BIOORGANIC CHEMISTRY 8, 399–407 (1979)

# Is the Vasoactive Intestinal Peptide (VIP) a Prohormone?[1]

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*Received January 29, 1979*

The possibility that the vasoactive intestinal peptide (VIP) is a prohormone, which through enzymic fragmentation gives rise to shorter chains with, yet unknown, hormonal activities is suggested by the occurrence of two pairs of adjoining basic residues in its sequence. (A similar pattern can be recognized in proinsulin.) Synthesis of one of the hormone-candidates, L-pyroglutamyl-L-methionyl-L-alanyl-L-valyl-L-lysyl-L-lysyl-L-tyrosyl-L-leucyl-L-asparaginyl-L-seryl-L-valyl-L-leucyl-L-threoninamide corresponding to the *C*-terminal 13-peptide sequences of chicken VIP is reported.

The vasoactive intestinal peptide (VIP) was discovered and isolated in pure form from porcine intestines by Said and Mutt (*1*, *2*). The sequence of the 28 amino acids constituting its single chain was determined by the same authors (*3*), and was corroborated through synthesis by Bodanszky and his associates (*4*). Isolation and sequence determination of a very similar peptide from chicken was also reported (*5*). The sequence of the porcine and avian peptides are shown in Fig. 1.

The pharmacological properties of VIP which led to its discovery and guided its isolation, e.g., relaxation of smooth muscle preparations such as rat gastric fundus and guinea pig trachea, and increase of blood flow in peripheral and splanchnic vessels, suggested a regulatory role for VIP in digestive and smooth muscle function. When VIP, or a closely related peptide, was subsequently detected by radioimmunoassay and immunofluorescence throughout the gastrointestinal tract (*6*) and later in the adrenals (*7*) in distinct areas of the brain (e.g., cerebral cortex and hypothalamus) and peripheral nerves (*8*), the possible physiological role of this compound had to be reexamined. The assumption that it may function as a neurotransmitter was an auxiliary hypothesis (*9*) which still lacks confirmation. The wide distribution of VIP (or VIP-like peptides) in different organs and the multiplicity of its actions lead us to suggest a possible alternative, namely, that VIP could be a prohormone.

[1] This paper is dedicated to the memory of Professor George W. Kenner, a great peptide chemist and dear friend.

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| | 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 |
|---|---|
| Pork | His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-**Arg-Lys**- |
| Chicken | His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Ser-Arg-Phe-**Arg-Lys**- |

| | 16 17 18 19 20 21 22 23 24 25 26 27 28 |
|---|---|
| Pork | Gln-Met-Ala-Val-**Lys-Lys**-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-$NH_2$ |
| Chicken | Gln-Met-Ala-Val-**Lys-Lys**-Tyr-Leu-Asn-Ser-Val-Leu-Thr-$NH_2$ |

FIG. 1. The amino acid sequences of porcine and chicken VIP. The basic residues participating in the assumed cleavage are printed in bold letters.

Phe-Val-Asn-Gln-His-Leu-Cys-Gly-Ser-His-Leu-Val-Glu-Ala-Leu-Tyr-Leu-
Val-Cys-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala-**Arg-Arg**-Glu-Val-
Glu-Gly-Pro-Gln-Val-Gly-Ala-Leu-Glu-Leu-Ala-Gly-Gly-Pro-Gly-Ala-Gly-
Gly-Leu-Glu-Gly-Pro-Pro-Gln-**Lys-Arg**-Gly-Ile-Val-Glu-Gln-Cys-Cys-Ala-
Ser-Val-Cys-Ser-Leu-Tyr-Gln-Leu-Glu-Asn-Tyr-Cys-Asn

FIG. 2. The amino acid sequence of porcine proinsulin [C. Nolan, E. Margoliash, J. D. Peterson, and D. F. Steiner, *J. Biol. Chem.* **246**, 1780 (1971)]. The basic residues removed in the conversion to insulin are printed in bold letters.

The prohormone concept was established through the discovery of proinsulin by Steiner (*10*). Conversion of proinsulin (Fig. 2) to insulin takes place by enzymic hydrolysis which cleaves the chain at two points, and also removes the *two pairs* of basic amino acids which are at the *C*-terminus of the newly formed fragments. These residues are indicated by bold type in Fig. 2. An inspection of the sequences of both porcine and avian VIP reveals a certain analogy with respect to the distribution of basic amino acids. *Two pairs* of such residues occur in VIP as well, Arg-Lys in positions 14, 15 and Lys-Lys in positions 20 and 21. It is tempting to assume a specific enzymic cleavage of VIP similar to the one that occurs with proinsulin and to hypothesize hormonal functions for the resulting fragments. To test this hypothesis several potential fragments were synthesized. An inherent difficulty in the planned experiments was caused by our ignorance of the possible physiological activities of these fragments.[3] Therefore, in this paper no data can be reported on such activities. One purpose of this communication is to call attention to the suggested prohormone nature of VIP and to the availability of the synthetic fragments in the expectation that this will stimulate experiments which could eventually reveal their yet unknown physiological properties.

The design of VIP-fragments was guided by the pattern of conversion of insulin to proinsulin. An analogous enzymic cleavage of VIP between residues 15 and 16 and removal of the pair of basic residues from the *C*-terminus of the newly formed *N*-terminal fragment should produce two 13-peptides,[4] $VIP_{1-13}$ and $VIP_{16-28}$. Further fragmentation of the latter between residues 21 and 22, followed by similar degradation, could result in the 4-peptide $VIP_{16-19}$ and the 7-peptide $VIP_{22-28}$. So far we have

[3] The VIP-like activity of *C*-terminal peptides of VIP was demonstrated earlier [Ref. (*4*)]. Their potency increases with increasing chain length.

[4] The designation of 13-peptide (rather than tridecapeptide) was used [cf. Ref. (*11*)].

prepared the peptides with sequences $VIP_{16-19}$, $VIP_{16-28}$, and $VIP_{22-28}$. Of these $VIP_{16-19}$ was obtained by conventional methods; $VIP_{22-28}$ (in protected form) was available as an intermediate of the synthesis of $VIP_{16-28}$ and also in previous syntheses of VIP peptides (*12*). In the experimental section we describe here only the synthesis of chicken $VIP_{16-28}$. This 13-peptide was secured by the condensation of two segments, *t*butyloxycarbonyl-L-glutaminyl-L-methionine, and the 11-peptide L-alanyl-L-valyl-$N^{\epsilon}$-*t*-butyloxycarbonyl-L-lysyl-$N^{\epsilon}$-*t*-butyloxycarbonyl-L-lysyl-L-tyrosyl-L-leucyl-l-asparaginyl-*O*-*t*-butyl-L-seryl-L-valyl-L-leucyl-L-threoninamide. This 11-peptide derivative was built stepwise (*13*) with active esters as reactive derivatives of the constituent amino acid residues. The benzyloxycarbonyl group (*14*) was used for $\alpha$-amino protection and hydrogenolysis in the presence of a tertiary amine (*15*) for partial deprotection. Final removal of protecting groups by acidolysis yielded the trifluoroacetate salt of the 13-peptide amide $VIP_{16-28}$. The inherent lability caused by the *N*-terminal glutaminyl residue prompted us to convert a major portion of the product to the corresponding pyroglutamyl peptide. This was done also in the expectation that a peptide with pyroglutamic acid as *N*-terminal residue is more suitable for biological tests than the analogous glutamine derivative because it is resistant to common aminopeptidases. In fact, pyroglutamic acid is the *N*-terminal residue of several biologically active peptides such as gastrins, caerulein, TRH, LH/RH, etc. The synthesis of $VIP_{16-28}$ is summarized in Chart I.

The synthetic peptides are being tested in various pharmacological experiments in the hope that more light will be shed on the biological role of VIP.

## EXPERIMENTAL

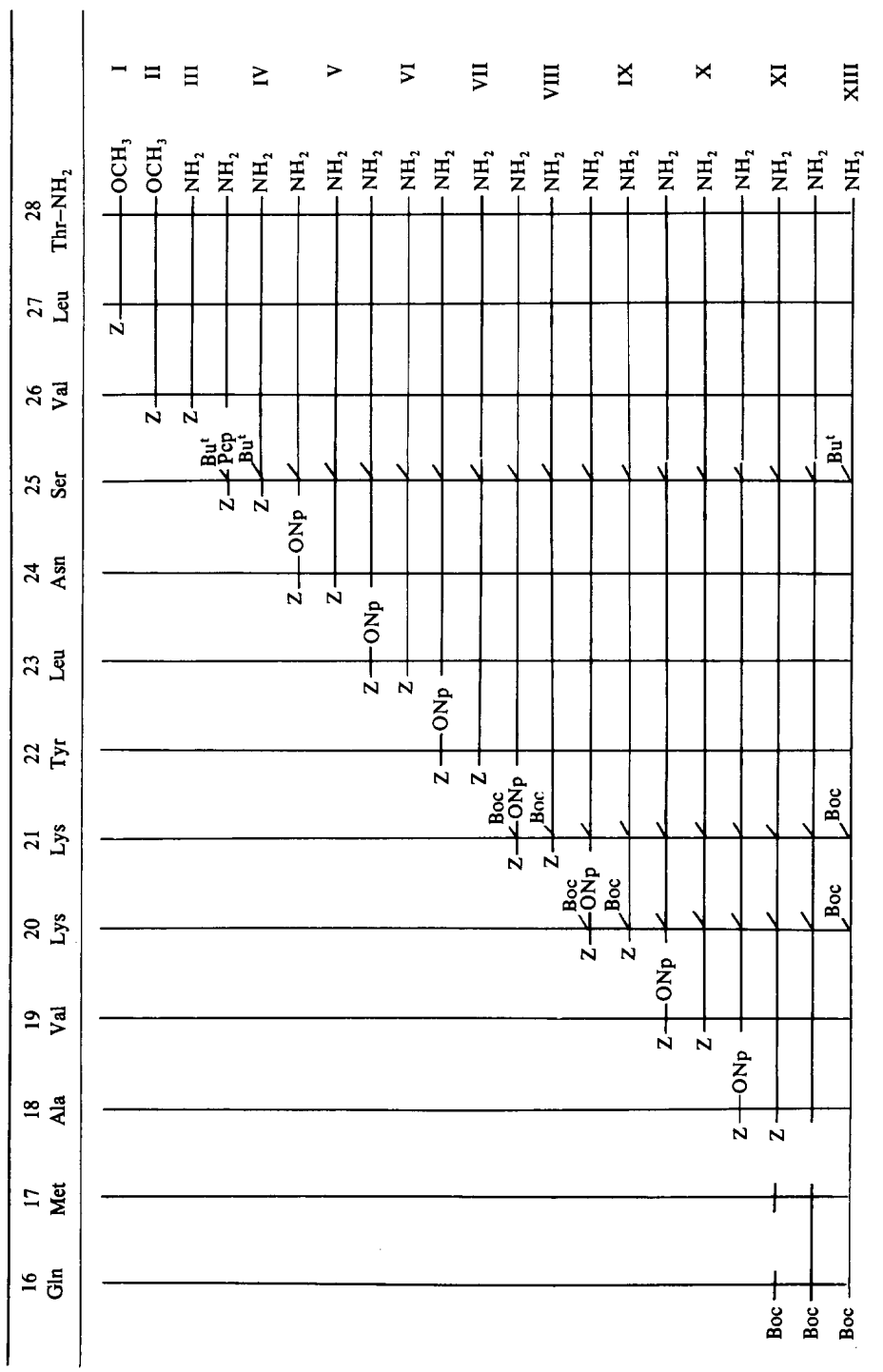
Capillary melting points are reported uncorrected. On tlc spots were revealed by charring, ninhydrin, or fluorescamine or by *t*-butyl hypochlorite-KI-starch reagent. The following solvent systems were used on silica gel plates: (A) $CHCl_3$–MeOH (9:1); (B) $CHCl_3$–MeOH (8:2); (C) *n*-BuOH–AcOH–$H_2O$ (4:1:1); (D) benzene–EtOAc (19:1); (E) EtOH–$H_2O$ (7:3); (F) *t*-AmOH–iso-PrOH–$H_2O$ (*16*) (51:21:28).

For amino acid analysis, samples were hydrolyzed with constant boiling hydrochloric acid in evacuated, sealed ampoules at 110°C for 16 hr and analyzed on a Beckman-Spinco 102C instrument. The $NH_3$ values were reported uncorrected for blanks.[5]

*Benzyloxycarbonyl-L-valyl-L-leucyl-L-threonine methyl ester* (*II*). Benzyloxycarbonyl-L-leucyl-L-threonine methyl ester (*12*) (I, 3.8 g, 10 mmol) was dissolved in a mixture of 95% EtOH (50 ml) and 1 *N* HCl (10 ml) and hydrogenated in the presence of a 10% Pd on charcoal catalyst (0.8 g). After removal of the catalyst and the solvent the residue was dissolved in DMF (35 ml), treated with DIEA (1.6 ml, 10 mmol), benzyloxycarbonyl-L-valine *p*-nitrophenyl ester (*17*) (4.3, g, 12 mmol) and HOBt (*18*) (1.5 g 10 mmol). After a negative spot test with ninhydrin indicated the completion of the reaction, the solvent was removed *in vacuo* and the residue triturated with $CHCl_3$

[5] The following abbreviations were used: DCC, dicyclohexylcarbodiimide; DIEA, diisopropylethylamine; DMF dimethylformamide; HOBt, 1-hydroxybenzotriazole monohydrate; TFA, trifluoroacetic acid; THF, tetrahydrofurane.

CHART I


16 Gln
17 Met
18 Ala
19 Val
20 Lys
21 Lys
22 Tyr
23 Leu
24 Asn
25 Ser
26 Val
27 Leu
28 Thr–NH2
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XIII
Z
Boc
ONp
But
Pcp
OCH3
NH2

(40 ml). The resulting solid was washed with $CHCl_3$ (20 ml) and dried: 4.5 g (94%, mp 217–218°C, unchanged after extraction with solvents, $[\alpha]_D^{24}$ −11.7° (*c* 1.5, DMF); $R_f$C 0.75; Thr, 1.08; Val, 0.98; Leu, 1.0.

*Anal.* Calcd for $C_{24}H_{37}N_3O_7$ (479.6): C, 60.1; H, 7.8; N, 8.8. Found: C, 59.9; H, 7.7; N, 8.8.

*N-Benzyloxycarbonyl-O-t-butyl-*L*-seryl-*L*-valyl-*L*-leucyl-*L*-threoninamide* (*IV*). A solution of the protected 3-peptide methyl ester II (3.5 g, 7.3 mmol) in MeOH (500 ml) was cooled in an ice-water bath while a stream of $NH_3$ was passed through it. After 1 h the flask was stoppered and kept at room temperature overnight. The protected 3-peptide amide (III) was collected by filtration, washed with $CH_3OH$ and dried: 2.7 g (79%) mp 240–242°C; $R_f$A, 0.24. A second crop (0.4 g, mp 238–240°C) obtained from the filtrate and washings was not used in the next step.

A sample of compound III (1.4 g, 3 mmol) was hydrogenated in a mixture of DMF (40 ml) and DIEA (2 ml) in the presence of palladium black (0.10 g) in a closed system. After 20 hr the catalyst was removed and the solution concentrated *in vacuo* to ca. 30 ml, and treated with *N*-benzyloxycarbonyl-*O-t*-butyl-L-serine pentachlorophenyl ester[6] (1.93 g, 3.6 mmol), HOBt (1.5 g, 3 mmol), and DIEA (0.48 ml, 3 mmol). After about 20 min the ninhydrin spot-test was negative. The solvent was removed *in vacuo* and trituration of the residue (a gel) with ether (30 ml) yielded a solid which was reprecipitated from DMF with ether. The product (homogeneous on tlc, system A) weighed 1.56 g (86%); mp 215–218°C. For analysis a sample was once more reprecipitated from DMF with ether: mp 217–219°C; $[\alpha]_D^{24}$ −1.0° (*c* 2, DMF); $R_f$A, 0.32; $R_f$C, 0.66; Thr, 0.97; Ser, 1.06; Val, 1.0; Leu, 1.0.

*Anal.* Calcd for $C_{30}H_{49}N_5O_8$ (607.7): C, 59.3; H, 8.1; N, 11.5. Found C, 59.0; H, 8.0; N, 11.4.

*Benzyloxycarbonyl-*L*-asparaginyl-O-t-butyl-*L*-seryl-*L*-valyl-*L*-leucyl-*L*-threoninamide* (*V*). Compound IV (1.5 g, 2.57 mmol) was hydrogenated in DMF (30 ml) in the presence of DIEA (1.8 ml) and a palladium black catalyst (0.10 g). The filtrate from the catalyst was concentrated *in vacuo* to ca. 25 ml and treated with Z-L-Asn-ONp(20) (1.49 g, 3.85 mmol). On standing at room temperature overnight the reaction went to completion (tlc). During the concentration the mixture formed a semisolid mass. This was treated with EtOAc (30 ml), filtered, washed with EtOAc and dried: 1.69 g (91%) mp 263–266°C dec.; $[\alpha]_D^{24}$ −11.3° (*c* 2, DMF); $R_f$A, 0.10; $R_f$C, 0.54. Asp, 1.02; Thr, 0.98; Ser, 0.97; Val, 1.0; Leu, 1.0.

*Anal.* Calcd for $C_{34}H_{55}N_7O_{10}$ (721.8) C, 56.6; H, 7.7; N, 13.6. Found: C, 56.6; H, 7.9; N, 13.4.

*Benzyloxycarbonyl-*L*-leucyl-*L*-asparaginyl-O-t-butyl-*L*-seryl-*L*-valyl-*L*-leucyl-*L*-threoninamide* (*VI*). Hydrogenation of compound V (1.68 g, 2.33 mmol) in DMF (50 ml) in the presence of DIEA (1.6 ml) and a palladium black catalyst (0.1 g) was followed by filtration and concentration *in vacuo* to ca. 20 ml. The amine was treated with Z-L-Leu-ONp (*20*) (1.31 g, 3.4 mmol) overnight. The semisolid mixture was diluted with EtOAc (60 ml), filtered, washed with EtOAc and dried: 1.86 g (95%), dec.

[6] Benzyloxycarbonyl-L-serine pentachlorophenyl ester (*19*) (6.1 g, 14.3 mmol) was suspended in $CH_2Cl_2$ (70 ml); conc. $H_2SO_4$ (0.5 ml) was slowly added and isobutene was passed through the stirred suspension. oil that failed to crystallize, $[\alpha]_D^{25}$ − 7.1° (*c* 2.6, $CHCl_3$), $R_f$C 0.54, showed traces of pentachlorophenol on tlc. It was used in this form for the incorporation of serine.

at about 265°C; $[\alpha]_D^{24}$ −15.5 (*c* 1.5, DMF); $R_f$C, 0.67; $R_f$D, 0.44. Asp, 0.96; Thr, 0.93; Ser, 0.92; Val, 1.01; Leu, 2.0.

*Anal.* Calcd for $C_{40}H_{66}N_8O_{11}$ (835.0): C, 57.5; H, 8.0; N, 13.4. Found: C, 57.3; H, 7.8; N, 12.9.

*Benzyloxycarbonyl-L-tyrosyl-L-leucyl-L-asparaginyl-O-t-butyl-L-seryl-L-valyl-L-leucyl-L-threoninamide* (*VII*). Compound VI (0.84 g, 1 mmol) was hydrogenated in DMF (50 ml) in the presence of DIEA (1.3 ml) and palladium black (60 mg) and after the removal of the catalyst the solution was concentrated to ca. 10 ml. Z-L-Tyr-ONp(*21*) (0.57 g, 1.3 mmol) was added and the mixture kept overnight at room temperature. The resulting gel was triturated with EtOAc (30 ml) to yield a solid, 0.92 g (92%) mp 261–263°C dec.; $R_f$A, 0.42; $R_f$C, 0.70; Asp, 1.01; Thr, 0.46; Ser, 0.43; Val, 1.04; Leu, 2.0; Tyr, 0.87.

*Anal.* Calcd for $C_{49}H_{75}N_9O_{13}$ (998.2): C, 59.0; H, 7.6; N, 12.6. Calcd for $C_{49}7_{75}N_9O_{13}\cdot H_2O$ (1016.2): C, 57.9; H, 7.6; N, 12.4. Found: C, 57.5; H, 7.8; N, 12.3.

*$N^{\alpha}$-Benzyloxycarbonyl-$N^{\epsilon}$-t-butyloxycarbonyl-L-lysyl-L-tyrosyl-L-leucyl-L-asparaginyl-O-t-butyl-L-seryl-L-valyl-L-leucyl-L-threoninamide* (*VIII*). Starting with hydrogenation of compound VII (0.915 g, 0.92 mmol) in DMF (40 ml) and DIEA (0.7 ml) in the presence of Pd-black (70 mg) the title compound was prepared by acylation with Z-L-Lys(Boc)-ONp (*22*) (0.60 g, 1.2 mmol). The reaction was complete in 2 days. Trituration of the mixture (a gel) with EtOAc (30 ml) and washing of the precipitate with EtOAc and with ether yielded 1.10 g (98%) of VIII, mp 251–253°C dec.; $R_f$B, 0.43; $R_f$C, 0.71; Asp, 1.09; Thr, 1.03; Ser, 1.0; Val, 1.02; Leu, 2.0; Tyr, 0.78.

*Anal.* Calcd for $C_{60}H_{95}N_{11}O_{16}$ (1226.5): C, 58.8; H, 7.8; N, 12.6. Found: C, 58.6; H, 7.8; N, 12.3.

*$N^{\alpha}$-Benzyloxycarbonyl-$N^{\epsilon}$-t-butyloxycarbonyl-L-lysyl-$N^{\epsilon}$-t-butyloxycarbonyl-L-lysyl-L-tyrosyl-L-leucyl-L-asparaginyl-O-t-butyl-L-seryl-L-valyl-L-leucyl-L-threoninamide* (*IX*). The protected 8-peptide VIII (1.1 g, 0.9 mmol) was hydrogenated as described above, in DMF (40 ml) in the presence of DIEA (0.7 ml). Hydrogenation took 72 hr to completion and hence each day more catalyst was added (a total 170 mg). After the removal of the catalyst and concentration to about 15 ml, the solution was treated with the active ester of lysine mentioned above (0.6 g, 1.2 mmol); HOBt and DIEA (both 0.4 mmol) were added to catalyze the acylation. The reaction still required 3 days for completion. The thick gel was triturated with EtOAc and dried: 1.78 g (97%) decomposing without melting at 247–252°C; $R_f$B, 0.41; $R_f$C, 0.72. Asp, 0.99; Thr, 0.95; Ser, 0.91; Val, 0.95; Leu, 2.0; Tyr, 0.88, Lys, 2.1.

*Anal.* Calcd for $C_{71}H_{115}N_{13}O_{19}$ (1454.7): C, 58.6; H, 8.0; N, 12.5. Calcd for $C_{71}H_{115}N_{13}O_{19}\cdot H_2O$ (1472.7): C, 57.9; H, 8.0; N, 12.4. Found: C, 57.2; H, 8.0; N, 12.3.

*Benzyloxycarbonyl-L-valyl-$N^{\epsilon}$-t-butyloxycarbonyl-L-lysyl-$N^{\epsilon}$-t-butyloxycarbonyl-L-lysyl-L-tyrosyl-L-leucyl-L-asparaginyl-O-t-butyl-L-seryl-L-valyl-leucyl-L-threoninamide* (*X*). The 9-peptide derivative IX (1.76 g, 0.86 mmol) was dissolved in DMF (25 ml). DIEA (1.3 ml) and Pd-black (0.10 g) were added and the mixture hydrogenated for about 4 hr. Removal of the catalyst and concentration of the solution to ca. 15 ml was followed by the addition of Z-L-Val-ONp (*17*) (0.67 g, 1.8 mmol), HOBt (0.12 g, 0.9 mmol), and DIEA (0.15 ml, 0.9 mmol). After overnight at room temperature acylation

was complete (tlc, system B). Trituration of the gel with ether (200 ml) yielded 1.11 g (81%); no mp could be observed up to 300°C; $R_f$B, 0.39; $R_f$C, 0.73.

*Anal.* Calcd for $C_{76}H_{124}N_{14}O_{20}$ (1553.9): C, 58.7; H, 8.0; N, 12.6. Calcd for $C_{76}H_{124}N_{14}O_{20} \cdot H_2O$ (1589.9): C, 57.4; H, 8.1; N, 12.3. Found: C, 57.2; N, 8.0; N, 12.7.

*Benzyloxycarbonyl-*L*-alanyl-*L*-valyl-$N^{\epsilon}$-t-butyloxycarbonyl-*L*-lysyl-$N^{\epsilon}$-t-butyloxycarbonyl-*L*-lysyl-*L*-tyrosyl-*L*-leucyl-*L*-asparaginyl-O-t-butyl-*L*-seryl-*L*-valyl-*L*-leucyl-*L*-threoninamide* (*XI*). Compound X (1.55 g, 0.97 mmol) was hydrogenated in DMF (100 ml) in the presence of DIEA (5 ml) and Pd-black (0.10 g) overnight. The catalyst and about 2/3 of the solvent were removed and acylation of the resulting amine was carried out with Z-L-Ala-ONp (*23*), (0.73 g, 2.1 mmol, Fox), HOBt and DIEA (1 mmol each). After overnight at room temperature the reaction was complete (tlc). The mixture was diluted with ether and washed with ether (total of 1 liter). The dried material, 1.34 g (84%) (Asp, 0.90; Thr, 0.90; Sr, 0.80; Ala, 1.10; Val, 1.40; Leu, 2.00; Tyr, 1.0; Lys, 1.90) was too insoluble for tlc or for purification and did not give entirely satisfactory values on elemental analysis.

*Anal.* Calcd for $C_{79}H_{129}N_{15}O_{21}$ (1624.9): C, 58.4; H, 8.0; N, 12.9. Found C, 57.8; H, 7.9; N, 13.3.

*tert-Butyloxycarbonyl-*L*-glutaminyl-*L*-methionine* (*XII*). A solution of Boc-L-Gln (Bachem, 4.92 g, 20 mmol) in THF (45 ml) was cooled to −15°C, neutralized with *N*-methylmorpholine (2.2 ml, 20 mmol), and treated with isobutylchlorocarbonate 2.74 g (20 mmol). After 5 min a solution of L-methionine (4.48 g, 30 mmol) and *N*-methylmorpholine (3.3 ml, 30 mmol) in $H_2O$ (45 ml) was added. (Some warming was necessary to obtain a clear solution of the methionine salt.) The mixture was stirred and allowed to warm up to room temperature; gradually a clear solution formed. After 1.5 hr the solution was concentrated *in vacuo* to remove most of the THF and then acidified with a 20% solution of citric acid in $H_2O$. Saturation with NaCl and extraction with EtOAc (3 × 50 ml) was necessary to isolate the desired material. The organic layer was washed with $H_2O$ and evaporated with a stream of $N_2$. The solid residue was suspended in warm EtOAc (100 ml), stirred for 1 hr, filtered, washed with EtOAc, and dried. The product (4.75 g, 63%) melted at 149–150°C; $[\alpha]_D^{20}$ −11.8° (*c* 2, DMF); $R_f$B, 0.60; $R_f$E, 0.73. Glu, 1.0; Met. 1.0. The proton nmr spectrum, in $CD_3COOD$, showed the expected resonances.

*Anal.* Calcd for $C_{15}H_{27}N_3O_6S \cdot H_2O$ (395.5): C, 45.5; H, 7.4; N, 10.6. Found: C, 45.3; H, 6.9; N, 10.6.

*L-Pyroglutamyl-*L*-methionyl-*L*-alanyl-*L*-valyl-*L*-lysyl-*L*-lysyl-*L*-tyrosyl-*L*-leucyl-*L*-asparaginyl-*L*-seryl-*L*-valyl-*L*-leucyl-*L*-threoninamide* (*XIII*). To a solution of compound XII (0.113 g, 0.3 mmol) and partially deprotected (by hydrogenation as described in previous paragraphs) compound XI (0.37 g, 0.35 mmol) in DMF (12 ml), HOBt (34 mg, 0.25 mmol), and DCC (0.5 ml of a 0.55 *M* solution in DMF) were added. After 1 day at room temperature EtOAc (20 ml) was added, the precipitate collected by centrifugation, washed with EtOAc, 95% EtOH, ether, and once more with EtOAc. The dried product weighed 0.38 g; no mp to 300°C; $R_f$B, 0.68; $R_f$F, 0.62.

A sample of the protected 13-peptide amide (0.15 g) was dissolved in TFA (ca. 3 ml) containing 10% anisole. After 50 min at room temperature the solution was evaporated

to dryness *in vacuo*, the residue triturated with ether, with EtOAc, and dried: 0.15 g, not melting up to 250°C; Asp, 0.96; Thr, 0.90; Ser, 0.88; Glu, 0.94; Ala, 1.1; Val, 2.04, Met, 0.87; Leu, 2.00, Tyr, 0.90; Lys, 2.1.

*Anal.* Calcd for $C_{67}H_{116}N_{18}O_{18}S \cdot 3CF_3COOH$ (1835.8): C, 47.8; H, 6.5; N, 13.7. Found: C, 47.5; H, 6.7; N, 13.5.

Notwithstanding the satisfactory amino acid analysis and elemental analysis the 13-peptide with glutamine as *N*-terminal residue showed two spots on tlc (cellulose, system C), one presumably the pyroglutamyl derivative. Therefore, the crude material was converted to the pyroglutamyl peptide by dissolving a sample (0.13 g) in 2 *N* AcOH (10 ml) and storing the solution at room temperature for 6 days (*24*). The solution was evaporated to dryness *in vacuo* and the residue distributed in an automatic Craig apparatus (3 ml phases) in the solvent system *n*-BuOH–EtOH-1% AcOH (5:1:4) through 201 transfers. A minor peak ($K = 0.14$) and a major peak ($K = 0.72$) were detected by absorption at 275 nm. Compound XIII was secured by concentration *in vacuo* of the contents of tubes No. 70 to 100 to a small volume and lyophilization. The purified 13-peptide (62 mg, di-trifluoroacetate salt) gave a single spot on tlc (cellulose, $R_f$C, 0.70) and also on paper chromatograms run with the upper phase of the solvent system used for countercurrent distribution. The uv spectrum of XIII in $H_2O$ corresponded to that of tyrosine; Asp, 0.93; Thr, 0.86; Ser, 0.86; Glu, 0.97; Ala, 1.1; Val, 1.9; Met, 1.0; Leu, 2.00; Tyr, 0.97, Lys, 2.03; $NH_3$, 2.8. For analysis a sample was dried over $P_2O_5$ at 60°C *in vacuo* (ca. 0.1 mm) for 4 hr. Nevertheless, the analysis indicates retention of acetic acid.

*Anal.* Calcd for $C_{67}H_{113}N_{17}O_{18}S \cdot 2CF_3COOH \cdot 4CH_3COOH$ (1944.9): C, 48.8; H, 6.8; N, 12.2. Found: C, 48.7; H, 6.8; N, 12.0.

## ACKNOWLEDGMENT

This study was supported by grants from the U.S. Public Health Service (NIH-1-P17-HE 14187 and NHI-AM 12473).

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