[Chem. Pharm. Bull. 28(6)1873—1883(1980)]

Studies on Peptides. XCV.[1,2] Alternative Synthesis of Porcine Vasoactive Intestinal Polypeptide (VIP)

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(Received January 25, 1980)

Two alternative deprotecting procedures were employed for the synthesis of porcine vasoactive intestinal polypeptide (VIP). Besides HF, TFA-thioanisole was found to cleave all the protecting groups employed, Z, Bzl and Mts, suppressing a newly found side reaction, *i.e.*, acid-catalyzed succinimide formation from Asp residues with the free carboxyl group. A small amount of the N-terminal His residue linked to Ser–Asp was found to be released on standing at pH 6.

Keywords——synthesis of porcine vasoactive intestinal polypeptide (VIP); N^G-mesitylene-2-sulfonylarginine; ring closure of Asp-Asn by acids; release of His from VIP; HF–*m*-cresol as a deprotecting system; trifluoroactic acid–thioanisole–*m*-cresol as a deprotecting system; pancreatic blood flow activity

The structure of porcine vasoactive intestinal polypeptide (VIP) was elucidated by Mutt and Said[4] in 1974. The first synthesis of this octacosapeptide amide was achieved by Bodanszky *et al.*[5] using TFA as a deprotecting reagent in the final step. Later, an alternative synthesis of VIP was orally presented by Sakagami *et al.*[6] In both syntheses, the protecting group of arginine was removed at an early stage, since no suitable protecting groups acidolytically removable TFA were available at the time.

Following the synthesis[7] of the structurally related chicken VIP,[8] we wish to report an alternative synthesis of porcine VIP in which our newly devised arginine derivative, Arg (Mts),[9] was again successfully employed. This protecting group was removed by HF[10] in the final step of the synthesis of chicken VIP. In the present synthesis, this protecting group was cleaved together with other protecting groups, not only by HF, but also by a modified TFA procedure in the presence of thioanisole.[11]

Porcine and chicken VIPs differ from each other by 4 amino acid residues at positions, 11, 13, 26 and 28, so among the six peptide fragments we employed for the present synthesis,

1) Part XCIV: H. Yajima, H. Ogawa, H. Ueda, and H. Takagi, *Chem. Pharm. Bull.*, **28**, 1935 (1980).
2) Amino acids, peptides and their derivatives mentioned in this paper are of the L-configuration. Abbreviations used: Z=benzyloxycarbonyl, Z(OMe)=*p*-methoxybenzyloxycarbonyl, Bzl=benzyl, Mts=mesitylene-2-sulfonyl, NP=*p*-nitrophenyl, DCC=dicyclohexylcarbodiimide, HOBT=1-hydroxybenzotriazole, TFA=trifluoroacetic acid, DMF=dimethylformamide, DMSO=dimethylsulfoxide, HMPA=hexamethylphosphoramide, MSA=methanesulfonic acid.
3) Location: *Sakyo-ku, Kyoto, 606, Japan.*
4) V. Mutt and S.I. Said, *Eur. J. Biochem.*, **42**, 581 (1974).
5) M. Bodanszky, Y.S. Klausner, and S.I. Said, *Proc. Natl. Acad. Sci. USA*, **70**, 382 (1973).
6) M. Sakagami, T. Hashimoto, and N. Yanaihara, *Abst. of the 98th Meeting of Japan Pharm. Soc.*, **1978**, p. 241.
7) H. Yajima, M. Takeyama, K. Koyama, T. Tobe, K. Inoue, T. Kawano, and H. Adachi, *Int. J. Peptide Protein Res.*, in press.
8) A. Nilsson, *FEBS Lett.*, **60**, 322 (1975).
9) H. Yajima, M. Takeyama, J. Kanaki, and K. Mitani, *J.C.S. Chem. Comm.*, **1978**, 482; H. Yajima, M. Takeyama, J. Kanaki, O. Nishimura, and M. Fujino, *Chem. Pharm. Bull.*, **26**, 3752 (1978).
10) S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada, and H. Sugihira, *Bull. Chem. Soc. Japan*, **40**, 2164 (1967).
11) Y. Kiso, K. Ukawa, S. Nakamura, K. Ito, and T. Akita, *Chem. Pharm. Bull.*, **28**, 673 (1980).

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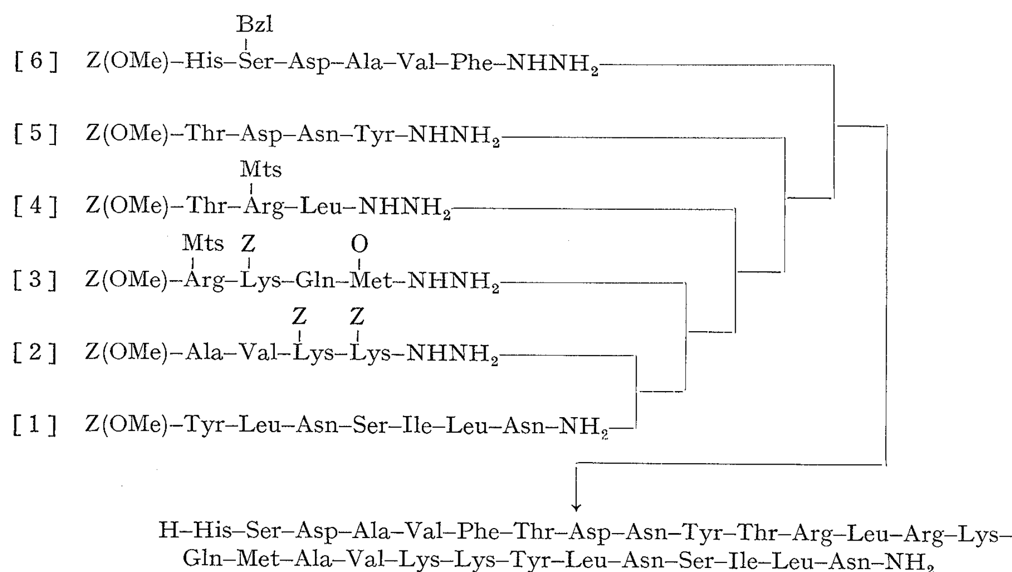

Fig. 1. Synthetic Route to Porcine Vasoactive Intestinal Polypeptide

four fragments were identical with those employed for the previous synthesis. Two fragments [1] and [4], which cover the area of species variation, were newly synthesized (Fig. 1).

As mentioned previously,[7] the TFA-labile Z(OMe) group[12] was employed as the N^{α}-protecting group in combination with Lys(Z) and Arg(Mts). The methionine residue was protected as its sulfoxide.[13,14] The β-carboxyl protecting group of aspartic acid residues (positions 3 and 8) was removed by hydrogenolysis immediately after the incorporation of Asp (OBzl) in order to suppress the undesired aminosuccinimide formation.[15]

The fragment [1], Z(OMe)–Tyr–Leu–Asn–Ser–Ile–Leu–Asn–NH_2 [Z(OMe)–(porcine VIP 22—28)-NH_2] was synthesized according to Fig. 2. In addition to the known dipeptide hydrazide, Z(OMe)–Tyr–Leu–$NHNH_2$,[7] two protected peptide hydrazides, Z(OMe)–Asn–Ser–$NHNH_2$ and Z(OMe)–Ile–Leu–$NHNH_2$, were newly synthesized by the NP[16] and DCC[17] procedures, respectively, followed by the usual hydrazine treatment of the corresponding dipeptide esters. The three hydrazides were successively condensed with a TFA-treated

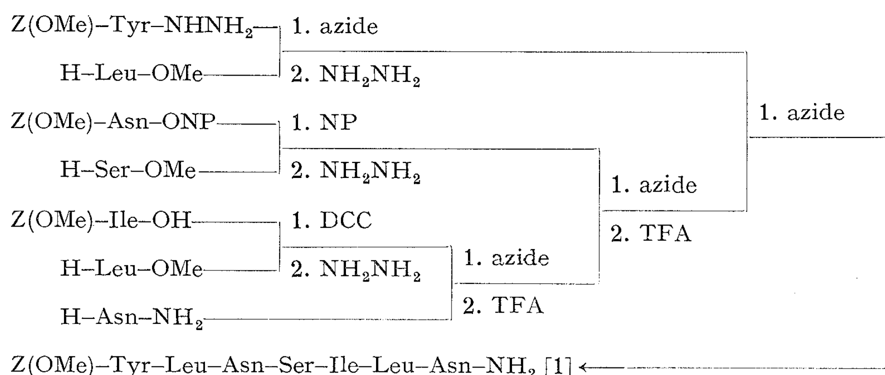

Fig. 2. Synthetic Scheme for the Protected Heptapeptide Amide Z(OMe)–(porcine VIP 22—28)-NH_2

12) F. Weygand and K. Hunger, *Chem. Ber.*, **95**, 1 (1962).
13) B. Iselin, *Helv. Chim. Acta*, **44**, 61 (1961).
14) N. Fujii, T. Sasaki, S. Funakoshi, H. Irie, and H. Yajima, *Chem. Pharm. Bull.*, **26**, 650 (1978).
15) M. Bodanszky and J.Z. Kwei, *Int., J. Peptide Protein Res.*, **12**, 69 (1978) and references cited therein.
16) M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959).
17) J.C. Sheehan and G.P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955).

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sample of Z(OMe)–Asn–NH_2 prepared by exposing Z(OMe)–Asn–ONP to ammonia, as shown in Fig. 2.

The fragment [4], Z(OMe)–Thr–Arg(Mts)–Leu–$NHNH_2$ [Z(OMe)-(porcine VIP 11—13)-$NHNH_2$], was synthesized according to Fig. 3 in a stepwise manner starting with H–Leu–OMe. Z(OMe)–Arg(Mts)–OH was incorporated by the DCC procedure in the presence of HOBT[18] and subsequently Z(OMe)–Thr–$NHNH_2$ was incorporated by the azide procedure.[19] Attempts to crystallize the resulting tripeptide ester, Z(OMe)–Thr–Arg(Mts)–Leu–OMe, were unsuccessful. This peptide ester was therefore converted to the corresponding hydrazide, which permitted us to check its homogeneity by elemental analysis.

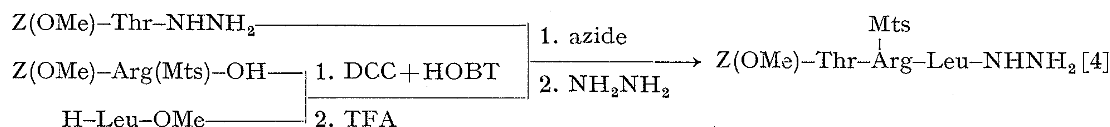

Fig. 3. Synthetic Scheme for the Protected Tripeptide Hydrazide, Z(OMe)-(porcine VIP 11—13)-$NHNH_2$

Six peptide fragments were assembled successively according to Fig. 1 by the azide procedure. The C-terminal undecapeptide amide was prepared by two alternative routes; by condensations of the fragments [1] and [2] and by successive azide condensations of Z(OMe)–Lys(Z)–Lys(Z)–$NHNH_2$[20] and Z(OMe)–Ala–Val–$NHNH_2$.[7] Owing to the insolubility of the protected heptapeptide amide in DMF, these condensations had to be performed in DMF–DMSO–HMPA, and in DMSO–HMPA in the case of the condensation of [3]. After incorporation of the fragment [3] bearing protecting groups, Mts and Z, the solubility of the amino component was improved and thus the subsequent condensation of fragment [4] was performed in DMSO–DMF and the condensations of fragments, [5] and [6], were performed in DMF. The insolubility of the protected products has some advantages in their purification. Up to the octadecapeptide amide, all protected products were purified easily by precipitation

Table I. Amino Acid Ratios in 6 N HCl Hydrolysates of Porcine VIP and Protected Intermediates

Position	Protected peptides						VIP
	22—28	18—28	14—28	11—28	7—28	1—28	1—28
Asp	2.01 (2)	2.04 (2)	2.02 (2)	2.06 (2)	4.56 (4)	5.27 (5)	5.25 (5)
Thr				0.89 (1)	2.04 (2)	1.92 (2)	1.95 (2)
Ser	0.86 (1)	0.86 (1)	0.86 (1)	0.87 (1)	0.86 (1)	1.73 (2)	1.76 (2)
Glu			1.08 (1)	1.07 (1)	1.09 (1)	1.21 (1)	1.13 (1)
Ala		0.98 (1)	1.02 (1)	0.93 (1)	0.95 (1)	2.01 (2)	2.03 (2)
Val		1.00 (1)	1.05 (1)	1.05 (1)	0.97 (1)	2.09 (2)	2.06 (2)
Met			0.88 (1)	0.87 (1)	0.83 (1)	0.84 (1)	0.96 (1)
Ile	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)
Leu	2.02 (2)	2.07 (2)	2.05 (2)	3.08 (3)	3.34 (3)	3.13 (3)	3.11 (3)
Tyr	0.93 (1)	1.00 (1)	1.04 (1)	0.93 (1)	2.16 (2)	1.98 (2)	2.06 (2)
Phe						1.03 (1)	1.04 (1)
Lys		2.08 (2)	3.20 (3)	3.07 (3)	3.20 (3)	3.24 (3)	3.04 (3)
His						0.95 (1)	0.90 (1)
Arg			1.04 (1)	2.01 (2)	2.24 (2)	2.08 (2)	2.11 (2)
Recovery	88%	90%	94%	99%	94%	93%	89%

18) W. König and R. Geiger, *Chem. Ber.*, **103**, 788 (1970).
19) J. Honzl and J. Rudinger, *Coll. Czech. Chem. Comm.*, **26**, 2333 (1961).
20) H. Yajima, K. Koyama, Y. Kiso, A. Tanaka, and M. Nakamura, *Chem. Pharm. Bull.*, **24**, 492 (1976).

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from DMSO or DMF with methanol, because of the great difference in solubility between the desired products and the small acyl components used in excess during each coupling reaction. However, gel-filtration was required for purification of the protected docosapeptide amide and the final protected octacosapeptide amide. Gelfiltration on Sephadex LH-60 effectively provided the desired compounds, each as a sharp single spot on TLC. All the protected intermediates and the protected octacosapeptide amide were confirmed to be homogeneous by TLC, acid hydrolysis (Table I) and elemental analysis. In the acid hydrolysis, amino acid recovery of Ile was selected as the basis of calculation, since this amino acid occurs only once in porcine VIP. With this standard, newly incorporated amino acids could be accurately assessed after each coupling step.

In a preliminary run, we treated the protected octacosapeptide amide with MSA[21] to remove all protecting groups, Z(OMe), Mts and Z, and incubated the product with mercaptoethanol to reduce the Met(O) residue. Acid hydrolysis of the product gave a somewhat low recovery of histidine, and aminopeptidase digestion gave a low recovery of aspartic acid. Thus, we examined the deprotection and incubation conditions using model peptides.

Deprotection of Z(OMe)–His–Ser(Bzl)–Asp–Ala–Val–Phe–$NHNH_2$ [6] with MSA or HF gave a product which afforded satisfactory recovery of His, after enzymic digestion. The product, H–His–Ser–Asp–Ala–Val–Phe–$NHNH_2$, was then incubated in an aqueous solution at pH 6.0 at 65° for 24 hr, with or without mercaptoethanol and the solutions were examined with an amino acid analyzer. About 1.6 to 3% of His was detected in both cases. Release of His amounting to 0.85% was also detected, when the solution was kept standing at room temperature for 90 hr (Tables II and III). These results indicate that the deprotecting reagents employed were not responsible for the release of His, but that slow hydrolysis of the His–Ser bond occurred during the incubation. It is interesting that the N-terminal tripeptide unit of porcine and chicken VIPs, His–Ser–Asp, is a unique combination of three functional amino acids which forms the active center of serine protease.[22] The instability of structurally related secretin in solution has been pointed out,[23] and aminosuccinimide formation of Asp (position 3) was considered to be the reason for this phenomenon.[24] Slow hydrolysis of His may be an additional reason for the instability of secretin in solutions, and presumably VIP also. As in the synthesis of chicken VIP, we reduced the Met(O) residue of the protected octacosapeptide amide, prior to deprotection, in order to avoid the release of His during the incubation.

Deprotection of Z(OMe)–Thr–Asp–Asn–Tyr–$NHNH_2$ [5] with MSA afforded a product with low recovery of Asp and Asn on aminopeptidase digestion. HBr–AcOH deprotection

Table II. Enzymic Examination of the Products formed by Deprotection of Z(OMe)–His–Ser(Bzl)–Asp–Ala–Val–Phe–$NHNH_2$

Reagent	*Rf* main and (minor)	Temp °C	Scavenger	Recovery of amino acids in AP-M digest					
				His	Ser	Asp	Ala	Val	Phe+Ser(Bzl)
TFA	0.50	0	Anisole	1.00		0.98	1.01	0.97	2.12 (as Phe)
HBr–AcOH	0.32 (0.42)	20	Anisole	1.00	0.23	0.72	0.80	0.90	1.00+Ser(OAc)
HBr–TFA	0.30	0	Anisole	1.00	0.97	0.95	0.97	0.93	0.96
MSA	0.30 (0.40)	20	Anisole	1.00	1.00	0.74	0.78	0.85	0.97
HF	0.30	0	Anisole	1.00	1.00	1.00	1.01	1.01	0.99
TFA (24 hr)	0.30	20	Thioanisole	1.00	0.98	0.99	0.97	0.98	1.00

21) H. Yajima, Y. Kiso, H. Ogawa, N. Fujii, and H. Irie, *Chem. Pharm. Bull.*, **23**, 1164 (1975).
22) W.W. Bachovchin and J.D. Roberts, *J. Am. Chem. Soc.*, **100**, 8041 (1978) and references cited therein.
23) M.I. Grossman, *Gastroenterology*, **57**, 767 (1969).
24) W. König, R. Geiger, H. Wissmann, M. Bickel, R. Obermeider, W. Teetz, and R. Uhmann, *Gastroenterology*, **72**, 797 (1977).

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Table III. Release of His from H–His–Ser–Asp–Ala–Val–Phe–$NHNH_2$ at pH 6.0

Mercaptoethanol	His released (%)			
	at 26°		at 65°	
	45 hr	90 hr	12 hr	24 hr
None	0.59	0.75	1.61	2.84
100 eq.		0.85	2.53	3.50

was similar, as mentioned briefly in connection with the synthesis of chicken VIP.[7] The possibility of aminosuccinimide formation of the Asp residue with a free carboxyl group was previously predicted by Bodanszky *et al.*[25] during the structural elucidation of a peptide antibiotic, amphomycin. This side reaction was found to occur preferentially at the Asp residue linked to Asn, and the degree of this side reaction seemed to depend on the deprotecting reagents employed, as shown in Table IV. In the HF-treated sample, more than 94% of Asp was recovered after enzymic digestion, and recovery was nearly quantitative in the TFA-treated sample. It is interesting to note that the HBr–TFA treatment gave a much better result than HBr–AcOH and such ring closure of Asp–Ala appeared to take place to some degree during the MSA deprotection of the fragment [6]. Bodanszky *et al.*[5] employed prolonged treatment with TFA for the removal of the Z group in the synthesis of porcine VIP. This seemed efficient, in terms of obtaining a homogeneous product. They also mentioned that HBr treatment produced a product with the same level of activity, but this deprotection was not applied in their preparative experiment. It seems likely that the HBr-deprotected product would be a mixture of isomers at position 8.

Table IV. Enzymic Examination of the Products formed by Deprotection of Z(OMe)–Thr–Asp–Asn–Tyr–OMe

Reagent	*Rf* main and (minor)	Temp °C	Scavenger	Recovery of amino acids			
				Thr	Asp	Asn	Tyr
TFA	0.37	0	Anisole	1.00	0.97	0.90	0.97
HBr–AcOH	(0.37) 0.50	20	Anisole	0.78	0.30	0.37	1.00
HBr–TFA	0.37 (0.50)	0	Anisole	1.00	0.96	0.94	0.99
MSA	(0.37) 0.50	20	Anisole	1.00	0.25	0.26	0.95
HF	0.37 (0.50)	0	Anisole	1.00	0.94	0.93	1.01
TFA (24 hr)	0.37 (0.50)	20	Thioanisole	1.00	0.95	0.97	0.98

Considering the side reaction at the Asp residue, as mention above, we decided to attempt two deprotection procedures in the present synthesis of porcine VIP; HF deprotection and TFA deprotection. In the latter instance, some modification was required to ensure removal of all the protecting groups employed, Mts, Bzl, Z and Z(OMe), as described later.

The protected octacosapeptide amide was treated with thiophenol[26] to reduce the Met(O) residue as mentioned above and the reduced peptide was treated with HF to remove all protecting groups. As a cation scavenger. *m*-cresol was employed to suppress a possible side reaction at the Tyr residue, *i.e.*, O-mesitylene-2-sulfonylation.[9] The deprotected peptide was converted to the corresponding acetate by Amberlite CG-4B (acetate form) treatment and purified by gel-filtration on Sephadex G-25, followed by ion-exchange chromatography

25) M. Bodanszky, G.F. Sigler, and A. Bodanszky, *J. Am. Chem. Soc.*, **95**, 2352 (1973).
26) H. Yajima, S. Funakoshi, N. Fujii, K. Akaji, and H. Irie, *Chem. Pharm. Bull.*, **27**, 1060 (1979).

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on CM-cellulose. In the latter step, gradient elution with pH 8.0, 0.1 M ammonium bicarbonate buffer was employed, according to the procedure used for the purification of natural porcine VIP by Said and Mutt.[27] The chromatographic pattern (Fig. 4) obtained here was quite similar to those observed in the purification of chicken VIP.[7] After the elution of small two peaks, the desired product was isolated. Disc-electrophoretic examination of the isolated product revealed the presence of a faint impurity, which seemed to be a succinimide derivative (at position 8). This impurity was removed by preparative isoelectric focusing[28] in Ampholine, pH 9—11 (LKB), as applied for the purification of synthetic chicken VIP.[7] The desired product appeared at pH 10.1 (Fig. 5).

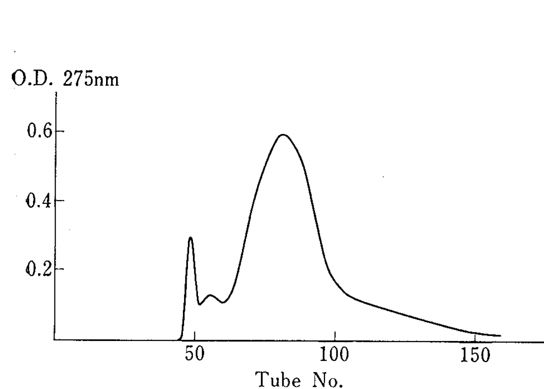

Fig. 4. Purification of Synthetic Porcine VIP on CM-Cellulose

Column: 2.5×6.0 cm.
Gradient: 0.1 M NH_4HCO_3 (pH 8.0), mixing flask (H_2O 200 ml).
Fraction: 6.6 ml.

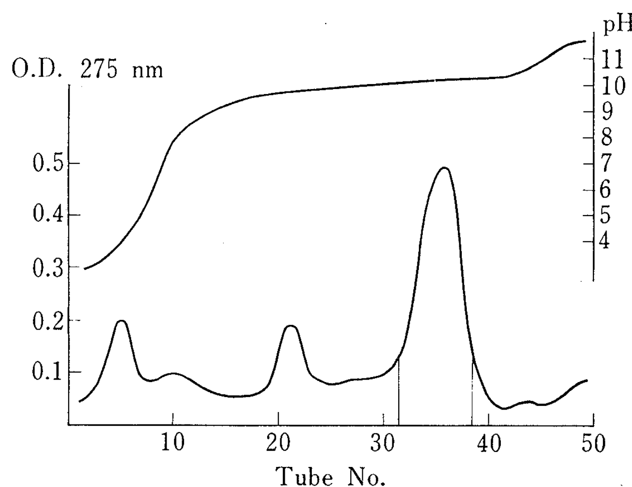

Fig. 5. Purification of Synthetic Porcine VIP by Isoelectric Focusing in Ampholine, pH 9—11(LKB)

The product thus purified gave a sharp single spot on TLC in two different solvent systems and behaved as a single component in disc-electrophoresis at pH 4.0. Amino acid ratios in the acid hydrolysate were in excellent agreement with the theoretical values (Table I), and satisfactory recovery of Asp, together with other constituent amino acids, was confirmed by aminopeptidase digestion.

Next, we wish to describe the application of a new deprotecting procedure for the synthesis of porcine VIP. As mentioned previously, Bodanszky *et al.*[5] exposed the protected octacosapeptide amide to the action of TFA at room temperature for 2.5 days to remove the Z group from Lys(Z). Recently Kiso *et al.*[11,29] found that the acidolytic deprotection of Tyr(Bzl) with TFA was greatly accelerated by the presence of thiol compounds which possess potent cation capture properties, and confirmed further that the Z group could be completely cleaved by TFA within 3 to 4 hr at room temperature in the presence of thioanisole. Complete removal of Ser(Bzl) and Arg(Mts) under these conditions was difficult. However, we found that deprotection of these groups was accelerated by thioanisole, and within 24 hr, 96 to 99% cleavage was detected in both cases. Recently the role of cation scavengers in the TFA deprotection of the Boc (*tert*-butoxycarbonyl) group was examined in detail by Lundt *et al.*[30] Thioanisole accepts the *tert*-butyl cation to form the sulfonium compound, which still has the ability to alkylate other functional amino acids. In order to trap all alkylating capability

27) S.I. Said and V. Mutt, *Eur. J. Biochem.*, **28**, 199 (1972).
28) G.R. Finlayson and A. *Chrambach*, *Anal. Biochem.*, **40**, 292 (1971).
29) Y. Kiso, S. Nakamura, K. Ito, K. Ukawa, K. Kitagawa, T. Akita, and H. Moritoki, *J.C.S. Chem. Comm.*, **1979**, 971.
30) B.F. Lundt, N.L. Johansen, A. Volund, and J. Markussen, *Int. J. Pept. Prot. Res.*, **12**, 258 (1978).

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in acidolytic deprotecting media, an additional scavenger system may be desirable, such as the TFA–thioanisole–*m*-cresol system or the TFA–thioanisole–dimethoxyresorcinol system, since such additional scavengers trap the alkyl cation at the aromatic ring system. The Mts group can be cleaved by HBr–AcOH in 70% within 60 minutes. However, we did not examine this deprotection in the present synthesis for the reason mentioned earlier.

Considering the reduced extent of the Asp–Asn side reaction on TFA treatment, we applied the above modified TFA deprotection for an alternative synthesis of porcine VIP. The reduced protected octacosapeptide amide was exposed to TFA in the presence of thioanisole and *m*-cresol (300 eq. each) at room temperature for 10 hr, then dry ether was added. The precipitated peptide was again treated under identical conditions to carry out deprotection as thoroughly as possible. The deprotected peptide was then purified by gel-filtration on Sephadex G-25 as described above. Though we predicted the presence of a small front peak due to partially deprotected peptide, the chromatographic pattern obtained here was quite similar to that of the HF deprotected product, indicating that the protecting groups, Bzl and Mts, were removed by TFA under these conditions to much the same extent as by HF deprotection. The main product that emerged from the column was then purified by ion-exchange chromatography on CM-cellulose, followed by isoelectric focusing as mentioned above. Identity of the product with that obtained by HF deprotection was ascertained by comparison of their *Rf* values on TLC and their mobilities on disc-electrophoresis. Its homogeneity was further confirmed by digestion with aminopeptidase.

Two samples of synthetic VIP prepared by HF deprotection and by the modified TFA deprotection gave an *Rf* value identical with that of natural VIP supplied by Professor V. Mutt, Karolinska Institute, Sweden, and accelerated the pancreatic blood flow in dogs to the same extent as natural porcine VIP.

We were thus able to synthesize porcine and chicken VIPs. Synthesis of VIP involves considerable difficulty in obtaining a homogeneous synthetic peptide free from side reaction at Asp, particularly that linked to Asn. Schön and Kisfaludy also reported acid catalyzed succinimide formation from Asp residues with the free carboxyl group, using H–Asp–Phe–NH_2 as an example.[31]

Experimental

General methods employed are essentially the same as those described in Part LXXXVII of this series.[7] Thin-layer chromatography was performed on silica gel (Kiesel G, Merck) and *Rf* values refer to the following solvent systems: Rf_1 $CHCl_3$–MeOH–H_2O (8:3:1), Rf_2 *n*-BuOH–AcOH–pyridine–H_2O (4:1:1:2), Rf_3 *n*-BuOH–AcOH–H_2O (4:1:5), Rf_4 *n*-BuOH–AcOH–pyridine–H_2O (30:6:20:24).

Z(OMe)-Asn-NH$_2$——Ammonia gas was bubbled through a solution of Z(OMe)–Asn–ONP (2.70 g, 6.47 mmol) in THF (80 ml) for 2 hr. The resulting crystalline mass was collected by filtration and precipitated from DMF with AcOEt; yield 1.81 g (95%), mp 198—199°, $[\alpha]_D^{20}$ +11.0° (c=0.4, DMF), Rf_1 0.32. *Anal.* Calcd for $C_{13}H_{17}N_3O_5$: C, 52.87; H, 5.80; N, 14.23. Found: C, 52.64; H, 5.82; N, 14.18.

Z(OMe)-Ile-Leu-NHNH$_2$——DCC (19.40 g, 94 mmol) was added to a stirred solution of Z(OMe)–Ile–OH (25.0 g, 85 mmol) and H–Leu–OMe [prepared from 30.0 g (0.17 mol) of the hydrochloride with 22.9 ml (0.17 mol) of Et_3N] in DMF–THF (130 ml–120 ml). After 24 hr, the solution was filtered, the filtrate was concentrated and the residue was dissolved in AcOEt. The extract was washed with 5% citric acid, 5% Na_2CO_3 and H_2O–NaCl, dried over Na_2SO_4 and concentrated. The residue was dissolved in MeOH (200 ml) and 80% hydrazine hydrate (51 ml, 0.85 mol) was added. After standing overnight, the resulting mass was collected by filtration, washed with EtOH and precipitated from DMF with EtOH; yield 19.75 g (55%), mp 196—197°, $[\alpha]_D^{20}$ −6.0° (c=0.8, DMF), Rf_1 0.64. *Anal.* Calcd for $C_{21}H_{34}N_4O_5$: C, 59.69; H, 8.11; N, 13.27. Found: C, 59.96; H, 8.26; N, 13.31.

Z(OMe)-Ile-Leu-Asn-NH$_2$——Z(OMe)–Asn–NH_2 (5.91 g, 20 mmol) was treated with TFA (15 ml) in the presence of anisole (3.0 ml) in an ice-bath for 60 min, then dry ether was added. The resulting powder was collected by filtration, washed with ether, dried over KOH pellets *in vacuo* for 3 hr and dissolved in DMSO–HMPA (1:1, 40 ml) containing Et_3N (2.8 ml, 20 mmol). The azide [prepared from 11.0 g (26 mmol)

31) I. Schön and L. Kisfaludy, *Int. J. Pept. Prot. Res.*, 14, 485 (1979).

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of Z(OMe)-Ile-Leu-NHNH$_2$] in DMF (100 ml) and Et$_3$N (3.6 ml, 26 mmol) were added to the above ice-chilled solution, and the mixture was stirred at 4° for 40 hr. The solvent was evaporated off and the residue was treated with 5% citric acid and ether. The resulting powder was washed with 5% citric acid and H_2O and precipitated from DMSO with MeOH; yield 9.39 g (90%), mp 272—273°, $[\alpha]_D^{20}$ +16.0 (c=0.3, HMPA), Rf_1 0.55. *Anal.* Calcd for $C_{25}H_{39}N_5O_7$: C, 57.56; H, 7.54; N, 13.43. Found: C, 57.47; H, 7.58; N, 13.40.

Z(OMe)-Asn-Ser-OMe——A mixture of Z(OMe)-Asn-ONP (17.53 g, 42 mmol) and H-Ser-OMe [prepared from 15.50 g (100 mmol) of the hydrochloride with 19.7 ml (142 mmol) of Et$_3$N] in DMF (200 ml) was stirred at room temperature for 30 hr. The solvent was evaporated off and the residue was treated with 5% citric acid and ether. The resulting powder was purified by washing with 5% citric acid, 5% Na_2CO_3 and H_2O, followed by precipitation from DMF with AcOEt; yield 7.67 g (46%), mp 161—163°, $[\alpha]_D^{20}$ +8.6° (c=0.5, DMF), Rf_1 0.48. *Anal.* Calcd for $C_{17}H_{23}N_3O_8$: C, 51.38; H, 5.83; N, 10.58. Found: C, 51.31; H, 6.00; N, 10.28.

Z(OMe)-Asn-Ser-NHNH$_2$——Z(OMe)-Asn-Ser-OMe (7.60 g, 19 mmol) in DMF (70 ml) was treated with 80% hydrazine hydrate (12 ml, 0.2 mol) at room temperature overnight. The resulting mass was collected by filtration, washed with EtOH and precipitated from DMSO with EtOH; yield 5.75 g (76%), mp 214—215°, $[\alpha]_D^{20}$ +33.2° (c=0.5, DMSO), Rf_1 0.24. *Anal.* Calcd for $C_{16}H_{23}N_5O_7 \cdot 1/2H_2O$: C, 47.28; H, 5.95; N, 17.23. Found: C, 46.85; H, 5.95; N, 17.58.

Z(OMe)-Asn-Ser-Ile-Leu-Asn-NH$_2$——Z(OMe)-Ile-Leu-Asn-NH$_2$ (8.0 g, 15.3 mmol) was treated with TFA-anisole (25 ml-5 ml) and the deprotected peptide isolated as described above was dissolved in DMSO-HMPA (1:1, 80 ml) containing Et$_3$N (2.1 ml, 15.3 mmol). The azide [prepared from 8.0 g (20 mmol) of Z(OMe)-Asn-Ser-NHNH$_2$] in DMF (60 ml) and Et$_3$N (2.8 ml, 20 mmol) were added to the above ice-chilled solution. After stirring at 4° for 40 hr, the solution was concentrated and the residue was treated with ether. The resulting powder was washed with 5% citric acid and MeOH and precipitated from DMSO with MeOH; yield 9.96 g (90%), mp 253—255°, $[\alpha]_D^{20}$ +27.9° (c=0.5, HMPA), Rf_1 0.25, Amino acid ratios in 6 N HCl hydrolysate: Asp 1.99, Ser 0.88, Ile 1.00, Leu 0.99 (average recovery 94%). *Anal.* Calcd for $C_{32}H_{50}N_8O_{11} \cdot 1/2H_2O$: C, 52.52; H, 7.02; N, 15.31. Found: C, 52.55; H, 7.29; N, 15.03.

Z(OMe)-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH$_2$ [1], Z(OMe)-(porcine VIP 22—28)-NH$_2$——Z(OMe)-Asn-Ser-Ile-Leu-Asn-NH$_2$ (5.35 g, 7.4 mmol) was treated with TFA-anisole (13 ml-3.5 ml) and the deprotected peptide isolated as described above was dissolved in DMSO-HMPA (1:1, 50 ml) containing Et$_3$N (1.03 ml, 7.4 mmol). The azide [prepared from 4.91 g (10.4 mmol) of Z(OMe)-Tyr-Leu-NHNH$_2$] in DMF (10 ml) and Et$_3$N (1.88 ml, 10.4 mmol) were added to this ice-chilled solution, and the mixture was stirred at 4° for 24 hr. The solvent was evaporated off and the residue was treated with ether. The resulting powder was purified by washing as described above, followed by precipitation from DMSO with MeOH; yield 5.69 g (77%), mp 255—257°, $[\alpha]_D^{20}$ −9.8° (c=0.4, HMPA), Rf_1 0.25. Amino acid ratios in 6 N HCl (with phenol) hydrolysate: Tyr 1.00, Leu 2.05, Asp 2.00, Ser 0.86, Ile 1.00, (average recovery, 86%). *Anal.* Calcd for $C_{47}H_{70}N_{10}O_{14} \cdot 1/2H_2O$: C, 55.99; H, 7.10; N, 13.90. Found: C, 56.01; H, 7.10; N, 13.63.

Z(OMe)-Arg(Mts)-Leu-OMe——DCC (4.40 g, 21.2 mmol) was added to a mixture of Z(OMe)-Arg(Mts)-OH (10.10 g, 19.3 mmol), HOBT (3.25 g, 21.2 mmol) and H-Leu-OMe [prepared from 6.80 g (37.4 mmol) of the hydrochloride with 5.2 ml (37.4 mmol) of Et$_3$N] in DMF (50 ml) and the whole was stirred at room temperature for 24 hr. The solution was filtered, the filtrate was concentrated and the residue was extracted with AcOEt. The extract was washed with 5% citric acid, 5% Na_2CO_3 and H_2O-NaCl, then dried over Na_2SO_4 and concentrated. The residue was triturated with ether and n-hexane and the resulting powder was recrystallized from AcOEt and n-hexane; yield 10.62 g (85%), mp 63—65°, $[\alpha]_D^{20}$ −6.8° (c=0.4, MeOH), Rf_1 0.70. *Anal.* Calcd for $C_{31}H_{45}N_5O_8S$: C, 57.48; H, 7.00; N, 10.81. Found: C, 57.48; H, 7.18; N, 10.76.

Z(OMe)-Thr-Arg(Mts)-Leu-NHNH$_2$ [4], Z(OMe)-(porcine VIP 11—13)-NHNH$_2$——Z(OMe)-Arg(Mts)-Leu-OMe (8.05 g, 12.4 mmol) was treated with TFA-anisole (20 ml-5 ml) as usual and the excess TFA was removed by evaporation. The oily residue was washed with n-hexane, dried over KOH pellets *in vacuo* for 3 hr and converted to the corresponding hydrochloride by treatment with 5.78 N HCl-dioxane (2.15 ml, 12.4 mmol). Trituration with ether and n-hexane afforded a powder, which was dissolved in DMF (40 ml) containing Et$_3$N (1.72 ml, 12.4 mmol). The azide [prepared from 4.46 g (15 mmol) of Z(OMe)-Thr-NHNH$_2$] in DMF (40 ml) and Et$_3$N (2.09 ml, 15 mmol) were added to the above ice-chilled solution. After stirring at 4° for 24 hr, the solvent was evaporated off and the residue was dissolved in AcOEt. The organic phase was washed with 5% citric acid, 5% Na_2CO_3 and H_2O-NaCl, dried over Na_2SO_4 and concentrated. The oily residue was dissolved in MeOH (50 ml) and 80% hydrazine hydrate (12 ml, 0.2 mol) was added. After standing overnight, the solution was concentrated, and the residue was treated with H_2O. The resulting powder was precipitated from DMF with ether; yield 6.04 g (65%), mp 124—126°, $[\alpha]_D^{20}$ −1.6° (c=0.6, DMF), Rf_1 0.59. Amino acid ratios in 6 N HCl hydrolysate: Thr 0.96, Leu 1.00, Arg 1.01 (average recovery 86%). *Anal.* Calcd for $C_{34}H_{52}N_8O_9S$: C, 54.53; H, 7.00; N, 14.96. Found: C, 54.25; H, 7.08; N, 14.80.

Z(OMe)-Lys(Z)-Lys(Z)-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH$_2$, Z(OMe)-(porcine VIP 20—28)-NH$_2$——Z(OMe)-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH$_2$ (1.81 g, 1.81 mmol) was treated with TFA-anisole (5.0 ml-1.2 ml) as usual, then dry ether was added. The resulting powder was dissolved in DMSO-HMPA-DMF (1:1:1, 15 ml) containing Et$_3$N (0.25 ml, 1.81 mmol). The azide [prepared from 1.70 g (2.35 mmol) of Z(OMe)-Lys(Z)-Lys(Z)-NHNH$_2$] in DMF (16 ml) and Et$_3$N (0.33 ml, 2.35 mmol) were added to the above

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ice-chilled solution and the mixture was stirred at 4° for 24 hr. The solvent was evaporated off and the residue was treated with 5% citric acid and AcOEt. The resulting powder was purified by washing with 5% citric acid, 5% Na_2CO_3 and H_2O followed by precipitation from DMSO with MeOH; yield 2.57 g (93%), mp 244—245°, $[\alpha]_D^{20}$ +6.8° (c=0.3, HMPA), Rf_1 0.31. Amino acid ratios in acid hydrolysate: Lys 2.06, Tyr 0.98, Leu 2.07, Asp 2.04, Ser 0.85, Ile 1.00 (average recovery 80%). *Anal.* Calcd for $C_{75}H_{106}N_{14}O_{20}$: C, 59.12; H, 7.01; N, 12.87. Found: C, 58.91: H, 6.99; N, 12.79.

Z(OMe)-Ala-Val-Lys(Z)-Lys(Z)-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH_2, Z(OMe)-(porcine VIP 18—28)-NH_2——(a) Z(OMe)–Lys(Z)–Lys(Z)–Tyr–Leu–Asn–Ser–Ile–Leu–Asn–NH_2 (2.50 g, 1.64 mmol) was treated with TFA–anisole (6.5 ml–1.6 ml) and the N^α-deprotected peptide isolated as described above was dissolved in DMSO–HMPA–DMF (2: 1: 1, 20 ml) containing Et_3N (0.23 ml, 1.64 mmol). The azide [prepared from 0.78 g (2.13 mmol) of Z(OMe)–Ala–Val–$NHNH_2$] in DMF (7 ml) and Et_3N (0.3 ml, 2.13 mmol) were added to the above ice-chilled solution, and the mixture was stirred at 4° for 30 hr. The solvent was removed by evaporation and the residue was treated with 5% citric acid and AcOEt. The resulting powder was purified by washing as described above, followed by precipitation from DMSO with MeOH; yield 2.54 g (92%), mp 270—271°, $[\alpha]_D^{20}$ −7.4° (c=0.4, HMPA). Rf_1 0.28. Amino acid ratios in acid hydrolysate: Asp 2.05, Ser 0.84, Ala 0.97, Val 1.00, Ile 1.00, Leu 1.90, Tyr 1.05, Lys 2.04 (average recovery 90%). *Anal.* Calcd for $C_{83}H_{120}N_{16}O_{22}$: C, 58.85; H, 7.14; N, 13.23. Found: C, 58.79; H, 6.92; N, 13.06.

(b) Z(OMe)–Tyr–Leu–Asn–Ser–Ile–Leu–Asn–NH_2 (5.70 g, 5.71 mmol) was treated with TFA–anisole (15 ml–4 ml) and the N^α-deprotected peptide isolated as described above was dissolved in DMSO–HMPA (1: 1, 50 ml) containing Et_3N (0.8 ml, 5.71 mmol). The azide [prepared from 6.70 g (7.52 mmol) of Z(OMe)–Ala–Val–Lys(Z)–Lys(Z)–$NHNH_2$] in DMF (60 ml) and Et_3N (1.05 ml, 7.52 mmol) were added to the above ice-chilled solution. After stirring at 4° for 40 hr, the solution was concentrated and the residue was treated with ether. The resulting powder was purified by washing as described above, followed by precipitation from DMSO with MeOH; yield 7.75 g (80%), mp 270—271°, $[\alpha]_D^{20}$ −7.7° (c=0.5, HMPA), Rf_1 0.28, Rf_3 0.73. Amino acid ratios were given in Table I.

Z(OMe)-Arg(Mts)-Lys(Z)-Gln-Met(O)-Ala-Val-Lys(Z)-Lys(Z)-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH_2, Z(OMe)-(porcine VIP 14—28)-NH_2——The above protected undecapeptide amide (3.39 g, 2 mmol) was treated with TFA–anisole (10 ml–2.5 ml) and the N^α-deprotected peptide was isolated as described above and dissolved in DMSO–HMPA (1: 1, 20 ml) containing Et_3N (0.28 ml, 2 mmol). The azide [prepared from 3.22 g (3 mmol) of Z(OMe)–Arg(Mts)–Lys(Z)–Gln–Met(O)–$NHNH_2$] in DMF (30 ml) and Et_3N (0.42 ml, 3 mmol) were added to the above ice-chilled solution and the mixture was stirred at 4° for 24 hr. The solvent was evaporated off and the residue was treated with ether. The resulting powder was purified by washing as described above, followed by precipitation from DMSO with MeOH; yield 3.91 g (76%), mp 253—256°, $[\alpha]_D^{20}$ −14.3° (c=0.4, DMSO), Rf_1 0.34, Rf_3 0.81. *Anal.* Calcd for $C_{122}H_{177}N_{25}O_{32}S_2 \cdot 3H_2O$: C, 55.84; H, 7.03; N, 13.35. Found: C, 55.68; H, 6.95; N, 13.07.

Z(OMe)-Thr-Arg(Mts)-Leu-Arg(Mts)-Lys(Z)-Gln-Met(O)-Ala-Val-Lys(Z)-Lys(Z)-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH_2, Z(OMe)-(porcine VIP 11—28)-NH_2——The above protected pentadecapeptide amide (1.03 g, 0.4 mmol) was treated with TFA–anisole (3.0 ml–0.8 ml) as usual and the N^α-deprotected peptide was isolated as described above and dissolved in DMSO–DMF (3 ml–8 ml) containing Et_3N (0.06 ml, 0.4 mmol). To this ice-chilled solution, the azide [prepared from 0.60 g (0.8 mmol) of Z(OMe)–Thr–Arg(Mts)–Leu–$NHNH_2$] in DMF (6 ml) and Et_3N (0.11 ml, 0.8 mmol) were added and the mixture, after stirring at 4° for 40 hr, was concentrated. The residue was treated with 5% citric acid and AcOEt and the resulting powder was purified by washing as described above, followed by precipitation from DMF with AcOEt; yield 1.12 g (90%), mp 140—143°, $[\alpha]_D^{20}$ +14.1° (c=0.3, DMF). *Anal.* Calcd for $C_{147}H_{217}N_{31}O_{38}S_3 \cdot 3H_2O$: C, 55.58; H, 7.08; N, 13.67. Found: C, 55.65; H, 6.83; N, 13.55.

Z(OMe)-Thr-Asp-Asn-Tyr-Thr-Arg(Mts)-Leu-Arg(Mts)-Lys(Z)-Gln-Met(O)-Ala-Val-Lys(Z)-Lys(Z)-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH_2, Z(OMe)-(porcine VIP 7—28)-NH_2——The above protected octadecapeptide amide (1.26 g, 0.4 mmol) was treated with TFA–anisole (3 ml–1 ml) and the N^α-deprotected peptide isolated as described above was dissolved in DMF (10 ml) containing Et_3N (0.06 ml, 0.4 mmol). To this ice-chilled solution, the azide [prepared from 0.48 g (0.7 mmol) of Z(OMe)–Thr–Asp–Asn–Tyr–$NHNH_2$ in DMF (5 ml) and Et_3N (0.1 ml, 0.7 mmol)] were added and the mixture, after stirring at 4° for 48 hr, was concentrated. The residue was treated with 5% citric acid and AcOEt. The resulting powder was dissolved in a small amount of DMF and the solution was applied to a column of Sephadex LH-20 (3.0×132 cm), which was eluted with the same solvent. Individual fractions (5 ml each) were collected and the absorption at 275 nm was determined. Fractions corresponding to the front main peak (tube Nos. 72—82) were combined and the solvent was removed by evaporation. Trituration with AcOEt afforded a powder; yield 0.80 g (55%), mp 171—174°, $[\alpha]_D^{27}$ +8.4° (c=0.7, DMF), Rf_1 0.19. *Anal.* Calcd for $C_{168}H_{244}N_{36}O_{47}S_3 \cdot 7H_2O$: C, 53.92; H, 6.95; N, 13.47. Found: C, 54.20; H, 6.90; N, 12.90.

Z(OMe)-His-Ser(Bzl)-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg(Mts)-Leu-Arg(Mts)-Lys(Z)-Gln-Met(O)-Ala-Val-Lys(Z)-Lys(Z)-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH_2, Protected Porcine VIP——The above protected docosapeptide amide (0.80 g, 0.22 mmol) was treated with TFA–anisole (3 ml–0.5 ml) and the N^α-deprotected peptide, isolated as described above, was dissolved in DMF (7 ml) containing Et_3N (0.12 ml, 0.44 mmol). To this ice-chilled solution, the azide[prepared from 0.42 g (0.44 mmol) of Z(OMe)–His–Ser–

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(Bzl)–Asp–Ala–Val–Phe–$NHNH_2$ in DMF (4.5 ml) and Et_3N (0.06 ml, 0.44 mmol)] were added. After stirring at 4° for 48 hr, the solution was concentrated and the residue was treated with 5% citric acid and AcOEt. The resulting powder was dissolved in a small amount of DMF and the solution was applied to a column of Sephadex LH-60 (3.0×132 cm), which was eluted with the same solvent. Individual fractions (5 ml each) were collected and the front main fractions (tube Nos. 98—118) determined as described above were combined. The solvent was removed by evaporation and the residue was treated with AcOEt to afford a powder; yield 0.68 g (70%), mp 161—164°, $[\alpha]_D^{25}$ +5.0° (c=0.4, DMF), Rf_1 0.21. *Anal.* Calcd for $C_{205}H_{290}N_{44}O_{56}S_3 \cdot 7H_2O$: C, 54.85; H, 6.83; N, 13.73. Found: C, 54.78; H, 6.73; N, 13.74.

Enzymic Examination of the Products formed by Deprotection of Z(OMe)-His-Ser(Bzl)-Asp-Ala-Val-Phe-$NHNH_2$——Z(OMe)–His–Ser(Bzl)–Asp–Ala–Val–Phe–$NHNH_2$ (10 mg) was treated for 60 min with one of the following reagents; TFA, 25% HBr–AcOH, HBr bubbled into TFA, MSA and HF in the presence of anisole (10 eq). Each product was precipitated with ether, converted to the acetate by treatment with Amberlite CG-4B (acetate form), treated with 3% NH_4OH at pH 8 for 30 min to reverse the possible N→O shift[32] and lyophilized. When examined by IR, the products derived by deprotection with MSA or HBr–AcOH in particular exhibited an additional carbonyl band (IR ν_{max}^{Nujol} cm^{-1} 1775, besides 1680) typical of aminosuccinyl derivatives. No change was observed on TLC when these two compounds were exposed to 3% NH_4OH in an ice-bath for 30 min. On TLC, the products were assigned as follows: Rf_2 0.50 (H–His–Ser(Bzl)–Asp–Ala–Val–Phe–$NHNH_2$, a), 0.30 (H–His–Ser–Asp–Ala–Val–Phe–$NHNH_2$, b), 0.40 (a succinimide compound of b), 0.32 (a mixture of b and its Ser (OAc)), 0.42 (a mixture of a 0.40 compound and its Ser (OAc) compound). Each product was exposed to the action of aminopeptidase (AP-M, Merck, Lot No. 9652457) at 37° for 24 hr. The results are listed in Table II.

In order to examine the TFA–thioanisole deprotection, the protected peptide was treated for 24 hr and the product was enzymatically examined. Recovery of Asp was quantitative, as shown in Table II.

Release of His from H-His-Ser-Asp-Ala-Val-Phe-$NHNH_2$——H–His–Ser–Asp–Ala–Val–Phe–$NHNH_2$ (2.15 mg, prepared by hydrogenolysis of the protected hexapeptide described above) in 0.01 M $AcONH_4$ buffer (3 ml) at pH 6.0 was kept standing at room temperature or incubated at 65° with or without 2-mercaptoethanol, then a part of the solution was examined with an amino acid analyzer. The results are listed in Table III.

Enzymic Examination of the Products formed by Deprotection of Z(OMe)-Thr-Asp-Asn-Tyr-OMe——Z(OMe)–Thr–Asp–Asn–Tyr–OMe (10 mg) was treated for 60 min with one of the following reagents: TFA, 25% HBr–AcOH, HBr bubbled into TFA, MSA and HF. Each product was isolated as described above and exposed to the action of AP-M. The results are listed in Table IV. In addition, the protected peptide was treated with TFA in the presence of thioanisole at room temperature for 24 hr and the product was enzymatically examined. A quantitative recovery of Asp was confirmed, as listed in Table IV.

H-His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH_2 (Porcine VIP)——The protected octacosapeptide amide (515 mg, 118 mmol) dissolved in DMF (5 ml) was incubated at 60° for 24 hr in the presence of thiophenol (1.21 ml, 100 eq). The solvent was evaporated off and the residue was treated with AcOEt to form a powder, which was collected by filtration and dried over P_2O_5 for 3 hr; yield 485 mg (94%).

The reduced peptide (276 mg) was treated with HF (approximately 3 ml) in the presence of *m*-cresol (0.47 ml, 70 eq) in an ice-bath for 60 min. After removal of the HF, dry ether was added and the resulting powder was dissolved in H_2O (5 ml). The solution was treated with Amberlite CG-4B (acetate form, approximately 3 g) for 30 min, then filtered. The pH of the solution was adjusted to 9 with 5% NH_4OH, then after 30 min, to 6 with AcOH. After lyophilization, the resulting powder was dissolved in 0.1 N AcOH and the solution was applied to a column of Sephadex G-25 (3×108 cm). Individual fractions (6.6 ml each) were collected and the absorption at 275 nm was determined. Fractions corresponding to the front main peak (tube Nos. 65—88) were combined and the solvent was removed by lyophilization. The fluffy powder thus obtained was next purified by column chromatography on CM-cellulose (2.5×6.0 cm), which was eluted by gradient elution with 0.1 M NH_4HCO_3 buffer at pH 8.0 (1000 ml) through a mixing flask containing H_2O (200 ml). Individual fractions (6.6 ml each) were collected and the absorption at 275 nm was determined (Fig. 4). After elution of two minor peaks, the main peak was eluted. Fractions corresponding to this main peak (tube Nos. 62—95) were collected and the solvent and NH_4HCO_3 were removed by repeated lyophilization to give a fluffy white powder: yield 134 mg (54%).

Disc electrophoretic examination of the CM-cellulose purified sample at pH 4.0 revealed the presence of a main band with a minor impurity band. The sample (9.1 mg) was then purified by preparative isoelectric focusing with Ampholine pH 9—11 (LKB) according to the procedure reported by Finlayson and Chrambach.[28] After running at 800 V at 3° for 48 hr, the UV absorption at 275 nm and the pH of the fractions (2 ml each) were measured. Two peaks were detected (Fig. 5); F-1 (tube Nos. 18—23, at pH 9.3) and F-2 (tube Nos. 31—38, at pH 10.1). The contaminating Ampholine was removed from F-2 by gel-filtration

32) S. Sakakibara, "Chemistry and Biochemistry of Amino Acids, Peptides and Proteins," Vol. 1, ed. B. Weinstein, Marcel Dekker, New York, 1971, p. 51.

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on Sephadex G-25 (1.5×133 cm) with 0.1 N AcOH as an eluent. After lyophilization, the purified product was obtained as a fluffy white powder; yield 8.1 mg (89%), $[\alpha]_D^{27}$ −62.0° (c=0.2, 0.1 N AcOH), Rf_2 0.23, identical with that of natural porcine VIP (on DC-Alufolien Kieselgel 60, Merck), Rf_4 0.50; single band in 15% polyacrylamide gel at pH 4.0 (mobility, 4.1 cm from the origin toward the cathode, after running at 5 mA per tube for 90 min). Amino acid ratios in 6 N HCl hydrolysate (Table I) and AP-M digest (numbers in parentheses are theoretical values): Asp 1.97 (2), Ala 2.05 (2), Val 1.96 (2), Met 0.93 (1), Ile 1.00 (1), Leu 3.14 (3) Tyr 1.96 (2), Phe 0.99 (1), Lys 3.14 (3), His 0.97 (1), Arg 2.03 (2), (recovery of Ile 92%). *Anal.* Calcd for $C_{147}H_{238}N_{44}O_{42}S \cdot 5CH_3COOH \cdot 14H_2O$: C, 48.62; H, 7.43; N, 15.89. Found: C, 48.51; H, 7.04; N, 16.05.

Deprotection of Ser(Bzl), Lys(Z), and Arg(Mts) with TFA in the Presence of Various Sulfur Compounds——H-Ser(Bzl)-OH, H-Lys(Z)-OH, and H-Arg(Mts)-OH (20 mg each) were each treated with TFA (0.6 ml each) in the presence of various sulfur compounds (50 eq) at room temperature (28°). Each solution was examined by TLC and the ninhydrin color intensity was determined by a Shimadzu dual-wavelength TLC scanner. The results are listed in Table V.

TABLE V. Deprotection of Ser(Bzl), Lys(Z) and Arg(Mts) by TFA in the Presence of Various Sulfur Compounds

Scavenger	Ser regenerated		Lys regenerated		Arg regenerated	
	1 hr	24 hr	1 hr	24 hr	1 hr	24 hr
Thioanisole	2.5	96	76.5	100	1.8	99
Dimethylsulfide	0	36	77.2	100	0	55
Ethanedithiol	0	33	45.0	98	0	22
Mercaptoethanol	0	3	5.0	14	0	2

Deprotection of Z(OMe)-(porcine VIP 1—28)-NH_2 by TFA-thioanisole——The protected octacosapeptide amide (reduced with thiophenol as described above; 57 mg, 13 μmol) was dissolved in TFA (6 ml) in the presence of thioanisole (0.46 ml, 300 eq) and *m*-cresol (0.41 ml, 300 eq). The solution was kept at 28° for 10 hr, then dry ether was added. The resulting powder was retreated with the same reagents for 14 hr. The deprotected peptide was precipitated with ether, then dissolved in H_2O (5 ml), and the solution was treated with Amberlite CG-4B (acetate form, approximately 2 g) for 30 min. The resin was removed by filtration; the filtrate was adjusted to pH 9 with 5% NH_4OH and, after 30 min, to 6 with AcOH. After lyophilization, the residue was purified by gel-filtration on Sephadex G-25 (1.5×130 cm) with 0.1 N AcOH as an eluent. As described above, the UV absorption at 275 nm was determined in each fraction (5 ml). Besides two peaks due to the scavengers, the desired material (tube Nos. 41—51) was obtained as a single peak. The solvent was removed by lyophilization and the residue was subsequently purified by ion-exchange chromatography on CM-cellulose (1.8×6.0 cm), using gradient elution with 0.1 M NH_4HCO_3 buffer at pH 8.0 (1000 ml) through a mixing flask containing H_2O (200 ml). UV absorption at 275 nm was determined in each fraction (5 ml). After elution of two minor peaks, the main peak (tube Nos. 93—120) was detected. The fractions corresponding to the main peak were combined and ammonium bicarbonate was removed by repeated lyophilization to give a fluffy white powder; yield 22.0 mg (43%). The purity of the sample (4.84 mg) was examined by preparative isoelectric focusing with Ampholine, pH 9—11 (800 V at 3° for 48 hr), as described above. The desired peptide appeared at pH 10.1, and no significant impurity was detected. After gel-filtration on Sephadex G-25, the sample was recovered; 4.4 mg (91%). $[\alpha]_D^{18}$ −66.8° (c=0.2, 0.1 N AcOH), Rf_2 0.23 (on DC-Alufolien Kieselgel 60, Merck), identical with that of natural porcine VIP. Amino acid ratios in 6 N HCl hydrolysate: Asp 5.26, Thr 1.91, Ser 1.69, Glu 1.17, Ala 2.00, Val 2.13, Met 0.98, Ile 1.00, Leu 3.11, Tyr 2.06, Phe 1.04, Lys 3.24, His 0.90, Arg 2.11 (recovery of Ile, 76%). Amino acid ratios in AP-M digest: Asp 1.97, Ala 2.00, Val 1.99, Met 1.05, Ile 1.00, Leu 3.03, Tyr 2.07, Phe 0.99, Lys 3.28, His 1.07, Arg 2.05 (recovery of Ile, 92%).

Acknowledgement This investigation was supported in part by a grant (No. 477927) from the Ministry of Education, Science and Culture, Japan. The authors are grateful to Professor Viktor Mutt, Karolinska Institute, Stockholm, Sweden for his generous supply of natural porcine VIP and to Professor Miklos Bodanszky, Case Western Reserve University, Cleveland Ohio, U.S.A., for his valuable advice.

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