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Studies on Peptides. CLVII.[1,2] Synthesis of a Frog-Skin Peptide, Sauvagine

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A tetracontapeptide corresponding to the entire amino acid sequence of a frog skin peptide, sauvagine, was synthesized by assembling eight peptide fragments of established purity followed by deprotection with 1 M trimethylsilyl trifluoromethanesulfonate–thioanisole in trifluoroacetic acid. The synthetic peptide stimulated secretion of immunoreactive corticotropin from rat pituitary cells and its potency was estimated to be equivalent to that of synthetic ovine corticotropin releasing factor. Synthetic sauvagine displayed long-lasting hypotensive action in rats.

Keywords——sauvagine synthesis; frog-skin peptide; thioanisole-mediated deprotection; trimethylsilyl trifluoromethanesulfonate deprotection; hard acid deprotection; corticotropin-releasing activity; hypotensive action

Sauvagine is a 40-residue peptide isolated from the skin of a South American frog, *Phyllomedusa sauvagei*, by Montecucchi *et al.*[3] in 1980 and its structure was elucidated by the same group of investigators[4] in 1981. This largest form of frog-skin peptides so far known is reported to display a number of biological activities, *i.e.*, hypotensive action in the cardiovascular system, inhibition of release of prolactin, thyrotropin and growth hormone, but release of corticotropin and β-endorphin in mammalian anterior pituitary.[4] Indeed, sauvagine has significant structural homologies with a hypothalamic corticotropin-releasing factor (CRF),[5] as well as urotensin I isolated from the carp (*Cyprinus carpio*) urophysis.[6] Solid-phase syntheses of sauvagine[7] and its partial sequence[8] were reported preliminarily, but no detailed information is available at the present time.

Following the synthesis of human CRF(hCRF)[9] and ovine CRF(oCRF),[10] we wish to report the solution-phase synthesis of a 40-residue peptide corresponding to the entire amino acid sequence of sauvagine (Fig. 1). The synthesis was accomplished by assembling eight peptide fragments, followed by deprotection with 1 M TMSOTf–thioanisole in TFA.[11]

The C-terminal pentapeptide amide, Z(OMe)–Leu–Leu–Asp(OBzl)–Thr–Ile–NH_2 (fragment [1]), was prepared by the azide condensation[12] of Z(OMe)–Leu–Leu–$NHNH_2$ with a TFA-treated sample of Z(OMe)–Asp(OBzl)–Thr–Ile–NH_2 as shown in Fig. 2. The former dipeptide hydrazide was prepared by the usual hydrazine treatment of Z(OMe)–Leu–Leu–OMe.[13] The latter tripeptide amide was prepared in a stepwise manner starting with H–Ile–NH_2. The respective amino acid residues, Thr and Asp(OBzl), were introduced by the azide and the Np[14] methods, respectively. The purity of fragment [1] was ascertained by thin layer chromatography (TLC), elemental analysis and amino acid analysis after acid hydrolysis, as was done with other fragments.

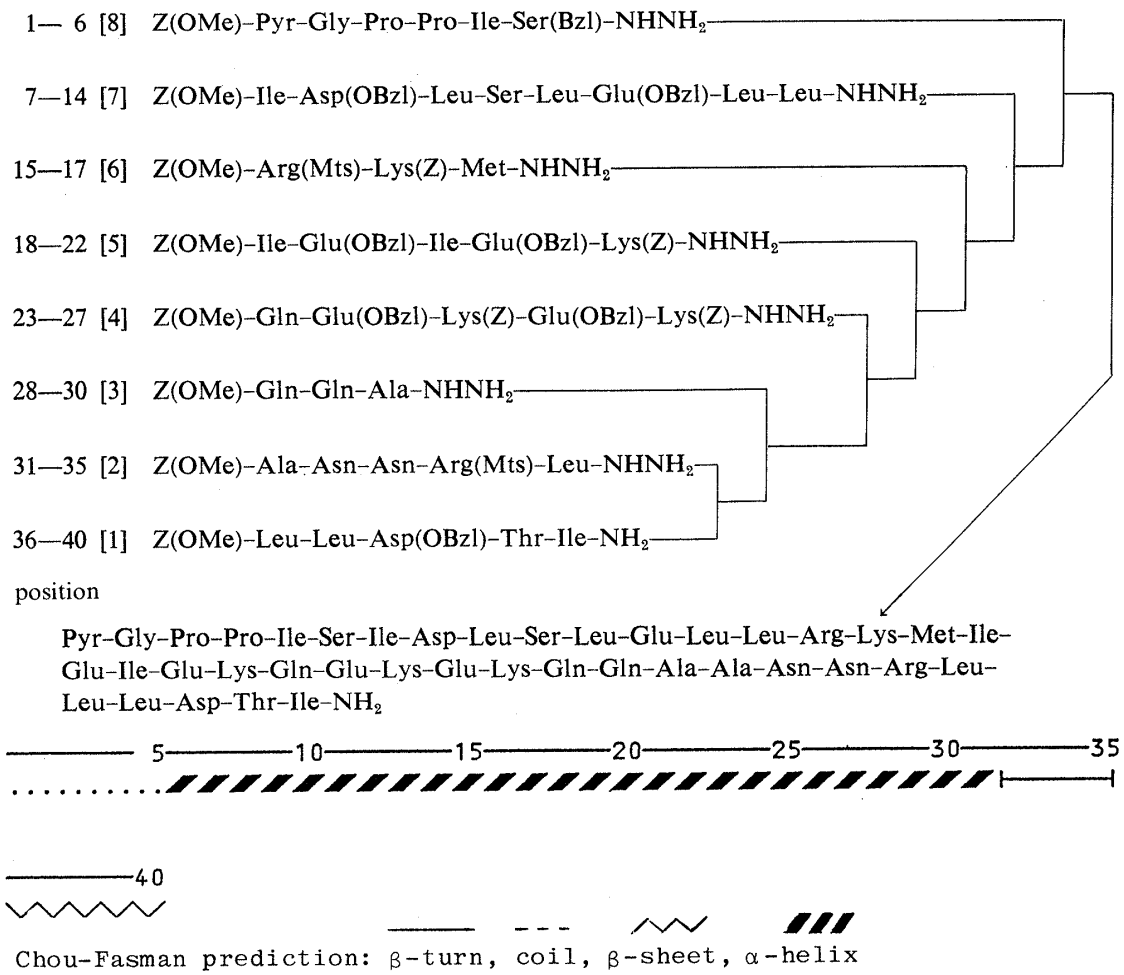

Fig. 1. Synthetic Route to Sauvagine

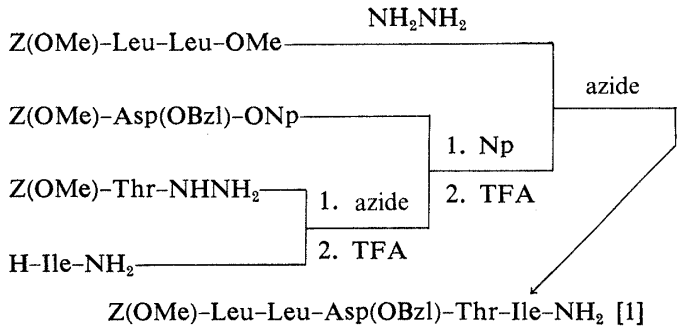

Fig. 2. Synthetic Scheme for the C-Terminal Pentapeptide Amide [1] (Positions 36—40)

Fragment [2], Z(OMe)–Ala–Asn–Asn–Arg(Mts)–Leu–$NHNH_2$, was prepared in a stepwise manner starting with a TFA-treated sample of Z(OMe)–Arg(Mts)–Leu–OMe[15] as shown in Fig. 3. Two Asn residues were introduced by the Np method and the Ala residue by the mixed anhydride (MA) method.[16] The resulting pentapeptide ester was converted to [2] by the usual hydrazine treatment.

Fragment [3], Z(OMe)–Gln–Gln–Ala–$NHNH_2$, was prepared by the usual hydrazine treatment of the corresponding methyl ester, used for our previous synthesis of hCRF.[9] Fragment [4], Z(OMe)–Gln–Glu(OBzl)–Lys(Z)–Glu(OBzl)–Lys(Z)–$NHNH_2$, was prepared

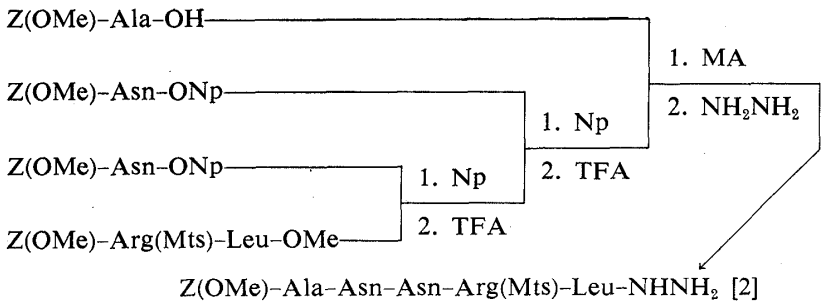

Fig. 3. Synthetic Scheme for the Protected Pentapeptide Hydrazide [2] (Positions 31—35)

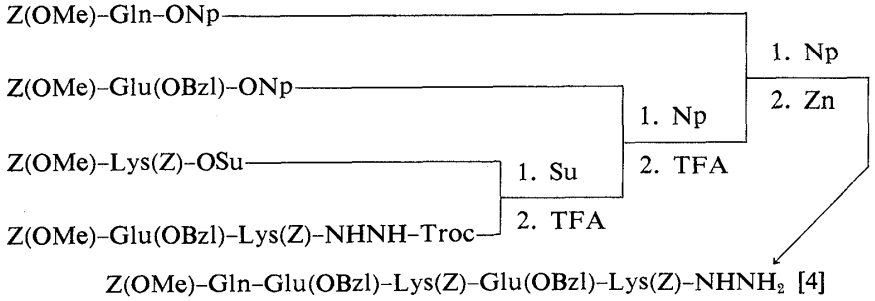

Fig. 4. Synthetic Scheme for the Protected Pentapeptide Hydrazide [4] (Positions 23—27)

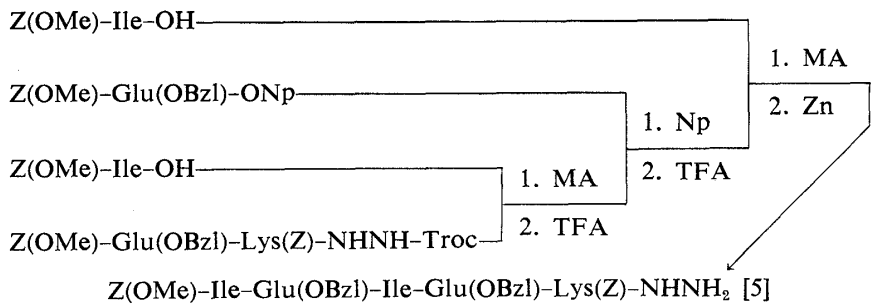

Fig. 5. Synthetic Scheme for the Protected Pentapeptide Hydrazide [5] (Positions 18—22)

in a stepwise manner starting from a TFA-treated sample of Z(OMe)–Glu(OBzl)–Lys(Z)–NHNH–Troc,[17] as shown in Fig. 4. The Su method[18] was employed for condensation of the Lys(Z) residue and the Np method for the subsequent two residues, Glu(OBzl) and Gln. From the resulting protected pentapeptide derivative, the Troc group[19] was removed by treatment with Zn powder.[20]

Fragment [5], Z(OMe)–Ile–Glu(OBzl)–Ile–Glu(OBzl)–Lys(Z)–$NHNH_2$, was prepared in a stepwise manner starting also from a TFA-treated sample of Z(OMe)–Glu(OBzl)–Lys(Z)–NHNH–Troc[16] as shown in Fig. 5. The MA method was employed for condensation of two Ile residues and the Np method for the Glu(OBzl) residue. From the resulting protected pentapeptide derivative, the Troc group was removed by the use of Zn powder as described above.

Fragment [6], Z(OMe)–Arg(Mts)–Lys(Z)–Met–$NHNH_2$, was prepared by condensation of Z(OMe)–Arg(Mts)–OH with a TFA- treated sample of Z(OMe)–Lys(Z)–Met–OMe,[21] followed by the usual hydrazine treatment. Next, a relatively large fragment, Z(OMe)–Ile–

Asp(OBzl)–Leu–Ser–Leu–Glu(OBzl)–Leu–Leu–$NHNH_2$ [7], was prepared by condensation of Z(OMe)–Ile–Asp(OBzl)–Leu–$NHNH_2$ (component 1) with a TFA-treated sample of Z(OMe)–Ser–Leu–Glu(OBzl)–Leu–Leu–NHNH–Troc (component 2) followed by removal of the Troc group (Fig. 6). Prior to this preparation, we attempted to elongate the peptide chain of sauvagine by successive condensations of the above two components. However, the azide condensation reaction of component 1 was unsatisfactory, even when a large excess was used. Such an unusual phenomenon was also observed in the chain elongation steps of the structurally related hCRF[9] and oCRF syntheses.[10] Both sauvagine and CRF are predicted to have a high degree of α-helical conformation[22] (Fig. 1). This particular steric feature may contribute to the unusual azide reaction mentioned above. Thus, we decided to unite these two components into one fragment. In order to prepare component 2, Z(OMe)–Glu(OBzl)–Leu–Leu–NHNH–Troc was first prepared in a stepwise manner starting from H–Leu–NHNH–Troc,[23] onto which the two residues, Leu and Glu(OBzl), were successively condensed by the MA method and the Np method, respectively. This tripeptide, after TFA treatment, was condensed with Z(OMe)–Ser–Leu–$NHNH_2$ by the azide method to give component 2. Component 1 was prepared by the MA condensation of Z(OMe)–Ile–OH with a TFA-treated sample of Z(OMe)–Asp(OBzl)–Leu–NHNH–Troc,[9] followed by removal of the Troc group by treatment with Zn powder. After condensation of the two components as described above, the Troc group was removed from the resulting protected octapeptide derivative by treatment with Cd powder,[24] instead of Zn powder. In this case, the reaction took longer than the Zn treatment, but a more homogeneous product [7] was obtained.

For preparation of the N-terminal fragment [8], Z(OMe)–Pyr–Gly–Pro–Pro–Ile–Ser(Bzl)–$NHNH_2$, an available tetrapeptide derivative, Z(OMe)–Pro–Pro–Ile–Ser(Bzl)–NHNH–Troc,[9] served as an amino component. The Np and the Su methods were employed to condense the Gly and the Pyr residues respectively. From the resulting protected hexapeptide, the Troc group was removed by treatment with Zn powder to give [8].

Eight peptide fragments thus obtained were condensed successively by the azide method (Fig. 1) to minimize racemization. As a solvent, DMF or DMF–DMSO (1 : 1) was employed. The fragment condensation reactions from [1] to [6] proceeded smoothly as usual, when each acyl component was employed in a slight excess (1.1 to 2 eq). However, condensations of fragments [7] and [8] had to be performed at lower temperature (−18 °C) than usual (+4 °C) in order to minimize Curtius rearrangement[25] of the corresponding azide. In addition, in

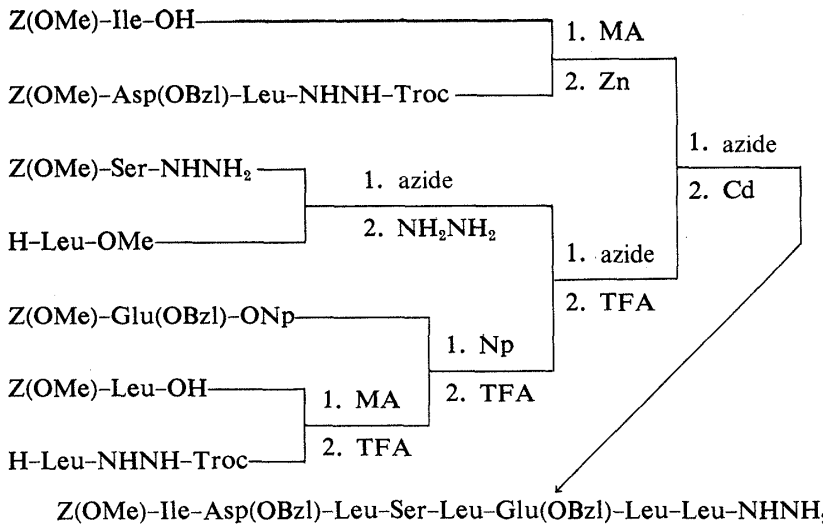

Fig. 6. Synthetic Scheme for the Protected Octapeptide Hydrazide [7] (Positions 7—14)

order to bring the reaction in completion, the acyl component was used in excess (5 eq) and the reaction was continued for 72 h in both cases. With these particular precautions, we were able to construct the entire peptide backbone of sauvagine. Like the hCRF and oCRF syntheses,[9,10] this sauvagine synthesis suffered considerable difficulty. As stated above, the circular dichroism (CD) spectra of CRF and sauvagine indicated predominantly α-helical character.[22] Such a conformational feature may still be retained even in protected peptides, and this may account more or less for the difficulty in the coupling reactions mentioned above. Protected sauvagine and its protected intermediates were purified by either precipitation from DMF or DMSO with appropriate solvents, such as MeOH or AcOEt, or by gel-filtration on Sephadex LH-20 using DMF as an eluant. Throughout this synthesis, Ala was used as a diagnostic amino acid in acid hydrolysis. By comparison of the recovery of Ala with those of newly incorporated amino acids, satisfactory incorporation of each fragment was ascertained. (Table I).

In the final step, protected sauvagine was treated with 1 M TMSOTf–thioanisole in TFA in the presence of *m*-cresol in an ice-bath for 120 min to remove all protecting groups employed. The deprotected peptide was treated with diluted NH_4OH and NH_4F at pH 8.0 to

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

	Protected peptides							Synthetic sauvagine
	31—40	28—40	23—40	18—40	15—40	9—40	1—40	
Asp	2.87	2.92	3.04	3.23	3.11	3.88	4.10	3.92 (4)
Thr	0.90	0.88	0.88	0.95	0.96	0.89	0.96	1.00 (1)
Ser						0.91	1.69	1.85 (2)
Glu		1.78	5.28	7.07	6.99	8.06	9.68	8.40 (9)
Pro							1.97	1.87 (2)
Gly							1.08	1.22 (1)
Ala	1.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00 (2)
Met[a)]					0.64	0.69	0.82	0.96 (1)
Ile	0.94	0.93	0.97	3.20	2.76	3.82	5.02	4.71 (5)
Leu	2.82	2.84	2.90	3.14	3.03	6.91	7.44	6.93 (7)
Lys			2.17	3.34	4.05	4.13	4.30	3.70 (4)
Arg	0.95	0.95	0.99	0.92	2.02	1.96	1.98	1.95 (2)
Recov. (%)	89	96	87	80	81	73	83	79

a) Met(O): N.D.

TABLE II. Activity of Synthetic Sauvagine. Release of Immunoreactive Adrenocorticotropin from Rat Pituitary Cells (Control Well, 100%)

	Dose	Exp. 1 ($n=4$) mean ± S.E. %	Exp. 2 ($n=4$) mean ± S.E. %
oCRF	0.1 nM	152.6 ± 2.6	
	1.0 nM	202.9 ± 14.7	
Sauvagine	0.1 nM	96.1 ± 8.2	116.9 ± 8.9
	1.0 nM	220.0 ± 11.2	180.5 ± 6.8
Rat CRF	0.1 nM		126.9 ± 10.0
	1.0 nM		190.6 ± 11.5

Rat CRF was purchased from Peninsula Laboratories (Belmont, California, U.S.A.) for comparison.

reverse any possible N→O shift[26] and to ensure complete hydrolysis of the trimethylsilyl moieties and then incubated with dithiothreitol at pH 6.0 for 12 h to reduce Met(O), possibly formed during manipulations. The product was purified by gel-filtration on Sephadex G-50, followed by partition chromatography[27] on Sephadex G-25 using *n*-BuOH–AcOH–H_2O (4 : 1 : 5). In the previous synthesis of oCRF,[1] the product was purified by ion-exchange chromatography on CM-cellulose, for which $AcONH_4$ buffer containing urea had to be applied to obtain a satisfactory recovery of the desired product. Otherwise most of the product was retained in the column, presumably due to its steric character. Thus, partition chromatography as employed here seems to be a rational method of choice for purification of such a compound. The product was finally purified by high performance liquid chromatography (HPLC) on Cosmosil 5C4-300 using gradient elution with acetonitrile in 0.1% TFA. The main product thus purified exhibited a sharp spot on TLC and gave a single band on disk isoelectrofocusing (Pharmalyte pH 3—10). Its acid hydrolysis gave the amino acids in the ratios predicted by theory and its purity was further ascertained by enzymatic digestion.

When tested by *in vitro* assay according to Fukata *et al.*[28] our synthetic sauvagine stimulated secretion of immunoreactive corticotropin from rat pituitary cells and its response was judged to be equivalent to those of synthetic oCRF and rat CRF (Table II).

Next, arterial blood pressure was measured directly in pentobarbitone-anesthetized rats. The intravenous administration of synthetic sauvagine (23.6×10^{-7} g/kg) displayed a dose-dependent and long-lasting hypotensive action. With regard to the changes in blood pressure (molar basis), synthetic sauvagine is about twice as potent as synthetic substance P.

Experimental

General experimental procedures employed in this investigation are essentially the same as described in the hCRF synthesis.[9] For brevity, experimental scales in the preparations of di- and tripeptides are omitted.

N^{α}-Deprotection——The N^{α}-protecting group, Z(OMe), was cleaved by TFA (*ca.* 10 ml per 1 g of a peptide) in the presence of anisole (2 mol eq or more) at ice-bath temperature for 60 min. After evaporation of TFA *in vacuo* at 30 °C or below, the residue was treated with dry ether. If a powder was obtained, it was collected by filtration, dried over KOH pellets *in vacuo* for 3 h and then used for the condensation reaction. If an oily precipitate was obtained, it was washed with *n*-hexane, dried over KOH pellets *in vacuo* for 3 h and then used for the condensation reaction.

Condensation Reactions——Every reaction was continued until the solution became negative to the ninhydrin test. The active ester reaction was performed at room temperature (17—25 °C). The azide was prepared with isoamyl nitrite and usually the reaction was conducted at 4 °C. An MA was prepared with isobutyl chloroformate and the reaction was performed in an ice-bath.

Purification——Unless otherwise stated, products were purified by one of the following procedures.

Procedure A: For purification of protected peptide esters soluble in AcOEt, the extract was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O, dried over Na_2SO_4 and concentrated. The residue was recrystallized or precipitated from appropriate solvents.

Procedure B: For purification of protected peptides less soluble in AcOEt, the crude product was triturated with ether and 5% citric acid. The resulting powder was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O and recrystallized or precipitated from appropriate solvents.

TLC was performed on silica gel (Kieselgel G, Merck). *Rf* values refer to the following solvent systems (v/v): Rf_1 $CHCl_3$–MeOH–H_2O (8 : 3 : 1), Rf_2 $CHCl_3$–MeOH (10 : 0.5), Rf_3 $CHCl_3$–MeOH (9 : 1), Rf_4 *n*-BuOH–AcOH–pyridine–H_2O (4 : 1 : 1 : 2), Rf_5 *n*-BuOH–AcOH–AcOEt–H_2O (1 : 1 : 1 : 1).

HPLC was conducted with a Waters compact model 204 and leucine-aminopeptidase (LAP) was purchased from Sigma (lot No. 62F-8000).

Z(OMe)–Thr–Ile–NH_2——The title compound was prepared by the azide procedure and purified by procedure B, followed by precipitation from DMF with AcOEt; yield 83%, mp 194—196 °C, $[\alpha]_D^{15}$ +17.0° (c = 1.0, DMF), Rf_1 0.78. *Anal.* Calcd for $C_{19}H_{29}N_3O_6$: C, 57.70; H, 7.39; N, 10.63. Found: C, 57.72; H, 7.45; N, 10.46.

Z(OMe)–Asp(OBzl)–Thr–Ile–NH_2——The title compound was prepared by condensation of Z(OMe)–Asp(OBzl)–ONp with a TFA-treated sample of the above dipeptide amide and purified by procedure B, followed by precipitation from DMF with AcOEt; yield 74%, mp 189—191 °C, $[\alpha]_D^{15}$ +2.1° (c = 0.9, DMF), Rf_1 0.70. *Anal.* Calcd for $C_{30}H_{40}N_4O_9 \cdot H_2O$: C, 58.24; H, 6.84; N, 9.06. Found: C, 58.20; H, 6.87; N, 9.24.

Z(OMe)–Leu–Leu–$NHNH_2$——The title compound was prepared by treatment of Z(OMe)–Leu–Leu–OMe in

MeOH with 80% hydrazine hydrate (5 eq) overnight and purified by recrystallization from MeOH and ether; yield 81%, mp 150—152 °C, $[\alpha]_D^{15}$ −17.4° (*c* = 1.0, DMF), Rf_1 0.76. *Anal.* Calcd for $C_{21}H_{34}N_4O_5$: C, 59.69; H, 8.11; N, 13.26. Found: C, 59.84; H, 8.17; N, 13.23.

Z(OMe)–Leu–Leu–Asp(OBzl)–Thr–Ile–NH$_2$ [1] (Positions 36—40)——The azide [prepared from 5.91 g (14.0 mmol) of Z(OMe)–Leu–Leu–NHNH$_2$] in DMF (30 ml) and Et_3N (2.3 ml, 16.8 mmol) were added to an ice-chilled solution of a TFA-treated sample of the above tripeptide amide (7.00 g, 11.7 mmol) in DMF (30 ml) and the solution was stirred overnight. The solution was concentrated and the residue was purified by procedure B, followed by precipitation from DMF with MeOH; yield 7.25 g (75%), mp 209—212 °C, $[\alpha]_D^{15}$ −24.2° (*c* = 1.0, DMF), Rf_1 0.67. Amino acid ratios in a 6 N HCl hydrolysate: Leu 2.04, Asp 1.03, Thr 0.94, Ile 1.00 (recovery of Ile, 86%). *Anal.* Calcd for $C_{42}H_{62}N_6O_{11} \cdot H_2O$: C, 59.70; H, 7.63; N, 9.95. Found: C, 59.86; H, 7.78; N, 10.02.

Z(OMe)–Asn–Arg(Mts)–Leu–OMe——The title compound was prepared by reaction of Z(OMe)–Asn–ONp and a TFA-treated sample of Z(OMe)–Arg(Mts)–Leu–OMe and purified by procedure B followed by precipitation from DMF with AcOEt; yield 6.25 g (67%), mp 129—131 °C, $[\alpha]_D^{15}$ −15.8° (*c* = 1.0, DMF), Rf_1 0.72. *Anal.* Calcd for $C_{35}H_{51}N_7O_{10}S$: C, 55.17; H, 6.75; N, 12.87. Found: C, 55.17; H, 6.80; N, 13.04.

Z(OMe)–Asn–Asn–Arg(Mts)–Leu–OMe——A mixture of a TFA-treated sample of the above tripeptide ester (15.00 g, 19.7 mmol), Z(OMe)–Asn–ONp (9.90 g, 23.6 mmol) and Et_3N (6.04 ml, 43.3 mmol) in DMF (100 ml) was stirred overnight. The solvent was removed by evaporation and the residue was purified by procedure B, followed by precipitation from DMF with MeOH; yield 14.81 g (86%), mp 183—185 °C, $[\alpha]_D^{15}$ −15.3° (*c* = 0.9, DMF), Rf_1 0.68. *Anal.* Calcd for $C_{39}H_{57}N_9O_{12}S$: C, 53.47; H, 6.56; N, 14.39. Found: C, 53.22; H, 6.84; N, 14.60.

Z(OMe)–Ala–Asn–Asn–Arg(Mts)–Leu–OMe——An MA [prepared from 1.73 g (6.85 mmol) of Z(OMe)–Ala–OH] in THF (30 ml) was added to an ice-chilled solution of a TFA-treated sample of the above tetrapeptide ester (5.00 g, 5.71 mmol) in DMF (20 ml) containing Et_3N (0.8 ml, 5.71 mmol) and the solution was stirred for 4 h. The solvent was removed by evaporation and the residue was purified by procedure B, followed by precipitation from DMF with MeOH; yield 4.10 g (76%), mp 208—209 °C, $[\alpha]_D^{15}$ −18.1° (*c* = 1.0, DMF), Rf_1 0.64. *Anal.* Calcd for $C_{42}H_{62}N_{10}O_{13}S$: C, 53.26; H, 6.60; N, 14.79. Found: C, 52.99; H, 6.65; N, 14.93.

Z(OMe)–Ala–Asn–Asn–Arg(Mts)–Leu–NHNH$_2$ [2] (Positions 31—35)——The above pentapeptide ester (3.35 g, 3.54 mmol) in DMF–MeOH (30 ml–20 ml) was treated with 80% hydrazine hydrate (1.08 ml, 5 eq) overnight. The solvent was removed by evaporation and the residue was precipitated from DMF with MeOH; yield 2.94 g (88%), mp 206—209 °C, $[\alpha]_D^{15}$ −14.2° (*c* = 0.8, DMF), Rf_1 0.42. Amino acid ratios in a 6 N HCl hydrolysate: Ala 1.09, Asp 2.04, Arg 1.03, Leu 1.00 (recovery of Leu, 88%). *Anal.* Calcd for $C_{41}H_{62}N_{12}O_{12}S$: C, 51.99; H, 6.60; N, 17.75. Found: C, 51.81; H, 6.66; N, 17.70.

Z(OMe)–Gln–Gln–Ala–NHNH$_2$ [3] (Positions 28—30)——Z(OMe)–Gln–Gln–Ala–OMe in DMSO–MeOH (1 : 1) was treated with 80% hydrazine hydrate (5 eq) overnight. After evaporation of the solvent, the residue was treated with EtOH and the resulting powder was precipitated from DMSO with MeOH; yield 93%, mp 235—238 °C, $[\alpha]_D^{15}$ −22.4° (*c* = 0.6, DMSO), Rf_4 0.65. Amino acid ratios in a 6 N HCl hydrolysate: Glu 1.96, Ala 1.00 (recovery of Ala, 84%). *Anal.* Calcd for $C_{22}H_{33}N_7O_8 \cdot H_2O$: C, 48.80; H, 6.51; N, 18.11. Found: C, 48.76; H, 6.20; N, 18.20.

Z(OMe)–Lys(Z)–Glu(OBzl)–Lys(Z)–NHNH–Troc——The title compound was prepared by reaction of Z(OMe)–Lys(Z)–OSu with a TFA-treated sample of Z(OMe)–Glu(OBzl)–Lys(Z)–NHNH–Troc and purified by procedure B, followed by recrystallization from MeOH and ether; yield 60%, mp 122—124 °C, $[\alpha]_D^{20}$ −13.1° (*c* = 1.0, DMF), Rf_2 0.31. *Anal.* Calcd for $C_{52}H_{62}Cl_3N_7O_{14}$: C, 55.99; H, 5.60; N, 8.79. Found: C, 55.77; H, 5.78; N, 9.04.

Z(OMe)–Glu(OBzl)–Lys(Z)–Glu(OBzl)–Lys(Z)–NHNH–Troc——A mixture of a TFA-treated sample of the above tripeptide derivative (16.40 g, 14.7 mmol), Z(OMe)–Glu(OBzl)–ONp (9.22 g, 17.6 mmol) and Et_3N (4.51 ml 32.3 mmol) was stirred for 15 h and concentrated. The residue was purified by procedure B, followed by precipitation from DMF with AcOEt; yield 16.70 g (85%), mp 142—144 °C, $[\alpha]_D^{20}$ −9.8° (*c* = 1.0, DMF), Rf_2 0.32. *Anal.* Calcd for $C_{64}H_{75}Cl_3N_8O_{17}$: C, 57.59; H, 5.66; N, 8.40. Found: C, 57.55; H, 5.61; N, 8.67.

Z(OMe)–Gln–Glu(OBzl)–Lys(Z)–Glu(OBzl)–Lys(Z)–NHNH–Troc——A mixture of a TFA-treated sample of the above tetrapeptide derivative (3.10 g, 2.32 mmol), Z(OMe)–Gln–ONp (1.20 g, 2.78 mmol) and Et_3N (0.71 ml, 5.10 mmol) was stirred for 15 h and concentrated. The residue was purified by procedure B, followed by precipitation from DMF with MeOH; yield 2.30 g (68%), mp 188—190 °C, $[\alpha]_D^{15}$ −7.2° (*c* = 1.0, DMF), Rf_1 0.89. *Anal.* Calcd for $C_{69}H_{83}Cl_3N_{10}O_{19}$: C, 56.65; H, 5.72; N, 9.58. Found: C, 56.35; H, 5.76; N, 9.87.

Z(OMe)–Gln–Glu(OBzl)–Lys(Z)–Glu(OBzl)–Lys(Z)–NHNH$_2$ [4] (Positions 23—27)——The above pentapeptide derivative (2.62 g, 1.79 mmol) in DMF–AcOH (30 ml–5 ml) was treated with Zn powder (1.17 g, 10 eq) at room temperature for 6 h. The solution was filtered, the filtrate was concentrated and the residue was treated with 5% EDTA to form a powder, which was washed with H_2O and precipitated from DMF with MeOH; yield 1.65 g (72%), mp 204—206 °C, $[\alpha]_D^{15}$ −8.0° (*c* = 1.0, DMF), Rf_1 0.70. Amino acid ratios in a 6 N HCl hydrolysate: Glu 3.21, Lys 2.00 (recovery of Lys, 82%). *Anal.* Calcd for $C_{66}H_{82}Cl_3N_{10}O_{17} \cdot H_2O$: C, 60.21; H, 6.51; N, 10.64. Found: C, 60.43; H, 6.30; N, 10.30.

Z(OMe)–Ile–Glu(OBzl)–Lys(Z)–NHNH–Troc——An MA [prepared from 3.53 g (12.0 mmol) of Z(OMe)–Ile–OH] in THF (30 ml) was added to an ice-chilled solution of a TFA-treated sample of Z(OMe)–Glu(OBzl)–Lys(Z)–NHNH–Troc (8.50 g, 10.0 mmol) in DMF (50 ml) containing Et_3N (1.39 ml, 10.0 mmol) and the solution was stirred

in an ice-bath for 4 h. The solvent was removed by evaporation and the residue was purified by procedure B, followed by recrystallization from MeOH and ether; yield 6.27 g (65%), mp 126—127 °C, $[\alpha]_D^{15}$ −11.6° (c=1.0, DMF), Rf_2 0.32. *Anal.* Calcd for $C_{44}H_{55}Cl_3N_6O_{12}$: C, 54.69; H, 5.74; N, 8.70. Found: C, 54.96; H, 5.72; N, 8.69.

Z(OMe)–Glu(OBzl)–Ile–Glu(OBzl)–Lys(Z)–NHNH–Troc——A mixture of a TFA-treated sample of Z(OMe)–Ile–Glu(OBzl)–Lys(Z)–NHNH–Troc (14.40 g, 14.9 mmol), Z(OMe)–Glu(OBzl)–ONp (9.34 g, 17.9 mmol) and Et_3N (4.57 ml, 32.8 mmol) in DMF (70 ml) was stirred for 15 h and concentrated. The residue was purified by procedure B, followed by precipitation from DMF with AcOEt; yield 15.60 g (88%), mp 148—150 °C, $[\alpha]_D^{15}$ −15.5° (c=0.8, DMF), Rf_2 0.27. *Anal.* Calcd for $C_{56}H_{68}Cl_3N_7O_{15}$: C, 56.73; H, 5.78; N, 8.27. Found: C, 57.29; H, 5.92; N, 8.45.

Z(OMe)–Ile–Glu(OBzl)–Ile–Glu(OBzl)–Lys(Z)–NHNH–Troc——An MA [prepared from 4.48 g (15.2 mmol) of Z(OMe)–Ile–OH] in THF (50 ml) was added to an ice-chilled solution of a TFA-treated sample of the above tetrapeptide derivative (15.00 g, 12.7 mmol) in DMF (50 ml) and the solution was stirred for 4 h. The residue was purified by procedure B, followed by precipitation from DMF with MeOH; yield 11.90 g (72%), mp 182—183 °C, $[\alpha]_D^{20}$ −7.2° (c=1.0, DMF), Rf_2 0.26. *Anal.* Calcd for $C_{62}H_{79}Cl_3N_8O_{16}$: C, 57.34; H, 6.13; N, 8.63. Found: C, 57.60; H, 6.31; N, 8.80.

Z(OMe)–Ile–Glu(OBzl)–Ile–Glu(OBzl)–Lys(Z)–NHNH$_2$ [5] (Positions 18—22)——The above Troc-derivative (5.00 g, 3.85 mmol) in DMF–AcOH (50 ml–5 ml) was treated with Zn powder (2.5 g, 10 eq) at room temperature for 6 h. The solution was filtered, the filtrate was concentrated and the residue was treated with 5% EDTA to form a powder, which was washed with H_2O and precipitated from DMF with MeOH; yield 3.48 g (81%), mp 217—218 °C, $[\alpha]_D^{15}$ −8.0° (c=1.0, DMF), Rf_1 0.63. Amino acid ratios in a 6 N HCl hydrolysate: Glu 2.09, Ile 2.11, Lys 1.00 (recovery of Lys, 81%). *Anal.* Calcd for $C_{59}H_{78}N_8O_{14}\cdot H_2O$: C, 62.09; H, 7.07; N, 9.82. Found: C, 62.32; H, 6.98; N, 9.86.

Z(OMe)–Arg(Mts)–Lys(Z)–Met–OMe——Z(OMe)–Arg(Mts)–OH was condensed with a TFA-treated sample of Z(OMe)–Lys(Z)–Met–OMe *via* the MA and the product was purified by procedure A, followed by recrystallization from MeOH and ether; yield 88%, mp 106—107 °C, $[\alpha]_D^{15}$ −15.5° (c=1.0, MeOH), Rf_1 0.85. *Anal.* Calcd for $C_{44}H_{61}N_7O_{11}S_2$: C, 56.94; H, 6.63; N, 10.57. Found: C, 56.65; H, 6.54; N, 10.55.

Z(OMe)–Arg(Mts)–Lys(Z)–Met–NHNH$_2$ [6] (Positions 15—17)——The above protected tripeptide derivative in MeOH was treated with 80% hydrazine hydrate (5 eq) overnight, then the solvent was removed by evaporation. Treatment of the residue with H_2O afforded a powder, which was precipitated from DMF with AcOEt; yield 76%, mp 133—135 °C, $[\alpha]_D^{15}$ −5.7° (c=1.1, DMF), Rf_1 0.70. Amino acid ratios in a 6 N HCl hydrolysate: Met 0.79, Lys 1.00, Arg 1.03 (recovery of Lys, 85%). *Anal.* Calcd for $C_{43}H_{61}N_9O_{10}S_2$: C, 55.64; H, 6.62; N, 13.58. Found: C, 55.70; H, 6.57; N, 13.54.

Z(OMe)–Leu–Leu–NHNH–Troc——Z(OMe)–Leu–OH was condensed with a TFA-treated sample of Z(OMe)–Leu–NHNH–Troc *via* the MA and the product was purified by procedure A, followed by recrystallization from AcOEt and isopropyl ether; yield 63%, mp 122—124 °C, $[\alpha]_D^{15}$ −44.1° (c=1.0, MeOH), Rf_2 0.60. *Anal.* Calcd for $C_{24}H_{35}Cl_3N_4O_7$: C, 48.21; H, 5.90; N, 9.37. Found: C, 48.61; H, 5.96; N, 9.61.

Z(OMe)–Glu(OBzl)–Leu–Leu–NHNH–Troc——Z(OMe)–Glu(OBzl)–OH was condensed with a TFA-treated sample of the above dipeptide derivatives by the Np method and the product was purified by procedure A, followed by recrystallization from AcOEt and ether; yield 57%, mp 104—105 °C, $[\alpha]_D^{15}$ −51.0° (c=1.0, MeOH), Rf_2 0.52. *Anal.* Calcd for $C_{36}H_{48}Cl_3N_5O_{10}$: C, 52.91; H, 5.92; N, 8.57. Found: C, 53.20; H, 6.10; N, 8.67.

Z(OMe)–Ser–Leu–OMe——The title compound was prepared by the azide method and purified by procedure A, followed by recrystallization from AcOEt and isopropyl ether; yield 76%, mp 71—72 °C, $[\alpha]_D^{15}$ −9.1° (c=1.0, DMF), Rf_2 0.28. *Anal.* Calcd for $C_{19}H_{28}N_2O_7$: C, 57.56; H, 7.12; N, 7.07. Found: C, 57.65; H, 7.05; N, 7.07.

Z(OMe)–Ser–Leu–NHNH$_2$——The above dipeptide ester in MeOH was treated with 80% hydrazine hydrate (5 eq) as usual and the product was precipitated from DMF with MeOH; yield 85%, mp 212—214 °C, $[\alpha]_D^{15}$ −18.4° (c=0.8, DMF), Rf_1 0.56. *Anal.* Calcd for $C_{18}H_{28}N_4O_6$: C, 54.53; H, 7.12; N, 14.13. Found: C, 54.27; H, 7.05; N, 14.11.

Z(OMe)–Ser–Leu–Glu(OBzl)–Leu–Leu–NHNH–Troc——The azide [prepared from 4.12 g (10.4 mmol) of Z(OMe)–Ser–Leu–NHNH$_2$] in DMF (20 ml) was added to an ice-chilled solution of a TFA-treated sample of Z(OMe)–Glu(OBzl)–Leu–Leu–NHNH–Troc (7.71 g, 9.44 mmol) in DMF (50 ml) containing Et_3N (3.05 ml, 21.9 mmol) and the solution was stirred for 48 h. The solvent was evaporated off and the residue was purified by procedure B, followed by precipitation from DMF with AcOEt; yield 6.02 g (63%), mp 200—202 °C, $[\alpha]_D^{15}$ −33.7° (c=1.0, DMF), Rf_3 0.62. *Anal.* Calcd for $C_{45}H_{64}Cl_3N_7O_{13}$: C, 53.12; H, 6.34; N, 9.64. Found: C, 53.05; H, 6.39; N, 9.78.

Z(OMe)–Ile–Asp(OBzl)–Leu–NHNH–Troc——Z(OMe)–Ile–OH was condensed with a TFA-treated sample of Z(OMe)–Asp(OBzl)–Leu–NHNH–Troc by the MA method and the product was purified by procedure B, followed by recrystallization from MeOH and ether; yield 12.80 g (55%), mp 168—170 °C, $[\alpha]_D^{15}$ −27.2° (c=1.1, DMF), Rf_2 0.33. *Anal.* Calcd for $C_{35}H_{46}Cl_3N_5O_{10}$: C, 52.34; H, 5.77; N, 8.72. Found: C, 52.53; H, 5.83; N, 8.54.

Z(OMe)–Ile–Asp(OBzl)–Leu–NHNH$_2$——The above Troc derivative in DMF–AcOH (10 : 1) was treated with Zn powder as described in the preparation of fragment [5] and purified by precipitation from DMF with MeOH–AcOEt (1 : 1); yield 68%, mp 214—216 °C, $[\alpha]_D^{15}$ −27.9° (c=1.0, DMF), Rf_1 0.68. Amino acid ratios in a 6 N HCl

hydrolysate: Asp 0.97; Ile 0.97; Leu 1.00 (recovery of Leu, 92%). *Anal.* Calcd for $C_{32}H_{45}N_5O_8 \cdot H_2O$: C, 59.52; H, 7.34; N, 10.90. Found: C, 59.32; H, 7.18; N, 11.29.

Z(OMe)–Ile–Asp(OBzl)–Leu–Ser–Leu–Glu(OBzl)–Leu–Leu–NHNH–Troc——The azide [prepared from 2.96 g (4.72 mmol) of Z(OMe)–Ile–Asp(OBzl)–Leu–$NHNH_2$] in DMF–DMSO (1 : 1, 20 ml) was added to a solution of a TFA-treated sample of Z(OMe)–Ser–Leu–Glu(OBz)–Leu–Leu–NHNH–Troc (4.00 g, 3.93 mmol) in DMF (20 ml) containing Et_3N (1.34 ml, 9.59 mmol) and the mixture was stirred for 72 h. The solvent was removed by evaporation and the product was purified by procedure B, followed by precipitation from DMF with AcOEt; yield 3.02 g (53%), mp 267 °C dec., $[\alpha]_D^{15}$ −32.5°, Rf_3 0.31. *Anal.* Calcd for $C_{68}H_{97}Cl_3N_{10}O_{18}$: C, 56.37; H, 6.75; N, 9.67. Found: C, 56.07; H, 6.95; N, 9.86.

Z(OMe)–Ile–Asp(OBzl)–Leu–Ser–Leu–Glu(OBzl)–Leu–Leu–$NHNH_2$ [7] (Positions 7—14)——The above Troc-derivative (3.00 g, 2.07 mmol) in DMF–AcOH (30 ml–5 ml) was treated with Cd powder (2.33 g, 10 eq) at room temperature for 40 h. The product was isolated as described in the preparation of fragment [5] and purified by precipitation from DMF with MeOH; yield 1.63 g (62%), mp 285 °C dec., $[\alpha]_D^{15}$ −33.4° (c=0.7, DMF), Rf_1 0.72. Amino acid ratios in a 6 N HCl hydrolysate: Asp 1.02, Ser 0.99, Glu 1.05, Ile 1.00, Leu 4.17 (recovery of Ile, 98%). *Anal.* Calcd for $C_{65}H_{96}N_{10}O_{16} \cdot H_2O$: C, 60.44; H, 7.65; N, 10.85. Found: C, 60.30; H, 7.57; N, 10.97.

Z(OMe)–Gly–Pro–Pro–Ile–Ser(Bzl)–NHNH–Troc——A mixture of Z(OMe)–Gly–ONp (3.03 g, 8.41 mmol), Et_3N (2.15 ml, 15.4 mmol) and a TFA-treated sample of Z(OMe)–Pro–Pro–Ile–Ser(Bzl)–NHNH–Troc (6.00 g, 7.0 mmol) in DMF (50 ml) was stirred for 24 h and concentrated. The product was purified by procedure A, followed by recrystallization from MeOH and ether; yield 4.35 g (68%), mp 147—148 °C, $[\alpha]_D^{15}$ −88.0° (c=0.8, MeOH), Rf_3 0.60. *Anal.* Calcd for $C_{40}H_{52}Cl_3N_7O_{11}$: C, 52.60; H, 5.74; N, 10.74. Found: C, 52.76; H, 5.73; N, 10.79.

Z(OMe)–Pyr–Gly–Pro–Pro–Ile–Ser(Bzl)–NHNH–Troc——A mixture of a TFA-treated sample of the above pentapeptide derivative (3.38 g, 3.70 mmol), Et_3N (1.14 ml, 8.14 mmol) and Z(OMe)–Pyr–OSu (1.00 g, 4.44 mmol) in DMF (50 ml) was stirred for 24 h and concentrated. The product was purified by procedure A, followed by column chromatography on silica gel (4.3 × 12 cm) using $CHCl_3$–MeOH (10 : 0.5) as an eluant. The product was finally recrystallized from MeOH and ether; yield 2.13 g (56%), mp 127—129 °C, $[\alpha]_D^{15}$ −114.1° (c=1.0, MeOH), Rf_3 0.67. *Anal.* Calcd for $C_{45}H_{57}Cl_3N_8O_{13}$: C, 52.76; H, 5.61; N, 10.94. Found: C, 52.89; H, 5.61; N, 10.76.

Z(OMe)–Pyr–Gly–Pro–Pro–Ile–Ser(Bzl)–$NHNH_2$ [8] (Positions 1—6)——The above Troc-derivative (1.80 g, 1.76 mmol) in MeOH–AcOH (10 ml–5 ml) was treated with Zn powder (1.15 g, 10 eq) for 24 h. The solution was filtered, the filtrate was concentrated and the residue was treated with 5% EDTA and 5% $NaHCO_3$. The resulting gummy precipitate was extracted with *n*-BuOH. The organic phase was washed with H_2O, dried over $MgSO_4$ and concentrated. Treatment of the residue with isopropyl ether afforded a powder, which was recrystallized from MeOH and ether; yield 0.97 g (65%), mp 124—126 °C, $[\alpha]_D^{15}$ −96.7° (c=1.0, MeOH), Rf_3 0.59. Amino acid ratios in a 6 N HCl hydrolysate: Ser 0.91, Glu 1.07, Pro 2.20, Gly 0.96, Ile 1.00 (recovery of Ile, 99%). *Anal.* Calcd for $C_{42}H_{56}N_8O_{11} \cdot H_2O$: C, 58.18; H, 6.74; N, 12.93. Found: C, 58.19; H, 6.97; N, 12.40.

Z(OMe)–Ala–Asn–Asn–Arg(Mts)–Leu–Leu–Leu–Asp(OBzl)–Thr–Ile–NH_2 (Position 31—40)——The azide [prepared from 8.82 g (9.31 mmol) of fragment [2]] in DMF (50 ml) and Et_3N (1.56 ml, 11.2 mmol) were added to a TFA-treated sample of fragment [1] (7.00 g, 8.46 mmol) in DMF (35 ml) containing Et_3N (1.18 ml, 8.46 mmol). The mixture was stirred for 24 h and concentrated. The residue was purified by procedure B followed by precipitation from DMF with MeOH; yield 10.32 g (77%), mp 241—242 °C, $[\alpha]_D^{15}$ −15.0° (c=1.0, DMF), Rf_1 0.52. *Anal.* Calcd for $C_{74}H_{112}N_{16}O_{20}S \cdot 2H_2O$: C, 55.07; H, 7.25; N, 13.89. Found: C, 54.98; H, 7.15; N, 14.15.

Z(OMe)–Gln–Gln–Ala–Ala–Asn–Asn–Arg(Mts)–Leu–Leu–Leu–Asp(OBzl)–Thr–Ile–NH_2 (Positions 28—40)——The azide [prepared from 2.98 g (5.70 mmol) of fragment [3]] in DMF–DMSO (1 : 1, 40 ml) and Et_3N (0.95 ml, 6.84 mmol) were added to an ice-chilled solution of a TFA-treated sample of the above decapeptide amide (6.00 g, 3.80 mmol) in DMF (30 ml) containing Et_3N (0.53 ml, 3.80 mmol) and the mixture was stirred for 24 h. H_2O (100 ml) was added and the resulting powder was precipitated from DMSO with MeOH; yield 5.12 g (71%), mp 267 °C dec., $[\alpha]_D^{15}$ −32.3° (c=0.9, DMSO), Rf_1 0.21. *Anal.* Calcd for $C_{87}H_{133}N_{21}O_{25}S \cdot 3H_2O$: C, 53.33; H, 7.15; N, 15.01. Found: C, 53.07; H, 7.01; N, 14.78.

Z(OMe)–Gln–Glu(OBzl)–Lys(Z)–Glu(OBzl)–Lys(Z)–Gln–Gln–Ala–Ala–Asn–Asn–Arg(Mts)–Leu–Leu–Leu–Asp(OBzl)–Thr–Ile–NH_2 (Position 23—40)——The azide [prepared from 1.52 g (1.18 mmol) of fragment [4]] in DMSO–DMF (1 : 1, 10 ml) and Et_3N (0.20 ml, 1.42 mmol) were added to an ice-chilled solution of a TFA-treated sample of the above tridecapeptide amide (1.50 g, 0.79 mmol) in DMF (10 ml) containing Et_3N (0.11 ml, 0.79 mmol) and the mixture was stirred for 24 h. H_2O (100 ml) was added and the resulting powder was precipitated from DMF with AcOEt. This partially purified product was dissolved in a small amount of DMF and the solution was applied to a column of Sephadex LH-20 (3.1 × 134 cm), which was eluted with DMF. Individual fractions (9 ml each) were examined by measurement of the ultraviolet absorption (UV) at 285 nm. The fractions corresponding to the front main peak (tube Nos. 39—48) were combined and the solvent was removed by evaporation. The residue was treated wih AcOEt to afford a powder; yield 1.61 g (68%), mp 252—253 °C, $[\alpha]_D^{15}$ −4.1° (c=1.0, DMF), Rf_1 0.64 *Anal.* Calcd for $C_{144}H_{203}N_{29}O_{39}S \cdot 3H_2O$: C, 56.70; H, 6.91; N, 13.32. Found: C, 56.47; H, 6.85; N, 13.72.

Z(OMe)–Ile–Glu(OBzl)–Ile–Glu(OBzl)–Lys(Z)–Gln–Glu(OBzl)–Lys(Z)–Glu(OBzl)–Lys(Z)–Gln–Gln–Ala–Ala–Asn–Asn–Arg(Mts)–Leu–Leu–Leu–Asp(OBzl)–Thr–Ile–NH_2 (Position 18—40)——The azide [prepared from

869 mg (0.77 mmol) of fragment [5]] in DMF (20 ml) and Et_3N (129 μl, 0.93 mmol) were added to an ice-chilled solution of a TFA-treated sample of the above octadecapeptide amide (1.16 g, 0.39 mmol) in DMF (10 ml) containing Et_3N (54 μl, 0.39 mmol) and the mixture was stirred for 24 h. H_2O (100 ml) was added and the resulting powder was purified by precipitation from DMF with AcOEt, followed by gel-filtration on Sephadex LH-20 as described above; yield 1.03 g (68%), mp 238—240 °C, $[\alpha]_D^{15}$ −4.3° (c=0.9, DMF), Rf_1 0.67. *Anal.* Calcd for $C_{194}H_{269}N_{35}O_{50}S \cdot 5H_2O$: C, 58.05; H, 7.04; N, 12.22. Found: C, 58.12; H, 6.94; N, 12.31.

Z(OMe)–Arg(Mts)–Lys(Z)–Met–Ile–Glu(OBzl)–Ile–Glu(OBzl)–Lys(Z)–Gln–Glu(OBzl)–Lys(Z)–Glu(OBzl)–Lys(Z)–Gln–Gln–Ala–Ala–Asn–Asn–Arg(Mts)–Leu–Leu–Leu–Asp(OBzl)–Thr–Ile–NH_2 (Positions 15—40)——The azide [prepared from 473 mg (0.51 mmol) of fragment [6]] in DMF (5 ml) and Et_3N (85 μl, 0.61 mmol) were added to an ice-chilled solution of a TFA-treated sample of the above tricosapeptide amide (1.00 g, 0.26 mmol) in DMF (10 ml) containing Et_3N (36 μl, 0.225 mmol) and the mixture was stirred for 48 h. H_2O (100 ml) was added and the residue was purified by precipitation from DMF with AcOEt, followed by gel-filtration on Sephadex LH-60. The product was isolated as stated above; yield 720 mg (61%), mp 125—127 °C, $[\alpha]_D^{15}$ −28.3° (c=0.7, DMF), Rf, 0.82. *Anal.* Calcd for $C_{228}H_{318}N_{42}O_{57}S_3 \cdot 2H_2O$: C, 58.39; H, 6.92; N, 12.54. Found: C, 58.10; H, 7.02; N, 12.55.

Z(OMe)–Ile–Asp(OBzl)–Leu–Ser–Leu–Glu(OBzl)–Leu–Leu–Arg(Mts)–Lys(Z)–Met–Ile–Glu(OBzl)–Ile–Glu(OBzl)–Lys(Z)–Gln–Glu(OBzl)–Lys(Z)–Glu(OBzl)–Lys(Z)–Gln–Gln–Ala–Ala–Asn–Asn–Arg(Mts)–Leu–Leu–Leu–Asp(OBzl)–Thr–Ile–NH_2 (Positions 7—40)——The azide [prepared from 499 mg (0.39 mmol) of fragment [7]] in DMF–DMSO (1 : 1, 20 ml) and Et_3N (66 μl, 0.47 mmol) were added to an ice-chilled solution of a TFA-treated sample of the above hexacosapeptide amide (365 mg, 78.4 μmol) in DMF (10 ml) containing Et_3N (11 μl, 78.4 μmol) and the mixture was stirred at −18 °C for 72 h. H_2O (100 ml) was added and the resulting powder was purified by precipitation from DMF with AcOEt, followed by gel-filtration on Sephadex LH-60. The product was isolated as stated above; yield 325 mg (72%), mp 136—138 °C, $[\alpha]_D^{15}$ −4.7° (c=0.9, DMF), Rf_1 0.86. *Anal.* Calcd for $C_{284}H_{402}N_{50}O_{70}S_3 \cdot 9H_2O$: C, 57.89; H, 7.18; N, 11.88. Found: C, 57.70; H, 6.96; N, 12.32.

Z(OMe)–Pyr–Gly–Pro–Pro–Ile–Ser(Bzl)–Ile–Asp(OBzl)–Leu–Ser–Leu–Glu(OBzl)–Leu–Leu–Arg(Mts)–Lys(Z)–Met–Ile–Glu(OBzl)–Ile–Glu(OBzl)–Lys(Z)–Gln–Glu(OBzl)–Lys(Z)–Glu(OBzl)–Lys(Z)–Gln–Gln–Ala–Ala–Asn–Asn–Arg(Mts)–Leu–Leu–Leu–Asp(OBzl)–Thr–Ile–NH_2 (Positions 1—40, Protected Sauvagine)——The azide [prepared from 358 mg (0.42 mmol) of fragment [8]] in DMF (10 ml) and Et_3N (70 μl, 0.51 mmol) were added to an ice-chilled solution of a TFA-treated sample of the above protected tetratriacontapeptide amide (483 mg, 84.3 μmol) in DMF (7 ml) containing Et_3N (12 μl, 84.3 μmol) and the mixture was stirred at −18 °C for 72 h. H_2O (100 ml) was added and the resulting powder was purified by precipitation from DMF with AcOEt followed by gel-filtration on Sephadex LH-60 as stated above; yield 240 mg (45%), mp 140—141 °C, $[\alpha]_D^{15}$ −19.3° (c=0.2, DMF), Rf_1 0.70. *Anal.* Calcd for $C_{317}H_{446}N_{56}O_{78}S_3 \cdot 7H_2O$: C, 58.47; H, 7.12; N, 12.05. Found: C, 58.17; H, 7.15; N, 12.41.

Synthetic Sauvagine——Protected sauvagine (40 mg) was treated with 1 M TMSOTf–thioanisole/TFA (4 ml) in the presence of *m*-cresol (46 μl, 70 eq) in an ice-bath for 120 min, then dry ether was added. The resulting powder was collected by centrifugation, dried over KOH pellets *in vacuo* for 2 h and dissolved in MeOH–H_2O (1 ml–5 ml). The pH of the solution was adjusted to 8.0 with 5% NH_4OH in an ice-bath and 1 M NH_4F (300 μl, 48 eq) was added. After 30 min, the pH was readjusted to 6.0 with 1 N AcOH and the solution was incubated with dithiothreitol (97 mg, 100 eq) at 37 °C for 12 h. The solution was applied to a column of Sephadex G-50 (2.4 × 133 cm), which was eluted with 1 N AcOH. The fractions (8 ml each) corresponding to the front main peak (tube Nos. 25—33, monitored by Folin–Lowry test[29]) were collected and the solvent was removed by lyophilization to give a powder; yield 25 mg (87%). The product was next applied to a column of Sephadex G-25 (4.5 × 20 cm) equilibrated with the lower phase of *n*-BuOH–AcOH–H_2O (4 : 1 : 5) and the column was developed with the upper phase of the above solvent (336 ml), then with the lower phase (200 ml). The fractions (8 ml each) corresponding to the main peak (Fig. 7, tube Nos. 51—63, monitored by Folin–Lowry test) were combined, the solvent was removed by evaporation and the residue was lyophilized to give a powder; yield 9 mg (31%). The final purification was carried out by HPLC. The sample (5 mg) in 1 N AcOH (1 ml) was applied to a Nucleosil 5C18 column (10 × 250 mm), which was eluted with a gradient of acetonitrile (35—50%, 45 min) in 0.1% TFA at a flow rate of 2 ml/min. The eluate corresponding to the main peak (Fig. 8a; retention time, 32.8 min, monitored by UV absorption measurement at 233 nm) was collected, the solvent

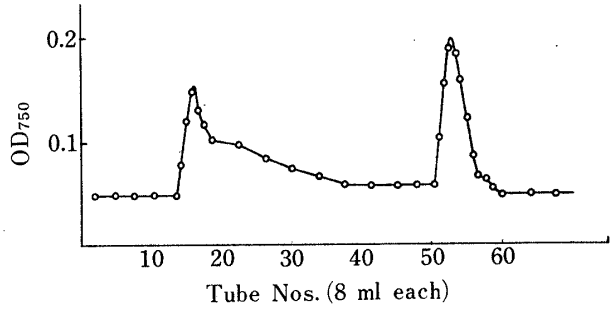

Fig. 7. Partition Chromatography of Synthetic Sauvagine

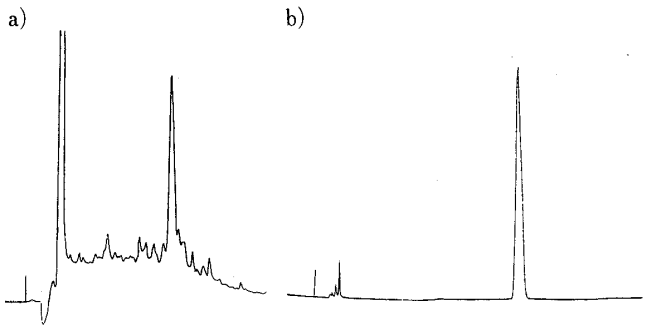

Fig. 8. HPLC of Synthetic Sauvagine (OD at 233 nm)

a) Partition-purified sample.
b) HPLC purified sample.

was removed by evaporation and the residue was lyophilized to give a fluffy white powder; yield 0.95 mg (19%). The rest of the sample was similarly purified; total yield 1.71 mg (5.1%, from the protected peptide); single spot on TLC: Rf_4 0.35, Rf_5 0.46, $[\alpha]_D^{20}$ $-66.2°$ ($c=0.3$, 1 N AcOH); retention time on an analytical Nucleosil 5C18 column (4 × 150 mm): 20.8 min (Fig. 8b) on gradient elution with acetonitrile (30—50%, 30 min) in 0.1% TFA at a flow rate of 1.0 ml/min; mobility on polyacrylamide gel containing pharmalyte, pH 3.0—10.0, was 4.6 cm from the origin toward the cathodic end of the gel, after running for 4 h at 200 V (stained with Coomassie Brilliant Blue G-250, Sigma). Amino acid ratios in a 6 N HCl hydrolysate are listed in Table I. Amino acid ratios in a LAP digest: Asp 1.92 (2), Ser 1.29 (2), Glu 4.13 (5), Gly 0.87 (1), Ala 2.00 (2), Met 0.84 (1), Ile 3.39 (5), Leu 7.05 (7), Lys 3.85 (4), Arg 1.96 (2), Thr, Pro. Asn and Gln were not determined (recovery of Ala 68%; low recovery of Ile linked to Pro seems to be due to poor prolidase-like activity of this LAP preparation).

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2) Amino acids used in this investigation are of the L-configuration. The following abbreviations are used: Z = benzyloxycarbonyl, Z(OMe) = *p*-methoxybenzyloxycarbonyl, Mts = mesitylenesulfonyl, Bzl = benzyl, Troc = 2,2,2-trichloroethyloxycarbonyl, Np = *p*-nitrophenyl, Su = *N*-hydroxysuccinimidyl, TFA = trifluoroacetic acid, TMSOTf = trimethylsilyl trifluoromethanesulfonate, DMF = dimethylformamide, DMSO = dimethylsulfoxide, THF = tetrahydrofuran, Pyr = pyroglutamyl, CM = carboxymethyl, EDTA = ethylenediaminetetraacetic acid.
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