Synthesis of the Vasoactive Intestinal Peptide (VIP)

Miklos Bodanszky,* Yakir S. Klausner, Cynthia Yang Lin, Viktor Mutt, and Sami I. Said

Contribution from the Department of Chemistry, Case Western Reserve University, Cleveland, Ohio 44106, Department of Biochemistry, Medicinska Nobelinstitutet, Karolinska Institutet, Stockholm, Sweden, and the Southwestern Medical School at Dallas, The University of Texas, Dallas, Texas 75235. *Received December 21, 1973*

Abstract: An octacosapeptide with the amino acid sequence proposed for the vasoactive intestinal peptide (VIP) was synthesized in solution through isolated intermediates. The protected heptapeptide derivative, *tert*-butyloxycarbonyl-L-threonyl-L-aspartyl-L-asparaginyl-L-tyrosyl-L-threonyl-L-arginyl-L-leucine azide, was coupled to the partially deprotected pentadecapeptide L-arginyl-N^ϵ-benzyloxycarbonyl-L-lysyl-L-glutaminyl-L-methionyl-L-alanyl-L-valyl-N^ϵ-benzyloxycarbonyl-L-lysyl-N^ϵ-benzyloxycarbonyl-L-lysyl-L-tyrosyl-L-leucyl-L-asparaginyl-L-seryl-L-isoleucyl-L-leucyl-L-asparaginamide (hydrochloride). The resulting protected docosapeptide, corresponding to sequence VIP_{7-28}, was partially deprotected and acylated with *tert*-butyloxycarbonyl-L-histidyl-L-seryl-L-aspartyl-L-alanyl-L-valyl-L-phenylalanine azide to afford a protected octacosapeptide encompassing the entire sequence of VIP. The protecting groups were removed in a single operation with trifluoroacetic acid. After purification by countercurrent distribution, the product was compared with natural (porcine) VIP. The synthetic peptide showed the characteristic biological activities of the natural material in systemic vasodilation and reduction of arterial blood pressure in intact dogs and relaxation of several isolated smooth muscle preparations. Comparisons of the fragments formed in side-by-side degradations of samples of natural and synthetic VIP with specific enzymes support the amino acid sequence (Figure 1) proposed for porcine VIP.

A vasoactive intestinal peptide (VIP) was isolated from hog intestines by Said and Mutt.[1,2] Its effects on peripheral and splanchnic blood flow and on smooth muscle preparations have been described by Said and Mutt[3] and by Piper, Said, and Vane.[4] The amino acid sequence of the single chain octacosapeptide was determined by Mutt and Said[5] and is shown in Figure 1. The synthesis of VIP was carried out with the principal aim of providing independent evidence for the correctness of the sequence elucidated by degradation. Even the scheme of the synthesis was influenced in part by considerations involving proof of structure by synthesis. *E.g.*, a hexapeptide sequence, VIP_{1-6},[6] with phenylalanine at its N-terminus was selected as a fragment because this is one of the points where chymotrypsin cleaves the molecule of VIP and a comparison of a fragment from natural VIP with the corresponding synthetic peptide was an attractive possibility. For similar reasons, the C-terminal cyanogen bromide fragment, VIP_{18-28},[7] was chosen as a larger building unit. The scheme of the synthesis is shown in Chart I.

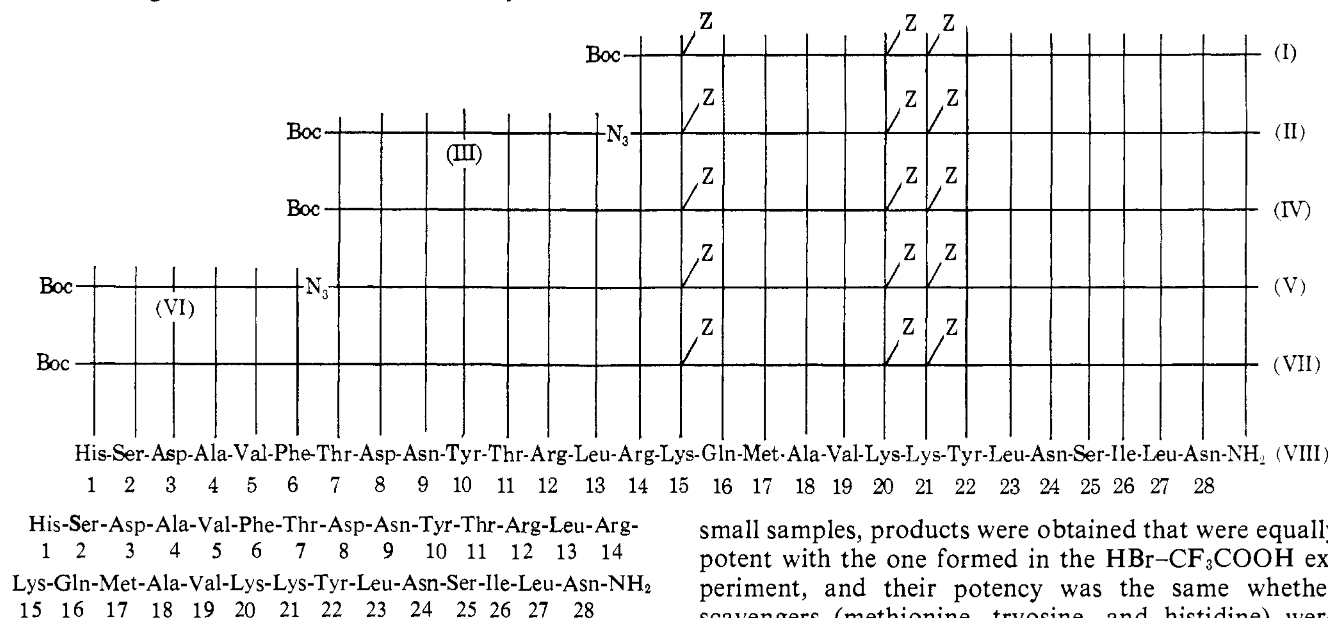
Strategy. The stepwise approach, building a peptide chain from its C-terminal residue with the addition of a single amino acid at a time, preferentially introduced as an active ester, is often the method of choice, for reasons described earlier.[8] In the synthesis of VIP, technical difficulties rendered an attempted stepwise building of the chain impractical. The protected intermediates, from the C-terminal tripeptide on, were extremely insoluble in the solvents commonly used in peptide synthesis. Acylations—with active esters—were carried out in viscous gels. Unusually long reaction times were necessary to achieve complete conversion of the amino components. A high excess[9] of active esters provided some improvement but the execution of the reactions remained both time consuming and inconvenient. A careful completion of each acylation became mandatory because the insolubility of the intermediates excluded purification by methods such as chromatography or countercurrent distribution, and therefore all effort had to be made to obtain each protected peptide, simply by washing with solvents, in sufficient purity to be used in the following step.

The significance of unimolecular side reactions is greatly increased in such situations. *E.g.*, after the incorporation of the glutamine residue in position 16, the formation of a pyroglutamyl peptide during the subsequent acylation had to be anticipated. Therefore, after the synthesis of the C-terminal hendecapeptide 18–28,[7] the stepwise strategy was abandoned. Glycine and proline are not constituents of VIP. Thus, the points where the sequence should be dissected were not obvious. Fragments corresponding to sequences 1–6,[6] 7–13,[10] and 14–17[11] were prepared stepwise and combined (*cf.* Chart I) by the azide procedure[12] under the conditions developed by Honzl and Rudinger.[13] The reasons for choosing VIP_{1-6} and VIP_{18-28} as fragments in synthesis have already been explained; VIP_{7-13} was selected because its C-terminal residue, leucine, seemed unlikely to suffer racemization during coupling.

Tactics. In the preparation of the C-terminal fragment,[7] VIP_{18-28}, the benzyloxycarbonyl (Z) group was

(1) S. I. Said and V. Mutt, *Nature* (*London*), **225**, 863 (1970).
(2) S. I. Said and V. Mutt, *Eur. J. Biochem.*, **28**, 199 (1972).
(3) S. I. Said and V. Mutt, *Science*, **169**, 1217 (1970).
(4) P. J. Piper, S. I. Said, and J. R. Vane, *Nature* (*London*), **225**, 1144 (1970).
(5) V. Mutt and S. I. Said, *Eur. J. Biochem.*, in press.
(6) Y. S. Klausner, V. Mutt, and M. Bodanszky, *Bioorg. Chem.*, **2**, 87 (1972).
(7) M. Bodanszky, Y. S. Klausner, and V. Mutt, *Bioorg. Chem.*, **2**, 30 (1972).
(8) M. Bondanszky, *Ann. N. Y. Acad. Sci.*, **88**, 655 (1960).
(9) M. Bodanszky in "Prebiotic and Biochemical Evolution," A. P. Kimball and J. Oro, Ed., North-Holland Publishing Co., Amsterdam, 1971, p 217.
(10) Y. S. Klausner, C. Yang Lin, V. Mutt, and M. Bodanszky, *Bioorg. Chem.*, **2**, 345 (1973).
(11) Y. S. Klausner and M. Bodanszky, *Bioorg. Chem.*, **2**, 354 (1973).
(12) T. Curtius, *Ber.*, **35**, 3226 (1902).
(13) J. Honzl and J. Rudinger, *Collect. Czech. Chem. Commun.*, **26**, 2333 (1961).

Chart I. Fragment Condensation Scheme in the Synthesis of VIP



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

Figure 1. The sequence of the porcine vasoactive intestinal peptide (VIP).

used for the protection of α-amino groups until the heptapeptide stage and hydrogenolysis for the removal of this protection. For the protection of the amino function in the side chain of lysine residues, the Z group was used and therefore, after introduction of the lysine in position 21, derivatives of *tert*-butyloxycarbonyl amino acids were applied. To keep the partial cleavage of the Z groups at a minimum, a mixture containing 70% trifluoroacetic acid and 30% acetic acid was used for deprotection. For the same reason, mixtures of trifluoroacetic acid with water were used by Schnabel and his associates.[14]

Before the coupling of fragments, side chain protecting groups of the carboxyl components were removed by catalytic hydrogenation and their C-terminal ester group hydrazinolyzed. After completion of the final coupling reaction and purification of the protected octacosapeptide by chromatography on Sephadex LH-20 in methanol, the benzyloxycarbonyl groups protecting the side chain amino functions of the lysine residues and the *tert*-butyloxycarbonyl group attached to the N-terminal histidine were simultaneously cleaved by acidolysis. Initially, hydrobromic acid in trifluoroacetic acid was used for this purpose with anisole and methionine added as scavengers. The hydrobromide of the crude octacosapeptide was converted to the acetate with the help of an ion-exchange resin and the product subjected to countercurrent distribution. Because of difficulties encountered in this procedure (*cf.* below), attempts were made to remove the protecting groups by a prolonged exposure to the action of trifluoroacetic acid.[15] In preliminary experiments on small samples, products were obtained that were equally potent with the one formed in the HBr–CF_3COOH experiment, and their potency was the same whether scavengers (methionine, tryosine, and histidine) were added or not. Therefore, trifluoroacetic acid alone was used for the final deprotection. The reaction was carried out at room temperature and was allowed to proceed for 2.5 days. It seems reasonable to assume that benzyl trifluoroacetate and *tert*-butyl trifluoroacetate formed in the acidolytic cleavage are less potent alkylating agents than benzyl bromide and that this advantage is not offset by a longer exposure of the peptide to the conditions of deprotection. Because of difficulties encountered in the purification of the free octacosapeptide, it is not possible at this time to evaluate the general usefulness of final deprotection with trifluoroacetic acid.

The Synthetic Product. In the first attempt for the purification of the free octacosapeptide, its hydrobromide was converted to an acetate salt with the help of the ion-exchange resin Dowex 1 (acetate), and the material was distributed in an automatic Craig apparatus through 487 transfers. The solvent system 1-butanol–0.1 *M* NH_4HCO_3, used in the isolation of porcine VIP,[2] was employed. However, in the case of the synthetic material, no satisfactory distribution could be achieved. The irregular features of the distribution curve suggested an aggregation of the peptide. An attempt to recover the product by concentration and lyophilization produced considerable amounts of insoluble material and resulted also in a drastic loss of biological activity. Subsequently, purifications were carried out on the trifluoroacetate salts of the octacosapeptide deprotected with trifluoroacetic acid. A short countercurrent distribution in the system 1-butanol–ethanol–dilute (0.008%) trifluoroacetic acid (4:1:5) was used. About half of the peptide was recovered from a band corresponding to a distribution coefficient of about 0.3. The purified product gave the expected amino acid analysis, and migrated on paper chromatograms and paper electropherograms with the mobility of VIP, but in the 1-butanol–0.1 *M* NH_4HCO_3 system it still showed a tendency for aggregation. Its biological activities

(14) E. Schnabel, H. Klostermeyer, and H. Berndt, *Justus Liebigs Ann. Chem.*, 749, 90 (1971).

(15) F. Weygand and W. Steglich (*Z. Naturforsch. B*, 14, 472 (1959)) recommended the use of boiling trifluoroacetic acid for this purpose. Model experiments revealed that when trifluoroacetic acid is applied at room temperature, no substitution occurs on the aromatic ring of tyrosine. For the (undesired) removal of benzyloxycarbonyl groups from the ϵ-amino groups of lysine residues by trifluoroacetic acid at room temperature, *cf.* D. A. Ontjes and C. B. Anfinsen in "Peptides: Chemistry and Biochemistry," B. Weinstein and S. Lande, Ed., Marcel Dekker, New York, N. Y., 1970, p 79; B. C. Erickson and R. B. Merrifield in "Chemistry and Biology of Peptides," J. Meienhofer, Ed., Ann Arbor Publishers, Ann Arbor, Mich., 1972, p 191; D. Yamashiro, R. L. Noble, and C. H. Li, *ibid.*, p 197.

Figure 2. (1) Synthetic VIP, 80 μg; (2) synthetic VIP, 80 μg, degraded with kallikrein; (3) natural VIP, 80 μg, degraded with kallikrein; (4) natural VIP, 80 μg. Electrophoresis conditions: 90 min at 50 V cm in pyridine–acetic acid–water (300:11.5:2700 4 v/v) at pH 6.4 using Whatman 3MM paper. Some loss of the synthetic peptide, probably due to aggregation, occurred in these experiments.

Figure 3. (1) Leucylarginine (10 n*M*); (2) 30 μg of N-terminal kallikreinic tetradeccapeptide of synthetic VIP degraded with trypsin; (3) 30 μg of N-terminal kallikreinic tetradecapeptide of natural VIP degraded with trypsin; (4) 10 n*M* leucylarginine. Electrophoresis conditions as described in Figure 2. For an explanation of the differences in intensities, *cf.* caption for Figure 2.

Table I. Bioassay of Natural and Synthetic VIP[a]

	VIP, natural		VIP, synthetic	
Tissue[b]	2 μg/ml	5 μg/ml	2 μg/ml	5 μg/ml
GPT	300	700	250	600
RSS	600	1500	500	1200
RC	20	80	7	30
GPGB	90	160	100	140

[a] Numbers, in arbitrary units, are products of amplitude and duration of response. The natural (porcine) VIP preparation used for comparisons was of the highest purity obtained so far (*cf.* ref 2). Concentrations of the solutions of the natural and synthetic peptides were determined by their respective absorptions at 275 nm. [b] Tissues: GPT, guinea pig trachea; RSS, rat stomach strip; RC, rat colon; GPGB, guinea pig gallbladder.

(Table I) were comparable to those of natural porcine VIP.[16]

Enzymic Degradation of Natural and Synthetic VIP. In previous studies,[6,7,10] the correctness of the partial sequences proposed[5] for the N-terminal hexapeptide (VIP_{1-6}), the consecutive heptapeptide (VIP_{7-13}), and the C-terminal hendecapeptide (VIP_{18-28}) portions of the molecule was ascertained by comparisons of fragments obtained from natural VIP with the corresponding synthetic peptides and further confirmed by enzymic hydrolysis of the fragment and comparison of the smaller peptides obtained from natural and synthetic materials. These studies, however, provided no evidence about sequence 14–17. Therefore, and also to ascertain that no drastic side reactions had taken place during the fragment coupling and the subsequent deprotection of the synthetic material, the following comparisons were made between samples of the synthetic octacosapeptide VIII and of pure natural VIP. The peptides were exposed to the hydrolytic action of kallikrein, which had previously[5] been found to cleave, between residues 14 and 15, the N-terminal heptadecapeptide, obtained by cleavage of VIP with cyanogen bromide. Cleavage of the same bond in VIP was found to take place in a fairly selective manner. The two tetradecapeptides formed, the N-terminal V-K-N and the C-terminal V-K-C, migrated with different mobilities on paper electrophoresis at pH 6.4, but the corresponding peptides from the natural and synthetic octacosapeptides migrated indistinguishably (Figure 2). Separation and subsequent degradation of the N-terminal tetradecapeptides with trypsin produced the dodecapeptide V-K-N-Tr-N (VIP_{1-12}) and leucylarginine (VIP_{13-14}). A comparison of these peptides from synthetic and natural VIP revealed no difference on paper electrophoresis (Figure 3).

After isolation, the dodecapeptides were cleaved further with chymotrypsin, and the degradation products from the natural and synthetic materials were again found to be indistinguishable on paper electropherograms (Figure 4).

The C-terminal tetradecapeptides, V-K-C, from the natural and synthetic octacosapeptides were cleaved with cyanogen bromide. The fragments formed, VIP_{15-17} and VIP_{18-28}, migrated with different mobilities both on paper electrophoresis and on paper chromatog-

(16) These results were reported in a preliminary form: M. Bodanszky, Y. S. Klausner, and S. I. Said, *Proc. Nat. Acad. Sci. U. S.*, **70**, 382 (1973).

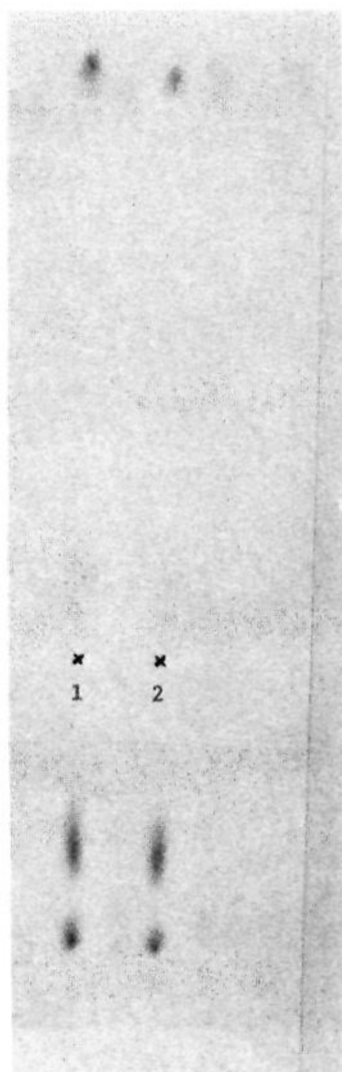


Figure 4. (1) Products obtained on degrading the N-terminal tryptic dodecapeptide of natural VIP (the acidic material of Figure 3) with chymotrypsin (approximately 20 μg of dodecapeptide, 0.4 μg of enzyme, degradation for 2 hr at 21°). (2) The same as lead 1, except that the dodecapeptide was derived from synthetic VIP. Electrophoresis conditions as in Figure 2.

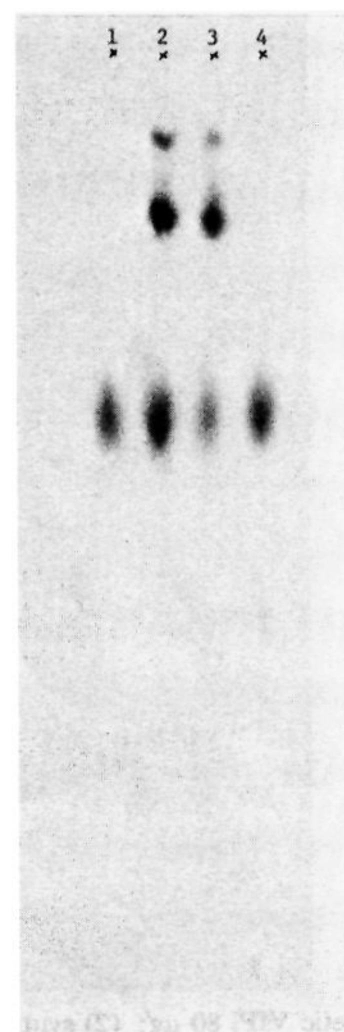


Figure 5. (1) VIP$_{18-28}$ (30 μg) from the natural octacosapeptide; (2) CNBr cleavage products of 30 μg of the C-terminal kallikreinic fragment V-K-C (VIP$_{15-28}$) of natural VIP; (3) CNBr cleavage products of 30 μg of the C-terminal kallikreinic fragment of synthetic VIP; (4) 30 μg of synthetic VIP$_{18-28}$. Descending paper chromatography on Whatman 42 paper in the system of Waley and Watson[22] (1-butanol–acetic acid–pyridine–water (30:6:20:24)).

raphy. The corresponding fragments from the natural and synthetic materials were, however, indistinguishable (Figure 5). The VIP$_{18-28}$ fragments were also indistinguishable from the natural and synthetic preparations of this peptide described previously.[7] In a separate experiment, the C-terminal fragments (VIP$_{15-28}$) of the kallikrein cleavage were treated with chymotrypsin. A complex mixture of peptides was seen on the electropherogram (Figure 6), but similar patterns of these products were formed from the natural and the synthetic materials.

The results of these comparisons give no reason to doubt the correctness of the sequence (Figure 1) proposed[5] for porcine VIP. Yet, because of some imperfections[17] in the final product, attempts are being made in our laboratory toward a new synthesis of VIP, with different protecting groups and also on a solid support.

Experimental Section

Capillary melting points are reported uncorrected. Thin-layer chromatograms (silica gel, Merck) were developed with the solvent systems: A, 1-butanol–acetic acid–water (4:1:1); B, 1-butanol–pyridine–acetic acid–water (30:20:6:24). Spots were revealed by uv, charring with ammonium sulfate,[18] modified Rydon–Zahn reagent,[19] Sakaguchi, and Pauly reagents. For amino acid analysis, samples were hydrolyzed with constant-boiling hydrochloric acid in evacuated sealed ampoules at 110° for 16 hr, and analyzed by the method of Spackman, Stein, and Moore[20] on a Beckman-Spinco 120C instrument.

***tert*-Butyloxycarbonyl-L-threonyl-L-aspartyl-L-asparaginyl-L-tyrosyl-L-threonyl-L-arginyl-L-leucyl-L-arginyl-N^{ϵ}-benzyloxycarbonyl-L-lysyl-L-glutaminyl-L-methionyl-L-alanyl-L-valyl-N^{ϵ}-benzyloxycarbonyl-L-lysyl-N^{ϵ}-benzyloxycarbonyl-L-lysyl-L-tyrosyl-L-leucyl-L-asparaginyl-L-seryl-L-isoleucyl-L-leucyl-L-asparaginamide (Hydrochloride) (IV).** The protected pentadecapeptide, *tert*-butyloxycarbonyl-L-arginyl-N^{ϵ}-benzyloxycarbonyl-L-lysyl-L-glutaminyl-L-methionyl-L-alanyl-L-valyl-N^{ϵ}-benzyloxycarbonyl-L-lysyl-N^{ϵ}-benzyloxycarbonyl-L-lysyl-L-tyrosyl-L-leucyl-L-asparaginyl-L-seryl-L-isoleucyl-L-leucyl-L-asparaginamide, hydrochloride[11] (I, 310 mg), was suspended in acetic acid (0.9 ml). The suspension was cooled in an ice–water bath and TFA (2.1 ml) was added. After 25 min at room temperature, the acids were removed *in vacuo*, dry ether (40 ml) was added, and the product was filtered, washed with ether (30 ml), and dried *in vacuo* over NaOH and P_2O_5 to give II, 270 mg; mp 118–120°; $[\alpha]^{25}D$ $-30°$ (*c* 1, 80% AcOH).

A solution of *tert*-butyloxycarbonyl-L-threonyl-L-aspartyl-L-asparaginyl-L-tyrosyl-L-threonyl-L-arginyl-L-leucine azide (III) was prepared in the following manner: the heptapeptide hydrazide[10] (228 mg) was suspended in DMF (3.5 ml) and the suspension cooled to $-30°$; HCl in dioxane (4.7 *N*, 0.24 ml) was added, followed by *tert*-butyl nitrite (41 μl). After 30 min at -25 to $-30°$, the solution was cooled to $-60°$, and diisopropylethylamine (196 μl) was slowly added, followed by the pentadecapeptide trifluoroacetate III as a fine powder and more base (20 μl). The suspended material dissolved after *ca.* 15 min. The reaction mixture was allowed to warm up to 0° and then stirred at about 4°. After 3 days an addi-

(17) The biological activity of the material obtained on removal of the protecting groups was found, in several experiments, to be equal to and sometimes even higher than that of natural VIP. After countercurrent distribution, however, the purified product seems to be slightly less active than natural VIP (Table I), although the differences are within experimental error. Also, the tendency of synthetic VIP for aggregation, possibly because of some loss of side chain amide groups in part of the material, is more pronounced than in natural VIP.

(18) T. Ziminski and E. Borowski, *J. Chromatogr.*, 23, 480 (1966).

(19) R. H. Mazur, B. W. Ellis, and P. Cammarata, *J. Biol. Chem.*, 237, 1619 (1962).

(20) D. H. Spackman, W. H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1962).

tional amount of the hydrazide (114 mg) was converted to the azide and added to the reaction mixture. After a total of 5 days, ethanol (40 ml) was added and a small amount of insoluble material was removed by filtration. The ethanol was removed *in vacuo* and ice-water (40 ml) was added. The precipitate was disintegrated, filtered, and washed with water (20 ml) and dried *in vacuo* over P_2O_5 to give IV, 303 mg (82%); shrinks at 160°, gradual darkening with no melting to 310°; tlc, R_fA 0.49 (with minor spots of R_fA 0.41 and 0.56), R_fB 0.73 (with some streaking). No solvent system was found for the purification of this material on a preparative scale.

Amino acid analysis: Lys, 3.0; NH_3, 5.6; Arg, 2.0; Asp, 4.8; Thr, 2.0; Ser, 1.0; Glu, 1.0; Ala, 1.0; Val, 1.1; Met, 0.9; Ile, 1.0; Leu, 3.2; Tyr, 2.0.

Anal. Calcd for $C_{146}H_{226}N_{36}O_{41}Cl_2S$: C, 54.0; H, 7.0; N, 15.5. Found: C, 54.3; H, 7.2; N, 14.8.

***tert*-Butyloxycarbonyl-L-histidyl-L-seryl-L-aspartyl-L-alanyl-L-valyl-L-phenylalanyl-L-threonyl-L-aspartyl-L-asparaginyl-L-tyrosyl-L-threonyl-L-arginyl-L-leucyl-L-arginyl-N^ϵ-benzyloxycarbonyl-L-lysyl-L-glutaminyl-L-methionyl-L-alanyl-L-valyl-N^ϵ-benzyloxycarbonyl-L-lysyl-N^ϵ-benzyloxycarbonyl-L-lysyl-L-tyrosyl-L-leucyl-L-asparaginyl-L-seryl-L-isoleucyl-L-leucyl-L-asparaginamide (Hydrochloride) (VII).** The protected docosapeptide IV (160 mg) was partially deblocked with 70% TFA–30% acetic acid mixture (2 ml) as described earlier. The yield of the trifluoroacetate V was 163 mg (quantitative); mp 132–134°; tlc, R_fA 0.30, with minor spots at R_fA 0.1 and at the origin.

The entire amount of crude V was applied in a small volume of methanol to a column of Sephadex LH-20 (2.5 × 77 cm). Fractions of 3.5 ml were collected at a flow rate of 30 ml/hr, with monitoring at 280 nm. The desired material was located in fractions 50–70. These were pooled and evaporated to give V; 130 mg; mp 134–136°; $[\alpha]^{25}D$ +3° (*c* 1, AcOH); tlc, R_fA 0.30, R_fB 0.55.

Amino acid analysis: Lys, 3.0; NH_3, 5.3; Arg, 2.0; Asp, 4.2; Thr, 1.9; Ser, 1.0; Glu, 0.95; Ala, 1.0; Val, 0.90; Met, 0.85; Ile, 1.0; Leu, 3.2; Tyr, 1.9.

A solution of *tert*-butoxycarbonyl-L-histidyl-L-seryl-L-aspartyl-L-alanyl-L-valyl-L-phenylalanine azide (VI) was prepared in the following manner: the partially protected hexapeptide hydrazide[6] (85 mg) was suspended in DMF (1.5 ml) and the suspension cooled to −30°; HCl in dioxane (4.7 N, 0.11 ml) was added, followed by *tert*-butyl nitrite (18 µl). After 30 min at −25 to −30°, the solution was cooled to −60°, diisopropylethylamine (86 µl) was slowly added, followed by the docosapeptide trifluoroacetate V (160 mg) and more base (10 µl). A clear solution resulted in about 10 min. It was allowed to warm up to 0° and then stirred in a cold room, while the pH was maintained slightly basic. After 3 days, ice-water (30 ml) was added slowly. The precipitate was disintegrated, filtered, washed with water (15 ml), and dried *in vacuo* over P_2O_5 to give crude VII, 150 mg; melting with decomposition at about 175°; tlc, R_fA 0.33 (with minor spots at 0.2, 0.45, and 0.5), R_fB 0.6 (with two minor spots).

A sample (29 mg) of the crude octacosapeptide derivative was dissolved in a mixture of DMF (0.2 ml) and methanol (0.8 ml), a small insoluble residue was removed by centrifugation, and the solution was applied to a column of Sephadex LH-20 (2.5 × 74 cm) equilibrated with methanol. The same solvent was used for elution. Fractions (3 ml) were collected at 10-min intervals; the elution was monitored by absorption at 280 nm. The purified product was obtained from fractions 30–45.[21] From five such chromatograms and a total of 148 mg of crude peptide, 52 mg of purified VII was collected. The product has no well-defined melting point; it sinters and gradually decomposes at about 185°; tlc, R_fA 0.33 (with a minor spot at 0.6), R_fB 0.60. Amino acid analysis: Lys, 2.8; His, 0.7; NH_3, 6.0; Arg, 1.9; Asp, 5.2; Thr, 1.9; Ser, 2.1; Glu, 1.0; Ala, 2.0; Val, 2.0; Met, 0.6 (some sulfoxide also present); Ile, 0.9; Leu, 2.8; Tyr, 1.6; Phe, 0.9.

Anal. Calcd for $C_{176}H_{266}N_{44}O_{50}Cl_2S$: C, 54.2; H, 6.9; N, 15.8. Found: C, 53.8; H, 7.1; N, 15.3. (The values of elemental analysis suggest that the sample, in spite of thorough drying (60°, 0.1 mm), retained some moisture, perhaps methanol.)

L-Histidyl-L-seryl-L-aspartyl-L-alanyl-L-valyl-L-phenylalanyl-L-threonyl-L-aspartyl-L-asparaginyl-L-tyrosyl-L-threonyl-L-arginyl-L-leucyl-L-arginyl-L-lysyl-L-glutaminyl-L-methionyl-L-alanyl-L-valyl-L-lysyl-L-lysyl-L-tyrosyl-L-leucyl-L-asparaginyl-L-seryl-L-isoleucyl-L-leucyl-L-asparaginamide (VIP, trifluoroacetate salt, VIII). The protected octacosapeptide amide VII (29 mg) was dissolved in TFA (2.5 ml) and kept, protected from light, at room temperature for about 2.5 days. The acid was removed *in vacuo* and the residue triturated with dry ether; the precipitate was separated by centrifugation, washed with ether, and dried *in vacuo*. The trifluoroacetate of crude VIP was dissolved in H_2O (*ca.* 1 ml) and lyophilized: 28 mg was obtained. A sample (23 mg) was dissolved in 3 ml of the lower phase of the solvent system 1-butanol–ethanol (95%)–0.008% TFA (4:1:5) and distributed in a 10-tube Craig apparatus, with 3-ml lower and 3-ml upper phases. After 19 transfers (single withdrawal technique), the distribution pattern was determined by evaporation and weighing the residues from individual tubes. The contents of tubes 0–2 (A, 7.2 mg) and those of 3–7 (B, 11.2 mg) were pooled, respectively. Quantitative amino acid analysis of A and B showed no significant difference. The hydrolysate of fraction A contained some methionine sulfoxide; this was absent in the hydrolysate of fraction B. On paper chromatograms in the Waley–Watson system,[22] both materials gave somewhat elongated spots (R_f *ca.* 0.6).

(21) Only the center part of a broad peak of the chromatogram was used: the side fractions also contained the desired protected octacosapeptide, but in lesser purity.

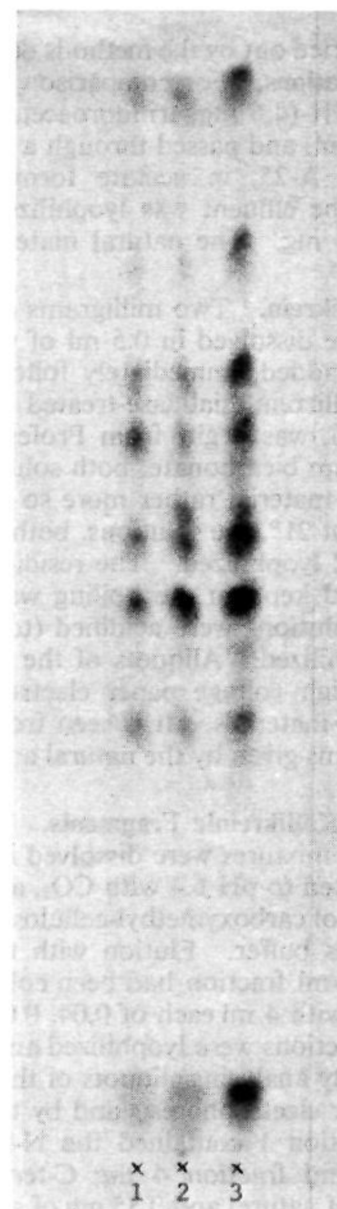


Figure 6. (1) V-K-C (VIP_{15-28}) (40 µg) from the natural octacosapeptide degraded with 0.8 µg of chymotrypsin for 2 hr at 21°; (2) as in lead 1, but two more additions of the enzyme, and a degradation time of 6 hr; (3) as in lead 2, but 60 µg of synthetic V-K-C. Electrophoresis conditions as described in Figure 2.

Amino acid analyses (* after digestion with aminopeptidase M)

	Lys	His	NH_3	Arg	Asp	Thr	Ser	Glu
Calcd	3.0	1.0	5.0	2.0	5.0	2.0	2.0	1.0
Found, A	2.7	1.0	5.8	2.1	5.1	1.9	1.9	1.1
Found, B	2.9	1.0	6.5	2.2	4.7	1.8	2.0	1.0
Found,* B	3.0	1.2		1.8	2.0			

	Ala	Val	Met	Ile	Leu	Tyr	Phe
Calcd	2.0	2.0	1.0	1.0	3.0	2.0	1.0
Found, A	2.1	1.8	0.8	0.9	2.8	1.7	0.9
Found, B	2.0	1.8	0.9	1.0	2.9	2.0	1.0
Found,* B	1.9	1.9	0.7	1.1	2.8	1.8	1.0

(22) S. G. Waley and J. Watson, *Biochem. J.*, **57**, 529 (1954).

Bioassays were carried out by the methods described in ref 1–4.

Enzymatic Degradations. For comparison of the synthetic and natural materials, VIII (4.6 mg, trifluoroacetate) was dissolved in 0.2 *M* acetic acid (1 ml) and passed through a 0.6 × 10 cm column of DEAE-Sephadex A-25, in acetate form. The peptide-containing fraction of the effluent was lyophilized. The lyophilized material weighed 2.4 mg. The natural material was prepared as described previously.[2]

Cleavage with Kallikrein. Two milligrams each of the synthetic and natural VIP were dissolved in 0.5 ml of water, and 0.5 ml of 2% NH_4HCO_3 was added, immediately followed by 20 μl of an 0.2% solution of kallicrein (sialidase-treated pig pancreatic kallikrein B. batch H376, was a gift from Professor E. Werle). On addition of ammonium bicarbonate, both solutions turned cloudy, that of the synthetic material rather more so than that of the natural. After 30 min at 21°, the solutions, both of which had clarified, were frozen and lyophilized. The residues were taken up in 0.25 ml of water and kept on the boiling water bath for 6 min. After cooling, the solutions were acidified (to about 0.2 *M*) with acetic acid and lyophilized. Aliquots of the lyophilized products were subjected to high voltage paper electrophoresis in parallel with the undegraded materials. It is seen from Figure 2 that the electrophoretic patterns given by the natural and synthetic products are essentially similar.

Separation of the Kallikreinic Fragments. The lyophilized kallikreinic degradation mixtures were dissolved in 0.25 ml of 0.02 *M* NH_4HCO_3, preadjusted to pH 6.4 with CO_2, and passed through a 0.6 × 12 cm column of carboxymethyl-cellulose (Whatman CM-22) equilibrated with this buffer. Elution with the same buffer was carried out until a 4-ml fraction had been collected. The elution was then continued with 4 ml each of 0.04, 0.08, 0.16, and 0.32 *M* NH_4HCO_3. The fractions were lyophilized and relyophilized from 0.2 *M* acetic acid. By analyzing aliquots of the fractions by means of high-voltage paper electrophoresis and by the Pauly reaction, it was found that fraction 1 contained the N-terminal kallikreinic fragment, V-K-N, and fraction 4 the C-terminal V-K-C. The yields were 0.64 mg of natural and 0.35 mg of synthetic V-K-N, and 0.52 mg of natural and 0.25 mg of synthetic V-K-C.

Comparison of Natural and Synthetic V-K-N. Synthetic and natural V-K-N (0.25 mg each) were degraded with trypsin (TRTPCK from Worthington) under the conditions given above for the degradation of VIP with kallikrein, for 2 hr. Figure 3 shows the electrophoretic patterns obtained on high-voltage paper electrophoresis.

The two tryptic fragments V-K-N-Tr-N and V-K-N-Tr-C, the latter identical with leucylarginine, were separated as follows. The degradation mixture was dissolved in 0.1 ml of 0.02 *M* NH_4HCO_3, preadjusted to pH 6.4 with CO_2, and followed by the same buffer, passed through a 0.3 × 8 cm column of carboxymethyl-cellulose. A fraction of 1.5 ml was collected, whereupon the buffer was changed to 0.2 *M* NH_4HCO_3, and a second 1.5-ml fraction was collected. Both fractions were lyophilized and relyophilized from 0.2 *M* acetic acid. Fraction 1 contained V-K-N-Tr-N and fraction 2, V-K-N-Tr-C.

Degradation of V-K-N-Tr-N with Chymotrypsin. The samples of natural and synthetic V-K-N-Tr-N were degraded with chymotrypsin (TLCK-treated chymotrypsin from Merck) under the conditions given above for the degradation of V-K-N with trypsin. Figure 5 shows the electrophoretic pattern at pH 6.4 of the degradation products.

Comparison of V-K-C from Natural and Synthetic VIP. Natural and synthetic C-terminal kallikreinic fragments, V-K-C, of VIP (150 μg each) were dissolved in 150 μl of 1% CNBr in 0.1 *M* HCl.[23] The solutions were kept for 16 hr at 21°, whereupon they were passed through 0.3 × 5 cm columns of DEAE-Sephadex equilibrated with 0.2 *M* acetic acid; 0.5-ml volumes of each effluent were lyophilized, and aliquots of the lyophilized material were subjected to paper electrophoresis and paper chromatography in the Waley–Watson system.[19] In neither case was any difference observed in the split products derived from the natural and synthetic materials. A paper chromatogram is shown in Figure 5. It may also be seen from this chromatogram that the fastest migrating product is indistinguishable in mobility from the natural and synthetic C-terminal hendecapeptides of VIP described previously.[7]

In another experiment, synthetic and natural preparations of V-K-C were degraded with chymotrypsin under conditions identical with those described above for the degradation of V-K-N-Tr-N with this enzyme, and with prolonged exposure to larger amounts of it. An electropherogram of aliquots of the lyophilized degradation products is shown in Figure 6.

Acknowledgments. This work was supported by a Center Award from the U. S. Public Health Service (HL-14187) and by a grant from the Swedish Medical Research Council (13X-1010). The authors thank Mrs. Delores J. Gaut for the amino acid analyses. Elemental analyses were carried out by The Baron Consulting Co., Orange, Conn.

(23) E. Gross and B. Witkop, *J. Amer. Chem. Soc.*, **83**, 1510 (1961).

Removal of the N^α-Benzyloxycarbonyl Group from Cysteine-Containing Peptides by Catalytic Hydrogenolysis in Liquid Ammonia, Exemplified by a Synthesis of Oxytocin[1]

Kenji Kuromizu and Johannes Meienhofer*[2]

Contribution from the Children's Cancer Research Foundation and Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115. *Received September 25, 1973*

Abstract: Cleavage of the benzyloxycarbonylamine protecting group from *S*-benzylcysteine-containing peptides can be attained by palladium-catalyzed hydrogenation when liquid ammonia is used as a solvent. The efficacy of the procedure is demonstrated by a synthesis of oxytocin *via* incremental chain elongation.

Development of adequate combinations of selectively removable protecting groups is still a major concern in peptide synthesis. For conventional synthesis in solution, combination of the *tert*-butyloxycarbonyl (Boc)[3] group[4] for α-amino and the benzyloxy-

(1) Paper V in the series "Reactions in Liquid Ammonia"; for paper IV, see ref 19. This work was supported in part by Research Grants C-6516 from the National Cancer Institute and RR-05526 from the Division of Research Facilities and Resources, National Institutes of Health.

(2) Address correspondence to J. Meienhofer, Chemical Research Department, Hoffmann-La Roche Inc., Nutley, N. J. 07110.

(3) Abbreviations for amino acids (all of L configuration) follow the rules of the IUPAC-IUB Commission on Biochemical Nomenclature in *Biochemistry*, **5**, 1445, 2885 (1966); **6**, 362 (1967); *J. Biol. Chem.*, **247**, 977 (1972). Boc, *tert*-butyloxycarbonyl; Bpoc, 2-(*p*-biphenylyl)isopropyloxycarbonyl; Bzl, benzyl; Fmoc, 9-fluorenylmethyloxycarbonyl; O-*t*-Bu, *tert*-butyl ester; ONph, *p*-nitrophenyl ester; Z, benzyloxycarbonyl.

(4) G. W. Anderson and A. C. McGregor, *J. Amer. Chem. Soc.*, **79**, 6180 (1957); F. C. McKay and N. F. Albertson, *ibid.*, **79**, 4686 (1957).