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Studies on Peptides. CXII.[1,2] Alternative Synthesis of Heptacosapeptide, a New Gastrointestinal Polypeptide

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A recently found gastrointestinal hormone, PHI (heptacosapeptide), was synthesized in a different manner from that of Moroder *et al.* [*Z. Naturforsch.*, **37b**, 772 (1982)]. Our synthesis was carried out by successive azide condensation of six peptide fragments (23—27, 19—22, 15—18, 11—14, 7—10, and 1—6), followed by deprotection with 1 M trifluoromethanesulfonic acid–thioanisole in trifluoroacetic acid. The deprotected peptide was purified by gel-filtration on Sephadex G-25, followed by ion-exchange chromatography on CM-Biogel A and reverse phase high-performance liquid chromatography on μBondapak C_{18}. The homogeneous product thus obtained produced a remarkable decrease in systemic blood pressure in anesthetized rats. In addition, N^{α}-biotinyl-PHI was synthesized for histochemical receptor-binding studies.

Keywords——gastrointestinal peptide; synthesis of heptacosapeptide; N^{α}-biothinyl-heptacosapeptide; mesitylenesulfonylarginine; side reaction of aspartic acid; *m*-cresol as cation scavenger; thioanisole-mediated deprotection; 1 M trifluoromethanesulfonic acid/TFA deprotection; effect on systemic blood pressure

Recently, Tatemoto and Mutt[3] discovered a new heptacosapeptide, designated PHI (PHI-27, H=N-terminal His, I=C-terminal Ile) in porcine upper intestinal tissue by using a new chemical assay method for finding peptides with C-terminal amide structure.[4] When sequenced, PHI showed remarkable structural homology with vasoactive intestinal polypeptide (VIP), secretin and glucagon (Fig. 1).[5] This peptide can thus be structurally classified as a new member of the glucagon–secretin family of gastrointestinal polypeptides, and was reported to have several biological activities[6-9] similar to those of VIP and secretin.

The first synthesis of PHI was reported in 1982 by Moroder *et al.*,[10] who employed TFA-labile protecting groups based on *tert*-butanol. We decided to synthesize this new gastrointestinal polypeptide by an alternative route, because of its presumed biological importance (the structural homology of PHI to human growth hormone-releasing peptide[11] has been pointed out). In addition, N^{α}-biotinyl-PHI was synthesized for use in histochemical studies on receptor binding.[12]

Our synthetic scheme for PHI is shown in Fig. 2. Six relatively small peptide fragments (five peptide hydrazides and the C-terminal pentapeptide amide) were selected as building blocks to construct its entire amino acid sequence by means of the azide procedure.[13]

The TFA-labile Z(OMe)[14] or Boc group was used as a temporal N^{α}-protecting group and amino acid derivatives bearing protecting groups removable by 1 M TFMSA/TFA[15] were employed, *i.e.*, Lys(Z), Glu(OBzl) and Arg(Mts).[16] This peptide contains two residues of Asp, which from the synthetic viewpoint involves some difficulty regarding its functional group. In order to minimize the base-catalyzed ring closure of Asp(OBzl),[17] this protecting group was

	1				5					10					15					20					25			
PHI	H	A	D	G	V	F	T	S	D	F	S	R	L	L	G	Q	L	S	A	K	K	Y	L	E	S	L	I[a]
VIP	H	S	D	A	V	F	T	D	N	Y	T	R	L	R	K	Q	M	A	V	K	K	Y	L	N	S	I	L	N[a]
secretin	H	S	D	G	T	F	T	S	E	L	S	R	L	R	D	S	A	R	L	Q	R	L	L	Q	G	L	V[a]	
glucagon	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	S	R	R	A	Q	D	F	V	Q	W	L	M	N	T

Fig. 1. Structures of PHI and Related Peptides

Identical residues in corresponding positions are underlined. A=Ala, D=Asp, E=Glu, F=Phe, G=Gly, H=His, I=Ile, K=Lys, L=Leu, M=Met, N=Asn, Q=Gln, R=Arg, S=Ser, T=Thr, V=Val, W=Trp, Y=Tyr. *a*) Amidated COOH terminus.

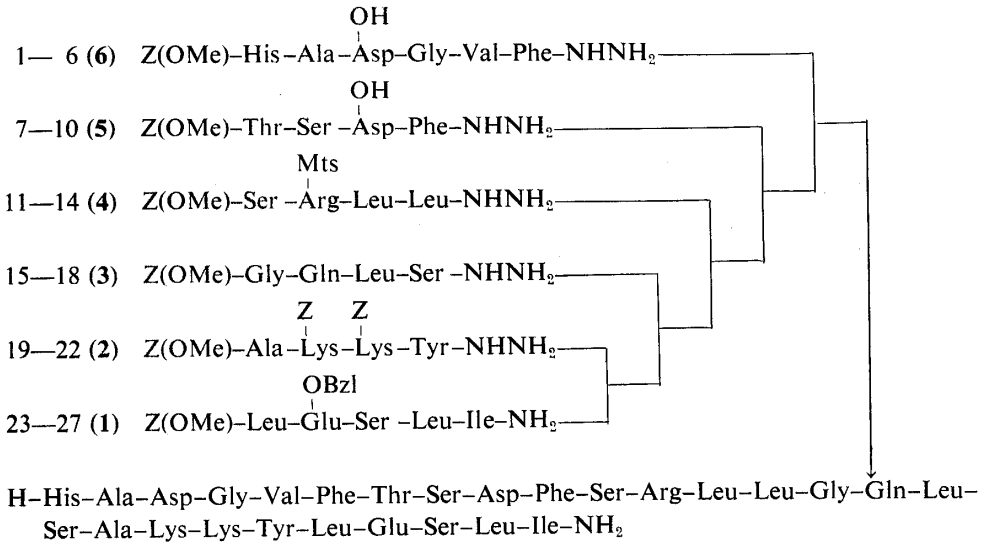

Fig. 2. Synthetic Route to PHI

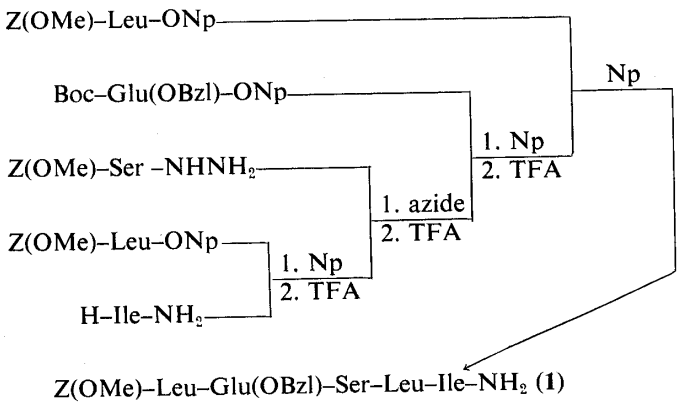

Fig. 3. Synthetic Scheme for the Protected Pentapeptide Amide, Z(OMe)-(PHI 23—27)-NH_2

removed by hydrogenolysis during the preparation of necessary fragments, as will be described later.

The C-terminal pentapeptide amide, Z(OMe)-Leu-Glu(OBzl)-Ser-Leu-Ile-NH_2 (**1**), was prepared in a stepwise manner starting with H-Ile-NH_2 either by the Np method[18] or the azide method as shown in Fig. 3. Each product was purified by precipitation from DMF with MeOH or AcOEt. Throughout this fragment synthesis and later syntheses, the purity of every fragment and intermediate was confirmed by thin layer chromatography (TLC), acid hydrolysis and elemental analysis.

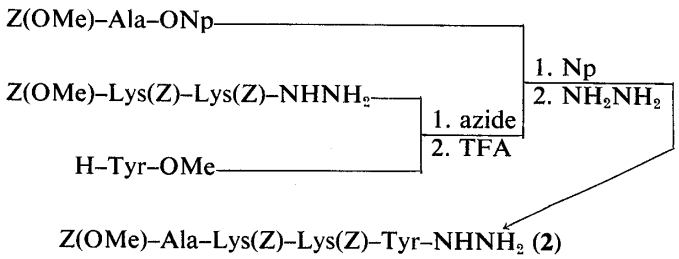

Fig. 4. Synthetic Scheme for the Protected Tetrapeptide Hydrazide, Z(OMe)–(PHI 19—22)–$NHNH_2$

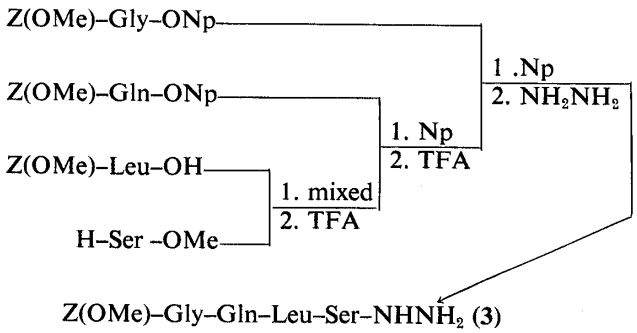

Fig. 5. Synthetic Scheme for the Protected Tetrapeptide Hydrazide, Z(OMe)–(PHI 15—18)–$NHNH_2$

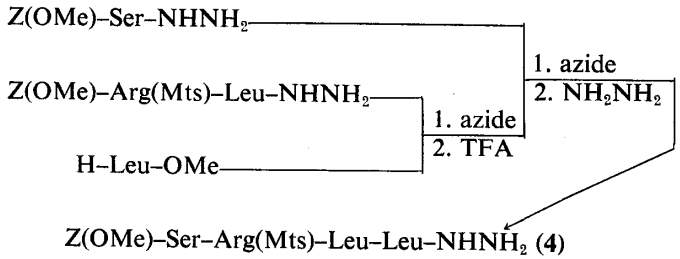

Fig. 6. Synthetic Scheme for the Protected Tetrapeptide Hydrazide, Z(OMe)–(PHI 11—14)–$NHNH_2$

In order to prepare Z(OMe)–Ala–Lys(Z)–Lys(Z)–Tyr–$NHNH_2$ (**2**), an available dipeptide hydrazide, Z(OMe)–Lys(Z)–Lys(Z)–$NHNH_2$,[19] was first condensed, *via* the azide, with H–Tyr–OMe as shown in Fig. 4. After TFA treatment, the tripeptide ester was allowed to react with Z(OMe)–Ala–ONp to give Z(OMe)–Ala–Lys(Z)–Lys(Z)–Tyr–OMe, which was smoothly converted to (**2**) by the usual hydrazine treatment.

The next fragment, Z(OMe)–Gly–Gln–Leu–Ser–$NHNH_2$ (**3**), was prepared in a stepwise manner, as shown in Fig. 5, starting with H–Ser–OMe. First, Z(OMe)–Leu–OH was introduced by the mixed anhydride method[20] and then two residues, Gln and Gly, were introduced successively by the Np method. The resulting tetrapeptide ester, Z(OMe)–Gly–Gln–Leu–Ser–OMe, was converted to (**3**) in the usual manner, as described above. Pyrrolidone formation of the N-terminal Gln residue[21] is a troublesome phenomenon often observed during the fragment condensation reaction. Thus, this fragment was terminated at the Gly residue.

In order to prepare Z(OMe)–Ser–Arg(Mts)–Leu–Leu–$NHNH_2$ (**4**), an available methyl ester, Z(OMe)–Arg(Mts)–Leu–OMe,[22] was first converted to the corresponding hydrazide and this hydrazide was next condensed, *via* the azide, with H–Leu–OMe. The product was purified by column chromatography on silica gel using $CHCl_3$–MeOH (10 : 0.5) as an eluant.

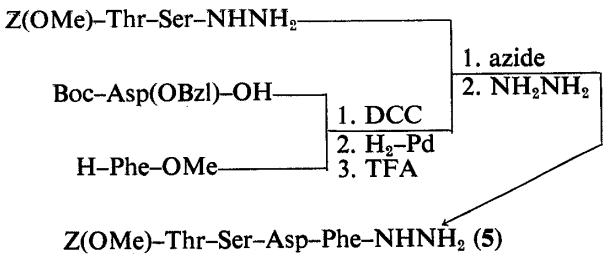

Fig. 7. Synthetic Scheme for the Protected Tetrapeptide Hydrazide, Z(OMe)–(PHI 7—10)–$NHNH_2$

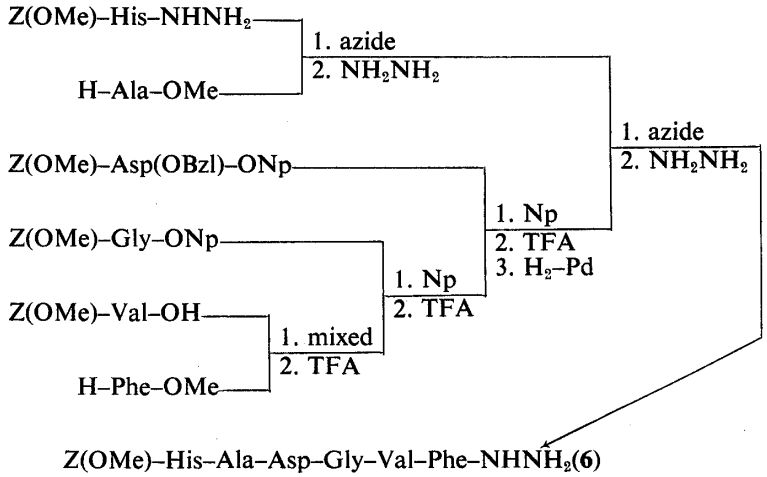

Fig. 8. Synthetic Scheme for the Protected Hexapeptide Hydrazide, Z(OMe)–(PHI 1—6)–$NHNH_2$

Next, the Ser residue was introduced by the azide procedure. The resulting tetrapeptide ester, Z(OMe)–Ser–Arg(Mts)–Leu–Leu–OMe, is soluble in AcOEt. Thus, after purification by the usual extraction procedure, it was converted to the corresponding hydrazide (**4**), as shown in Fig. 6.

The next fragment, Z(OMe)–Thr–Ser–Asp–Phe–$NHNH_2$ (**5**), contains the Asp residue. Base-catalyzed ring closure of the Asp(OBzl) residue seems to be sequence-dependent.[17b] In order to avoid the possibility of such succinimide formation, the Bzl ester group was removed from Boc–Asp(OBzl)–Phe–OMe by catalytic hydrogenation, prior to the next coupling reaction, as shown in Fig. 7. After deblocking of the N^{α}-Boc group by the usual TFA treatment, the resulting dipeptide, H–Asp–Phe–OMe, was subjected to the next azide condensation with Z(OMe)–Thr–Ser–$NHNH_2$ to form the tetrapeptide ester, Z(OMe)–Thr–Ser–Asp–Phe–OMe, which was smoothly converted to (**5**) by the usual hydrazine treatment.

Next, the N-terminal hexapeptide hydrazide, Z(OMe)–His–Ala–Asp–Gly–Val–Phe–$NHNH_2$ (**6**), was prepared on the basis of experience obtained in our previous syntheses of chicken[22] and porcine VIPs,[23] since PHI and VIP have striking structural homology, especially at their N-terminal portions. As shown in Fig. 8, the mixed anhydride procedure was employed to prepare Z(OMe)–Val–Phe–OMe, rather than the DCC procedure.[24] The latter procedure gave predominantly an acylurea compound. Next, the Gly and Asp(OBzl) residues were introduced successively by the Np method. The Z(OMe) and Bzl groups were removed from the resulting tetrapeptide ester, Z(OMe)–Asp(OBzl)–Gly–Val–Phe–OMe, by the TFA treatment, followed by hydrogenolysis. The resulting tetrapeptide ester, H–Asp–Gly–Val–Phe–OMe, was subsequently condensed *via* the azide with Z(OMe)–His–Ala–$NHNH_2$ to give the hexapeptide ester, which was converted to (**6**) as usual, without par-

Table I. Amino Acid Ratios in 6 N HCl Hydrolysates of Synthetic PHI and Protected Intermediates

	Protected peptide					Synthetic PHI	Residue
	19—27	15—27	11—27	7—27	1—27		
Asp				1.02	2.13	1.88	2
Thr				0.98	1.00	0.90	1
Ser	0.92	1.88	2.83	3.78	3.74	3.52	4
Glu	1.06	2.11	2.16	2.13	2.14	2.03	2
Gly		1.07	1.06	1.11	2.13	1.90	2
Ala	1.07	1.08	1.07	1.06	2.19	1.90	2
Val					1.03	0.88	1
Ile	1.00	1.00	1.00	1.00	1.00	1.00	1
Leu	2.02	3.07	5.10	5.09	5.09	5.04	5
Tyr	0.97	0.96	0.95	0.98	1.01	0.86	1
Phe				0.99	1.93	1.80	2
Lys	2.09	2.03	2.06	2.07	2.07	2.02	2
His					1.09	0.86	1
Arg			1.05	1.02	1.03	0.92	1
Rec.	92.9%	78.5%	83.0%	78.8%	85.2%	80.3%	

ticular difficulty.

The six peptide fragments thus obtained were then assembled successively by the azide procedure according to the route illustrated in Fig. 2. The amount of the acyl component in each fragment condensation was increased from 1.1 to 2 eq as the chain was elongated. Each intermediate was purified by repeated precipitation from DMF with MeOH or AcOEt and the protected PHI was purified by gel-filtration on Sephadex LH-60 using DMF as the eluant. Throughout this synthesis, Ile was taken as a diagnostic amino acid in acid hydrolysis. By comparison of the recovery of Ile with those of newly incorporated amino acids, satisfactory incorporation of each fragment in each condensation reaction was confirmed as shown in Table I.

In the final step of the synthesis, all protecting groups, Z, Z(OMe), Bzl, and Mts, were removed from the protected PHI by treatment with 1 M TFMSA–thioanisole in TFA in an ice-bath for 60 min. *m*-Cresol was used as an additional cation-scavenger to suppress a side reaction, *i.e.*, *O*-sulfonation of Tyr residue.[25] The treatment was repeated once more to ensure complete deprotection. Previously, we confirmed that under such conditions, the acid-catalyzed ring closure of the Asp residue with the free carboxyl group was minimized.[15b] The deprotected peptide, precipitated by ether, was treated with base at pH 8.6 for 30 min to reverse a possible N→O shift at the Ser and Thr residues and then purified by gel-filtration on Sephadex G-25, using 1 N AcOH as an eluant, followed by ion-exchange chromatography on CM-Biogel A using gradient elution with pH 5.8, 0.1 M NH_4OAc buffer and by reverse phase high-performance liquid chromatography (HPLC) using acetonitrile–0.05 M NH_4OAc–H_3PO_4 (34 : 65 : 1) as an eluant. Desalting on Sephadex G-25 gave a fluffy powder, which exhibited a sharp single spot on TLC in two different solvent systems and single band on disc electrophoresis. The purity of the product was further confirmed by amino acid analysis after acid hydrolysis and by enzymatic digestion as well.

When administered to anesthetized Schild rats, synthetic PHI (50—100 μg/kg) inhibited the basal acid secretion in the stomach, but no bethanechol-stimulated acid secretion. Thus, no significant effect on acid secretion was observed, but synthetic PHI (2.5—5 μg/kg) produced a remarkable decrease in the systemic blood pressure (Fig. 9), like porcine VIP.

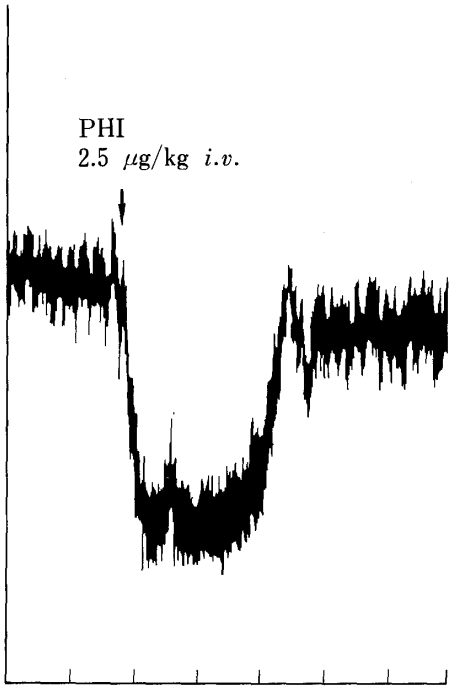

Fig. 9. Effect of Synthetic PHI on Systemic Blood Pressure (Rat)

In addition to PHI, we have prepared N^{α}-biotinyl-PHI according to the procedure of Becke *et al.*[26] Biotinyl *N*-hydroxysuccinimide[26] was allowed to react with a TFA-treated sample of the protected PHI. Deprotection was carried out in essentially the same manner as described above and the deprotected peptide was purified by repeated gel-filtrations on Sephadex G-25 and Sephadex G-50. This derivative retains the powerful effect on systemic blood presure. The effects of synthetic PHI on pancreatic functions, as well as its immunochemical properties, are under investigation. The usefulness of N^{α}-biotinyl-PHI for histochemical studies will be reported elsewhere.

Experimental

General experimental methods employed in this investigation are essentially the same as described in Part LXXXVIII[27] of this series. *Rf* values in TLC performed on silica gel (Kieselgel G, Merck) refer to the following solvent systems: Rf_1 $CHCl_3$–MeOH–H_2O (8:3:1), Rf_2 $CHCl_3$–MeOH–AcOH (9:1:0.5), Rf_3 $CHCl_3$–MeOH (10:0.5), Rf_4 *n*-BuOH–pyridine–AcOH–H_2O (4:1:1:2), Rf_5 *n*-BuOH–pyridine–AcOH–H_2O (30:20:6:24). HPLC was conducted with a Waters 204 compact model, using a μBondapak C_{18} (3.9 × 300 mm) column.

Z(OMe)–Ile–NH_2——Z(OMe)–Ile–OH (10.90 g, 36.9 mmol) was converted to the amide by the usual mixed anhydride procedure. The product was precipitated from DMF with MeOH; yield 9.77 g (90%), mp 224—225 °C, $[\alpha]_D^{18}$ +21.7° (c=0.4, DMF), Rf_1 0.76. *Anal.* Calcd for $C_{15}H_{22}N_2O_4$: C, 61.20; H, 7.53; H, 9.51. Found: C, 60.94; H, 7.50; N, 9.47.

Z(OMe)–Leu–Ile–NH_2——Z(OMe)–Ile–NH_2 (24.0 g, 81.5 mmol) was treated with TFA–anisole (36 ml–17.6 ml) in an ice-bath for 60 min, then TFA was removed by evaporation and dry ether was added. The resulting powder was dried over KOH pellets *in vacuo* for 3 h and dissolved in DMF (250 ml), together with Et_3N (22.66 ml, 163 mmol) and Z(OMe)–Leu–ONp (40.73 g, 97.8 mmol). After being stirred at room temperature for 12 h, the solution was concentrated and the residue was treated with 5% citric acid and ether. The resulting powder was washed with 5% citric acid, 3% $NaHCO_3$ and H_2O and precipitated from DMF with MeOH; yield 23.85 g (69%); mp 220—222 °C, $[\alpha]_D^{18}$ −4.1° (c=0.7, DMF), Rf_2 0.76. *Anal.* Calcd for $C_{21}H_{33}N_3O_5$: C, 61.89; H, 8.16; N, 10.31. Found: C, 61.94; H, 8.24; N, 10.21.

Z(OMe)–Ser–Leu–Ile–NH_2——Z(OMe)–Leu–Ile–NH_2 (3.98 g, 9.8 mmol) was treated with TFA–anisole (6 ml–2 ml) and the N^{α}-deprotected peptide isolated as stated above was dissolved in DMF (40 ml) containing Et_3N (1.31 ml, 9.4 mmol). The azide [prepared from 3.99 g (14.1 mmol) of Z(OMe)–Ser–$NHNH_2$] in DMF (30 ml) and Et_3N (1.96 ml, 14.1 mmol) were added to the above ice-chilled solution and the mixture, after being stirred at 4 °C for 24 h, was concentrated. The residue was treated with 5% citric acid and the resulting powder was precipitated from DMF with MeOH; yield 4.77 g (98%), mp 224—226 °C, $[\alpha]_D^{18}$ −5.7° (c=0.8, DMF), Rf_1 0.85. *Anal.* Calcd for

$C_{24}H_{38}N_4O_7 \cdot 0.5H_2O$: C, 57.24; H, 7.81; N, 11.12. Found: C, 57.38; H, 7.58; N, 11.40.

Boc–Glu(OBzl)–Ser–Leu–Ile–NH_2——Z(OMe)–Ser–Leu–Ile–NH_2 (4.77 g, 9.65 mmol) was treated with TFA–anisole (8 ml–2 ml) and the N^{α}-deprotected peptide isolated as described above was dissolved in DMF (40 ml), together with Et_3N (2.68 ml, 19.30 mmol) and Boc–Glu(OBzl)–ONp (4.42 g, 9.65 mmol). The solution was stirred at 4 °C for 24 h, the solvent was removed by evaporation, and the residue was treated with 5% citric acid. The resulting powder was washed with 5% citric acid, 3% $NaHCO_3$ and H_2O and precipitated from DMF with AcOEt; yield 3.79 g (60%), mp 198—200 °C, $[\alpha]_D^{18}$ −9.1° (c=1.0, DMF), Rf_1 0.75. *Anal.* Calcd for $C_{32}H_{51}N_5O_9$: C, 59.15; H, 7.91; N, 10.78. Found: C, 59.27; H, 8.08; N, 10.72.

Z(OMe)–Leu–Glu(OBzl)–Ser–Leu–Ile–NH_2 (2)——Boc–Glu(OBzl)–Ser–Leu–Ile–NH_2 (2.46 g, 3.79 mmol) was treated with TFA–anisole (4 ml–0.8 ml) and the N^{α}-deprotected peptide isolated as stated above was dissolved in DMF (20 ml), together with Et_3N (0.94 ml, 6.78 mmol) and Z(OMe)–Leu–ONp (1.58 g, 3.79 mmol). After being stirred at 4 °C for 24 h, the solution was concentrated and the residue was treated with H_2O. The resulting powder was washed with 3% $NaHCO_3$, 5% citric acid and H_2O, and precipitated from DMF with AcOEt; yield 2.55 g (81%), mp 242—244 °C, $[\alpha]_D^{18}$ −2.2° (c=1.4, DMF), Rf_1 0.88, Rf_2 0.67. Amino acid ratios in 6 N HCl hydrolysate; Ser 0.86, Glu 1.02, Leu 2.04, Ile 1.00 (recovery of Ile 90%). *Anal.* Calcd for $C_{42}H_{62}N_6O_{11} \cdot H_2O$: C, 59.70; H, 7.63; N, 9.95. Found: C, 59.80; H, 7.50; N, 10.00.

Z(OMe)–Lys(Z)–Lys(Z)–Tyr–OMe——The azide [prepared from 3.98 g (5.52 mmol) of Z(OMe)–Lys(Z)–Lys(Z)–$NHNH_2$[19]] in DMF (30 ml) and Et_3N (1.53 ml, 5.52 mmol) were added to an ice-chilled solution of H–Tyr–OMe [prepared from 1.92 g (8.28 mmol) of the hydrochloride], and the mixture was stirred at 4 °C for 24 h. The solvent was evaporated off and the residue was treated with ether. The resulting powder was washed with 5% citric acid, 3% $NaHCO_3$ and H_2O and recrystallized from MeOH and EtOH; yield 4.12 g (84%). mp 144—146 °C, $[\alpha]_D^{18}$ −4.6° (c=0.9, DMF), Rf_1 0.84. *Anal.* Calcd for $C_{47}H_{57}N_5O_{12}$: C, 62.58; H, 6.49; N, 7.65. Found: C, 62.47; H, 6.35; N, 7.85.

Z(OMe)–Ala–Lys(Z)–Lys(Z)–Tyr–OMe——Z(OMe)–Lys(Z)–Lys(Z)–Tyr–OMe (4.12 g, 4.63 mmol) was treated with TFA–anisole (10 ml–3 ml) and the N^{α}-deprotected peptide isolated as described above was dissolved in DMF (40 ml), together with Et_3N (1.29 ml, 9.26 mmol) and Z(OMe)–Ala–ONp (1.73 g, 4.63 mmol). The mixture was stirred for 24 h, then the solvent was evaporated off and the residue was treated with H_2O. The resulting powder was recrystallized from MeOH; yield 3.80 g (86%); mp 166—167 °C, $[\alpha]_D^{18}$ −3.2° (c=0.9, DMF), Rf_1 0.77. *Anal.* Calcd for $C_{50}H_{62}N_6O_{13}$: C, 62.88; H, 6.54; N, 8.80. Found: C, 62.91; H, 6.58; N, 8.98.

Z(OMe)–Ala–Lys(Z)–Lys(Z)–Tyr–$NHNH_2$ (2)——Z(OMe)–Ala–Lys(Z)–Lys(Z)–Tyr–OMe (3.80 g, 3.98 mmol) in DMF–MeOH (40 ml, 1 : 1) was treated with 80% hydrazine hydrate (1.24 ml, 5 eq) at room temperature for 24 h. The solvent was evaporated off and the residue was treated with MeOH. The resulting solid was precipitated from DMF with MeOH; yield 3.04 g (80%), mp 224—226 °C, $[\alpha]_D^{18}$ −26.1° (c=0.3, DMF), Rf_1 0.72. Amino acid ratios in 6 N HCl hydrolysate: Ala 1.00, Tyr 0.85, Lys 1.93 (recovery of Ala 93.2%). *Anal.* Calcd for $C_{49}H_{62}N_8O_{12}$: C, 61.62; H, 6.54; N, 11.73. Found: C, 61.78; H, 6.55; N, 11.50.

Z(OMe)–Leu–Ser–OMe——A mixed anhydride [prepared from 15.11 g (31.71 mmol) of Z(OMe)–Leu–OH·DCHA salt] in dry THF (100 ml) was added to an ice-chilled solution of H–Ser–OMe [prepared from 5.92 g (38.05 mmol) of the hydrochloride] in DMF (50 ml). The mixture was stirred in an ice-bath for 6 h, then was concentrated, and the residue was dissolved in AcOEt. The organic phase was washed with 3% $NaHCO_3$, 5% citric acid and H_2O–NaCl, dried over Na_2SO_4 and concentrated. The residue was recrystallized from MeOH and ether; yield 9.42 g (75%), mp 138—141 °C, $[\alpha]_D^{18}$ −5.9° (c=0.7, DMF), Rf_1 0.84, Rf_3 0.22. *Anal.* Calcd for $C_{19}H_{28}N_2O_7$: C, 57.56; H, 7.12; N, 7.07. Found: C, 57.57; H, 7.38; N, 7.21.

Z(OMe)–Gln–Leu–Ser–OMe——Z(OMe)–Leu–Ser–OMe (6.22 g, 15.68 mmol) was treated with TFA–anisole (12 ml–4 ml) and the N^{α}-deprotected peptide isolated as stated above was dissolved in DMF (60 ml), together with Et_3N (4.36 ml, 31.35 mmol) and Z(OMe)–Gln–ONp (6.76 g, 15.68 mmol). After being stirred for 24 h, the solution was concentrated and the residue was treated with H_2O. The resulting powder was washed with 3% $NaHCO_3$, 5% citric acid and H_2O and precipitated from DMF with MeOH; yield 6.45 g (77%), mp 229—230 °C, $[\alpha]_D^{18}$ −10.8° (c= 0.5, DMF), Rf_1 0.73. *Anal.* Calcd for $C_{24}H_{36}N_4O_9$: C, 54.02; H, 6.99; N, 10.50. Found: C, 53.97; H, 6.80; N, 10.49.

Z(OMe)–Gly–Gln–Leu–Ser–OMe——Z(OMe)–Gln–Leu–Ser–OMe (6.45 g, 12.29 mmol) was treated with TFA–anisole (13 ml–3 ml) and the N^{α}-deprotected peptide isolated as stated above was dissolved in DMF (60 ml), together with Et_3N (3.41 ml, 24.52 mmol) and Z(OMe)–Gly–ONp (4.43 g, 12.29 mmol). The solution was stirred for 24 h, the solvent was removed by evaporation, and the residue was treated with H_2O. The resulting powder was washed as stated above and recrystallized from MeOH and AcOEt; yield 4.29 g (60%), mp 180—182 °C, $[\alpha]_D^{18}$ −20.6° (c=1.1, DMF), Rf_1 0.71. *Anal.* Calcd for $C_{26}H_{39}N_5O_{10}$: C, 53.69; H, 6.76; N, 12.04. Found: C, 53.45; H, 6.97; N, 12.02.

Z(OMe)–Gly–Gln–Leu–Ser–$NHNH_2$ (3)——Z(OMe)–Gly–Gln–Leu–Ser–OMe (4.29 g, 7.38 mmol) in DMF (40 ml) was treated with 80% hydrazine hydrate (2.31 ml, 5 eq) overnight. The solvent was evaporated off, the residue was treated with MeOH, and the resulting solid was precipitated from DMF with MeOH; yield 3.60 g (83%), mp 235—237 °C, $[\alpha]_D^{18}$ −1.5° (c=0.6, DMSO), Rf_1 0.44. Amino acid ratios in 6 N HCl hydrolysate: Ser 0.92, Glu 1.02, Gly 1.02, Leu 1.00 (recovery of Leu 98.2). *Anal.* Calcd for $C_{25}H_{39}N_7O_9$: C, 51.62; H, 6.76, N, 16.86. Found: C, 51.49;

H, 6.72; N, 16.69.

Z(OMe)–Arg(Mts)–Leu–NHNH$_2$——Z(OMe)–Arg(Mts)–Leu–OMe (8.33 g, 12.86 mmol) in MeOH (90 ml) was treated with 80% hydrazine hydrate (6.5 ml, 6 eq) for 48 h. The solvent was removed by evaporation, the residue was treated with H_2O and the resulting powder was recrystallized from AcOEt and ether; yield 8.09 g (97%), mp 111—114 °C, $[\alpha]_D^{18}$ −6.0° (c=1.3, DMF), Rf_1 0.73. *Anal.* Calcd for $C_{30}H_{45}N_7O_7S$: C, 54.60; H, 7.08; N, 14.86. Found: C, 54.66; H, 7.26; N, 14.54.

Z(OMe)–Arg(Mts)–Leu–Leu–OMe——The azide [prepared from 6.99 g (10.8 mmol) of Z(OMe)–Arg(Mts)–Leu–NHNH$_2$] in DMF (10 ml) and Et_3N (3.31 ml, 23.8 mmol) were added to an ice-chilled solution of H–Leu–OMe [prepared from 2.94 g (16.2 mmol) of the hydrochloride] in DMF (5 ml) and the mixture was stirred at 4 °C for 24 h. After evaporation of the solvent, the residue was dissolved in AcOEt. The organic phase was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O, then dried over Na_2SO_4 and concentrated. The product was purified by column chromatography on silica (30 × 3.0 cm), using $CHCl_3$–MeOH (10 : 0.5) as an eluant, and precipitated from DMF with ether; yield 5.22 g (63%), mp 89—91 °C, $[\alpha]_D^{18}$ −12.2° (c=2.1, DMF), Rf_1 0.92. *Anal.* Calcd for $C_{37}H_{56}N_6O_9S$: C, 58.40; H, 7.42; N, 11.05. Found: C, 58.47; H, 7.71; N, 10.79.

Z(OMe)–Ser–Arg(Mts)–Leu–Leu–OMe——Z(OMe)–Arg(Mts)–Leu–Leu–OMe (5.22 g, 6.86 mmol) was treated with TFA–anisole (8 ml–1.48 ml) and the N^{α}-deprotected peptide (see above) was dissolved in DMF (5 ml) containing Et_3N (0.95 ml, 6.86 mmol). The azide [prepared from 2.33 g (8.24 mmol) of Z(OMe)–Ser–NHNH$_2$] in DMF (15 ml) and Et_3N (1.15 ml, 8.24 mmol) were added to the above ice-chilled solution and the mixture was stirred at 4 °C for 24 h. After evaporation of the solvent, the product was purified by extraction with AcOEt as described for the preparation of Z(OMe)–Leu–Ser–OMe and recrystallized from AcOEt and ether; yield 4.11 g (71%), mp 97—99 °C, $[\alpha]_D^{18}$ −3.2° (c=0.6, DMF), Rf_1 0.75. *Anal.* Calcd for $C_{40}H_{61}N_7O_{11}S$: C, 55.47; H, 7.33; N, 11.32. Found: C, 55.59; H, 7.31; N, 11.30.

Z(OMe)–Ser–Arg(Mts)–Leu–Leu–NHNH$_2$ (4)——Z(OMe)–Ser–Arg(Mts)–Leu–Leu–OMe (4.11 g, 4.85 mmol) in MeOH (40 ml) was treated with 80% hydrazine hydrate (1.46 ml, 6 eq) overnight. The solvent was removed by evaporation, the residue was triturated with EtOH–ether and the resulting powder was recrystallized from MeOH–EtOH–ether; yield 3.28 g (80%), mp 175—177 °C, $[\alpha]_D^{18}$ −11.0° (c=1.2, DMF), Rf_2 0.55. Amino acid ratios in 6 N HCl hydrolysate: Ser 0.96, Leu 2.00, Arg 0.99 (recovery of Leu 92.2%). *Anal.* Calcd for $C_{39}H_{61}N_9O_{10}S$: C, 54.65; H, 7.29; N, 14.71. Found: C, 54.66; H, 7.11; N, 14.76.

Boc–Asp–Phe–OMe——Boc–Asp(OBzl)–OH (5.47 g, 16.92 mmol) and H–Phe–OMe [prepared from 5.47 g (25.38 mmol) of the hydrochloride] in tetrahydrofuran (THF)–DMF (50 ml–40 ml) were condensed by the use of DCC (4.18 g, 20.3 mmol) in the usual manner. After being stirred for 24 h, the solution was filtered, then the filtrate was concentrated and the residue was extracted with AcOEt. The organic phase was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O, dried over Na_2SO_4 and concentrated. The residue (5.22 g, Rf_1 0.86, Rf_3 0.67) was dissolved in MeOH (50 ml) containing a few drops of AcOH and hydrogenated over a Pd catalyst for 3 h. After filtration, the filtrate was concentrated. The residue was treated with isopropylether and the resulting powder was recrystallized from AcOEt–ether; yield 3.0 g (66%), mp 149—151 °C $[\alpha]_D^{18}$ −34.5° (c=0.5, DMF), Rf_1 0.80, Rf_3 0.21. *Anal.* Calcd for $C_{19}H_{26}N_2O_7$: C, 57.86; H, 6.64; H, 7.10. Found: C, 58.00; H, 6.76; N, 6.99.

Z(OMe)–Thr–Ser–Asp–Phe–OMe——Boc–Asp–Phe–OMe (3.0 g, 7.61 mmol) was treated with TFA–anisole (12 ml–3 ml) and the N^{α}-deprotected peptide isolated as usual was dissolved in DMF (30 ml) containing Et_3N (2.12 ml, 15.22 mmol). The azide [prepared from 3.22 g (8.37 mmol) of Z(OMe)–Thr–Ser–NHNH$_2$[28]] in DMF (25 ml) and Et_3N (1.16 ml, 8.37 mmol) were added to the above ice-chilled solution and the mixture was stirred at 4 °C for 24 h. After evaporation of the solvent, the residue was treated with 5% citric acid. The resulting powder was washed with H_2O and precipitated from DMF with MeOH; yield 3.21 g (65%), mp 159—161 °C, $[\alpha]_D^{18}$ −19.1° (c=0.4, DMF), Rf_1 0.64. *Anal.* Calcd for $C_{30}H_{38}N_4O_{12}$: C, 55.72; H, 5.92; N, 8.60. Found: C, 55.57; H, 6.05, N, 8.55.

Z(OMe)–Thr–Ser–Asp–Phe–NHNH$_2$ (5)——Z(OMe)–Thr–Ser–Asp–Phe–OMe (3.21 g, 4.96 mmol) in MeOH (40 ml) was treated with 80% hydrazine hydrate (1.55 ml, 5 eq) overnight. The crystalline mass that formed on standing at room temperature overnight was precipitated from DMF with MeOH; yield 1.90 g (59%), mp 195—197 °C, $[\alpha]_D^{18}$ −26.9° (c=0.7, DMF), Rf_1 0.26. Amino acid ratios in 6 N HCl hydrolysate: Asp 1.03, Thr 1.00, Ser 0.95, Phe 0.90 (recovery of Thr 82.0%). *Anal.* Calcd for $C_{29}H_{38}N_6O_{11}\cdot 0.5H_2O$: C, 53.12; H, 6.00; N, 12.82. Found: C, 52.76; H, 6.25; N, 13.03.

Z(OMe)–Val–Phe–OMe——A mixed anhydride [prepared from 15.52 g (55.20 mmol) of Z(OMe)–Val–OH] in THF (120 ml) was added to an ice-chilled solution of H–Phe–OMe [prepared from 15.48 g (71.80 mmol) of the hydrochloride] in DMF (120 ml) and the mixture, after being stirred in an ice-bath for 3 h, was concentrated. The residue was treated with H_2O and the resulting powder, after being washed with 5% citric acid, 5% $NaHCO_3$ and H_2O, was recrystallized from MeOH and ether; yield 19.94 g (82%), mp 161—162 °C, $[\alpha]_D^{18}$ −7.5° (c=1.6, DMF), Rf_1 0.94. *Anal.* Calcd for $C_{24}H_{30}N_2O_6$: C, 65.14; H, 6.83; N, 6.33. Found: C, 64.96; H, 6.76; N, 6.26.

Z(OMe)–Gly–Val–Phe–OMe——Z(OMe)–Val–Phe–OMe (10.53 g, 23.79 mmol) was treated with TFA–anisole (20 ml–5 ml), then n-hexane was added. The resulting oily precipitate (soluble in ether) was dried over KOH pellets *in vacuo* for 3 h and dissolved in DMF (50 ml), together with Et_3N (6.61 ml, 47.58 mmol) and Z(OMe)–Gly–ONp (10.29 g, 28.55 mmol). After being stirred for 24 h, the solution was concentrated, and the residue was treated with

H_2O. The resulting powder was washed with 5% citric acid and 5% $NaHCO_3$ as described above and recrystallized from MeOH; yield 8.30 g (70%), mp 154—155 °C, $[\alpha]_D^{18}$ −8.1 ° (c = 1.1, DMF), Rf_1 0.74. *Anal.* Calcd for $C_{26}H_{33}N_3O_7$: C, 62.51; H, 6.66; N, 8.41. Found: C, 62.37; H, 6.69; N, 8.38.

Z(OMe)–Asp(OBzl)–Gly–Val–Phe–OMe——Z(OMe)–Gly–Val–Phe–OMe (4.21 g, 8.42 mmol) was treated with TFA–anisole (8 ml–2 ml) and the N^{α}-deprotected peptide (isolated as usual) was dissolved in DMF (40 ml), together with Et_3N (1.29 ml, 9.27 mmol) and Z(OMe)–Asp(OBzl)–ONp (4.71 g, 9.27 mmol). NMM (0.85 ml, 8.42 mmol) was added and the mixture was stirred at 4 °C for 24 h. After addition of a few drops of AcOH, the solvent was removed by evaporation and the residue was treated with H_2O. The resulting powder was washed with 5% citric acid and H_2O and precipitated from DMF with MeOH; yield 5.50 g (90%), mp 198—200 °C, $[\alpha]_D^{18}$ −19.1 ° (c = 0.4, DMF), Rf_1 0.84. *Anal.* Calcd for $C_{37}H_{44}N_4O_{10}$: C, 63.05; H, 6.29; N, 7.95. Found: C, 63.35; H, 6.18; N, 7.93.

Z(OMe)–His–Ala–OMe——The azide [prepared from 10.78 g (33.3 mmol) of Z(OMe)–His–$NHNH_2$] in DMF (150 ml) and Et_3N (4.50 ml, 32.33 mmol) were added to an ice-chilled solution of H–Ala–OMe [prepared from 6.77 g (48.50 mmol) of the hydrochloride] in DMF (50 ml). After being stirred at 4 °C for 24 h, the solution was concentrated and the residue was treated with 5% $NaHCO_3$. The resulting powder was washed with H_2O and recrystallized from MeOH; yield 6.32 g (48%), mp 178—180 °C, $[\alpha]_D^{18}$ −20.1 ° (c = 1.2, DMF), Rf_1 0.66. *Anal.* Calcd for $C_{19}H_{29}N_4O_6$: C, 56.42; H, 5.98; N, 13.86. Found: C, 56.42; H, 5.81; N, 13.94.

Z(OMe)–His–Ala–$NHNH_2$——Z(OMe)–His–Ala–OMe (6.32 g, 15.63 mmol) in DMF–MeOH (60 ml, 1 : 2) was treated with 80% hydrazine hydrate (4.89 ml, 5 eq) overnight. The solvent was removed by evaporation and the residue was precipitated from DMF with EtOH; yield 6.11 g (97%), mp 188—191 °C, $[\alpha]_D^{18}$ −10.0 ° (c = 0.4, DMF), Rf_1 0.48. *Anal.* Calcd for $C_{18}H_{24}N_6O_5$: C, 53.45; H, 5.98; N, 20.78. Found: C, 53.43; H, 5.98; N, 20.84.

Z(OMe)–His–Ala–Asp–Gly–Val–Phe–OMe——Z(OMe)–Asp(OBzl)–Gly–Val–Phe–OMe (5.50 g, 7.80 mmol) was treated with TFA–anisole (12 ml–3 ml). The N^{α}-deprotected peptide (isolated as usual) was dissolved in DMF (50 ml), then hydrogenated over a Pd catalyst at room temperature for 3 h. The solution was filtered and the filtrate was neutralized with Et_3N (0.92 ml, 7.80 mmol). The azide [prepared from 2.67 g (6.60 mmol) of Z(OMe)–His–Ala–$NHNH_2$] in DMF (15 ml) and Et_3N (0.92 ml, 6.60 mmol) were added to the above ice-chilled filtrate and the mixture was stirred at 4 °C for 24 h. Additional azide and Et_3N (0.3 eq each) in DMF (5 ml) were added and stirring was further continued for 24 h. The solvent was removed by evaporation and the residue was treated with H_2O and precipitated from DMF with MeOH; yield 5.35 g (83%), mp 217 °C (dec.), $[\alpha]_D^{18}$ −8.5 ° (c = 0.6, DMF), Rf_1 0.42. *Anal.* Calcd for $C_{39}H_{50}N_8O_{12}$. H_2O: C, 55.70; H, 6.23; N, 13.33. Found: C, 55.88; H, 6.24; N, 13.53.

Z(OMe)–His–Ala–Asp–Gly–Val–Phe–$NHNH_2$ (6)——Z(OMe)–His–Ala–Asp–Gly–Val–Phe–OMe (5.34 g, 6.49 mmol) in DMF (50 ml) was treated with 80% hydrazine hydrate (2.03 ml, 5 eq) for 24 h. The solvent was removed by evaporation, the residue was treated with MeOH and the resulting solid was precipitated from DMF with MeOH; yield 3.39 g (62%), mp 200—202 °C, $[\alpha]_D^{18}$ −40.4 ° (c = 0.6, DMF), Rf_1 0.34. Amino acid ratios in 6 N HCl hydrolysate: Asp 1.00, Gly 1.00, Ala 1.03, Val 0.86, Phe 0.79, His 0.98 (recovery of Gly 91%). *Anal.* Calcd for $C_{38}H_{50}N_{10}O_{11} \cdot H_2O$: C, 54.27; H, 6.23; N, 16.66. Found: C, 54.05; H, 6.42; N, 16.43.

Z(OMe)–Ala–Lys(Z)–Lys(Z)–Tyr–Leu–Glu(OBzl)–Ser–Leu–Ile–NH_2, Z(OMe)–(PHI 19—27)–NH_2——Z(OMe)–Leu–Glu(OBzl)–Ser–Leu–Ile–NH_2 (**1**) (2.55 g, 3.07 mmol) was treated with TFA–anisole (8 ml–2 ml) and the resulting N^{α}-deprotected peptide (isolated as usual) was dissolved in DMF (20 ml) containing Et_3N (0.43 ml, 3.07 mmol). The azide [prepared from 3.23 g (3.83 mmol) of Z(OMe)–Ala–Lys(Z)–Lys(Z)–Tyr–$NHNH_2$ (**2**)] in DMF (30 ml) and Et_3N (0.47 ml, 3.38 mmol) were added to the above ice-chilled solution and the mixture was stirred at 4 °C for 24 h. The solvent was removed by evaporation and the residue was treated with H_2O. The resulting powder was washed with 5% citric acid and H_2O and precipitated three times from DMF with MeOH; yield 3.48 g (71%), mp 251 °C (dec.), $[\alpha]_D^{18}$ −14.4 ° (c = 1.5, DMF), Rf_1 0.52. *Anal.* Calcd for $C_{82}H_{112}N_{12}O_{20} \cdot H_2O$: C, 61.40; H, 7.16; N, 10.48. Found: C, 61.36; H, 7.24; N, 10.56.

Z(OMe)–Gly–Gln–Leu–Ser–Ala–Lys(Z)–Lys(Z)–Tyr–Leu–Glu(OBzl)–Ser–Leu–Ile–NH_2, Z(OMe)–(PHI 15—27)–NH_2——The above protected nonapeptide amide (3.28 g, 2.07 mmol) was treated with TFA–anisole (10 ml–3 ml) and the N^{α}-deprotected peptide, isolated as usual, was dissolved in DMF–DMSO (30 ml, 1 : 1) containing Et_3N (0.29 ml, 2.07 mmol). The azide [prepared from 1.44 g (2.48 mmol) of Z(OMe)–Gly–Gln–Leu–Ser–$NHNH_2$ (**3**)] in DMF (10 ml) and Et_3N (0.35 ml, 2.48 mmol) were added to the above ice-chilled solution and the mixture was stirred at 4 °C for 24 h. The additional azide and Et_3N (0.3 eq each) in DMF (3 ml) were added and stirring was continued for 24 h. The solvent was removed by evaporation and the residue was treated with 5% citric acid to form a powder, which was washed with 5% citric acid and H_2O and precipitated twice from DMF with MeOH; yield 3.19 g (78%), mp 258 °C (dec.), $[\alpha]_D^{18}$ −20.8 ° (c = 0.4, DMF), Rf_1 0.61. *Anal.* Calcd for $C_{98}H_{139}N_{17}O_{26} \cdot H_2O$: C, 59.17; H, 7.14; N, 11.97. Found: C, 59.22; H, 7.47; N, 11.64.

Z(OMe)–Ser–Arg(Mts)–Leu–Leu–Gly–Gln–Leu–Ser–Ala–Lys(Z)–Lys(Z)–Tyr–Leu–Glu(OBzl)–Ser–Leu–Ile–NH_2, Z(OMe)–(PHI 11—27)–NH_2——The above protected tridecapeptide amide (3.19 g, 1.62 mmol) was treated with TFA–anisole (10 ml–3 ml) and the N^{α}-deprotected peptide, isolated as usual, was dissolved in DMF–DMSO (30 ml, 1 : 1) containing Et_3N (0.22 ml, 1.62 mmol). The azide [prepared from 1.66 g (2.42 mmol) of Z(OMe)–Ser–Arg(Mts)–Leu–Leu–$NHNH_2$ (**4**)] in DMF (10 ml) and Et_3N (0.34 ml, 2.43 mmol) were added and the mixture was stirred at 4 °C for 24 h. The solvent was removed by evaporation and the residue was treated with 5% citric acid to

form a powder, which was washed with H_2O and precipitated twice from DMF with MeOH; yield 3.17 g (75%), mp 267 °C (dec.), $[\alpha]_D^{18}$ −15.2° (c=0.4, DMF), Rf_1 0.54. *Anal.* Calcd for $C_{128}H_{188}N_{24}O_{33}S \cdot 2H_2O$: C, 57.81; H, 7.28, N, 12.64. Found: C, 57.83; H, 7.21; N, 12.61.

Z(OMe)–Thr–Ser–Asp–Phe–Ser–Arg(Mts)–Leu–Leu–Gly–Gln–Leu–Ser–Ala–Lys(Z)–Lys(Z)–Tyr–Leu–Glu(OBzl)–Ser–Leu–Ile–NH_2, Z(OMe)–(PHI 7—27)–NH_2——The above protected heptadecapeptide amide (2.18 g 0.83 mmol) was treated with TFA–anisole (8 ml–2 ml) and the N^{α}-deprotected peptide, isolated as usual, was dissolved in DMF–DMSO (20 ml, 1:2) containing Et_3N (0.12 ml, 0.83 mmol). The azide [prepared from 806 mg (1.25 mmol) of Z(OMe)–Thr–Ser–Asp–Phe–$NHNH_2$ (**5**)] in DMF (10 ml) and Et_3N (0.18 ml, 1.25 mmol) were added to the above ice-chilled solution and the mixture was stirred at 4 °C for 24 h. After addition of a few drops of AcOH, the solvent was removed by evaporation and the residue was treated with 5% citric acid to form a solid powder, which was washed with H_2O and precipitated three times from DMF with AcOEt; yield 2.38 g (93%), mp 252 °C (dec.), $[\alpha]_D^{18}$ +5.3° (c=1.0, DMF), Rf_1 0.52. *Anal.* Calcd for $C_{148}H_{214}N_{28}O_{41}S \cdot 2H_2O$: C, 57.16; H, 7.07; N, 12.61. Found: C, 57.11; H, 7.21; N, 12.36.

Z(OMe)–His–Ala–Asp–Gly–Val–Phe–Thr–Ser–Asp–Phe–Ser–Arg(Mts)–Leu–Leu–Gly–Gln–Leu–Ser–Ala–Lys(Z)–Lys(Z)–Tyr–Leu–Glu(OBzl)–Ser–Leu–Ile–NH_2, Z(OMe)–(PHI 1—27)–NH_2——The above protected heneicosapeptide amide (2.38 g, 0.77 mmol) was treated with TFA–anisole (12 ml–3 ml) and the N^{α}-deprotected peptide, isolated as usual, was dissolved in DMF–DMSO (20 ml, 1:1) containing Et_3N (0.22 ml, 1.55 mmol). The azide [prepared from 1.27 g (1.55 mmol) of Z(OMe)–His–Ala–Asp–Gly–Val–Phe–$NHNH_2$ (**6**)] in DMF (10 ml) and Et_3N (0.22 ml, 1.55 mmol) were added and the mixture was stirred at 4 °C for 24 h. After addition of a few drops of AcOH to the mixture, the solvent was removed by evaporation and the residue was treated with H_2O. The resulting powder was washed with H_2O and precipitated from DMF with AcOEt to form a powder; yield 2.60 g. A part of this partially purified product (443 mg) was dissolved in DMF (4 ml) and the solution was applied to a column of Sephadex LH-60 (3 × 144 cm), which was eluted with the same solvent. Each fraction (8 ml) was examined for UV absorption at 275 nm; fractions corresponding to the main peak (tube Nos. 74—92) were combined and the solvent was removed by evaporation. Treatment of the residue with AcOEt afforded a powder; yield 381 mg. The rest of the sample was similarly purified; total yield 2.30 g (88%), mp 230 °C (dec.), $[\alpha]_D^{18}$ −14.7° (c=0.5, DMSO), Rf_1 0.42. *Anal.* Calcd for $C_{177}H_{252}N_{36}O_{49}S \cdot 5H_2O$: C, 56.09; H, 6.97; N, 13.30. Found: C, 55.79, H, 6.98; N, 13.19.

H–His–Ala–Asp–Gly–Val–Phe–Thr–Ser–Asp–Phe–Ser–Arg–Leu–Leu–Gly–Gln–Leu–Ser–Ala–Lys–Lys–Tyr–Leu–Glu–Ser–Leu–Ile–NH_2 (PHI)——The above protected PHI (100 mg, 27 μmol) was treated with 1 M TFMSA–thioanisole/TFA (2.7 ml) in the presence of *m*-cresol (71 μmol, 5 eq) in an ice-bath for 60 min, then dry ether was added. The resulting powder was collected by filtration, washed with ether and again treated with 1 M TFMSA–thioanisole/TFA under the same conditions as stated above. The deprotected peptide, isolated by addition of dry ether, was dissolved in H_2O (5 ml). The pH of the solution was adjusted to 8.6 with 0.2 N NH_4OH, then after 30 min,

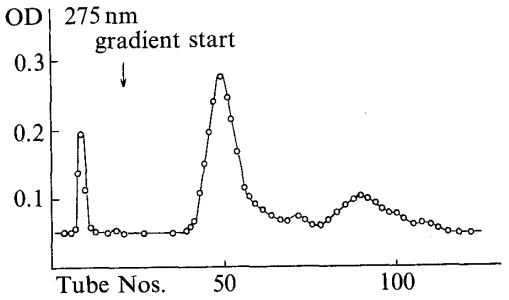

Fig. 10a. Purification of Synthetic PHI by Ion-Exchange Chromatography on CM-Biogel A

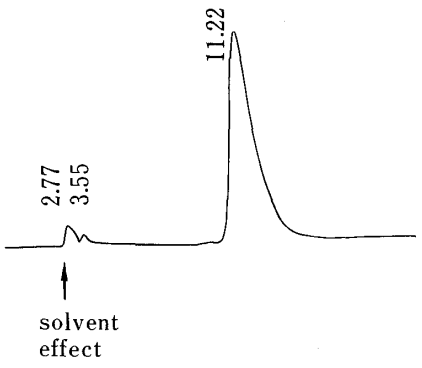

Fig. 10c. HPLC of Purified PHI

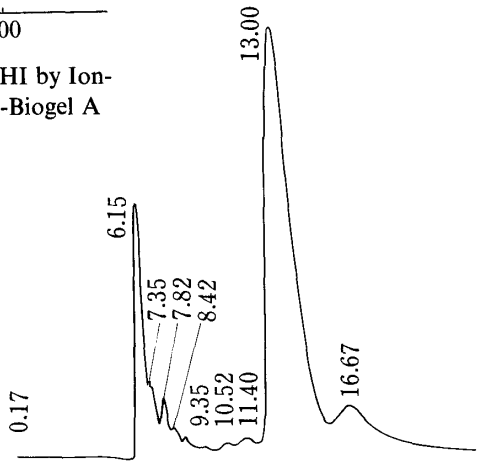

Fig. 10b. Purification of CM-Purified PHI by HPLC

to 6.5 with 0.2 N AcOH. After lyophilization, the residue was dissolved in 1 N AcOH (5 ml) and the solution was applied to a column of Sephadex G-25 (2.8 × 134 cm), which was eluted with the same solvent. Each fraction (3.5 ml) was examined for UV absorption at 275 nm; the fractions corresponding to the main peak (tube Nos. 42—57) were collected and the solvent was removed by lyophilization to give a fluffy powder; yield 65.6 mg (81.2%).

The crude product thus obtained was dissolved in pH 4.8, 0.02 M NH_4OAc (2 ml) and the solution was applied to a column of CM-Biogel A (2 × 9.5 cm), which was eluted first with the same buffer as described above (100 ml) and then with a gradient formed from pH 5.8, 0.1 M NH_4OAc (250 ml) through a mixing flask containing pH 4.8, 0.02 M NH_4OAc (250 ml). The UV absorption of each fraction (5 ml) was determined (Fig. 10a). The fractions corresponding to the main peak (tube Nos. 41—60) were combined and the solvent was removed by lyophilization to give a white powder; yield 30.2 mg (46%).

Next, a part of this powder (0.65 mg each) was purified by reverse phase HPLC on μBondapak C_{18} (3.9 × 300 mm) using acetonitrile–0.05 M NH_4OAc–H_3PO_4 (34 : 65 : 1) at pH 2.3. The eluate corresponding to the main peak (retention time 13.0 min, Fig. 10b) was collected. The rest of the sample was similarly purified. The combined eluates were neutralized with Et_3N and lyophilized. The residue was desalted by gel-filtration on Sephadex G-25 (2.8 × 134 cm) using 1 N AcOH as the eluant. The desired fractions were collected and the solvent was removed by lyophilization to give a fluffy white powder; yield 20.5 mg (68%); $[\alpha]_D^{17}$ −62.4° (c=0.2, 0.2 N AcOH), Rf_4 0.44, Rf_5 0.67. Single peak on HPLC (Fig. 10c). Disk isoelectrophoresis: single band in 7.5% polyacrylamide gel (Pharmalyte, pH 3—10), mobility 3.9 cm from the origin toward the cathode, after running at 200 V for 120 min. Amino acid ratios in 6 N HCl hydrolysate (Table I) and AP-M digest (numbers in parentheses are theoretical values): Asp 1.80 (2), Thr 1.01 (1), Gln N.D. (1), Ser 3.91 (4), Glu 1.10 (1), Gly 1.78 (2), Ala 2.00 (2), Val 0.95 (1), Ile 1.00 (1), Leu 5.00 (5), Tyr 1.02 (1), Phe 1.78 (2), Lys 2.00 (2), His 0.97 (1), Arg 0.95 (1) recovery of Ile 85.2%. *Anal.* Calcd for $C_{146}H_{236}N_{36}O_{40} \cdot 5CH_3COOH \cdot 5H_2O$: C, 51.78; H, 7.32; N, 14.90. Found: C, 51.44; H, 7.07; N, 15.28.

N^{α}-Biotinyl-PHI——Z(OMe)-(PHI 1—27)-NH_2 (200 mg, 0.05 mmol) was treated with TFA–anisole (1 ml–0.2 ml) in an ice-bath for 2 h and the N^{α}-deprotected peptide, isolated by precipitation with ether, was dissolved in DMSO–DMF (2 ml, 1 : 1), together with biotinyl-*N*-hydroxysuccinimide ester (22 mg, 0.065 mmol) and Et_3N (23 μl, 0.16 mmol). After being stirred overnight, the ninhydrin-negative solution was neutralized with AcOH, then concentrated *in vacuo* and the residue was treated with H_2O. The resulting powder was precipitated from DMSO with MeOH; yield 136 mg (67%), mp 233 °C (dec.), $[\alpha]_D^{18}$ +3.4° (c=0.6, MDSO), Rf_1 0.39. Amino acid ratios in 6 N HCl hydrolysate: Asp 2.12, Thr 1.00, Ser 3.62, Glu 2.06, Gly 2.17, Ala 2.35, Val 1.04, Ile 1.00, Leu 5.07, Tyr 0.96, Phe 1.98, Lys 1.95, His 1.02, Arg 1.08 (recovery of Ile 75.5%). *Anal.* Calcd for $C_{178}H_{256}N_{38}O_{48}S_2 \cdot 8H_2O$: C, 54.71; H, 7.09; N, 13.62. Found: C, 54.53; H, 6.86; N, 13.72.

The above protected form of N^{α}-biotinyl-PHI (100 mg, 27 μmol) was treated with 1 M TFMSA–thioanisole/TFA (2.1 ml) in the presence of *m*-cresol (110 μl) and Me_2S (39 μl) in an ice-bath for 60 min. This treatment was repeated twice more as stated above. The deprotected peptide precipitated with ether was dissolved in H_2O (5 ml). The pH of the solution was adjusted to 8.0 with 1 N NH_4OH, then after 30 min to 6 with 1 N AcOH. Next, the solution was incubated with DTT (81 mg, 20 eq) at 37 °C for 24 h, then lyophilized. The residue was dissolved in 1 N AcOH and applied to a column of Sephadex G-25 (1.8 × 135 cm), which was eluted with the same solvent. Individual fractions (5 ml each) were examined for the absorption at 275 nm. Fractions corresponding to the front main peak (tube Nos. 40—55) were combined and the solvent was removed by lyophilization to give a powder; yield 69.6 mg (81%). To remove a trace impurity (Rf_3 0.49), the crude product thus obtained was purified by gel-filtration on Sephadex G-50 (1.6 × 110 cm) using 1 N AcOH as an eluant. The UV absorption at 275 nm of each fraction (3 ml) was determined and the fractions corresponding to the main peak (tube Nos. 34—40) were combined and lyophilized to give a homogeneous product on TLC; yield 58.4 mg (60%), $[\alpha]_D^{18}$ −58.0° (c=0.6, 1 N AcOH), Rf_4 0.46, Rf_5 0.61. Amino acid ratios in 6 N HCl hydrolysate: Asp 2.05, Thr 1.00, Ser 3.93, Glu 2.09, Gly 2.17, Ala 2.22, Val 0.94, Ile 1.00, Leu 5.04, Tyr 0.92, Phe 1.88. Lys 1.94, His 0.97, Arg 1.03 (recovery of Ile 91%). *Anal.* Calcd for $C_{146}H_{231}N_{38}O_{42}S \cdot 2CH_3COOH \cdot H_2O$: C, 53.60; H, 7.23; N, 15.84. Found: C, 53.40; H, 7.15; N, 15.97.

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References and Notes

1) Part CXI: *Int. J. Pept. Protein Res.*, **20**, 276 (1982).
2) Amino acids, peptides and their derivatives mentioned in this report are of the L-configuration. The following abbreviations are used: Z = benzyloxycarbonyl, Z(OMe) = *p*-methoxybenzyloxycarbonyl, Boc = *tert*-butoxycarbonyl, Bzl = benzyl, Mts = mesitylene-2-sulfonyl, TFA = trifluoroacetic acid, TFMSA = trifluoromethanesulfonic acid, DCC = dicyclohexylcarbodiimide, HOBt = *N*-hydroxybenzotriazole, DTT = dithiothreitol, DMF = dimethylformamide, DMSO = dimethylsulfoxide, Np = *p*-nitrophenyl, Su = *N*-hydroxysuccinimide, NMM = *N*-methylmorpholine.
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