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Synthesis of the Vasoactive Intestinal Peptide (VIP)

IV.[1] The Sequence 14–28

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A protected pentadecapeptide with the C-terminal sequence of the vasoactive intestinal peptide (VIP) was prepared by coupling the tetrapeptide derivative *t*-butyloxycarbonyl-L-arginyl-N^{ε}-benzyloxycarbonyl-L-lysyl-L-glutaminyl-L-methionine azide to the partially deprotected hendecapeptide 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. The preparation of the protected tetradecapeptide *t*-butyloxycarbonyl-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 is also reported. The protecting groups were removed from samples of the tetradeca- and pentadecapeptides. The resulting free peptides showed, although at high dose levels, increase of visceral blood flow and reduction of blood pressure in the dog, and also relaxation of different smooth muscle preparations, which are the characteristic biological activities of VIP.

INTRODUCTION

In our continued effort toward the synthesis of the vasoactive intestinal peptide (VIP) (*1*, *2*), the preparation of the C-terminal hendecapeptide benzyloxycarbonyl-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, VIP_{18-28}, by the fragment condensation, was reported earlier (*3*). The present paper describes the entirely stepwise synthesis of the same sequence (Chart I). Furthermore, the protected tetrapeptide derivative *t*-butyloxycarbonyl-L-arginyl-N^{ε}-benzyloxycarbonyl-L-lysyl-L-glutaminyl-L-methionine hydrazide, VIP_{14-17} (XIV), was prepared (Chart II) and, after conversion to the corresponding azide, coupled to the partially deprotected hendecapeptide (VIII) to give *t*-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, a protected pentadecapeptide with the sequence VIP_{14-28} (compound XV). A tetradecapeptide with the sequence VIP_{15-28} (XVII) was prepared in the same manner (Fig. 1).

[1] For previous paper in this series, cf. *Bioorg. Chem.* **2**, 30 (1972).

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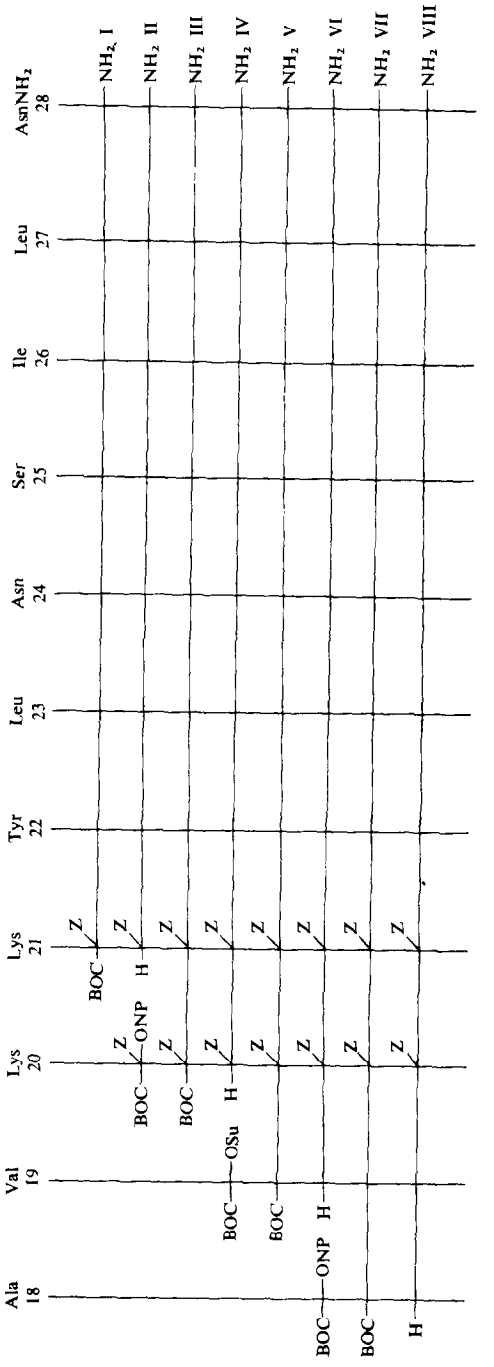
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Val 19
Lys 20
Lys 21
Tyr 22
Leu 23
Asn 24
Ser 25
Ile 26
Leu 27
AsnNH2 28
NH2 I
NH2 II
NH2 III
NH2 IV
NH2 V
NH2 VI
NH2 VII
NH2 VIII
BOC
ONP
OSu
Z
H

CHART I.

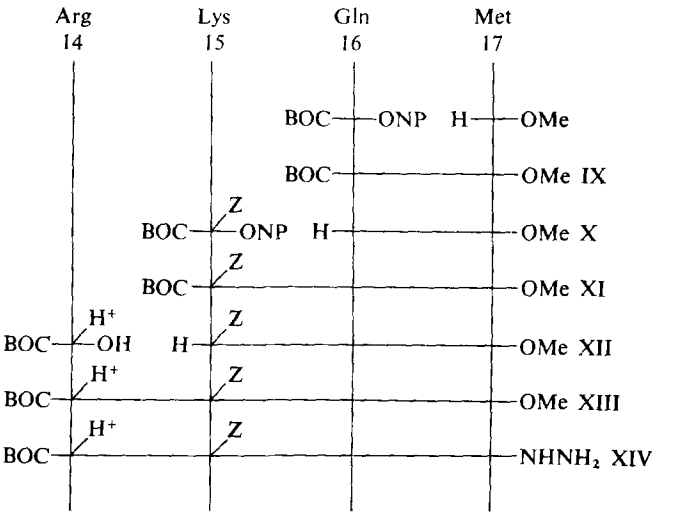

CHART II.

Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH_2
14 15 16 17 18 19 20 21 22 23 24 25 26 27 28

FIG. 1. The C-terminal pentadecapeptide sequence of VIP (VIP_{14-28}).

Strategy

The previous synthesis of sequence VII (*3*) was encumbered by an unusual lack of solubility of the intermediates in common organic solvents. In the coupling step linking the protected tripeptide with the sequence VIP_{18-20} to the C-terminal octapeptide, VIP_{21-28}, this difficulty was serious enough to warrant a new attempt, and the stepwise lengthening of the octapeptide to the hendecapeptide VII (described here) indeed turned out to be more practical. The intermediates of the synthesis formed gels in dimethylformamide, and in these gels the acylation reactions with active esters required prolonged reaction times. During such long periods, intramolecular side reactions can produce significant amounts of byproducts; e.g., the formation of a pyroglutamyl residue from N-terminal glutamine should be anticipated. Therefore, fragment condensation had to be preferred for the elongation of the hendecapeptide at this point. Methionine (in position 17) was chosen as the C-terminal residue rather than glutamine (in position 16) in order to avoid possible complications on the latter during hydrazinolysis.

Tactics

In the preparation of the hendecapeptide and tetrapeptide, after incorporation of N^{ε}-benzyloxycarbonyl-L-lysine residues, the α-amino protecting *t*-butyloxycarbonyl groups were removed by treatment with a mixture of 70% trifluoroacetic acid:30% acetic acid, at room temperature for 25 min. Under these conditions the loss of N^{ε}-benzyloxycarbonyl groups is kept at a minimum. A similar approach, with 70%

trifluoroacetic acid: 30% water, was proposed earlier by Schnabel (*4*). The arginine side chain was protected by protonation of the guanido group.

Biological Activities

On removal of the protecting groups from a sample of compound XV, the free peptide was purified by ion-exchange chromatography on a carboxymethyl cellulose column. The pentadecapeptide, VIP_{14-28} (XVI), revealed the biological properties of natural VIP (*5*, *6*); for the same effect, however, doses about 50 times higher were needed. The free tetradecapeptide, VIP_{15-28} (XVIII), also showed biological activity, but to an even lesser extent. Thus in VIP, even a part of the chain can have substantial biological effect, whereas in the related hormones, glucagon and secretin, the entire chain seems to be necessary for hormonal activity. The potencies determined in these experiments were reported elsewhere (*7*).

EXPERIMENTAL PART

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 (30:20:6:24); C, ethyl acetate–pyridine–acetic acid–water (60:20:6:11); D, chloroform–methanol (9:1). Spots were revealed by uv, charring with ammonium sulfate (*8*), modified Rydon-Zahn reagent (*9*), 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 (*10*) on a Beckman-Spinco 120C instrument[4].

t-Butyloxycarbonyl-N^{ε}-benzyloxycarbonyl-L-lysyl-N^{ε}-benzyloxycarbonyl-L-lysyl-L-tyrosyl-L-leucyl-L-asparaginyl-L-seryl-L-isoleucyl-L-leucyl-L-asparaginamide (III)

t-Butyloxycarbonyl-N^{ε}-benzyloxycarbonyl-L-lysyl-L-tyrosyl-L-leucyl-L-asparaginyl-L-seryl-L-isoleucyl-L-leucyl-asparaginamide I (*3*) (5.42 g) was suspended in acetic acid (15 ml), the suspension cooled in ice, and TFA (35 ml) added. After 25 min at room temperature, the acids were rapidly removed *in vacuo* and ether (250 ml) was added. The product was filtered, washed with ether (100 ml) and dried *in vacuo*. Yield: 5.32 g (97%); mp 247–249°C (dec., softens at 238°C); $[\alpha]_D^{25} - 26°$ (*c*1, 80% AcOH).

The octapeptide amide trifluoroacetate II (5.3 g) was powdered and suspended in DMF (40 ml). Triethylamine (0.61 ml) was added, followed by *t*-butyloxycarbonyl-N^{ε}-benzyloxycarbonyl-L-lysine *p*-nitrophenyl ester[5] (4.5 g). The reaction mixture was kept slightly alkaline by the addition of the same base. After 3 days, the semisolid mass was disintegrated under ethanol (150 ml). The product was filtered, washed with ethanol (150 ml), ethyl acetate (100 ml), and dried in air to give 5.86 g (92%); mp 249–250° (dec., softens at 246°C). The material was too insoluble for TLC. Amino acid analysis: Lys, 2.0; Tyr, 0.9; Leu, 2.2; Asp, 2.0; Ser, 0.9; Ile, 1.0; NH_3, 3.0.

[4] The following abbreviations are used: DMF (dimethylformamide); TFA (trifluoroacetic acid).

[5] Purchased from Fox Chemical Co., Los Angeles, Calif.

Anal. Calcd for $C_{71}H_{106}N_{14}O_{19}$ (1459.7): C, 58.4; H, 7.3; N, 13.4. Found: C, 58.5; H, 7.1; N, 13.8.

t-Butyloxycarbonyl-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 (V)

The protected nonapeptide amide III (1.46 g) was suspended in acetic acid (3 ml). The suspension was cooled and TFA (7 ml) was added. After 25 min at room temperature, the acids were removed *in vacuo*, dry ether (150 ml) was added and the product filtered, washed with ether (100 ml) and dried *in vacuo* over NaOH and P_2O_5 to give 1.47 g trifluoroacetate (quantitative); mp 246–250°C; $[\alpha]_D^{25} - 24°$ (*c*0.5, 80% AcOH). The trifluoroacetate (IV) was suspended in DMF (15 ml), triethylamine (0.13 ml) was added, followed by *t*-butyloxycarbonyl-L-valine *N*-hydroxysuccinimide ester (*11*) (1.41 g). The reaction mixture was kept slightly basic. After 6 days, the semisolid mixture was disintegrated under ethanol (60 ml). The product was filtered, washed with ethanol (50 ml), ethyl acetate (20 ml), and dried in air; 1.41 g (90%); mp 263–264°C; too insoluble for TLC. Amino acid analysis: Val, 0.9; Lys, 2.1; Tyr, 0.80; Leu, 2.2; Asp, 2.2; Ser, 1.0; Ile, 1.0; NH_3, 3.0.

Anal. Calcd for $C_{76}H_{115}N_{15}O_{20}$ (1558.8): C, 58.6; H, 7.4; N, 13.5. Found: C, 58.7; H, 7.4; N, 13.4.

t-Butyloxycarbonyl-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 (VII)

The *t*-butyloxycarbonyl group was removed from the protected decapeptide amide V (1.4 g) as described above. Yield: 1.38 g (98%); mp 230–233°C; $[\alpha]_D^{25} - 28°$ (*c*1, 80% AcOH). The trifluoroacetate (VI) was suspended in DMF (10 ml), triethylamine (0.12 ml) was added, followed by *t*-butyloxycarbonyl-L-alanine *p*-nitrophenyl ester[5]. After 3 days, the product was isolated as described for compound V. Yield: 1.34 g (94%); mp 271–273°C. Amino acid analysis: Ala, 1.0; Val, 1.0; Lys, 2.0; Tyr, 0.8; Leu, 2.1; Asp, 2.1; Ser, 0.9; Ile, 1.0; NH_3, 3.0.

Anal. Calcd for $C_{79}H_{120}N_{16}O_{21}$ (1629.9): C, 58.2; H, 7.4; N, 13.7. Found: C, 57.9; H, 7.4; N, 13.5.

t-Butyloxycarbonyl-L-glutaminyl-L-methionyl Methyl Ester (IX)

t-Butyloxycarbonyl-L-glutamine *p*-nitrophenyl ester[5] (7.34 g, 20 mmole) was added to a solution of L-methionine methyl ester hydrochloride[6] (4.2 g, 21 mmole) and triethylamine (2.94 ml) in DMF (30 ml). After 5 hr, ethyl acetate (300 ml) was added and the organic phase was washed with 2% citric acid (3 × 20 ml), 0.5 *M* ammonium hydroxide until free of nitrophenol, 2% citric acid (20 ml), water until neutral, and a saturated solution of NaCl (20 ml). The solution was dried over Na_2SO_4, filtered and concentrated *in vacuo*. Petroleum ether (100 ml, bp 40–60°C) was added, the precipitate filtered, washed with the same solvent (20 ml), and dried in air to give 7.0 g (90%), mp 110–111°C. Reprecipitation from ethyl acetate–petroleum ether did not change the mp; $[\alpha]_D^{25} - 17°$ (*c*1, DMF); TLC R_fA 0.72, R_fB 0.74.

[6] Purchased from Nutritional Biochemical Co., Cleveland, Ohio.

Anal. Calcd for $C_{16}H_{29}N_3O_6S$ (391.5): C, 49.1; H, 7.5; N, 10.7; S, 8.2. Found: C, 48.9; H, 7.6; N, 10.7; S, 7.9.

t-Butyloxycarbonyl-N^{ε}-benzyloxycarbonyl-L-lysyl-L-glutaminyl-L-methionine Methyl Ester (XI)

The protected dipeptide ester IX (3.13 g, 8 mmole) was dissolved in TFA (25 ml). After 10 min, the TFA was removed *in vacuo* and dry ether (200 ml) was added. The ether was decanted and the residue washed with ether (100 ml). The oily material was dried over NaOH and P_2O_5; TLC R_fA 0.42, R_fB 0.61. The dipeptide ester trifluoroacetate X (3.24 g, 8 mmole), triethylamine (1.12 ml) and *t*-butyloxycarbonyl-N^{ε}-benzyloxycarbonyl-L-lysine *p*-nitrophenyl ester[5] (4.8 g, 9.6 mmole) were dissolved in DMF (15 ml). The reaction mixture was kept basic with small amounts of triethylamine. After stirring overnight at room temperature, the mixture was stored at −15°C for several hours. The product was filtered, washed with ethyl acetate (25 ml) and dried in air to give 2.77 g (53%); mp 146–147°C; TLC R_fA 0.80, R_fB 0.70. The filtrate was concentrated *in vacuo* and treated with unsym. dimethylethylenediamine (*12*) (0.5 ml). After 2 hr, ethyl acetate (200 ml) was added and the product was isolated as described for compound IX. Yield: 1.26 g (24%), mp 146–147°C. A sample was recrystallized from ethanol, mp 146–147°C; $[\alpha]_D^{25} - 18°$ (*c*1, DMF). Amino acid analysis: Lys, 1.0; Glu, 1.0; Met, 0.9; NH_3, 1.1.

Anal. Calcd for $C_{30}H_{47}N_5O_9S$ (653.8): C, 55.1; H, 7.2; N, 10.7; S, 4.9. Found: C, 54.7; H, 7.2; N, 10.5; S, 5.0.

t-Butyloxycarbonyl-N^{ε}-benzyloxycarbonyl-L-lysyl-L-glutaminyl-L-methionyl Hydrazide (XIA)

The tripeptide methyl ester XI (2.61 g, 4 mmole) was suspended in methanol (90 ml), hydrazine (10 ml) was added, and the stirring continued for 6 hr. The product was filtered, washed with cold methanol (3 × 15 ml), water (8 × 10 ml), and dried over H_2SO_4 to give 2.33 g (89%), mp 202–204°C. After recrystallization from methanol: mp 203–204°C; $[\alpha]_D^{25}$ −29° (*c*1, AcOH); TLC R_fA 0.70, R_fB 0.76. Amino acid analysis: Lys, 1.0; Glu, 1.0; Met, 0.9; NH_3, 1.0.

Anal. Calcd for $C_{29}H_{47}N_7O_8S$ (653.8): C, 53.3; H, 7.2; N, 15.0; S, 4.9; Hydrazide N, 4.3. Found: C, 53.1; H, 7.2; N, 15.0; S, 5.0; Hydrazide N, 4.4.

t-Butyloxycarbonyl-L-arginyl-N^{ε}-benzyloxycarbonyl-L-lysyl-L-glutaminyl-L-methionine Methyl Ester Hydrochloride (XIII)

The protected tripeptide ester XI (955 mg) was suspended in a mixture of acetic acid (2.7 ml) and anisole (0.3 ml), TFA (6.3 ml) was added with cooling, and the resulting clear solution was allowed to stand for 25 min at room temperature. The acids were removed *in vacuo* and dry ether (50 ml) was added. The ether was decanted and the residue was dissolved in ice-cold water (40 ml). A cold solution of sodium carbonate (155 mg) in water (10 ml) was added and the free base was extracted into ethyl acetate (100 ml, then 4 × 30 ml). The organic phase was washed with water (2 × 20 ml), saturated sodium chloride solution (20 ml), dried over sodium sulfate, filtered and evaporated *in vacuo*. The yield was 602 mg (75%); mp 129–131°C; TLC R_fA 0.43, R_fB 0.60. The free amine XII (600 mg) was dissolved in DMF (3 ml). The solution was cooled

and then *t*-butyloxycarbonyl-L-arginine hydrochloride[7] (426 mg) and DCC (282 mg) were added. Two hours later a second portion of *t*-butyloxycarbonyl-L-arginine hydrochloride (155 mg) and DCC (103 mg) were added and the reaction allowed to proceed at room temperature. Next day the dicyclohexylurea was removed by filtration and washed with DMF (3 × 2 ml). The solvent was removed *in vacuo* and the residue applied in methanol to a Sephadex LH-20 column (2.5 × 80 cm) preequilibrated with the same solvent. Fractions of 5 ml were collected at a flow rate of 20 ml/hr. The fractions containing the desired product were pooled, evaporated and rechromatographed under the same conditions. Yield: 580 mg (60%); mp 107–108°C; $[\alpha]_D^{25}$ −26° (*c*1, methanol); TLC R_fA 0.56, R_fC 0.60. Amino acid analysis: Arg, 1.0; Lys, 1.0; Glu, 1.0; Met, 0.9; NH_3, 1.1.

Anal. Calcd for $C_{36}H_{60}N_9O_{10}SCl$ (864.5): C, 51.1; H, 7.2; N, 14.9; S, 3.8. Found: C, 50.8; H, 7.5; N, 15.2; S, 3.6.

t-Butyloxycarbonyl-L-arginyl-N$^{\varepsilon}$-benzyloxycarbonyl-L-lysyl-L-glutaminyl-L-methionyl Hydrazide Hydrochloride (XIV)

The protected tetrapeptide ester XIII (825 mg) was dissolved in methanol (4.5 ml). Hydrazine (0.5 ml) was added and after 3 hr at room temperature the solvent was removed *in vacuo* and the oily residue dried over H_2SO_4. Next day, the amorphous product was suspended in methanol (2 ml) and precipitated with ether (50 ml). It was filtered, washed with ether (20 ml) and dried over H_2SO_4 to give 780 mg (98%), mp 152–154°C. Reprecipitation of a sample from methanol–ether raised the mp to 155–157°C; $[\alpha]_D^{25}$ −14° (*c*1, DMF); according to its analysis, the product contains a molecule of methanol; TLC R_fA 0.52, R_fB 0.63, R_fC 0.60. Amino acid analysis: Arg, 10.; Lys, 0.9; Glu, 1.0; Met, 0.9; NH_3, 1.1.

Anal. Calcd for $C_{35}H_{60}N_{11}O_9SCl \cdot CH_3OH$ (878.5): C, 49.2; H, 7.3; N, 17.5; S, 3.7; Hydrazide N, 3.2; OCH_3, 3.5. Found: C, 49.1; H, 7.3; N, 17.4; S, 3.7; Hydrazide N, 3.1; OCH_3, 4.0.

t-Butyloxycarbonyl-L-arginyl-N$^{\varepsilon}$-benzyloxycarbonyl-L-lysyl-L-glutaminyl-L-methionyl-L-alanyl-L-valyl-N$^{\varepsilon}$-benzyloxycarbonyl-L-lysyl-N$^{\varepsilon}$-benzyloxycarbonyl-L-lysyl-L-tyrosyl-L-leucyl-L-asparaginyl-L-seryl-L-isoleucyl-L-leucyl-L-asparaginamide (Hydrochloride) (XV)

The protected hendecapeptide amide VII (1.3 g) was treated with a mixture of acetic acid (3 ml) and TFA (7 ml). The trifluoroacetate (VIII) was isolated as described for compound IV. Yield: 1.3 g (quantitative); mp 264–266°C (dec.); $[\alpha]_D^{25}$ −36° (*c*1, 80% AcOH).

A solution of *t*-butyloxycarbonyl-L-arginyl-N$^{\varepsilon}$-benzyloxycarbonyl-L-lysyl-L-glutaminyl-L-methionine azide was prepared as follows: the tetrapeptide hydrazide XIV (382 mg) was dissolved in DMF (5 ml) and the solution cooled to −30°C. HCl in dioxane (0.44 ml, 4.7 *M*) was added, followed by *t*-butylnitrite (60 μl). After 30 min at −25 to −30°C, the solution was cooled to −60°C, triethylamine (0.30 ml) was added, followed by the hendecapeptide amide trifluoroacetate VIII (560 mg) and more base (0.05 ml). The suspension was allowed to warm up to 0° and then stirred in a cold room. After

[7] This salt was prepared by adding the calculated amount of dilute aqueous HCl to a methanolic solution of the acetate (Fox Chemical Co.) and evaporating to dryness.

2 days, more tetrapeptide hydrazide (216 mg) was converted to the azide and added to the reaction mixture. After a total of 5 days, ethanol (50 ml) was added, and the product was filtered, washed with ethanol (5 × 15 ml), ethyl acetate (3 × 15 ml) and dried in air to give 600 mg (75%); mp 262–264°C (dec.), with softening at 250°C. Amino acid analysis: Arg, 0.9; Lys, 3.0; Glu, 0.9; Met, 0.9; Ala, 1.0; Val, 0.9; Tyr, 1.0; Leu, 2.2; Asp, 2.1; Ser, 0.9; Ile, 1.1; NH_3, 4.4.

Anal. Calcd for $C_{109}H_{168}N_{25}O_{28}SCl$ (2344.2): C, 55.9; H, 7.2; N, 14.9; S, 1.4. Found: C, 56.3; H, 7.5; N, 14.2; S, 1.4. (The value originally found for N was 13.9. On slow combustion, 14.2 was observed.)

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* (*Acetate Salt*) (*XVI*)

The protected pentadecapeptide XV (80 mg) and L-methionine (200 mg) were dissolved in TFA (4.5 ml) containing anisole (0.5 ml) and HBr gas was bubbled through the solution for 90 min. The acids were removed *in vacuo* and dry ether (25 ml) was added. The precipitate was filtered, washed with ether (30 ml) and dried *in vacuo* over NaOH and P_2O_5. It was dissolved in water (100 ml) and passed through a Dowex-1 column (18 × 2 cm) in the acetate form. The eluate was made 0.05 *M* with ammonium acetate and applied to a carboxymethyl cellulose column (45 × 1.1 cm) equilibrated with the same buffer. The column was washed with 0.05 *M* ammonium acetate solution (300 ml) and then developed with a linear gradient from 0.05 *M* ammonium acetate (500 ml) and 0.55 *M* ammonium acetate (500 ml). Fractions of 5 ml were collected, at a flow rate of 20 ml/hr, with uv monitoring at 280 nm. The desired material was located in tubes 130–180. These fractions were pooled, concentrated *in vacuo* to a small volume and lyophilized several times from water. The product (30 mg) moves as a single spot (ninhydrin and Sakaguchi positive) on paper chromatograms, with R_fB 0.32. Amino acid analysis: Arg, 1.0; Lys, 2.9; Glu, 0.9; Met, 0.8; Ala, 1.1; Val, 0.9; Tyr, 0.8; Leu, 1.9; Asp, 2.0; Ser, 1.1; Ile, 1.0. A sample (3.6 mg) was treated with aminopeptidase M (Röhm) under the conditions described by Hofmann and his coworkers (*13*). The following ratios of amino acids were found: Lys, 3.0; Arg, 1.1; Ala, 1.0; Val, 1.0; Met, 0.4; Ile, 1.0; Leu, 1.9; Tyr, 0.9. (Ser not separated from asparagine; methionine sulfoxides present but not determined.)

t-Butyloxycarbonyl-N$^{\varepsilon}$*-benzyloxycarbonyl*-L-*lysyl-N*$^{\varepsilon}$-L-*glutaminyl*-L-*methionyl*-L-*alanyl*-L-*valyl-N*$^{\varepsilon}$*-benzyloxycarbonyl*-L-*lysyl-N*$^{\varepsilon}$*-benzyloxycarbonyl*-L-*lysyl*-L-*tyrosyl*-L-*leucyl*-L-*asparaginyl*-L-*seryl*-L-*isoleucyl*-L-*leucyl*-L-*asparaginamide* (*XVII*)

A solution of *t*-butyloxycarbonyl-N^{ε}-benzyloxycarbonyl-L-lysyl-L-glutaminyl-L-methionine azide was prepared as follows: the tripeptide hydrazide XIA (655 mg) was suspended in DMF (8 ml) and the suspension cooled to −30°C. HCl in dioxiane (1.07 ml, 4.7 *M*) was added, followed by *t*-butylnitrite (0.15 ml). After 30 min at −25° to −30°C, the mixture was cooled to −60°C. Triethylamine (0.7 ml) was added, followed by the hendecapeptide trifluoroacetate VIII (1.3 g) in the form of fine powder and also additional base (0.11 ml). The suspension was allowed to reach 0° slowly and stirring was continued at 4°C. After 2 days the same amount of tripeptide azide was added, and after 2 more days one-half of the initial amount of azide was added. After a total of 6 days, the mixture was diluted with ethanol (75 ml) and the product isolated as de-

scribed for compound XV. Yield: 1.4 g (81%), mp above 275°. Amino acid analysis: Lys, 3.0; Gly, 1.0; Met, 0.8; Ala, 1.0; Val, 1.0; Tyr, 0.7; Leu, 2.2; Asp, 2.2; Ser, 1.0; Ile, 1.1; NH_3, 4.1. For analysis, a sample was dried at 80°C and 0.1 mm for 2 hr.

Anal. Calcd for $C_{103}G_{155}N_{21}O_{27}S$ (2151.5): C, 57.5; H, 7.3; N, 13.7; S, 1.5. Calcd for $C_{103}H_{155}N_{21}O_{27}S \cdot 3H_2O$ (2205.6): C, 56.1; H, 7.4; N, 13.3; S, 1.5. Found: C, 56.1; H, 7.3; N, 13.3; S, 1.6.

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

The protected tetradecapeptide (100 mg) and L-methionine (150 mg) were dissolved in TFA (3.8 ml) containing anisole (0.2 ml), and HBr gas was bubbled through the solution for 90 min. The acids were removed *in vacuo* and dry ether (100 ml) was added. The precipitate was filtered, washed with ether (50 ml) and dried *in vacuo* over NaOH and P_2O_5. It was then dissolved in water (5 ml) and neutralized with triethylamine. The water was evaporated and the residue was dried *in vacuo* over P_2O_5. It was extracted with chloroform (5 ml), filtered, washed with chloroform (15 ml) and dried. The crude peptide was applied in 1% acetic acid (2 ml) to a Sephadex G-25 column (70 × 2.5 cm), preequilibrated with 1% acetic acid. Fractions of 6 ml were collected, at a flow rate of 30 ml/hr. Monitoring at 280 nm showed that tubes 27–41 contained the desired product[8]. These were pooled and evaporated to give 35 mg. The product moves as a single spot on paper with R_fB 0.35. Amino acid analysis: Lys, 3.0; Glu, 1.0; Met, 0.9; Ala, 1.0; Val, 0.9; Tyr, 1.0; Leu, 2.0; Asp, 2.1; Ser, 0.9; Ile, 1.0.

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[8] The same product, but in somewhat lesser purity, was recovered also from a few tubes preceding tube no. 27 and from a few tubes following tube no. 41.