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Vasoactive Intestinal Peptide (VIP) from Chicken

Synthesis and Properties of the C-Terminal Hendecapeptide

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The hendecapeptide, Ala–Val–Lys–Lys–Tyr–Leu–Asn–Ser–Val–Leu–Thr–NH_2, corresponding to sequence 18–28 of chicken vasoactive intestinal peptide (VIP), was synthesized stepwise, starting with the C-terminal residue. The *in situ* technique was applied; *o*-nitrophenyl esters and *p*-nitrophenyl esters were used for acylation. The product was compared with, and found indistinguishable from, the C-terminal cyanogen bromide fragment of natural chicken-VIP. Some pharmacological properties of the hendecapeptide were also determined. In two separate experiments, the chain of the hendecapeptide was further lengthened to encompass residues 14–28 of chicken-VIP but with leucine and norleucine in place of methionine in position 17. The two pentadecapeptides showed biological activities comparable to those of the C-terminal pentadecapeptide fragment of porcine VIP or its 17-norleucine analog.

Isolation (*1*), structure elucidation (*2*), and synthesis (*3*) of the vasoactive intestinal peptide (VIP) from porcine tissues were reported earlier. More recently, an octacosapeptide with similar physiological–pharmacological properties was extracted from the intestines of chickens (*4*). The amino acid sequence of the avian peptide (*5*) closely resembles that of porcine-VIP (Fig. 1). We report here the synthesis and properties of

Chicken	His–	Ser–	Asp–	Ala–	Val–	Phe–	Thr–	Asp–	Asn–	Tyr–	Ser–	Arg–	Phe–	Arg–	
Pork	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	
Chicken	Lys–	Gln–	Met–	Ala–	Val–	Lys–	Lys–	Tyr–	Leu–	Asn–	Ser–	Val–	Leu–	Thr–	NH_2
Pork	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	

FIG. 1. Amino acid sequences of chicken- and porcine-VIP.

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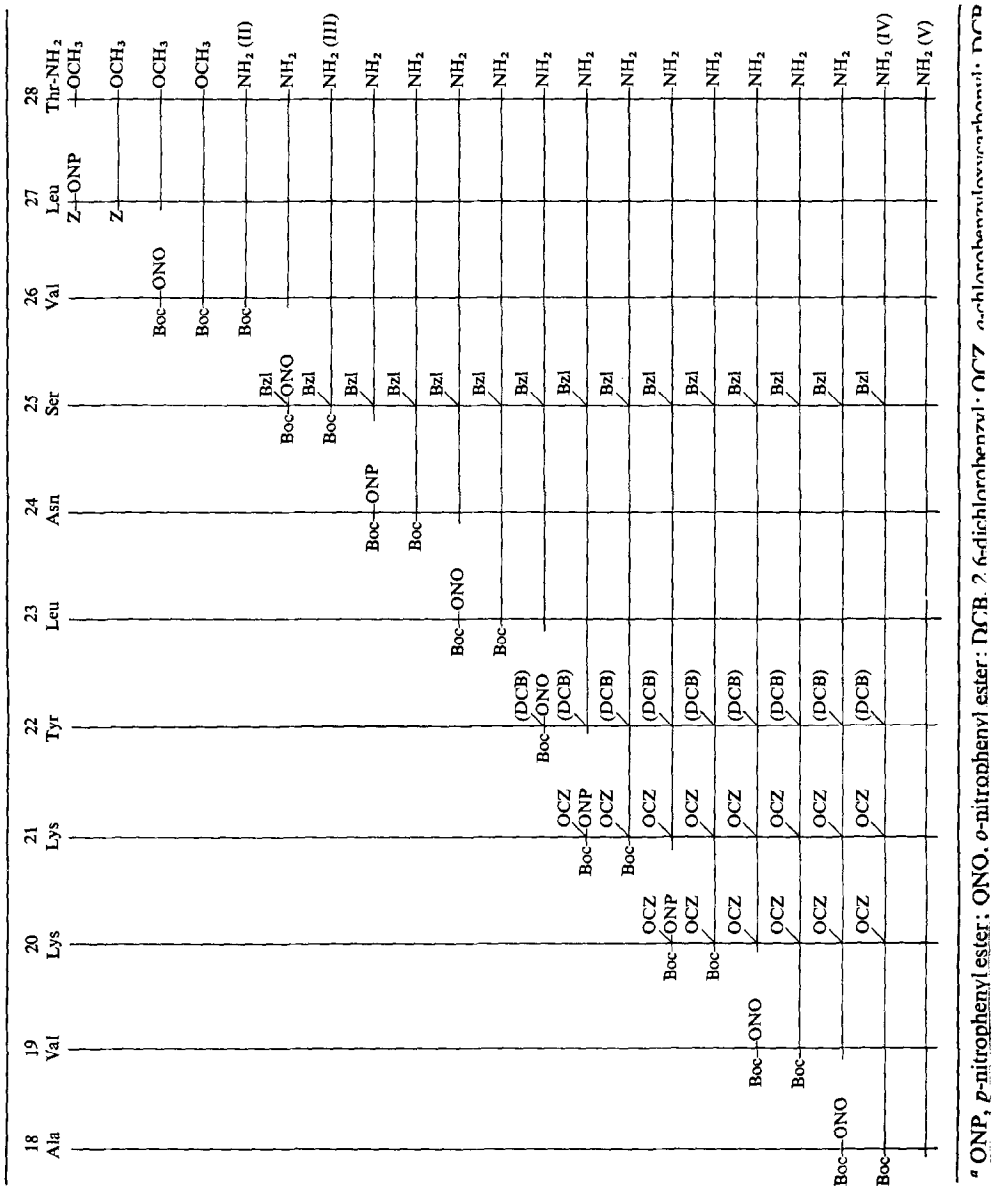
CHART I

SYNTHESIS OF THE C-TERMINAL HENDECAPEPTIDE OF HEN VIP[a]



[a] ONP, *p*-nitrophenyl ester; ONO, *o*-nitrophenyl ester; DCB, 2,6-dichlorobenzyl; OCZ, *o*-chlorobenzyloxycarbonyl; DCB

the C-terminal cyanogen bromide fragment, chicken-VIP_{18-28}. The chain of 11 amino acids was built by stepwise chain lengthening (*6*) starting with the C-terminal residue, threoninamide. Active esters (*7*, *8*) were used as acylating agents. Because of the poor solubility of several intermediates, reactions were slow in the gel-like mixtures. The acylations were catalyzed in such cases by the addition of 1-hydroxybenzotriazole (*9*). The side chains of the lysine residues were protected by the acid-resistant *o*-chlorobenzyloxycarbonyl group (*10*), the hydroxyl of the tyrosine residue with the 2,6-dichlorobenzyl group (*11*). In an alternative synthesis, however, the phenolic hydroxyl group was left unprotected. The intermediates of this second synthesis were more soluble and presented no problems during acylation. From the protected tetrapeptide stage on, the *in situ* technique was applied. The results were quite satisfactory: The protected hendecapeptide was prepared in high yield, and was sufficiently pure to give correct elemental and amino acid analysis. On removal of the protecting groups, the hendecapeptide showed only minor impurities on thin-layer and paper chromatograms and could be obtained in homogeneous form after a single purification step. The synthesis is summarized in Chart I.

The C-terminal hendecapeptide was prepared in order to provide corroboratory evidence for the corresponding cyanogen bromide fragment of chicken-VIP. It serves also as a fragment in the synthesis of the entire chain of 28 residues constituting the active peptides. Since the methionine residue in position 17 introduces severe restriction in the synthetic procedures, the possibilities of its replacement by norleucine and also by leucine were explored. Norleucine is the usual isosteric substituent for methionine. Leucine was used because, in case of application in medicine, peptides containing only the common amino acid constituents of proteins might be preferable. The chain lengthening from the protected hendecapeptide to the two pentadecapeptides, Arg–Lys–Gln–Nle–Ala–Val–Lys–Lys–Tyr–Leu–Asn–Ser–Val–Leu–Thr–NH_2 and Arg–Lys–Gln–Leu–Ala–Val–Lys–Lys–Tyr–Leu–Asn–Ser–Val–Leu–Thr–NH_2, was uneventful and is described in the experimental part.

A comparison of the synthetic hendecapeptide, chicken-VIP_{18-28}, with the C-terminal cyanogen bromide fragment of natural chicken-VIP, on paper chromatograms in the Waley–Watson (*12*) system and on high-voltage paper electropherograms (Fig. 2), showed them to be indistinguishable from each other, and the same was true of the fragments of these peptides obtained by degrading them with chymotrypsin.

The effect of chicken-VIP_{18-28} was studied on several isolated, superfused smooth muscle preparations (*14*) and compared with porcine- and chicken-VIP. Like the porcine peptide, the synthetic fragment of the chicken peptide relaxed gastric and colonic (rat) and tracheal (guinea pig) smooth muscle and contracted ileal (guinea pig) smooth muscle. Like chicken-VIP, the fragment was relatively more potent as a relaxant of the trachea than of the stomach, when compared to the porcine peptide. In these actions, the synthetic fragment had 0.4 to 1 % the potency of porcine- or chicken-VIP. The activities of the two pentadecapeptides, 17-norleucine chicken-VIP_{14-28} and 17-leucine chicken-VIP_{14-28}, on the same smooth muscle preparations were similar to those reported for porcine-VIP_{14-28} (*15*) and its 17-norleucine analog (*16*). In a radioimmunoassay for VIP, based on antibodies against the porcine peptide (*17*), chicken-VIP_{18-28} showed immunologic reactivity similar to that exhibited by porcine VIP_{18-28} and equivalent to approximately 1 % of the whole porcine peptide.

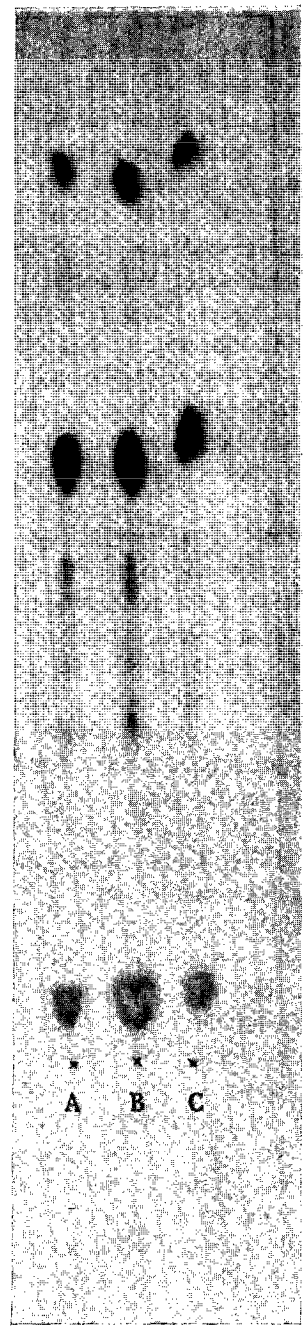


FIG. 2. Paper electrophoresis of chymotryptic degradation products of natural and synthetic C-terminal hendecapeptide amides of chicken-VIP. (A) Degradation products from 10 μg of natural substance. (B) Degradation products from mixture of 5 μg of natural and 5 μg of synthetic substance. (C) Degradation products from 10 μg of synthetic substance. Electrophoresis was performed for 90 min at 50 V/cm in pyridine–acetic acid–water (300 : 11.5 : 2700, by vol.) at pH 6.4 using Whatman 3MM paper. The spots were revealed by staining with the cadmium–ninhydrin reagent of Barollier *et al.* (*13*)

EXPERIMENTAL[2]

Capillary melting points are reported uncorrected. Reagent-grade solvents were used; DMF was dried over a molecular sieve (Linde 4A). On tlc, spots were revealed by charring, ninhydrin, or fluorescamine or by *t*-butyl hypochlorite–KI–starch reagent. The following solvent systems were used for tlc on silica gel plates: A, $CHCl_3$–MeOH (9:1); B, *n*-butanol–AcOH–H_2O (4:1:1); C, upper layer of *n*-butanol–AcOH–H_2O (4:1:5). On tlc on cellulose plates, system C was applied.

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 120C instrument. The NH_3 values are reported uncorrected for blanks.

N-t-butyloxycarbonyl-O-(2,6-dichlorobenzyl)-L-tyrosine o-nitrophenyl ester. This was prepared from the protected amino acid (*11*) (4.83 g, 11 mmoles) in pyridine (20 ml) as solvent with *o*-nitrophenol (2.78 g, 20 mmoles) and dicyclohexylcarbodiimide (2.06 g, 10 mmoles) as described for other *o*-nitrophenyl esters (*8*). The crude product (3.8 g, mp 136–138°C) was used. For analysis, a sample was recrystallized from boiling 95% EtOH: mp 136–139°C; $[\alpha]_D^{25}$ –45.0° (*c* 1.4, DMF containing 1% AcOH); tlc R_fB 0.85.

Anal. Calcd for $C_{27}H_{26}N_2O_7Cl_2$ (560.6): C, 57.8; H, 4.7; N, 5.0. Found: C, 57.9; H, 4.8; N, 5.0.

N^{α}-t-butyloxycarbonyl-N^{ϵ}-2-chlorobenzyloxycarbonyl-L-lysine p-nitrophenyl ester. The protected amino acid was liberated from the commercially available (Beckman) tert-butylammonium salt by acidification with H_2SO_4 in EtOAc–H_2O system as recommended by the manufacturer. It was converted (*18*) to the *p*-nitrophenyl ester: mp 88–91°C; $[\alpha]_D^{25}$ –23.8° (*c* 2, DMF containing 1% AcOH); tlc R_fA 0.46. For analysis, a sample was extracted with ether and dried: mp 93–95°C.

Anal. Calcd for $C_{25}H_{30}N_3O_8Cl$ (537.1): C, 56.0; H, 5.6; N, 7.8. Found: C, 56.3; H, 5.7; N, 7.8.

Benzyloxycarbonyl-L-leucyl–L-threonine methyl ester (*I*). L-Threonine methyl ester (*19*) (1.46 g, 11 mmoles) and benzyloxycarbonyl-L-leucine *p*-nitrophenyl ester (*18*) (3.87 g, 10 mmoles) were dissolved in EtOAc (30 ml) and the reaction was allowed to proceed overnight. The solvent was removed *in vacuo*, the residue dissolved in $CHCl_3$ (50 ml) and extracted with 0.5 *N* NH_4OH until the extracts became colorless. After washing with H_2O, 0.5 *N* HCl, and H_2O, the solution was dried over $MgSO_4$ and evaporated to dryness. The residue, an oil, was crystallized from EtOAc–hexane to give a crude product (2.9 g, 76%, mp 94–97°C) that was recrystallized from warm ether. The purified material (2.28 g, 60%) melted at 96–99°C; $[\alpha]_D^{25}$ –22.3° (*c* 2, MeOH); tlc R_fA 0.67, R_fB 0.80.

Anal. Calcd for $C_{19}H_{28}N_2O_6$ (380.4): C, 60.0; H, 7.4; N, 7.4. Found: C, 60.1; H, 7.4; N, 7.3.

The same product was obtained in 92% yield by the DCC method (*20*, *21*), but was contaminated with *N,N'*-dicyclohexylurea.

t-Butyloxycarbonyl-L-valyl-L-leucyl-L-threoninamide (*II*). Compound I (1.9 g, 5 mmoles) was hydrogenated in a mixture of EtOH (25 ml) and 1 *N* HCl (5 ml) in the

[2] The following abbreviations were used: DMF, dimethylformamide; DIEA, diisopropylethylamine; DCB, 2,6-dichlorobenzyl; DCZ, 2,6-dichlorobenzyloxycarbonyl; HOBt, 1-hydroxybenzotriazole; OCZ, *o*-chlorobenzyloxycarbonyl; TFA, trifluoroacetic acid; tlc, thin-layer chromatogram.

presence of a 10% palladium on charcoal catalyst (0.4 g). After removal of the catalyst and the solvent, the residue was dried *in vacuo* (1.34 g), dissolved in DMF (20 ml), and treated with DIEA (0.8 ml), followed by the addition of *t*-butyloxycarbonyl-L-valine *o*-nitrophenyl ester (*8*) (2.02 g, 6 mmoles) and HOBt (*9*) (0.92 g, 6 mmoles). The solvent was removed and the residue taken up in EtOAc. A solid (DIEA·HCl) separated. The mixture was extracted with H_2O, the organic layer concentrated to dryness, and the residue dissolved in $CHCl_3$ (100 ml) and washed with 0.5 *N* NH_4OH, water, 10% citric acid solution, and H_2O. After drying over $MgSO_4$, the solvent was evaporated *in vacuo* and the crude product was extracted with ether (150 ml). The tripeptide methyl ester (1.9 g, 85%, mp 158–161°C, R_fA 0.58; Amino acid analysis: Thr, 1.0; Val, 1.0; Leu 1.0) was used without further purification for the preparation of the amide. It was dissolved in MeOH (40 ml), cooled in an ice–water bath while a stream of NH_3 was passed through the solution. After 1 hr, the flask was closed with a stopper and left to stand overnight. The separated amide was collected, washed with methanol, and dried: 1.1 g, mp 198–200°C. Evaporation of the filtrate and washing of the residue with MeOH afforded a crude product that was recrystallized from boiling EtOAc to give 0.69 g, mp 199–202°C. Further recrystallization did not raise the mp; $[\alpha]_D^{25}$ −50.7° (*c* 2, MeOH); tlc R_fA 0.24. Amino acid analysis: Thr, 1.0; Val, 1.0; Leu, 1.0; NH_3, 1.25.

Anal. Calcd for $C_{20}H_{38}N_4O_6$ (430.5): C, 55.8; H, 8.9; N, 13.0. Found: C, 55.7; H, 8.9; N, 12.8.

N-t-Butyloxycarbonyl-O-benzyl-L-seryl–L-valyl–L-leucyl–L-threoninamide (*III*). The protected tripeptide amide II (0.65 g, 1.5 mmoles) was dissolved in distilled 98% trifluoroacetic acid (TFA). After about 15 min at room temperature, the TFA was removed *in vacuo* and the residue was triturated with ether, collected, and washed with ether. The dried material (0.655 g, R_fA 0.05, R_fB 0.23) was dissolved in DMF (7.5 ml), and DIEA (0.255 ml) and *N-t*-butyloxycarbonyl-*O*-benzyl-L-serine *o*-nitrophenyl ester (*8*) (0.83 g, 2 mmoles) were added. Next day, negative ninhydrin and fluorescamine spot tests showed that acylation of the amino component was complete. The solvent was removed *in vacuo* and the residue was triturated and washed with ether and dried (0.83 g, 93.5%, mp 183–186°C). The product was recrystallized from hot EtOH (10 ml); 0.78 g was recovered: mp 196–197°C dec; $[\alpha]_D^{25}$ −8.9° (*c* 2, DMF); tlc R_fA 0.32, R_fB 0.75. Amino acid analysis: Thr, 1.0; Ser, 0.95; Val, 1.0; Leu, 1.05; NH_3, 1.3.

Anal. Calcd for $C_{30}H_{49}N_5O_8$ (607.8): C, 59.3; H, 8.1; N, 11.5. Found: C, 59.6; H, 8.0; N, 11.6.

In situ synthesis of t-Butyloxycarbonyl-L-alanyl–L-valyl-N$^{\varepsilon}$-2-chlorobenzyloxycarbonyl–L-lysyl – N$^{\varepsilon}$-2-chlorobenzyloxycarbonyl – L-lysyl – O-2,6-dichlorobenzyl – L-tyrosyl – L-leucyl – L-asparaginyl – O-benzyl-L-seryl – L-valyl – L-leucyl – L-threoninamide (*IV*). A sample (0.61 g, 1 mmole) of the protected tetrapeptide amide III was placed in a 40-ml centrifuge tube provided with a 24/40 standard tapered joint (*22*). All the subsequent operations were carried out in this vessel without removing the intermediates from it. Trifluoroacetic acid (2 ml) was added and the solution was kept at room temperature for 15 min. After evaporation of the TFA *in vacuo*, the residue was triturated with ether, centrifuged, washed with ether, and dried. The partially deprotected product (0.58 g, 93%, R_fA 0.06, R_fB 0.45) was dissolved in DMF (5 ml) and treated with DIEA (0.15 ml, 0.93 mmole) and with *t*-Boc-Asn-ONP (*23*) (0.46 g, 1.3 mmoles). The mixture was kept slightly basic by the addition of small amounts of DIEA; alkalinity was tested with

a piece of moist universal indicator paper held close to the surface of the solution. Next day the solvent was removed *in vacuo* and the residue triturated with EtOAc, centrifuged, washed with EtOAc, and dried. The protected pentapeptide, Boc-Asn–Ser(Bzl)–Val–Leu–Thr-NH_2 (0.63 g), melts, after softening at 245°C, at 248–250°C with dec. It was deprotected with TFA as described above. The trifluoroacetate (R_fB 0.4) was dissolved in DMF (4.4 ml) and acylated with Boc-Leu-ONO (*8*) (0.4 g, 1.13 mmoles) in the presence of DIEA (0.14 ml). The protected hexapeptide amide, Boc-Leu–Asn–Ser(Bzl)–Val–Leu–Thr-NH_2 (0.70 g, mp 236–245°C dec), was recrystallized *in situ* from boiling 95% EtOH. The purified material was recovered by centrifugation and dried (0.62 g, mp 243–248°C dec).

Deprotection of the hexapeptide derivative was carried out as described in the previous paragraph. The trifluoroacetate salt (R_fB 0.41; amino acid analysis: Asp, 1.1; Thr, 1.0; Ser, 0.9; Val, 1.0; Leu, 2.0; NH_3, 2.6) was dissolved in DMF (3.7 ml) and acylated in the presence of DIEA (0.12 ml) with *N-t*-butyloxycarbonyl-O-2,6-dichlorobenzyl-L-tyrosine *o*-nitrophenyl ester (0.54 g, 0.96 mmole). The mixture gradually turned into a semisolid mass. This was treated with EtOAc and the solid formed was washed with the same solvent. The dried product, Boc-Tyr(2,6-DCB)–Leu–Asn–Ser(Bzl)–Val–Leu–Thr-NH_2 (0.85 g, 0.73 mmole, mp 248–260°C dec), was deprotected with TFA and the partially deblocked intermediate (R_fB 0.55; Amino acid analysis: Asp, 1.1; Thr, 1.1; Ser, 1.0; Val, 1.0, Leu, 2.0; Tyr, 0.9; NH_3, 2.8) was treated in DMF (5.5 ml) in the presence of DIEA (0.12 ml) with N^{α}-*t*-butyloxycarbonyl-N^{ε}-2-chlorobenzyloxycarbonyl-L-lysine *p*-nitrophenyl ester (0.505 g, 0.95 mmole). The product slowly separated, turning the reaction mixture into a semisolid mass. Three days later, addition of EtOAc yielded a solid that was washed with EtOAc to afford Boc-Lys(OCZ)–Tyr(2,6-DCB)–Leu–Asn–Ser(Bzl)–Val–Leu–Thr-NH_2 (1.04 g, 0.71 mmole, mp 255–267°C dec). After partial deprotection, amino acid analysis was Asp, 1.1; Thr, 1.0; Ser, 0.9; Val, 1.0; Leu, 2.1; Tyr, 0.9; Lys, 1.1; NH_3, 2.6.

The second lysine residue was incorporated similarly with 0.50 g, 0.93 mmole of the same active ester. The protected nonapeptide derivative, Boc-Lys(OCZ)–Lys(OCZ)–Tyr(DCB)–Leu–Asn–Ser(Bzl)–Val–Leu–Thr-NH_2, was isolated by precipitation with EtOAc etc.: 1.18 g, mp 252–264°C dec.

Partial deprotection of the nonapeptide derivative yielded the trifluoroacetate salt (Asp, 1.1; Thr, 1.1; Ser, 1.0; Val, 1.1; Leu, 2.0; Tyr, 0.9; Lys, 2.1; NH_3, 2.3) that was converted with Boc-Val-ONO (*8*) (0.30 g, 0.88 mmole) in the presence of DIEA (0.11 ml) and the catalyst 1-hydroxybenzotriazole (0.134 g, 0.87 mmole) to the protected decapeptide derivative Boc-Val–Lys(OCZ)–Lys(OCZ)–Tyr(DCB)–Leu–Asn–Ser(Bzl)–Val–Leu–Thr-NH_2 (1.24 g, 0.67 mmole) which, after partial deprotection with TFA and hydrolysis, gave the following amino acid analysis: Asp, 1.1; Thr, 0.9; Ser, 1.0; Val, 2.0; Leu, 2.2; Tyr, 0.9; Lys, 2.0; NH_3; 2.7. The next residue, alanine, was introduced, in the same manner with Boc-Ala-ONO (*8*) (0.27 g, 0.83 mmole), DIEA (0.11 ml) and 1-hydroxybenzotriazole (0.134 g, 0.87 mmole). The protected hendecapeptide (1.22 g, 0.63 mmole) melted at 275–285°C with dec. A sample was dried (110°C, 0.1 mm, 2 hr) for analysis.

Anal. Calcd for $C_{92}H_{127}N_{15}O_{21}Cl_4$ (1920.9): C, 57.5; H, 6.7; N, 10.9. Found: C, 57.7; H, 6.7; N, 10.8.

L-Alanyl–L-valyl–L-lysyl–L-lysyl–L-tyrosyl–L-leucyl–L-asparaginyl–L-seryl–L-valyl–

L-leucyl-L-threoninamide trifluoroacetate (*V*). Compound IV (300 mg) was suspended in 80% AcOH (45 ml) and hydrogenated overnight in the presence of a 10% palladium on charcoal catalyst (100 mg). Removal of the catalyst and the solvent was followed by lyophilization from AcOH. For final deprotection, the material was dissolved in 98% TFA (2 ml) and the solution evaporated to dryness after 15 min. Trituration of the residue with ether, washing with ether, and drying over P_2O_5 and NaOH *in vacuo* afforded the hendecapeptide (trifluoroacetate, 220 mg). The product had no well-defined mp, softened at about 170°C and melted with dec between 180 and 210°C. On thin-layer plates of cellulose, the peptide appeared essentially homogeneous with only traces of impurities: R_f 0.73 in the Waley–Watson system (*12*). On descending paper chromatograms (*n*-butanol–acetic acid–water, 4 : 1 : 5, upper phase), an R_f of 0.3 was observed, while in the Waley–Watson system (*12*), R_f 0.42 and faint spots with R_f 0.36 and 0.48 were also detected. A sample (40 mg) was purified by preparative paper chromatography on two sheets of Whatman No. 3 paper: The recovered material (22 mg) gave a single spot on rechromatography. This was used for comparisons with the fragment from natural chicken VIP and also in the biological tests. Amino acid analysis: Asp, 1.1; Thr, 0.90; Ser, 0.95; Ala, 1.1; Val, 2.1; Leu, 1.9; Tyr, 0.95; Lys, 2.2; NH_3, 2.7.

Alternative synthesis of compound V via intermediates with unprotected tyrosine side chain. A sample (0.60 g, 0.71 mmole) of the protected hexapeptide derivative, Boc-Leu–Asn–Ser(Bzl)–Val–Leu–Thr-NH_2 was placed in a 40-ml centrifuge tube (cf. above) and deprotected as described before. The trifluoroacetate was dissolved in DMF (4.0 ml) and treated with DIEA (0.104 ml) and *N-t*-butyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester (Bachem) (0.37 g, 0.92 mmole). Next day the protected heptapeptide derivative was isolated by removal of the solvent *in vacuo*, trituration of the residue with EtOAc, and washing with EtOAc. The product, Boc-Tyr–Leu–Asn–Ser(Bzl)–Val–Leu–Thr-NH_2 (0.72 g, mp 238–245°C dec, R_fB 0.80), was deprotected with TFA and the resulting salt (R_fB 0.54; Asp, 1.05; Thr, 0.95; Ser, 1.0; Val, 1.1; Leu, 2.1; Tyr, 0.95; NH_3, 2.7) converted to the protected octapeptide derivative, Boc-Lys(OCZ)–Tyr–Leu–Asn–Ser(Bzl)–Val–Leu–Thr-NH_2, (0.92 g, mp 247–257°C dec, R_fB 0.8). The protected nonapeptide (0.99 g, mp 244–264°C dec) was secured in the same manner and was lengthened, in the presence of 1-hydroxybenzotriazole (0.81 mmole), to the protected decapeptide (1.05 g, mp 240–260°C dec). The last added residue, alanine, also was incorporated with the help of the same catalyst (0.81 mmole). The protected hendecapeptide, Boc-Ala–Val–Lys(OCZ)–Lys(OCZ)–Tyr–Leu–Asn–Ser(Bzl)–Val–Leu–Thr-NH_2 (IVa, 1.02 g, mp 260–270°C dec, softening at about 250°C), was deprotected as described for the fully protected derivative. On tlc and paper chromatograms, the free hendecapeptide was indistinguishable from the product described in the preceding paragraph.

'In situ' synthesis of N^{α}-t-Butyloxycarbonyl-nitro-L-arginyl-N^{ε}-2,6-dichlorobenzyloxycarbonyl-L-lysyl – L-glutaminyl – L-norleucyl – L-alanyl – L-valyl – N^{ε}-2-chlorobenzyloxycarbonyl-L-lysyl – N^{ε}-2-chlorobenzyloxycarbonyl-L-lysyl – L-tyrosyl – L-asparaginyl – O-benzyl-L-seryl–L-valyl–L-leucyl–L-threoninamide (*VI*). Compound IVa (0.41 g, 0.23 mmole) was dissolved in TFA (2 ml). After 15 min at room temperature, most of the TFA was removed *in vacuo*, the residue triturated with ether, centrifuged, washed with ether, and dried over P_2O_5 and NaOH *in vacuo*. The dried trifluoroacetate salt was suspended in DMF (3.9 ml) and DIEA (0.037 ml, 0.23 mmole), *t*-butyloxycarbonyl-L-norleucine *o*-nitrophenyl ester (*16*) (0.1 g, 0.3 mmole) and HOBt (*16*) (0.045 g, 0.3

mmole) were added to the resulting gel, followed 4 hr later by more Boc-Nle-ONO (0.02 g) and DMF (1 ml). The reaction mixture was kept slightly basic by the addition of small amounts of DIEA. Three days later, the mixture was triturated with ether, centrifuged, washed with EtOAc, and dried. The protected dodecapeptide, Boc-Nle-Ala-Val-Lys(OCZ)-Lys(OCZ)-Tyr-Leu-Asn-Ser(Bzl)-Val-Leu-Thr-NH_2 (0.43 g, 0.23 mmole) was deprotected with TFA as described above. The trifluoroacetate (Asp, 1.0; Thr, 1.0; Ser, 1.0; Ala, 1.2; Val, 2.0; Leu, 2.0; Nle, 1.1; Tyr, 0.9; Lys, 2.1; NH_3, 2.6) was suspended in DMF (9 ml) and acylated in the presence of DIEA (0.037 ml, 0.23 mmole) with *t*-butyloxycarbonyl-L-glutamine *p*-nitrophenyl ester (*22*) (0.21 g, 0.58 mmole). The mixture was kept basic by the addition of small portions of DIEA. After 5 days, it was triturated with Et_2O–EtOAc (80:20), centrifuged, washed with EtOAc, and dried. The protected tridecapeptide, Boc-Gln-Nle-Ala-Val-Lys (OCZ)-Lys(OCZ)-Tyr-Leu-Asn-Ser(Bzl)-Val-Leu-Thr-NH_2 (0.43 g, 0.21 mmole) was dissolved in TFA (2 ml). After 15 min, most of the TFA was removed *in vacuo*, the residue triturated with ether, centrifuged, washed with ether, and dried over P_2O_5 and NaOH *in vacuo*. The trifluoroacetate salt (Asp, 1.0; Thr, 1.0; Ser, 1.0; Glu, 1.2; Ala, 1.2; Val, 2.0; Leu, 2.1; Nle, 1.1; Tyr, 0.9; Lys, 1.8; NH_3, 3.4) was suspended in DMF (10 ml) and DIEA (0.034 ml, 0.21 mmole), and N^{α}-*t*-butyloxycarbonyl-N^{ϵ}-2,6-dichlorobenzyloxycarbonyl-L-lysine *o*-nitrophenyl ester (*8*) (0.3 g, 0.53 mmole) were added. Five days later, an additional amount of active ester (0.12 g, 0.21 mmole) was added. Next day, the solvent was removed *in vacuo*, the residue triturated with EtOAc, centrifuged, washed with EtOAc, and dried. The product gave a slightly positive ninhydrin test. It was therefore suspended in DMF (5 ml) and treated with more Boc-Lys(DCZ)-ONO (0.24 g, 0.42 mmole) in the presence of a small amount of DIEA. After 3 days, the DMF was removed *in vacuo*, the residue triturated with EtOAc, etc., and the protected tetradecapeptide derivative, Boc-Lys(DCZ)-Gln-Nle-Ala-Val-Lys(OCZ)-Lys(OCZ)-Tyr-Asn-Ser(Bzl)-Val-Leu-Thr-NH_2 was isolated by precipitation with EtOAc, etc. (0.44 g, 0.2 mmole). The protected tetradecapeptide was deblocked (TFA, 2 ml) and the resulting trifluoroacetate salt (Asp, 1.0; Thr, 1.0; Ser, 0.9; Glu, 1.3; Ala, 1.3; Val, 1.9; Leu, 2.0; Nle, 1.1; Tyr, 0.8; Lys, 3.4; NH_3, 3.9) was suspended in DMF (6 ml), and DIEA (0.032 ml, 0.2 mmole) and N^{α}-*t*-butyloxycarbonyl-nitro-L-arginine 2,4-dinitrophenyl ester (*24*) (0.29 g, 0.6 mmole) were added. An additional amount of active ester (1.1 g, 0.84 mmole) was added in small portions during the following 4 days. The reaction mixture was kept slightly basic by the addition of DIEA. The solvent was removed *in vacuo* and the residue triturated with EtOAc, centrifuged, washed with EtOAc and EtOH (95%), and dried to give 0.39 g of the protected pentadecapeptide, Boc-Arg(NO_2)-Lys(DCZ)-Gln-Nle-Ala-Val-Lys(OCZ)-Lys(OCZ)-Tyr-Leu-Asn-Ser(Bzl)-Val-Leu-Thr-NH_2.

L-Arg-L-Lys-L-Gln-L-Nle-L-Val-L-Lys-L-Lys-L-Tyr-L-Leu-L-Asn-L-Ser-L-Val-Leu-L-Thr-NH_2 (*trifluoroacetate*) (*VII*). The protected pentadecapeptide (VI) (127 mg, 0.05 mmole) was suspended in 80% AcOH (12 ml) and hydrogenated for 48 hr in the presence of a palladium black catalyst (*25*) (~70 mg). The catalyst was removed by filtration and the filtrate evaporated *in vacuo* to dryness. The residue was dissolved in TFA (3 ml) and, after 15 min at room temperature, most of the TFA was removed *in vacuo*. Trituration of the residue with ether, washing with ether, and drying over P_2O_5 and NaOH *in vacuo* yielded the pentadecapeptide trifluoroacetate, 95 mg. On thin-

layer plates of cellulose (*n*-butanol–acetic acid–water, 4:1:5; upper phase, R_f 0.51), the peptide appeared essentially homogeneous with only trace amounts of impurities. On thin-layer plates of silica gel (*n*-butanol–acetic acid–water, 4:1:5; upper phase, R_f 0.07), there were some impurities at the origin and at R_f 0.21–0.32. Amino acid analysis: Asp, 1.0; Thr, 1.0; Ser, 0.95; Glu, 1.2; Ala, 1.2; Val, 1.95; Leu, 1.95; Nle, 1.10; Tyr, 0.85; Lys, 2.9; NH_3, 3.1; Arg, 1.0.

A sample (26 mg) was purified by preparative paper chromatography on a sheet of Whatman 3MM paper in the system *n*-butanol–acetic acid–water, 4:1:5, upper phase. The recovered material (14 mg) gave a single spot on thin-layer chromatograms of cellulose and one main component on silica gel with a trace of a second spot. Amino acid analysis: Asp, 1.0; Thr, 1.0; Ser, 1.0; Glu, 1.1; Ala, 1.2; Val, 1.9; Leu, 2.0; Nle, 1.0; Lys, 3.1; NH_3, 3.0; Arg, 1.0. A second sample (57.5 mg) of the crude pentadecapeptide was purified only by chromatography on Sephadex G-25 (1 × 31 cm, flow rate 23 ml/hr, eluant 1% AcOH). The recovered material (52 mg) gave the following amino acid analysis: Asp, 1.0; Thr, 0.95; Ser, 1.0; Glu, 1.2; Ala, 1.2; Val, 2.0; Leu, 1.9; Nle, 1.1; Tyr, 0.85; Lys, 2.8; NH_3, 3.3; Arg, 1.1.

'In situ' synthesis of N^{α}-t-butyloxycarbonyl-nitro-L-arginyl-N^{ε}-2,6-dichlorobenzyloxycarbonyl-L-lysyl-L-glutaminyl-L-leucyl-L-alanyl-L-valyl-N^{ε}-2-chlorobenzyloxycarbonyl-L-lysyl-N^{ε}-2-chlorobenzyloxycarbonyl-L-lysyl-L-tyrosyl-L-leucyl-L-asparaginyl-O-benzyl-L-seryl-L-valyl-L-leucyl-L-threoninamide (*VIII*). The same procedure was used as in the preparation of VI with the only difference that all acylations were carried out in the presence of 1-hydroxybenzotriazole. A sample of IVa (0.46 g, 0.26 mmole) was acylated with *t*-butyloxycarbonyl-L-leucine *p*-nitrophenyl ester (0.14g, 0.39 mmole) and HOBt (0.059 g, 0.39 mmole) to give the protected dodecapeptide Boc-Leu–Ala–Val–Lys(OCZ)–Lys(OCZ)–Tyr–Leu–Asn–Ser (Bzl)–Val–Leu–Thr-NH_2 (0.49 g, p.26 mmole). The trifluoroacetate salt of dodecapeptide (Asp, 1.0; Thr, 1.0; Ser, 1.0; Ala, 1.2; Val, 1.9; Leu, 3.3; Tyr, 0.9; Lys, 1.8; NH_3, 1.8) was treated with *t*-butyloxycarbonyl-L-glutamine *p*-nitrophenyl ester (0.14 g, 0.39 mmole) and HOBt (0.058 g, 0.39 mmole) to yield, after 4 days, the protected tridecapeptide Boc-Gln–Leu–Ala–Val–Lys(OCZ)–Lys(OCZ)–Tyr–Leu–Asn–Ser(Bzl)–Val–Leu–Thr-NH_2 (0.49 g, 0.24 mmole). The trifluoroacetate of this tridecapeptide (Asp, 1.1; Thr, 1.0; Ser, 1.0; Glu, 1.3; Ala, 1.25; Val, 2.0; Leu, 3.5; Tyr, 1.0; Lys, 1.8; NH_3, 3.0) was suspended in DMF (6 ml) and acylated with N^{α}-*t*-butyloxycarbonyl-N^{ε}-2,6-dichlorobenzyloxycarbonyl-L-lysine *o*-nitrophenyl ester (*8*) (0.2 g, 0.36 mmole) and HOBt (0.054 g, 0.36 mmole) to give the protected tetradecapeptide Boc-Lys(DCZ)–Gln–Leu–Ala–Val–Lys(OCZ)–Lys(OCZ)–Tyr–Leu–Asn–Ser(Bzl)–Val–Leu–Thr-NH_2 (0.56 g, 0.24 mmole). The trifluoroacetate salt of tetradecapeptide (Asp, 1.0; Thr, 1.0; Ser, 1.0; Glu, 1.2; Ala, 1.2; Val, 1.85; Leu, 3.2; Tyr, 0.95; Lys, 3.2; NH_3, 3.2) was acylated with N^{α}-*t*-butyloxycarbonyl-nitro-L-arginine 2,4-dinitrophenyl ester (0.6 g) and HOBt (0.072 g, 0.48 mmole) to yield the protected pentadecapeptide Boc-Arg(NO_2)–Lys(DCZ)–Gln–Leu–Ala–Val–Lys(OCZ)–Lys(OCZ)–Tyr–Leu–Asn–Ser(Bzl)–Val–Leu–Thr-NH_2 (0.53 g). A sample (127 mg, 0.05 mmole) of the protected pentadecapeptide in 80% AcOH (18 ml) was hydrogenated in the presence of a palladium black catalyst (*25*) (~70 mg) for 42 hr. After removal of the catalyst, the filtrate was evaporated *in vacuo* to dryness. The residue was dissolved in TFA (2 ml) and, after 15 min, most of the TFA was removed *in vacuo*. Trituration of the residue with ether, washing with

ether, and drying over P_2O_5 and NaOH *in vacuo* gave the pentadecapeptide as trifluoroacetate salt (94 mg). Amino acid analysis: Asp, 1.0; Thr, 1.0; Ser, 1.0; Glu, 1.2; Ala, 1.2; Val, 1.9; Leu, 2.9; Tyr, 1.0; Lys, 3.0; NH_3 3.3; Arg, 1.0. On thin-layer plates of cellulose and silica gel, the peptide was essentially homogeneous (R_fC 0.51 and R_fC 0.35, respectively), but minor impurities also were detected. An aliquot (48 mg) of the pentadecapeptide was purified by preparative paper chromatography on two sheets of Whatman 3MM paper in the system: *n*-butanol–acetic acid–water, 4:1:5 upper phase. The recovered material (30 mg) was further purified by chromatography on Sephadex G-25 (1 × 31 cm, flow rate 20 ml/hr, eluant 1% AcOH) to yield 15 mg. Amino acid analysis: Asp, 1.0; Thr, 1.0; Ser, 1.0; Glu, 1.0; Ala, 1.1; Val, 1.9; Leu, 3.2; Tyr, 0.9; Lys, 3.1; NH_3, 4.0; Arg, 1.0.

Comparison of natural and synthetic C-terminal hendecapeptide amides of chicken-VIP. Fragmentation of natural chicken-VIP with CNBr, according to Gross and Witkop, was carried out exactly as described previously for the porcine octacosapeptide amide (*2*). Attempts to separate the N-terminal and C-terminal fragments by chromatography on Sephadex G-25 in 1 *M* acetic acid were not completely successful, possibly because of the substitution of Phe-13 for Leu-13. The fragments were, however, separated in this step from a small quantity of undegraded VIP. They were recovered by lyophilization, and the C-terminal homoserine lactone of the N-terminal fragment was hydrolyzed (*2*). They were then clearly separated on a column, 0.6 × 26 cm, of carboxymethyl cellulose: The mixture of the peptides was applied to the column in 0.02 *M* NH_4HCO_3, and the chromatogram was developed in this solvent. The N-terminal fragment appeared in the 2–4.5-ml volume of the eluate. The eluant was then changed to 0.2 *M* NH_4HCO_3. The C-terminal hendecapeptide amide appeared in the 4.5–6.5-ml volume of the eluate after the change of buffer. It was recovered by lyophilization and relyophilized from 0.2 *M* acetic acid. When chromatographed, parallel or in mixture, on Whatman 42 paper in the Waley–Watson system (*12*), the natural and synthetic materials were indistinguishable (R_f 0.44), and the same was the case when they were subjected to high voltage paper electrophoresis at pH 6.4 (for 90 min at 50 V/cm in pyridine–acetic acid–water, 300:11.5:2700, on Whatman 3MM paper) where they migrated at 0.7 of the mobility of lysine. Aliquots of the natural and synthetic hendecapeptide amides were degraded with chymotrypsin as described earlier for the corresponding porcine materials (*26*). On paper electrophoresis, parallel or in mixture, the degradation products from the natural and synthetic materials were indistinguishable (Fig. 2), and the same was true when they were chromatographed in the Waley–Watson system. The R_f values for the three chymotryptic fragments were 0.25, 0.35, and 0.70.

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