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Studies on Peptides. CLXIII.[1,2] Synthesis of Guinea Pig Vasoactive Intestinal Polypeptide (gVIP)

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A 28-residue peptide corresponding to the entire amino acid sequence of vasoactive intestinal polypeptide of guinea pig origin (gVIP) was synthesized by assembling 6 peptide fragments, followed by thioanisole-mediated deprotection with 1 M trimethylsilyl trifluoromethanesulfonate in trifluoroacetic acid. The synthetic peptide was biologically as active as synthetic porcine VIP, but behaved immunologically in a different manner from the above mammalian VIP.

Keywords——guinea pig vasoactive intestinal polypeptide; VIP synthesis; β-cycloheptyl-aspartate; Asp-succinimide formation; hard acid deprotection; trimethylsilyl trifluoromethane-sulfonate; pancreatic blood flow; immunological properties

Mammalian vasoactive intestinal polypeptide (VIP) has been reported to have an identical amino acid sequence in four species, *i.e.*, pig,[3] cow,[4] human[5] and rat.[6] However, guinea pig VIP (gVIP) sequenced by Du *et al.*,[7] was found to differ from other mammalian VIP's by four amino acid substitutions at positions 5, 9, 19, and 26. In order to evaluate its biological and immunological properties, we undertook the solution phase synthesis of this unique mammalian VIP, according to the route illustrated in Fig. 1.

Different from our previous synthesis of porcine VIP,[8] our newly found thioanisole-mediated deprotecting procedure[9] with TMSOTf/TFA was applied in the final step of the present synthesis. The peptide chain was constructed by assembling six fragments. Of these,

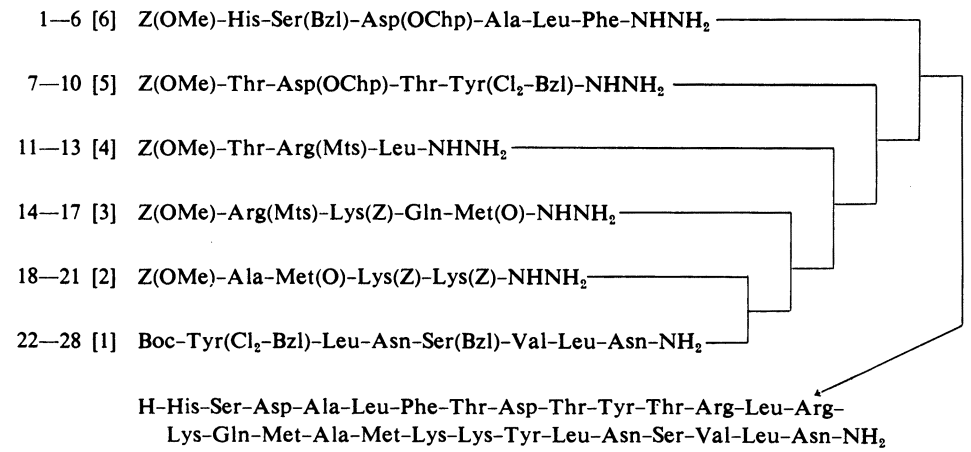

Fig. 1. Synthetic Route to Guinea Pig VIP (gVIP)

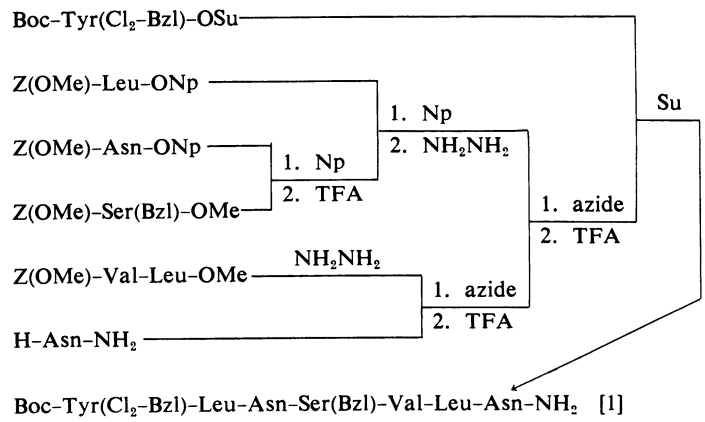

Fig. 2. Synthetic Scheme for the Protected C-Terminal Heptapeptide Amide (Positions 22—28)

two fragments, [3] and [4], are those employed for the previous porcine VIP synthesis. Like porcine VIP, gVIP possesses two Asp residues sensitive to base-catalyzed cyclization.[10] In order to suppress this type of side reaction, Asp(OChp)[11] was employed. In addition, in order to exclude the possibility of over-acylation during fragment condensation, a masked Tyr derivative, Tyr(Cl_2–Bzl),[12] was employed.

The C-terminal fragment [1], Boc–Tyr(Cl_2–Bzl)–Leu–Asn–Ser(Bzl)–Val–Leu–Asn–NH_2, was prepared by azide condensation[13] of two tripeptide units, Z(OMe)–Val–Leu–Asn–NH_2 and Z(OMe)–Leu–Asn–Ser(Bzl)–$NHNH_2$, followed by Su condensation[14] of Boc–Tyr(Cl_2–Bzl)–OH as shown in Fig. 2. The former unit was prepared by azide condensation of Z(OMe)–Val–Leu–$NHNH_2$[15] with a TFA-treated sample of Z(OMe)–Asn–NH_2 and the latter unit by Np condensation of the respective amino acids in a stepwise manner.

For preparation of fragment [2], Z(OMe)–Ala–Met(O)–Lys(Z)–Lys(Z)-$NHNH_2$, the known dipeptide, Z(OMe)–Ala–Met–OMe[16] was first converted to Z(OMe)–Ala–Met(O)–$NHNH_2$ by $NaIO_4$ oxidation, followed by usual hydrazine treatment. This hydrazide was then condensed with a TFA-treated sample of Z(OMe)–Lys(Z)–Lys(Z)–OMe[17] *via* the azide to give the protected tetrapeptide ester, which was smoothly converted to [2] by hydrazine treatment.

Fragment [5], Z(OMe)–Thr–Asp(OChp)–Thr–Tyr(Cl_2–Bzl)–$NHNH_2$, was prepared with the aid of substituted hydrazine, Troc–$NHNH_2$.[18] Starting with Boc–Tyr(Cl_2–Bzl)–NHNH–Troc prepared by the mixed anhydride procedure,[19] Z(OMe)–Thr–Asp(OChp)–Thr–Tyr(Cl_2–Bzl)–NHNH–Troc was prepared in a stepwise manner as shown in Fig. 3. From this tetrapeptide derivative, the Troc group was removed by treatment with Zn powder in AcOH–DMF to give [5].

The N-terminal fragment, Z(OMe)–His–Ser(Bzl)–Asp(OChp)–Ala–Leu–Phe–$NHNH_2$ [6], was also prepared with the aid of Troc–$NHNH_2$ as shown in Fig. 4. A TFA-treated sample of Z(OMe)–Phe–NHNH–Troc[20] was condensed with Z(OMe)–Ala–Leu–$NHNH_2$, derived from the corresponding Me ester.[21] The resulting tripeptide chain was elongated by successive condensations of Z(OMe)–Asp(OChp)–OH *via* the Su ester and Z(OMe)–His–Ser(Bzl)–$NHNH_2$[22] *via* the azide. From the resulting hexapeptide derivative, the Troc group was removed by Zn treatment as stated above to give [6].

Six fragments thus obtained were successively condensed by the azide procedure according to the route shown in Fig. 1. Every reaction was carried out in a mixture of DMF and DMSO and the amount of the acyl component was increased from 1.5 to 3 equivalents as

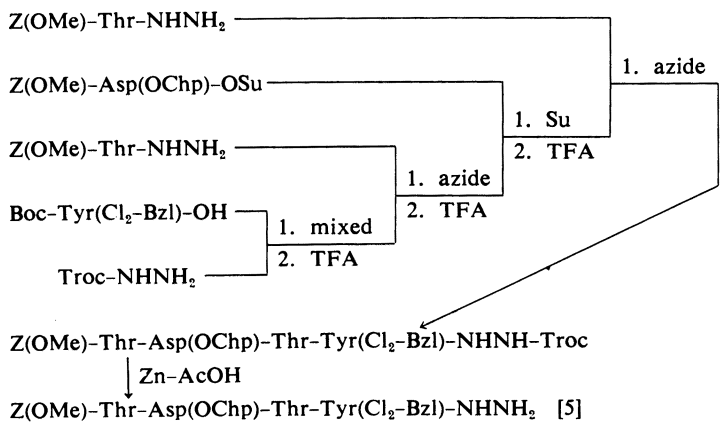

Fig. 3. Synthetic Scheme for the Protected Tetrapeptide Hydrazide (Positions 7— 10)

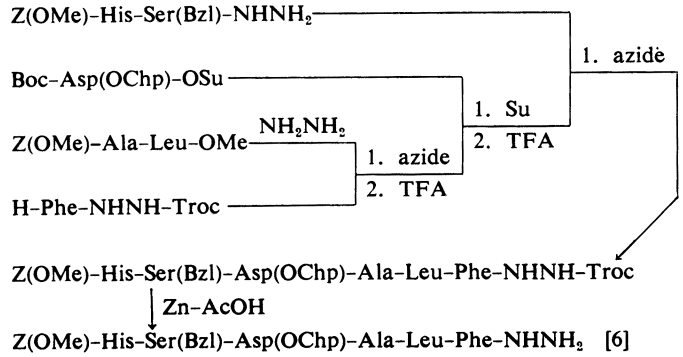

Fig. 4. Synthetic Scheme for the Protected N-Terminal Hexapeptide Hydrazide (Positions 1—6)

TABLE I. Amino Acid Ratios in 6 N HCl Hydrolysates of Synthetic gVIP and Its Intermediates

	Protected peptides						Syn. gVIP	Residue
	22—28	18—28	14—28	11—28	7—28	1—28		
Asp	1.93	2.08	1.95	2.00	3.58	4.17	4.12	4
Thr				0.91	3.63	2.95	3.12	3
Ser	0.87	0.78	0.82	0.87	1.09	1.88	1.71	2
Glu			0.95	0.92	1.12	1.07	1.06	1
Ala		0.87	0.91	0.90	1.11	1.99	1.93	2
Val	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1
Met		0.77	1.39	1.48	1.97	0.98	1.87	2
Leu	1.86	1.97	1.93	2.91	3.26	3.96	3.97	4
Tyr	0.94	0.92	0.94	0.98	2.26	1.93	2.10	2
Phe						0.86	0.82	1
Lys		1.91	2.70	2.64	3.17	3.14	3.17	3
His						1.10	0.92	1
Arg			0.90	1.82	2.23	2.26	2.14	2
Recovery (%)	89	90	90	92	92	95	85	

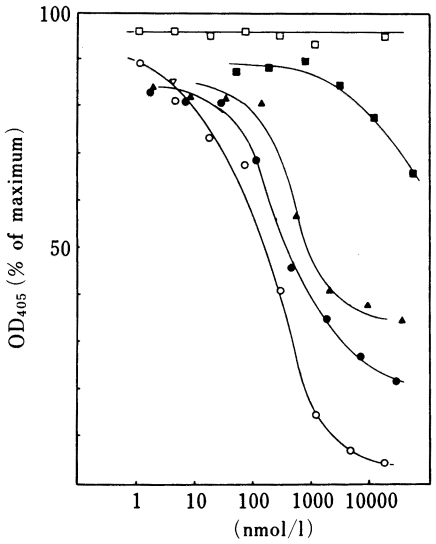

Fig. 5. Immunological Properties of Synthetic gVIP

○, porcine VIP; □, G-VIP; ●, p-VIP (11—28); ▲, p-VIP (14—28); ■, p-VIP (18—28).

the chain elongation progressed. Every product was purified by either precipitation from DMSO with MeOH or by gel-filtration on Sephadex LH-20 or LH-60. Throughout the synthesis, Val was used as a diagnostic amino acid in acid hydrolysis (Table I). By comparison of the recovery of Val with those of newly incorporated amino acids, satisfactory incorporation of each fragment was ascertained.

Protected gVIP thus obtained was first treated with phenylthiotrimethylsilane in DMF at room temperature for 12 h to reduce the Met(O) residue. The reduced peptide was then treated with 1 M TMSOTf–thioanisole/TFA in the presence of an additional scavenger, EDT, in an ice-bath for 2.5 h to remove all protecting groups employed. This additional scavenger was judged to give a more homogeneous product than *m*-cresol, which we previously employed.[9] The deprotected peptide was dissolved in an 0.2 M Tris–HCl buffer (pH 8.0) containing 6 M guanidine HCl and a small amount of NH_4F was added to hydrolyze the trimethylsilyl groups and to reverse any possible N→O[23] shift at the Ser and Thr residues. The solution was incubated with 2-mercaptoethanol at 36 °C for 15 h to ensure the complete reduction of the Met(O) residue. The treated product was then purified by gel-filtration on Sephadex G-25, followed by high-performance liquid chromatography (HPLC) on a Cosmosil 5C18 column with gradient elution (MeCN in 0.1% TFA). The homogeneous product was isolated in 24% yield from protected gVIP as a fluffy white powder. Its purity was ascertained by thin layer chromatography (TLC), analytical HPLC, and amino acid analyses after 6 N HCl hydrolysis and leucine-aminopeptidase (LAP) digestion.

For bioassay, a mongrel dog (*ca.* 10 kg) was anesthetized by intravenous administration of sodium pentobarbital. Left femoral arterial flow and cardiac output were measured by using an ultrasound transit time volume flow meter.[24] Pancreatic tissue blood flow was measured with a laser doppler perfusion monitor.[25] Synthetic gVIP (0.025 and 0.1 μg) was intravenously administered as a bolus. Graded doses of synthetic gVIP increased celiac arterial flow, cardiac output and pancreatic blood flow in a dose-related manner, but decreased systemic blood pressure. The potency of synthetic gVIP was judged to be equivalent to that of synthetic porcine VIP. Next, cross-reactivity of gVIP against antisera raised by synthetic porcine VIP was examined. Enzyme-linked immunosorbent assay (ELISA)[26,27] was conducted and amounts of *p*-nitrophenol liberated from *p*-nitrophenyl phosphate by alkaline phosphatase conjugated goat anti-rabbit immunoglobulin G (IgG) (Capel Laboratories Inc., USA) were determined. As shown in Fig. 5, synthetic gVIP did not cross-react with anti-

porcine VIP sera, even though gVIP is structurally different from porcine VIP by only four amino acid substitutions.

Experimental

General experimental methods employed here are essentially the same as described in Part CLXII[1] of this series. The N^{α}-protecting group was cleaved by TFA in the presence of anisole as usual. Active ester condensation was performed at room temperature, mixed anhydride condensation in an ice-bath and the azide reaction at 4 °C. Each reaction was continued until the reaction mixture became negative to ninhydrin, then the solvent was removed by evaporation.

Unless stated otherwise, products were purified by one of the following procedures. Procedure A: For purification of a product less soluble in AcOEt, the crude product was triturated with ether and 5% citric acid. The resulting powder was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O and recrystallized or precipitated from appropriate solvents. Procedure B: For purification of a product soluble in AcOEt, the product was dissolved in AcOEt. The organic phase was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O–NaCl, dried over Na_2SO_4, and concentrated. The residue was recrystallized or precipitated from appropriate solvents. Procedure C: Two fragment condensation products were purified by gel-filtration on Sephadex LH-20 or LH-60 using DMF as an eluant. The fractions corresponding to the front main peak, monitored by measuring the ultraviolet (UV) absorption at 275 nm, were combined and the solvent was removed by evaporation. The residue was precipitated from DMF with AcOEt.

HPLC was conducted with a Waters 204 compact model. TLC was performed on silica gel (Kieselgel G, Merck) and *Rf* values refer to the following solvent systems: Rf_1 $CHCl_3$–MeOH–H_2O (8 : 3 : 1), Rf_2 $CHCl_3$–MeOH (10 : 0.5), Rf_3 $CHCl_3$–MeOH–AcOH (9 : 1 : 0.5), Rf_4 *n*-BuOH–AcOH–pyridine–H_2O (4 : 1 : 1 : 2), and Rf_5 *n*-BuOH–AcOH–AcOEt–H_2O (1 : 1 : 1 : 1). LAP was purchased from Sigma Chem. Co. (Lot. No. 62-F-8000).

Z(OMe)–Val–Leu–Asn–NH_2 (Positions 26—28)——The azide [prepared from 9.13 g (1.2 eq) of Z(OMe)–Val–Leu–$NHNH_2$] in DMF (20 ml) and TEA (3.12 ml, 1.2 eq) were added to a TFA-treated sample of Z(OMe)–Asn–NH_2 (5.50 g, 18.6 mmol) in DMF (20 ml) containing TEA (2.60 ml, 1 eq) and the mixture was stirred for 18 h. The product was purified by procedure A, followed by precipitation from DMF with AcOEt; yield 9.17 g (97%), Rf_1 0.63. Analytical data and physical constants are listed in Table II, together with those of peptides related to fragments [1] and [3].

Z(OMe)–Asn–Ser(Bzl)–OMe (Positions 24—25)——A mixture of a TFA-treated sample of Z(OMe)–Ser(Bzl)–

Table II. Physical Constants and Analytical Data of Fragments [1] and [2], and Related Peptides

Protected peptide (positions)	mp (°C)	$[\alpha]_D^{20}$ (°)	Formula	Analysis (%) Calcd (Found)		
				C	H	N
Z(OMe)–(26—28)–NH_2	253—255	−9.2 DMF	$C_{24}H_{37}N_5O_7 \cdot H_2O$	54.84 (54.93)	7.48 7.24	13.33 13.35)
Z(OMe)–(24—25)–OMe	168—170	−7.5 DMF	$C_{24}H_{29}N_3O_8$	59.13 (59.11	6.00 5.88	8.62 8.57)
Z(OMe)–(23—25)–OMe	184—186	−19.6 DMF	$C_{30}H_{40}N_4O_9$	59.98 (59.72	6.71 6.71	9.33 9.38)
Z(OMe)–(23—25)–$NHNH_2$	225—227	−25.2 DMF	$C_{29}H_{40}N_6O_8$	57.98 (57.71	6.71 6.62	13.99 13.92)
Z(OMe)–(23—28)–NH_2	275—277	−14.5 DMSO	$C_{44}H_{65}N_9O_{12} \cdot 2H_2O$	55.74 (55.84	6.91 7.25	13.29 12.95)
Boc–(22—28)–NH_2 [1]	286—288	−13.9 DMSO	$C_{56}H_{78}Cl_2N_{10}O_{13} \cdot H_2O$	56.60 (56.77	6.79 6.67	11.79 11.97)
Z(OMe)–(18—19)–OMe sulfoxide	105—107	−22.5 DMSO	$C_{18}H_{26}N_2O_7S$	52.16 (52.01	6.32 6.41	6.76 6.87)
Z(OMe)–(18—19)–$NHNH_2$ sulfoxide	208—210	−16.3 DMF	$C_{17}H_{26}N_4O_6S$	49.26 (48.89	6.32 6.24	13.52 13.49)
Z(OMe)–(18—21)–OMe	153—155	−26.9 DMF	$C_{46}H_{62}N_6O_{13}S \cdot 1/2\,H_2O$	58.27 (58.19	6.70 6.61	8.87 8.98)
Z(OMe)–(18—21)–$NHNH_2$ [2]	212—214	−26.3 DMF	$C_{45}H_{62}N_8O_{12}S \cdot 1/2\,H_2O$	57.00 (57.08	6.69 6.42	11.82 11.89)

OMe (5.00 g, 13.4 mmol), TEA (3.74 ml, 2 eq) and Z(OMe)–Asn–ONp (6.72 g, 1.2 eq) in DMF (50 ml) was stirred for 18 h. The product was purified by procedure A, followed by precipitation from DMF with AcOEt; yield 4.16 g (64%), Rf_2 0.27.

Z(OMe)–Leu–Asn–Ser(Bzl)–OMe (Positions 23—25)——A mixture of a TFA-treated sample of the above dipeptide ester (4.00 g, 8.21 mmol), TEA (2.28 ml, 2 eq) and Z(OMe)–Leu–ONp (4.10 g, 1.2 eq) in DMF (40 ml) was stirred for 18 h. The product was purified by procedure A, followed by precipitation from DMF with AcOEt; yield 4.41 g (89%), Rf_1 0.69.

Z(OMe)–Leu–Asn–Ser(Bzl)–NHNH$_2$ (Positions 23—25)——The above tripeptide ester (4.41 g, 7.34 mmol) in DMF–MeOH (1 : 2, 30 ml) was treated with 80% hydrazine hydrate (2.3 ml, 5 eq) at room temperature for 10 h. The product was precipitated from DMF with MeOH; yield 3.48 g (79%), Rf_1 0.68.

Z(OMe)–Leu–Asn–Ser(Bzl)–Val–Leu–Asn–NH$_2$ (Positions 23—28)——The azide [prepared from 3.27 g (1.2 eq) of Z(OMe)–Leu–Asn–Ser(Bzl)–NHNH$_2$] in DMF (30 ml) and TEA (0.76 ml, 1.2 eq) were added to an ice-chilled solution of a TFA-treated sample of Z(OMe)–Val–Leu–Asn–NH$_2$ (2.30 g, 4.53 mmol) in DMF (20 ml) containing TEA (0.63 ml, 1 eq) and the mixture was stirred for 18 h. The product was purified by procedure A, followed by precipitation from DMSO with MeOH; yield 3.68 g (89%), Rf_1 0.56.

Boc–Tyr(Cl$_2$–Bzl)–Leu–Asn–Ser(Bzl)–Val–Leu–Asn–NH$_2$ [1] (Positions 22—28)——A mixture of a TFA-treated sample of the above hexapeptide (3.73 g, 4.09 mmol), TEA (1.14 ml, 2 eq) and Boc–Tyr(Cl$_2$–Bzl)–OSu (2.64 g, 1.2 eq) in DMF (30 ml) was stirred for 16 h. The product was purified by procedure A, followed by precipitation from DMSO with MeOH; yield 3.78 g (79%), Rf_1 0.58. Amino acid ratios in a 6 N HCl hydrolysate: Asp 2.00, Ser 0.90, Val 1.04, Leu 1.93, Tyr 0.97 (recovery of Asp, 89%).

Z(OMe)–Ala–Met(O)–OMe (Positions 18—19)——$NaIO_4$ (2.40 g, 1.1 eq) in H_2O (25 ml) was added dropwise to an ice-chilled solution of Z(OMe)–Ala–Met–OMe (4.00 g, 10.0 mmol) in MeOH (40 ml) and the mixture was stirred at room temperature for 20 h. The solvent was removed by evaporation and the residue was extracted with AcOEt. The organic phase was washed with H_2O–NaCl, dried over Na_2SO_4 and concentrated. The residue was triturated with *n*-hexane; yield 3.40 g (82%), Rf_2 0.35.

Z(OMe)–Ala–Met(O)–NHNH$_2$ (Positions 18—19)——The above sulfoxide (2.00 g, 4.83 mmol) in MeOH (20 ml) was treated with 80% hydrazine hydrate (1.5 ml, 5 eq). The solid formed on standing at room temperature for 18 h was collected by filtration and precipitated from DMF–DMSO (1 : 1) with MeOH; yield 1.51 g (75%), Rf_1 0.42.

Z(OMe)–Ala–Met(O)–Lys(Z)–Lys(Z)–OMe (Positions 18—21)——The azide [prepared from 0.97 g (1.2 eq) of Z(OMe)–Ala–Met(O)–NHNH$_2$] in DMF (9 ml) and TEA (0.32 ml, 1.2 eq) were added to an ice-chilled solution of a TFA-treated sample of Z(OMe)–Lys(Z)–Lys(Z)–OMe (1.40 g, 1.94 mmol), then the mixture was stirred for 24 h. The product was purified by procedure A, followed by precipitation from DMF with MeOH; yield 1.64 g (90%), Rf_1 0.65.

Z(OMe)–Ala–Met(O)–Lys(Z)–Lys(Z)–NHNH$_2$ [2] (Positions 18—21)——The above tetrapeptide ester (1.64 g, 1.75 mmol) in DMF–MeOH (2 : 1, 15 ml) was treated with 80% hydrazine hydrate (0.55 ml, 5 eq) at room temperature for 24 h. The solvent was removed by evaporation and the residue was precipitated from DMSO with MeOH; yield 1.19 g (73%), Rf_1 0.61. Amino acid ratios in a 6 N HCl hydrolysate: Ala 1.13, Lys 2.00, Met(O) N.D. (recovery of Lys 90%).

Boc–Tyr(Cl$_2$–Bzl)–NHNH–Troc——A mixed anhydride [prepared from 14.0 g (31.8 mmol) of Boc–Tyr(Cl$_2$–Bzl)–OH] in THF (100 ml) was added to an ice-chilled solution of Troc–NHNH$_2$ (7.92 g, 1.2 eq) in DMF (50 ml), then the solution was stirred for 6 h and the solvent was removed by evaporation. The product was recrystallized from MeOH and ether; yield 16.62 g (83%), Rf_2 0.59. $[\alpha]_D^{20}$ −11.8° (c=0.2, MeOH). *Anal.* Calcd for $C_{24}H_{26}Cl_5N_3O_6$: C, 45.77; H, 4.16; N, 6.67. Found: C, 45.85; H, 4.22; N, 6.41.

Z(OMe)–Thr–Tyr(Cl$_2$–Bzl)–NHNH–Troc (Positions 9—10)——The azide [prepared from 2.83 g (1.2 eq) of Z(OMe)–Thr–NHNH$_2$] in DMF (20 ml) and TEA (1.3 ml, 1.2 eq) were added to an ice-chilled solution of a TFA-treated sample of Boc–Tyr(Cl$_2$–Bzl)–NHNH–Troc (5.00 g, 7.94 mmol) in DMF (50 ml) containing TEA (1.1 ml, 1 eq) and the mixture was stirred for 18 h. The product was purified by procedure A, followed by precipitation from DMF with ether; yield 4.79 g (76%), Rf_1 0.71. Physical constants and analytical data are listed in Table III, together with those of protected peptides related to fragments [5] and [6].

Z(OMe)–Asp(OChp)–Thr–Tyr(Cl$_2$–Bzl)–NHNH–Troc (Positions 8—10)——Z(OMe)–Asp(OChp)–OSu [prepared from 3.72 g (1.5 eq) of the CHA salt as usual] in THF (30 ml) and NMM (0.83 ml, 1.5 eq) were added to a solution of a TFA-treated sample of the above dipeptide derivative (4.00 g, 5.03 mmol) in DMF (40 ml) containing TEA (0.55 ml, 1 eq), and the mixture was stirred at 4 °C for 18 h. The product was purified by procedure A, followed by precipitation from DMF with a mixture of MeOH and ether; yield 2.84 g (56%), Rf_2 0.40.

Z(OMe)–Thr–Asp(OChp)–Thr–Tyr(Cl$_2$–Bzl)–NHNH–Troc (Positions 7—10)——The azide [prepared from 0.99 g (1.2 eq) of Z(OMe)–Thr–NHNH$_2$] in DMF (10 ml) and NMM (0.37 ml, 1.2 eq) were added to an ice-chilled solution of a TFA-treated sample of the above tripeptide derivative (2.80 g, 2.78 mmol) in DMF (20 ml) containing NMM (0.31 ml, 1 eq) and the mixture was stirred for 36 h. The product was purified by precipitation from DMF with AcOEt; yield 2.49 g (81%), Rf_2 0.29.

Z(OMe)–Thr–Asp(OChp)–Thr–Tyr(Cl$_2$–Bzl)–NHNH$_2$ [5] (Positions 7—10)——The above tetrapeptide derivative (0.86 g, 0.78 mmol) in DMF–AcOH (9 ml–0.9 ml) was treated with Zn powder (1.0 g, 20 eq) at room

TABLE III. Physical Constants and Analytical Data of Fragments [5] and [6], and Related Peptides

Protected peptide (positions)	mp (°C)	$[\alpha]_D^{20}$ (°)	Formula	Analysis (%) Calcd (Found)		
				C	H	N
Z(OMe)-(9—10)-NHNH-Troc	133—135	−5.8 DMF	$C_{32}H_{33}Cl_5N_4O_9$	48.35 (48.54	4.18 4.30	7.05 6.83)
Z(OMe)-(8—10)-NHNH-Troc	104—106	−14.4 DMF	$C_{43}H_{50}Cl_5N_5O_{12}\cdot H_2O$	50.42 (50.38	5.12 5.05	6.84 6.85)
Z(OMe)-(7—10)-NHNH-Troc	141—143	−15.1 DMF	$C_{47}H_{57}Cl_5N_6O_{14}$	50.98 (50.71	5.19 5.27	7.59 7.34)
Z(OMe)-(7—10)-$NHNH_2$ [5]	198—200	−93.6 DMF	$C_{44}H_{56}Cl_2N_6O_{12}\cdot H_2O$	55.63 (55.65	6.15 6.05	8.85 8.77)
Z(OMe)-(4—5)-$NHNH_2$	188—190	−23.4 DMF	$C_{18}H_{28}N_4O_5$	56.82 (56.87	7.42 7.57	14.73 14.75)
Z(OMe)-(4—6)-NHNH-Troc	190—192	−23.5 DMF	$C_{30}H_{38}Cl_3N_5O_8$	51.25 (51.30	5.45 5.48	9.96 10.07)
Boc-(3—6)-NHNH-Troc	207—209	−43.7 MeOH	$C_{37}H_{55}Cl_3N_6O_{10}$	52.26 (52.00	6.52 6.45	9.89 10.06)
Z(OMe)-(1—6)-NHNH-Troc	166—168	−7.5 DMF	$C_{57}H_{73}Cl_3N_{10}O_{14}\cdot 3H_2O$	53.37 (53.39	6.21 5.83	10.92 10.72)
Z(OMe)-(1—6)-$NHNH_2$ [6]	235—237	+8.1 DMF	$C_{54}H_{72}N_{10}O_{12}\cdot 9H_2O$	53.36 (53.81	7.46 7.26	11.52 10.86)

temperature for 6 h, then the solution was filtered and the filtrate was concentrated. The residue was treated with 3% EDTA. The resulting powder was washed with H_2O and precipitated from DMF with EtOH; yield 0.51 g (70%), Rf_1 0.66. Amino acid ratios in a 6 N HCl hydrolysate: Thr 1.91, Asp 1.00, Tyr 1.00 (recovery of Asp, 92%).

Z(OMe)-Ala-Leu-$NHNH_2$ (Positions 4—5)——Z(OMe)-Ala-Leu-OMe (37.0 g, 97 mmol) in MeOH (300 ml) was treated with 80% hydrazine hydrate (30.4 ml, 5 eq) at room temperature for 15 h. The solution was concentrated and the resulting solid was precipitated from DMF with MeOH; yield 27.4 g (74%), Rf_1 0.61.

Z(OMe)-Ala-Leu-Phe-NHNH-Troc (Positions 4—6)——The azide [prepared from 4.40 g (1.2 eq) of Z(OMe)-Ala-Leu-$NHNH_2$] in DMF (30 ml) and TEA (1.62 ml, 1.2 eq) were added to an ice-chilled solution of a TFA-treated sample of Z(OMe)-Phe-NHNH-Troc (5.00 g, 9.64 mmol) in DMF (25 ml) containing TEA (1.34 ml, 1 eq) and the mixture was stirred for 15 h. The product was purified by procedure A, followed by precipitation from DMF with ether; yield 3.73 g (55%), Rf_2 0.30.

Boc-Asp(OChp)-Ala-Leu-Phe-NHNH-Troc (Positions 3—6)——A mixture of a TFA-treated sample of Z(OMe)-Ala-Leu-Phe-NHNH-Troc (3.70 g, 5.26 mmol), Boc-Asp(OChp)-OSu (2.70 g, 1.2 eq) and NMM (1.16 ml, 2 eq) in DMF (25 ml) was stirred at 4 °C for 18 h and the product was purified by procedure B, followed by recrystallization from MeOH and ether; yield 3.88 g (87%), Rf_3 0.78.

Z(OMe)-His-Ser(Bzl)-Asp(OChp)-Ala-Leu-Phe-NHNH-Troc (Positions 1—6)——The azide [prepared from 1.95 g (1.2 eq) of Z(OMe)-His-Ser(Bzl)-$NHNH_2$] in DMF (5 ml) and NMM (0.42 ml, 1.2 eq) were added to an ice-chilled solution of a TFA-treated sample of the above tetrapeptide derivative (2.70 g, 3.18 mmol) in DMF (10 ml) containing NMM (0.35 ml, 1 eq) and the mixture was stirred for 24 h. The product was purified by procedure A, followed by precipitation from DMF with ether; yield 2.89 g (74%), Rf_1 0.82.

Z(OMe)-His-Ser(Bzl)-Asp(OChp)-Ala-Leu-Phe-$NHNH_2$ [6] (Positions 1—6)——The above hexapeptide derivative (2.89 g, 2.74 mmol) in DMF-AcOH (20 ml-3.14 ml) was treated with Zn powder (3.6 g, 20 eq) at room temperature for 6 h, then the solution was filtered and the filtrate was concentrated. The residue was treated with 3% EDTA and the resulting powder was precipitated from DMF with EtOH; yield 2.21 g (89%), Rf_1 0.68. Amino acid ratios in a 6 N HCl hydrolysate: His 0.97, Ser 0.97, Asp 1.00, Ala 1.03, Leu 1.02, Phe 0.93 (recovery of Asp, 88%).

Z(OMe)-Ala-Met(O)-Lys(Z)-Lys(Z)-Tyr(Cl_2-Bzl)-Leu-Asn-Ser(Bzl)-Val-Leu-Asn-NH_2 (Positions 18—28)——The azide, prepared from 3.61 g (1.5 eq) of fragment [2], in DMF (15 ml) and TEA (0.59 ml, 1.7 eq) were added to an ice-chilled solution of a TFA-treated sample of fragment [1] (3.00 g, 2.56 mmol) in DMF-DMSO (1 : 1, 40 ml) containing TEA (0.35 ml, 1 eq) and the mixture was stirred for 36 h. The product was purified by procedure A, followed by precipitation from DMSO with MeOH; yield 3.61 g (71%), Rf_1 0.57.

Z(OMe)-Arg(Mts)-Lys(Z)-Gln-Met(O)-Ala-Met(O)-Lys(Z)-Lys(Z)-Tyr(Cl_2-Bzl)-Leu-Asn-Ser(Bzl)-Val-Leu-Asn-NH_2 (Positions 14—28)——The azide, prepared from 2.71 g (2 eq) of fragment [3], in DMF (10 ml) and TEA (0.39 ml, 2.2 eq) were added to an ice-chilled solution of a TFA-treated sample of the above undecapeptide

TABLE IV. Physical Constants and Analytical Data of Protected gVIP and Its Intermediates

Protected peptide (positions)	mp (°C)	$[\alpha]_D^{20}$ (°)	Formula	Analysis (%) Calcd (Found)		
				C	H	N
Z(OMe)-(18—28)-NH_2	289—291	−25.3 DMSO	$C_{96}H_{128}Cl_2N_{16}O_{23}S \cdot H_2O$	57.79 (57.72)	6.57 6.58	11.23 11.28)
Z(OMe)-(14—28)-NH_2	284—286	−36.5 DMSO	$C_{135}H_{185}Cl_2N_{25}O_{33}S_3 \cdot 6H_2O$	54.75 (54.54)	6.71 6.59	11.83 12.11)
Z(OMe)-(11—28)-NH_2	298—300	−23.1 DMSO	$C_{160}H_{225}Cl_2N_{31}O_{39}S_4 \cdot 4H_2O$	55.25 (55.04)	6.75 6.62	12.49 12.35)
Z(OMe)-(7—28)-NH_2	297—299	−14.0 DMF	$C_{194}H_{268}Cl_4N_{34}O_{47}S_4 \cdot 3H_2O$	56.11 (56.14)	6.65 6.60	11.53 11.23)
Z(OMe)-(1—28)-NH_2	272—274	−6.8 DMF	$C_{240}H_{329}Cl_4N_{43}O_{57}S_4 \cdot 7H_2O$	56.25 (56.08	6.75 6.65	11.86 11.75)

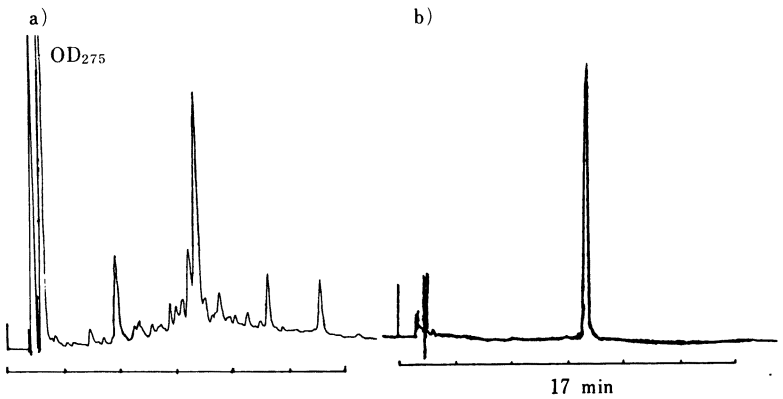

Fig. 6. HPLC (Cosmosil 5C18 Column) of Synthetic gVIP

a) Gel-filtered sample. b) Purified sample.

amide (2.50 g, 1.30 mmol) in DMF–DMSO (1 : 1, 40 ml) containing TEA (0.18 ml, 1 eq) and the mixture was stirred for 36 h. The product was purified by procedure A, followed by precipitation from DMSO with MeOH; yield 2.77 g (77%), Rf_1 0.53.

Z(OMe)-Thr-Arg(Mts)-Leu-Arg(Mts)-Lys(Z)-Gln-Met(O)-Ala-Met(O)-Lys(Z)-Lys(Z)-Tyr(Cl_2-Bzl)-Leu-Asn-Ser(Bzl)-Val-Leu-Asn-NH_2 (Positions 11—28)——The azide, prepared from 1.05 g (2 eq) of fragment [4], in DMF (5 ml) and TEA (0.22 ml, 2.2 eq) were added to an ice-chilled solution of a TFA-treated sample of the above pentadecapeptide amide (2.00 g, 0.70 mmol) in DMF–DMSO (1 : 1, 30 ml) containing TEA (0.10 ml, 1 eq) and the mixture was stirred for 48 h. The product was purified by procedure A, followed by precipitation from DMSO with MeOH; yield 1.72 g (72%), Rf_1 0.54.

Z(OMe)-Thr-Asp(OChp)-Thr-Tyr(Cl_2-Bzl)-Thr-Arg(Mts)-Leu-Arg(Mts)-Lys(Z)-Gln-Met(O)-Ala-Met-(O)-Lys(Z)-Lys(Z)-Tyr(Cl_2-Bzl)-Leu-Asn-Ser(Bzl)-Val-Leu-Asn-NH_2 (Positions 7—28)——The azide, prepared from 0.27 g (2 eq) of fragment [5], in DMF (3 ml) and NMM (32 μl, 2 eq) were added to an ice-chilled solution of a TFA-treated sample of the above octadecapeptide amide (0.50 g, 0.15 mmol) in DMF (2 ml) containing NMM (16 μl, 1 eq) and the mixture was stirred for 26 h. The product was purified by procedure C, followed by precipitation from DMF with AcOEt; yield 0.44 g (72%), Rf_1 0.56.

Z(OMe)-His-Ser(Bzl)-Asp(OChp)-Ala-Leu-Phe-Thr-Asp(OChp)-Thr-Tyr(Cl_2-Bzl)-Thr-Arg(Mts)-Leu-Arg(Mts)-Lys(Z)-Gln-Met(O)-Ala-Met(O)-Lys(Z)-Lys(Z)-Tyr(Cl_2-Bzl)-Leu-Asn-Ser(Bzl)-Val-Leu-Asn-NH_2 (Protected gVIP)——The azide, prepared from 0.22 g (2 eq) of fragment [6], in DMF (1 ml) and NMM (25 μl, 2.2 eq) were added to an ice-chilled solution of a TFA-treated sample of the above docosapeptide amide (0.44 g, 0.11 mmol) in DMF (2 ml) containing NMM (12 μl, 1 eq) and the mixture was stirred for 60 h. The additional azide and NMM (1 eq each) were added and stirring was continued for an additional 36 h. The product was purified by procedure C, followed by precipitation from DMF with AcOEt; yield 0.25 g (47%), Rf_1 0.57.

H–His–Ser–Asp–Ala–Leu–Phe–Thr–Asp–Thr–Tyr–Thr–Arg–Leu–Arg–Lys–Gln–Met–Ala–Met–Lys–Lys–Tyr–Leu–Asn–Ser–Val–Leu–Asn–NH_2 (gVIP)——Protected gVIP (100 mg, 20 μmol) in DMF (0.8 ml) was treated with phenylthiotrimethylsilane (0.38 ml, 100 eq) at room temperature for 12 h, then ether was added to form a powder. The reduced form of protected gVIP was treated with 1 M TMSOTf–thioanisole/TFA (10 ml) in the presence of *m*-cresol (0.5 ml, 240 eq) and EDT (0.59 ml, 350 eq) in an ice-bath for 150 min, then dry ether was added. The resulting powder was dissolved in 0.2 M Tris–HCl buffer (8 ml) containing 6 M guanidine–HCl and 1 M NH_4F (150 μl) was added. The pH of the solution was adjusted to 8.0 and after 30 min, to 6.0 with 1 N AcOH. The solution was incubated with 2-mercaptoethanol (140 μl, 100 eq) at 36 °C for 15 h and applied to a column of Sephadex G-25 (2 × 100 cm), which was eluted with 1 N AcOH. The fractions corresponding to the front main peak (6 ml each, tube Nos. 25—37, monitored by UV absorption measurement at 275 nm) were combined and the solvent was removed by lyophilization to give a powder; 60.1 mg (92%).

Next, the product was purified by HPLC on a Cosmosil 5C18 column (4.6 × 200 mm), which was eluted with a gradient of MeCN (24—37%, 30 min) in 0.1% TFA at a flow rate of 1.0 ml/min. The desired eluates (Fig. 6, retention time, 17 min) were collected and the solvent was removed by lyophilization to give a white fluffy powder; yield 16.0 mg (24%), $[\alpha]_D^{20}$ −35.0° (c = 0.1, 0.5 N AcOH), Rf_4 0.51, Rf_5 0.40. Amino acid ratios in a LAP digest (numbers in parentheses are theoretical): Asp 1.87 (2), Thr 3.85 (4), Ser 1.96 (2), Ala 1.78 (2), Val 1.00 (1), Met 1.87 (2), Leu 3.86 (4), Tyr 1.97 (2), Phe 0.90 (1), Lys 3.11 (3), His 0.91 (1), Arg 2.07 (2), Gln and Asn were not determined (recovery of Val, 87%).

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2) Amino acids used in this investigation are of the L-configuration. The following abbreviations are used: Z(OMe) = *p*-methoxybenzyloxycarbonyl, Z = benzyloxycarbonyl, Boc = *tert*-butoxycarbonyl, Bzl = benzyl, Cl_2-Bzl = 2,6-dichlorobenzyl, Chp = cycloheptyl, (O) = sulfoxide, DCC = dicyclohexylcarbodiimide, Troc = 2,2,2-trichloroethyloxycarbonyl, Mts = mesitylenesulfonyl, Su = *N*-hydroxysuccinimidyl, TFA = trifluoroacetic acid, TMSOTf = trimethylsilyl trifluoromethanesulfonate, DMF = dimethylformamide, DMSO = dimethylsulfoxide, NMM = *N*-methylmorpholine, DCHA = dicyclohexylamine, EDT = ethanedithiol, EDTA = ethylenediaminetetraacetic acid disodium salts.
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