49.6; H, 6.9; N, 16.7; hydrazide N, 3.6.

COMPARISON OF THE SYNTHETIC AND NATURAL HEXAPEPTIDES

Chymotrypsin (α-chymotrypsin, TLCK-treated) and thermolysine, crystalline, were obtained from Merck, Darmstadt, dipeptidyl aminopeptidase I from Schwarz/Mann. The techniques used for paper electrophoresis and paper chromatography have been described elsewhere (*19*, *20*).

Hydrolysis of the Hexapeptide Ester Bond

Two milligrams of the hexapeptide ester were suspended in 1 ml 1% NH_4HCO_3, and 20 μl of a 0.2% solution of chymotrypsin in m*M* AcOH was added. After 2 hr at 21°C, the now clear solution was frozen and lyophilized. The residue was dissolved in 0.5 ml of water and the solution kept on the boiling water bath for 3 min and then immediately cooled with ice-cold water. A barely distinguishable precipitate was centrifuged off and the clear supernatant fraction was lyophilized. On high voltage paper electrophoresis at pH 6.4 (90 min at 50 V/cm in pyridine/acetic acid/water, 300:11.5:2700 by volume, using Whatman 3 MM paper), an aliquot (20 μg) of the lyophilized material was found to have moved 4.2 cm toward the anode (not corrected for electroendosmosis). Under the same conditions, the unhydrolyzed material migrated equidistantly toward the cathode.

Degradation with thermolysine was carried out essentially under the conditions

recommended by Ambler and Meadway (*11*). Approximately 200-μg samples of the synthetic hexapeptide and of the *N*-terminal fragment of natural VIP, isolated from a chymotryptic digest of the octacosapeptide (*3*), were each dissolved in 100 μl of an ammonia/acetic acid buffer of pH 8.5 which was 0.2 *M* with respect to acetic acid, and contained 5 m*M* $CaCl_2$. To these solutions were added 3-μl aliquots of a 0.2% solution of thermolysine, in the same buffer, at intervals of 2 hr. The degradation was allowed to proceed at 37°C. Two hours after the third, and last, addition of the enzyme, the degradation mixtures were frozen and lyophilized.

On paper chromatography in the *n*-butanol/acetic acid/pyridine/water (30:6:20:24) system of Waley and Watson (*21*), it was found that the degradation products of the synthetic and natural hexapeptides gave identical pictures, as did the hexapeptides themselves (Fig. 1). On paper electrophoresis at pH 6.4, the fragment with the higher mobility in this chromatography was found to behave like a neutral, and the other fragment like an acidic substance.

Separation of the Thermolytic Split Products

The thermolytic split products were clearly separated on a 0.6 × 4-cm column of DEAE–cellulose (Whatman DE 22, standard). The column was equilibrated with 0.02 *M* NH_4HCO_3 which had been adjusted to pH 6.5 with CO_2. The degradation mixture was dissolved in 200 μl of this buffer and allowed to sink into the column, which was then developed with the same buffer. The neutral peptide was recovered by lyophilization from the first 3 ml of effluent. The acidic peptide which had been retained on the column was then eluted with 3 ml of 0.2 *M* NH_4HCO_3 and recovered by lyophilization. The neutral peptide was identified as valylphenylalanine, obviously VIP_{5-6}, and the acidic peptide consequently was identified as VIP_{1-4}. In this separation there was again no difference in behavior between the degradation products from the synthetic and natural hexapeptides.

Degradation of the Tetrapeptides into Histidylserine and Aspartylalanine

This degradation, with the use of dipeptidyl aminopeptidase I (*12*), was carried out essentially as described for the identification of histidylserine as the *N*-terminal dipeptidyl sequence of VIP (*2*). After 3 hr at 37°C with an enzyme to substrate ratio of about 0.1, it was found on paper electrophoresis (*11*) of an aliquot of the degradation mixture that most of the tetrapeptide had been split into two fragments. One fragment, which had migrated 11.6 cm toward the cathode, was indistinguishable from a sample of synthetic histidylserine (from Mann/Schwarz) run in parallel with it, and the other fragment, which had migrated 15.5 cm toward the anode, was indistinguishable from synthetic aspartylalanine (from Fox Chemical Company). Under these conditions, the original tetrapeptide migrated 6 cm toward the anode.

Except for the omission of the prefractionation on Sephadex G25, the histidylserine was isolated from the degradation mixture by chromatography at pH 6 on carboxymethyl cellulose, as described earlier for its isolation from the degradation mixture obtained on treating VIP itself with dipeptidyl aminopeptidase (*2*). The acidic fragment was not separated, under these conditions, from the small amount of tetrapeptide that had been left undegraded. However, they could be clearly separated by chromatography at pH 4.8 on a column (0.3 × 25 cm) of SE–Sephadex (*22*). The acidic fragment was

identified as aspartylalanine. In this, there was again no difference between the synthetic and the natural products.

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