Synthesis of a Protected Tridecapeptide Corresponding to Positions 17—29 of Human Lysozyme

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For a semi-synthesis of human lysozyme by coupling its natural fragment with synthetic peptides, a protected tridecapeptide corresponding to positions 17—29 of the enzyme was prepared from three protected peptide fragments (II, VI, and XI) by the conventional method in pure form on the basis of analyses.

Human lysozyme[1] contains two residues of methionine at the positions 17 and 29 of the peptide chain of the enzyme.[2,3] Cleavage by cyanogen bromide[4] of the methionyl bonds in the enzyme followed by reductive alkylation[5] of the disulfide linkages give a fragment lacking the 29 amino acid residues at the N-terminus of the enzyme. The natural fragment is considered to be a suitable material for a semi-synthesis,[6,7] as described in the preceding paper.[8] Coupling of the natural fragment with synthetic peptides *via* the formation of peptide linkages will give a covalent semi-synthesis of human lysozyme. For the aim, we have attempted to isolate the natural fragment[5] and synthesize complementary peptide fragments. The peptide fragments refer to the N-terminal region corresponding to positions 1—29 of human lysozyme, which is liberated from the enzyme by treatment with cyanogen bromide. The N-terminal section of the enzyme was divided into two parts and synthesized; a hexadecapeptide[8] corresponding to positions 1—16 and a tridecapeptide corresponding to positions 17—29. This paper reports the synthesis of the latter peptide.

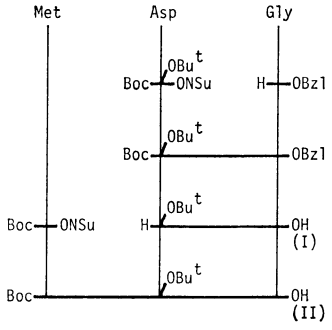

Fig. 1. Synthesis of sequence 17—19.

The peptide was constructed from three peptide fragments, Boc–Met–Asp(OBu^t)–Gly–OH (II),[9] Boc–Tyr–Arg(Tos)–Gly–Ile–Ser–OMe (VI), and Boc–Leu–Ala–Asn–Trp–Met–OMe (XI). The first fragment was synthesized by coupling Boc–Met–ONSu[10] with H–Asp(OBu^t)–Gly–OH (I) (Fig. 1). The dipeptide (I) was obtained by the catalytic hydrogenation of the syrupy material, Z–Asp(OBu^t)–Gly–OBzl, which was prepared by condensing Z–Asp(OBu^t)–ONSu[11] with H–Gly–OBzl. The synthesis of the second fragment, Boc–Tyr–Arg(Tos)–Gly–Ile–Ser–OMe (VI), was started by coupling Z–Ile–ONp[12] with H–Ser–OMe (Fig. 2). Z–Ile–Ser–OMe (III) thus obtained was catalytically hydrogenated and the resulting dipeptide methyl ester was

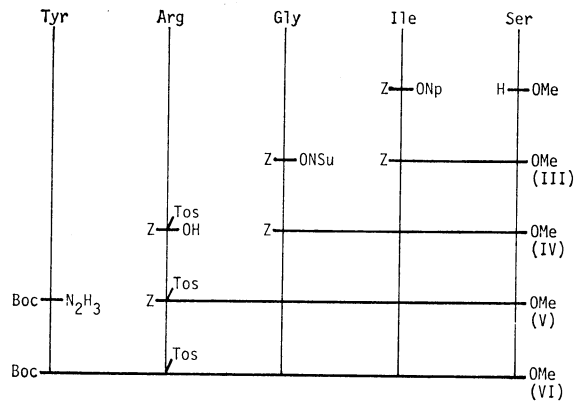

Fig. 2. Synthesis of sequence 20—24.

acylated with Z–Gly–ONSu[10] to give Z–Gly–Ile–Ser–OMe (IV). The tripeptide (IV) was catalytically hydrogenated and condensed with Z–Arg(Tos)–OH[13] by DCC in the presence of HOBt.[14] The resulting tetrapeptide, Z–Arg(Tos)–Gly–Ile–Ser–OMe (V), was catalytically hydrogenated. The tetrapeptide methyl ester was not isolated, but directly coupled with Boc–Tyr–N_3 prepared from the corresponding hydrazide,[15] to give Boc–Tyr–Arg(Tos)–Gly–Ile–Ser–OMe (VI).

For the synthesis of the last fragment, Boc–Leu–Ala–Asn–Trp–Met–OMe (XI), Boc–Asn–ONp[16] was first coupled with H–Trp–OMe. The resulting dipeptide, Boc–Asn–Trp–OMe (VII), was converted to the corresponding hydrazide (VIII) by usual hydrazinolysis. The hydrazide (VIII) was allowed to react with isopentyl nitrite by Rudinger's method,[17] the resulting

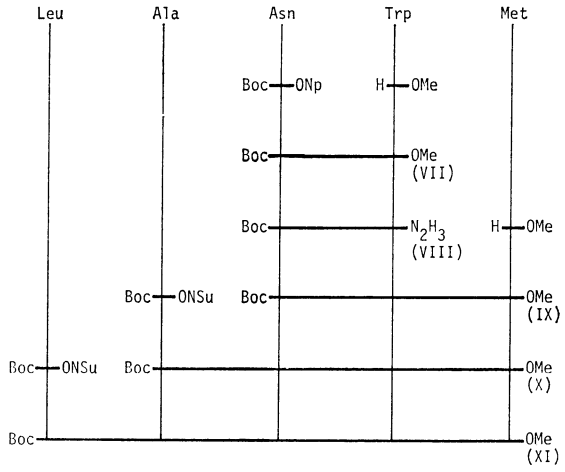

Fig. 3. Synthesis of sequence 25—29.

Table 1. Amino acid analyses of hydrolysates of oligopeptides containing the Asn–Trp linkage

1 2 3 4 5[a]	Acid hydrolysis[b]					Enzymatic hydrolysis				
	1	2	3	4	5	1	2	3	4	5
Boc–Asn–Trp–Gly–OEt			0.78	0.58	1.00					
H–Asn–Trp–Gly–OH			0.81	0.78	1.00			1.01	0.97	1.00
Z–Asn–Trp–Ile –OBzl			0.67	0.45	1.00					
Boc–Asn–Trp–Phe–OEt			0.65	0.50	1.00					
Z–Ala–Asn–Trp–OMe		1.00	0.82	—[c]						
Boc–Leu–Ala–Asn–Trp–OMe	0.97	1.00	0.69	—[c]						
Boc–Ala–Asn–Trp–Met–OMe		1.00	0.64	—[c]	0.59					
Boc–Leu–Ala–Asn–Trp–Met–OMe	0.99	1.00	0.48	—[c]	0.56					
	0.96	1.00	0.59	—[c]	0.59[d]					
H–Leu–Ala–Asn–Trp–Met–OH	0.92	1.00	0.84	—[c]	0.88	1.00	1.00	0.96	1.07	1.05
	0.92	1.00	0.79	—[c]	0.84[d]					

a) Residue number from N-terminus. b) Value obtained after hydrolysis for 24 h. c) Not determined.
d) Value obtained after hydrolysis for 72 h.

Table 2. Analytical data of synthetic peptides containing the Asn–Trp linkage

	Mp (°C)	$[\alpha]_D^{23}$		Analysis (%) C	H	N
Boc–Asn–Trp–Gly–OEt	106—108	−21.9° (*c* 1.0, DMF)	Found	57.18	6.69	13.58
			Calcd	57.24	6.61	13.19
Z–Asn–Trp–Ile–OBzl	207—208	−18.9° (*c* 1.0, DMF)	Found	65.35	6.22	10.53
			Calcd	65.94	6.30	10.68
Boc–Asn–Trp–Phe–OEt	129—131	−31.8° (*c* 1.0, DMF)	Found	62.41	6.72	11.56
			Calcd	62.71	6.62	11.80
Z–Ala–Asn–Trp–OMe	162—165 (dec)	+11.2° (*c* 0.5, DMF)	Found	60.03	5.86	12.89
			Calcd	60.32	5.81	13.03
Boc–Leu–Ala–Asn–Trp–OMe	146 (sintered) 153 (melted and dec)	+0.2° (*c* 1.0, DMF)	Found	57.98	7.36	13.39
			Calcd	58.42	7.19	13.63

azide being condensed *in situ* with H–Met–OMe to give Boc–Asn–Trp–Met–OMe (IX). The tripeptide (IX) was treated with formic acid for removal of the Boc group, and elongated by two single-step reactions using Boc–Ala–ONSu[10] and Boc–Leu–ONSu[10] for acylation, the pentapeptide, Boc–Leu–Ala–Asn–Trp–Met–OMe (XI), thus being obtained (Fig. 3). Amino acid analysis of the acid hydrolysate of pentapeptide (XI) showed that the recovery of aspartic acid is low. The same phenomenon was observed on analyses of the acid hydrolysates of the peptides containing Asn–Trp linkage given in Table 1, which were synthesized separately (Table 2). In contrast, amino acid analyses of enzymatic[18] hydrolysates of the corresponding free peptides gave a theoretical recovery of asparagine. The results are summarized in Table 1.

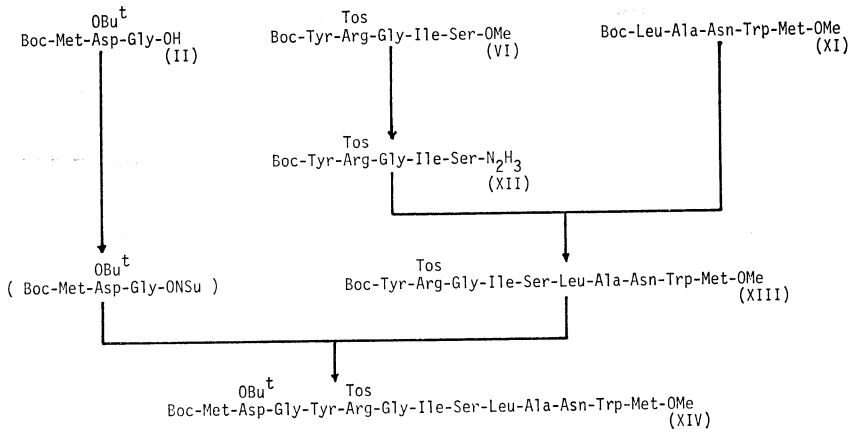

Fig. 4. Synthesis of the tridecapeptide corresponding to positions 17—29 of human lysozyme.

The tridecapeptide, Boc–Met–Asp(OBu^t)–Gly–Tyr–Arg(Tos)–Gly–Ile–Ser–Leu–Ala–Asn–Trp–Met–OMe (XIV), was obtained as shown in Fig. 4. First, the second fragment (VI) was combined with the third fragment (XI). The Boc group of the protected pentapeptide methyl ester (XI) was removed by treatment with formic acid in the presence of mercaptoacetic acid. The resulting pentapeptide with a free amino group was coupled with Boc–Tyr–Arg(Tos)–Gly–Ile–Ser–N_3, prepared from the corresponding hydrazide (XII) by the method of Honzl and Rudinger,[17] to give a decapeptide, Boc–Tyr–Arg(Tos)–Gly–Ile–Ser–Leu–Ala–Asn–Trp–Met–OMe (XIII). The decapeptide (XIII) thus obtained was treated with formic acid for removal of the Boc group and the resulting peptide was coupled with Boc–Met–Asp(OBu^t)–Gly–ONSu obtained from the first fragment (II) as an oily material and used without further purification. Thus, a protected tridecapeptide corresponding to positions 17—29 in the amino acid sequence of human lysozyme, Boc–Met–Asp(OBu^t)–Gly–Tyr–Arg(Tos)–Gly–Ile–Ser–Leu–Ala–Asn–Trp–Met–OMe (XIV), was obtained.

Experimental

The experimental and analytical methods are the same as described in the preceding paper.[8]

H–Asp(OBu^t)–Gly–OH (I). Z–Asp(OBu^t)–ONSu[11] (42.1 g, 0.1 mol) was added to a solution of TosOH·H–Gly–OBzl (33.7 g, 0.1 mol) and TEA (14.0 ml) in DMF (150 ml). The solution was stirred at room temperature for a day and then concentrated to an oily residue under reduced pressure. The residue was dissolved in AcOEt. The solution was washed successively with 0.5 M HCl, 5% aqueous $NaHCO_3$ and H_2O, and then dried over Na_2SO_4 and concentrated to an oil. The oil was dissolved in a mixture of EtOH (600 ml) and H_2O (150 ml), and hydrogenated over 5% palladium–charcoal catalyst under atmospheric pressure. The catalyst was filtered off and the filtrate was concentrated *in vacuo*. The residue was repeatedly flashed with benzene and then crystallized from MeOH and ether; wt 22.5 g (91.5%), mp 158—159 °C, $[\alpha]_D^{24}$ +18.5° (*c* 1.0, AcOH).

Found: C, 48.12; H, 7.45; N, 11.05%. Calcd for $C_{10}H_{18}O_5N_2$: C, 48.77; H, 7.37; N, 11.38%.

Boc–Met–Asp(OBu^t)–Gly–OH·DCHA (II). Boc–Met–ONSu[10] (24.2 g, 69.9 mmol) and TEA (8.4 ml) were added at 0 °C to a suspension of compound I (14.8 g, 60.1 mmol) in a mixture of DMF (200 ml) and H_2O (70 ml). The mixture was stirred at room temperature for 3.5 h to give a clear solution, which was concentrated to an oil under reduced pressure. The oil was redissolved in AcOEt and the solution was washed with 0.5 M HCl and H_2O, dried over Na_2SO_4, and concentrated to an oily residue *in vacuo*. The oil was dissolved in a mixture of ether (1 litre) and AcOEt (200 ml), and then mixed with DCHA (10.8 g). The resulting precipitate was filtered and recrystallized from EtOH, AcOEt, and hexane; wt 27.7 g (69.9%), mp 165—166 °C, $[\alpha]_D^{18}$ −19.4° (*c* 1.0, DMF).

Found: C, 58.08; H, 8.99; N, 8.41; S, 4.88%. Calcd for $C_{32}H_{58}O_8N_4S$: C, 58.33; H, 8.87; N, 8.50; S, 4.87%.

Z–Ile–Ser–OMe (III). Z–Ile–ONp[12] (38.6 g, 0.1 mol) was added to a solution of HCl·H–Ser–OMe (17.0 g, 0.11 mol) and TEA (15.4 ml) in DMF (100 ml). The solution was stirred at room temperature for 3 days, and then concentrated to an oily residue *in vacuo*. The residue was dissolved in AcOEt and H_2O. The AcOEt layer was separated, washed successively with 5% aqueous $NaHCO_3$, 0.7 M HCl and H_2O and dried over Na_2SO_4. The dried solution was concentrated to half its volume, and mixed with hexane and ether. Resulting crystals were separated and recrystallized from AcOEt; wt 31.1 g (85.0%), mp 179—181 °C, $[\alpha]_D^{18}$ +4.3° (*c* 1.0, DMF).

Found: C, 58.76; H, 7.11; N, 7.95%. Calcd for $C_{18}H_{26}O_6N_2$: C, 59.00; H, 7.15; N, 7.65%.

Z–Gly–Ile–Ser–OMe (IV). Compound III (22.0 g, 60.1 mmol) was dissolved in a mixture of MeOH (600 ml) and 4.74 M HCl in dioxane (14 ml), and then hydrogenated under atmospheric pressure over 5% palladium–charcoal catalyst. The catalyst was filtered off and the filtrate was concentrated to a solid residue *in vacuo*. The solid was dissolved in DMF (150 ml), and then mixed with TEA (8.4 ml) and Z–Gly–ONSu[10] (20.2 g, 66.0 mmol). The solution was stirred at room temperature for 2 days, and then concentrated to a syrup under reduced pressure. The syrup was dissolved in AcOEt, and washed successively with 0.7 M HCl, 5% aqueous $NaHCO_3$ and H_2O. The washed solution was dried over Na_2SO_4, and then concentrated to a syrup *in vacuo*. The material was then crystallized from AcOEt and hexane, and recrystallized from AcOEt, ether and hexane; wt 23.0 g (90.6%), mp 137—139 °C, $[\alpha]_D^{18}$ −0.2° (*c* 1.0, DMF).

Found: C, 56.29; H, 6.97; N, 9.81%. Calcd for $C_{20}H_{29}O_7N_3$: C, 56.72; H, 6.90; N, 9.92%.

Z–Arg(Tos)–Gly–Ile–Ser–OMe (V). Compound IV (12.7 g, 30.0 mmol) was dissolved in MeOH (1 litre) and hydrogenated over 5% palladium–charcoal catalyst at atmospheric pressure in a water bath at 30—35 °C. The catalyst was filtered off and the filtrate was concentrated to a solid under reduced pressure. The solid was dissolved with Z–Arg(Tos)–OH, obtained from the corresponding cyclohexylammonium salt[13] (18.4 g, 32.7 mmol), and HOBt (8.1 g, 60.0 mmol) in a mixture of DMF (100 ml) and tetrahydrofuran (200 ml). The solution was cooled to −20 °C in a cold bath, and mixed with a solution of DCC (6.8 g, 33.0 mmol) in tetrahydrofuran (40 ml). The mixture was stirred at −10 °C for an hour and at room temperature overnight, and then concentrated to a solid residue under reduced pressure. The residue was suspended in AcOEt, the insoluble material being filtered off. The filtrate was washed successively with 0.7 M HCl, 5% aqueous $NaHCO_3$ and H_2O. The washed solution was dried over Na_2SO_4, and then concentrated to an oily material *in vacuo*. The material was dissolved in a mixture of EtOH and AcOEt, and crystallized by adding hexane. The crude product was recrystallized from EtOH, AcOEt, and hexane; wt 18.8 g (85.5%), mp 138 °C (sintered) and 152 °C (melted), $[\alpha]_D^{18}$ −1.9° (*c* 1,1, DMF).

Found: C, 53.86; H, 6.52; N, 13.29; S, 4.58%. Calcd for $C_{33}H_{47}O_{10}N_7S$: C, 54.01; H, 6.46; N, 13.36; S, 4.37%.

Boc–Tyr–Arg(Tos)–Gly–Ile–Ser–OMe (VI). Compound V (14.7 g, 20.0 mmol) was dissolved in MeOH (350 ml) and hydrogenated over 5% palladium–charcoal catalyst under atmospheric pressure. The catalyst was filtered off and the filtrate was concentrated to a foaming residue *in vacuo*. Boc–Tyr–N_2H_3[15] (6.5 g, 22.0 mmol) was dissolved in DMF (80 ml). The solution was cooled below −20 °C in a cold bath and mixed with 4.74 M HCl in dioxane (21.6 ml) and then isopentyl nitrite (3.6 ml). The solution was stirred at the same temperature for 45 min and mixed with a solution of the foaming residue in DMF (20 ml) as described above and then with TEA (14.5 ml). The mixture was stirred at 0 °C for 5 days. The precipitate was then filtered off and the filtrate was concentrated to a small volume under reduced

pressure. The remaining solution was diluted with AcOEt, and washed successively with 0.4 M HCl, 5% aqueous $NaHCO_3$ and H_2O. The washed solution was dried over Na_2SO_4 and then concentrated to a foaming residue under reduced pressure, the residue being solidified in a mixture of EtOH, AcOEt, and hexane. The crude material was repeatedly crystallized from a mixture of EtOH, AcOEt, and ether, and then a mixture of CH_3CN, AcOEt, and ether; wt 13.7 g (77.8%), mp 138 °C (sintered) and 142 °C (melted), $[\alpha]_D^{18}$ −4.8° (*c* 1.2, DMF). Amino acid ratio in the acid hydrolysate: Tyr, 0.99 (1); Arg, 0.96 (1); Gly, 1.00 (1); Ile, 0.96 (1); Ser, 0.91 (1).

Found: C, 53.38; H, 6.72; N, 12.52; S, 3.81%. Calcd for $C_{39}H_{58}O_{12}N_8S \cdot H_2O$: C, 53.17; H, 6.86; N, 12.72; S, 3.64%.

Boc-Asn-Trp-OMe (VII). H-Trp-OMe·HCl (22.8 g, 89.4 mmol) was dissolved in DMF (150 ml) and mixed with TEA (12.6 ml). Boc-Asn-ONp[16] (28.2 g, 79.9 mmol) was added to the solution which was stirred at room temperature for a day and then concentrated to a syrup *in vacuo*. The syrup was dissolved in AcOEt and washed successively with 5% aqueous $NaHCO_3$, 0.5 M HCl, and H_2O. The washed solution was dried over Na_2SO_4 with active charcoal, and then concentrated to a solid *in vacuo*. The solid was dissolved in a mixture of EtOH and AcOEt and crystallized by adding hexane. The crude material was recrystallized from the same solvent mixture; wt 30.2 g (86.5%), mp 132—133 °C, $[\alpha]_D^{24}$ +14.3° (*c* 1.0, DMF).

Found: C, 57.75; H, 6.53; N, 13.00%. Calcd for $C_{21}H_{28}O_6N_4 \cdot 1/4H_2O$: C, 57.72; H, 6.57; N, 12.82%.

Boc-Asn-Trp-N_2H_3 (VIII). Compound VII (30.2 g, 69.1 mmol) was dissolved in MeOH (350 ml) and cooled to 0 °C in an ice-bath. Then 90% hydrazine hydrate (100 ml) was added to the chilled solution. The solution was stirred at room temperature for 2 h, and concentrated to an oil *in vacuo*. The oil was triturated with cold water, and washed thoroughly with water. The crude product was crystallized from EtOH, ether, and hexane; wt 26.6 g (88.4%), mp 151—153 °C, $[\alpha]_D^{24}$ −24.6° (*c* 1.0, DMF).

Found: C, 55.09; H, 6.64; N, 18.93%. Calcd for $C_{20}H_{28}O_5N_6 \cdot 1/4H_2O$: C, 54.97; H, 6.58; N, 19.23%.

Boc-Asn-Trp-Met-OMe (IX). Compound VIII (21.6 g, 49.4 mmol) was dissolved in DMF (120 ml) and cooled below −20 °C in a cold bath and mixed with 4.74 M HCl in dioxane (33 ml) and then isopentyl nitrite (7.5 ml). The solution was stirred at −20 °C for 45 min, and then mixed with H-Met-OMe·HCl (12.0 g, 60.0 mmol) and TEA (30.4 ml) below −20 °C and stirred at 0 °C for a day. The precipitate was filtered off and the filtrate was concentrated to a small volume under reduced pressure. The remaining solution was diluted with AcOEt and washed successively with 0.3 M HCl, 5% aqueous $NaHCO_3$ and H_2O. The washed solution was dried over Na_2SO_4 with active charcoal. The dried solution was concentrated under reduced pressure to a solid, which was crystallized from AcOEt, EtOH, and hexane. The crude material was recrystallized from EtOH, hexane, and ether; wt 21.9 g (77.9%), mp 140—144 °C, $[\alpha]_D^{14}$ −39.0° (*c* 1.0, DMF).

Found: C, 54.99; H, 6.70; N, 12.29; S, 5.95%. Calcd for $C_{26}H_{37}O_7N_5S \cdot 1/4H_2O$: C, 54.96; H, 6.65; N, 12.33; S, 5.64%.

Boc-Ala-Asn-Trp-Met-OMe (X). Compound IX (16.9 g, 29.8 mmol) was mixed with 98% formic acid in an ice-bath, and the mixture was kept at room temperature overnight. The clear solution obtained was evaporated *in vacuo* and the residue was mixed with 2.5 M HCl in AcOEt under cooling. The mixture was concentrated to dryness *in vacuo*, and then triturated with ether. The powder was filtered and dried *in vacuo*. The dried powder was dissolved in DMF (60 ml) and mixed with TEA (4.2 ml) and Boc-Ala-ONSu[10] (9.5 g, 33.2 mmol). The mixture was stirred at room temperature for 2 days, and then concentrated to a syrupy residue *in vacuo*. The residue was dissolved in AcOEt and washed successively with 0.4 M HCl, 5% aqueous $NaHCO_3$, and H_2O. The washed solution was dried over Na_2SO_4, and concentrated *in vacuo* to a syrup, which was triturated in a mixture of AcOEt, EtOH, and hexane. The crude product was repeatedly crystallized from CH_3CN and ether; wt 15.8 g (83.2%), mp 134—136 °C, $[\alpha]_D^{24}$ −44.8° (*c* 1.0, DMF).

Found: C, 54.52; H, 6.72; N, 12.96; S, 5.07%. Calcd for $C_{29}H_{42}O_8N_6S \cdot 1/4H_2O$: C, 54.48; H, 6.70; N, 13.15; S, 5.02%.

Boc-Leu-Ala-Asn-Trp-Met-OMe (XI). Compound X (12.7 g, 19.9 mmol) was mixed with 98% formic acid (40 ml) containing mercaptoacetic acid (4 ml) under cooling, and the mixture was left to stand at room temperature overnight. The solvent was evaporated under reduced pressure, and the residue was mixed with 2.5 M HCl in AcOEt (20 ml) under cooling. The mixture was concentrated under reduced pressure to a solid, which was collected with ether. The solid was dissolved in DMF (50 ml) and mixed with TEA (2.8 ml) and Boc-Leu-ONSu[10] (7.2 g, 22.0 mmol). The mixture was stirred at room temperature for 6 days, and then concentrated to a syrup *in vacuo*. The syrup was dissolved in AcOEt containing a small amount of EtOH. The solution was washed successively with 0.3 M HCl, 5% aqueous $NaHCO_3$, and H_2O. The washed solution was dried over Na_2SO_4 and concentrated *in vacuo* to a syrup, which was crystallized from EtOH and hexane. The crude product was recrystallized from CH_3CN and ether; wt 8.0 g (53.3%), mp 173—175 °C. The material thus obtained was further purified by chromatography on silica gel, using a solvent mixture of $CHCl_3$ and MeOH (v/v, 5/1); wt 6.6 g (44.0%), mp 174—176 °C, $[\alpha]_D^{24}$ −41.0° (*c* 1.0, DMF). Amino acid ratio in the acid hydrolysate: Leu, 0.99 (1); Ala, 1.00 (1); Asp, 0.48 (1); Trp, not determined (1); Met, 0.56 (1).

Found: C, 55.87; H, 7.15; N, 12.57; S, 4.38%. Calcd for $C_{35}H_{53}O_9N_7S \cdot 1/4H_2O$: C, 55.87; H, 7.17; N, 13.03; S, 4.26%.

Boc-Tyr-Arg(Tos)-Gly-Ile-Ser-N_2H_3 (XII). Compound VI (25.9 g, 29.4 mmol) was dissolved in EtOH (250 ml) by gentle heating. The solution was cooled to 0 °C in an ice-bath and mixed with 90% hydrazine hydrate (70 ml). The mixture was stirred at room temperature for a day, and then concentrated and flashed repeatedly with benzene. The resulting solid was filtered with a mixture of AcOEt and EtOH. The solid was crystallized from DMF, EtOH, and ether; wt 23.8 g (92.0%), mp 156—158 °C, $[\alpha]_D^{18}$ −7.2° (*c* 1.1, DMF).

Found: C, 51.78; H, 6.95; N, 15.64; S, 3.70%. Calcd for $C_{38}H_{58}O_{11}N_{10}S \cdot H_2O$: C, 51.85; H, 6.87; N, 15.90; S, 3.63%.

Boc-Tyr-Arg(Tos)-Gly-Ile-Ser-Leu-Ala-Asn-Trp-Met-OMe (XIII). Compound XI (4.75 g, 6.31 mmol) was dissolved in 98% formic acid (60 ml) containing mercaptoacetic acid (0.5 ml). The solution was stirred at room temperature for 3.5 h, and then concentrated to a syrupy residue *in vacuo*. The residue was mixed for 1 min with 4 M HCl in dioxane (1.5 ml) under cooling and concentrated *in vacuo* to a syrup, which was triturated in ether. The powder was dried over NaOH *in vacuo*. Compound XII (3.74 g, 4.25 mmol) was dissolved in DMF (20 ml). The solution was cooled below −20 °C in a cold bath and mixed with 4 M HCl in dioxane (7 ml) and isopentyl nitrite (0.78 ml). The solution was stirred at the same temperature for 30 min and then mixed with the powder described above in dimethyl sulfoxide (15 ml), and *N*-methylmorpholine (4.0 ml), and stirred at 0 °C for

7 days. The precipitate formed was filtered off, and the filtrate was concentrated to a small volume under reduced pressure. The residual solution was poured into ice-water, and the precipitate formed was filtered, washed thoroughly with H_2O and dried over P_2O_5 *in vacuo*. The crude material was dissolved in a mixture of MeOH (300 ml) and $CHCl_3$ (200 ml), and insoluble material was filtered off. The filtrate was concentrated *in vacuo* to a residue, which was boiled in a mixture of MeOH (100 ml) and AcOEt (300 ml) and mixed with ether under cooling. The precipitated material was boiled in a mixture of CH_3CN (100 ml) and MeOH (20 ml), and reprecipitated with ether under cooling; wt 3.70 g (57.5%), mp 228—230 °C, $[\alpha]_D^{26}$ −21.2° (*c* 1.0, DMF). Amino acid ratio in the acid hydrolysate: Tyr, 0.95 (1); Arg, 1.08 (1); Gly, 1.01 (1); Ile, 0.93 (1); Ser, 0.83 (1); Leu 0.90 (1); Ala, 1.00 (1); Asp, 0.55 (1); Trp, 0.62 (1); Met, 0.66 (1).

Found: C, 53.84; H, 6.73; N, 13.71; S, 4.40%. Calcd for $C_{68}H_{99}O_{18}N_{15}S_2 \cdot 2H_2O$: C, 53.92; H, 6.85; N, 13.87; S, 4.23%.

Boc–Met–Asp(OBu^t)–Gly–Tyr–Arg(Tos)–Gly–Ile–Ser–Leu–Ala–Asn–Trp–Met–OMe (XIV). Compound II (2.70 g, 4.10 mmol) was suspended in AcOEt (150 ml), and washed with 1 M H_2SO_4 and then H_2O. The washed solution was dried over Na_2SO_4, and then concentrated to an oily material under reduced pressure. The oil was dissolved in a mixture of AcOEt (50 ml) and dioxane (30 ml). The solution was cooled to 0 °C in an ice bath, and mixed with HONSu (0.52 g, 4.5 mmol) and DCC (0.93 g, 4.5 mmol). The mixture was stirred at room temperature for a day. The precipitate formed was filtered off and the filtrate was concentrated to an oily material *in vacuo*.

Compound XIII (3.70 g, 2.44 mmol) was dissolved in 98% formic acid (50 ml). The solution was stirred at room temperature for 3 h and then concentrated to a residue *in vacuo*. The residue was stirred with 2.5 M HCl in AcOEt (5 ml) for 1 min in an ice bath, and then concentrated *in vacuo* to a syrup, which was solidified in ether and dried over NaOH under reduced pressure. The dried material was dissolved in a mixture of dimethyl sulfoxide (10 ml) and DMF (5 ml) with TEA (0.35 ml). The solution was mixed with the oily substance obtained as described above from compound II in DMF (5 ml). The mixture was stirred at room temperature for 4 days and then concentrated to a syrup *in vacuo*. The syrup was triturated in a mixture of AcOEt and ether, and the crude product was crystallized from MeOH. The crystallized material was repeatedly precipitated from a mixture of DMF and ether; wt 3.30 g (72.2%), mp 203 °C, $[\alpha]_D^{24}$ −28.1° (*c* 1.0, DMF). Amino acid ratio in the acid hydrolysate: Met, 1.33 (2); Asp, 1.54 (2); Gly, 1.88 (2); Tyr, 0.99 (1); Arg, 0.98 (1); Ile, 0.99 (1); Ser, 0.85 (1); Leu, 0.99 (1); Ala, 1.00 (1); Trp, not determined.

Found: C, 52.91; H, 6.83; N, 13.31; S, 4.80%. Calcd for $C_{83}H_{123}O_{23}N_{18}S_3 \cdot 2H_2O$: C, 53.19; H, 6.88; N, 13.45; S, 5.13%.

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9) The abbreviations used are those recommended by IUPAC-IUB: *J. Biol. Chem.*, **247**, 977 (1972). Additional abbreviations: DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; DMF, *N,N*-dimethylformamide; TEA, triethylamine; DCHA, dicyclohexylamine.
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