Synthesis of a Protected Hexadecapeptide Corresponding to Positions 1—16 of Human Lysozyme

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For a semi-synthesis of human lysozyme by coupling its natural fragment with synthetic peptides, a protected hexadecapeptide corresponding to positions 1—16 of the enzyme was constructed from three protected peptide fragments (V, IX, and XII) by the conventional method in pure state on the basis of analyses.

Lysozyme, isolated from the urine of patients with monocytic or monomyelocytic leukemia[1] and from human milk,[2] is a basic single-chain protein, 130 amino acid residues in length, cross-linked by four disulfide bridges.[3-5] The three dimensional structure[6] of human lysozyme shows considerable homology with that of hen egg-white lysozyme, in spite of the insertion of a glycine residue between positions 47 and 48 and the substitutions of about 40% of the amino acid residues of the latter. The enzyme can be unfolded by reduction with dithiothreitol, and the rapid refolding of the reduced disordered enzyme can be achieved in a high yield in the presence of oxidized and reduced glutathione,[7] as in the case of hen egg-white lysozyme. Recently,[8] investigation was made on the influence of anhydrous liquid hydrogen fluoride (HF) on human lysozyme. It was found that the original intact lysozyme can be recovered in fair yield from HF-solutions of native lysozyme and its derivative substituted by the 2-chlorobenzyloxycarbonyl group.

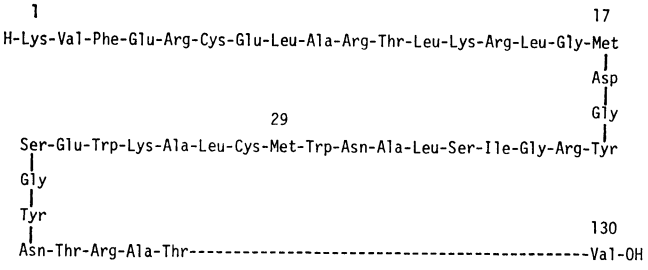

Fig. 1. N-Terminal region of human lysozyme.[3,4]

Human lysozyme contains two residues of methionine located in the N-terminal region of the peptide chain of the enzyme (Fig. 1). Cleavage by cyanogen bromide[9] of the methionyl bonds in the derivative[8] of the enzyme in which all the amino groups are substituted with 2-chlorobenzyloxycarbonyl group, followed by reductive alkylation[10] of the disulfide linkages gives a large fragment lacking the 29 amino acid residues at the N-terminal of the enzyne. The fragment seems to be a suitable material for a semi-synthesis[11,12] of the enzyme, because it has only one free amino group at the amino end. The coupling of the natural fragment with a synthetic peptide leads to the semi-synthesis of the enzyme. This prompted us to synthesize protected peptide fragments containing the N-terminal section of human lysozyme removed from the enzyme by cleavage with cyanogen bromide.

This paper describes the synthesis of a protected hexadecapeptide corresponding to positions 1—16 of human lysozyme. The procedure for synthesis of this peptide is outlined in Figs. 2—5. Figure 2 shows the synthesis of protected hexapeptide V with sqeuence [Lys[1]

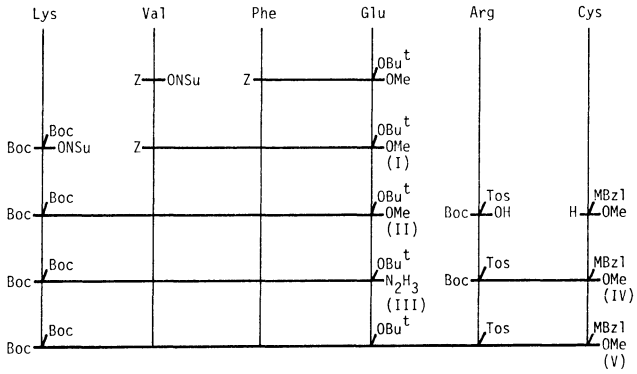

Fig. 2. Scheme for synthesis of sequence 1—6.

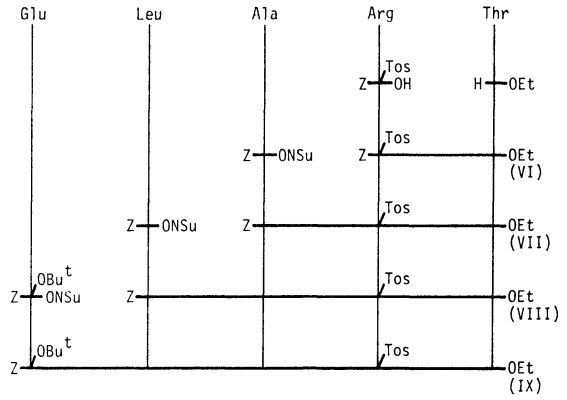

Fig. 3. Scheme for synthesis of sequence 7—11.

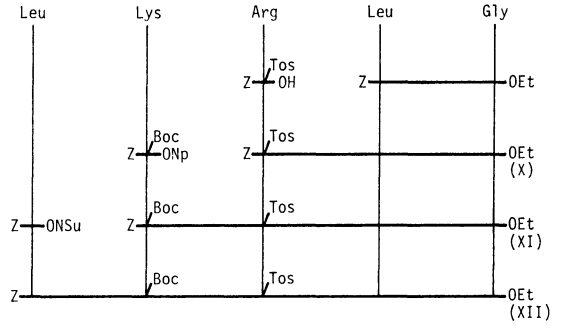

Fig. 4. Scheme for synthesis of sequence 12—16.

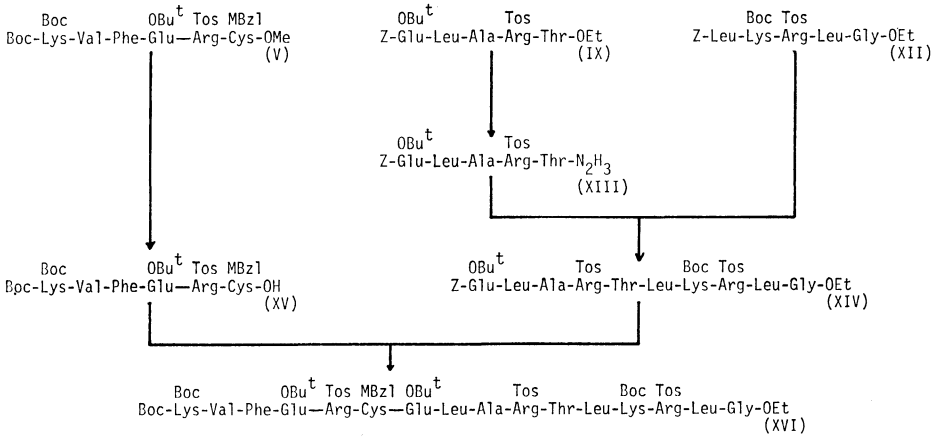

Fig. 5. Scheme for synthesis of the hexadecapeptide corresponding to positions 1—16 of human lysozyme.

to Cys[6].[13] Namely, Z–Phe–Glu(OBu^t)–OMe[14] was subjected to catalytic hydrogenation and the peptide ester obtained was not isolated but allowed to react with Z–Val–ONSu[15] to give Z–Val–Phe–Glu(OBu^t)–OMe (I). The protecting group was removed by catalytic hydrogenation and the resulting tripeptide methyl ester was condensed with Boc–Lys(Boc)–ONSu[12] to yield Boc–Lys(Boc)–Val–Phe–Glu(OBu^t)–OMe (II). The tetrapeptide methyl ester was converted to the corresponding hydrazide (III). Boc–Lys(Boc)–Val–Phe–Glu(OBu^t)–N_3 prepared from compound III was not isolated, but allowed to react directly with H–Arg(Tos)–Cys(MBzl)–OMe prepared by removal of the Boc group from Boc–Arg(Tos)–Cys(MBzl)–OMe (IV) by trifluoracetic acid. In this way Boc–Lys(Boc)–Val–Phe–Glu(OBu^t)–Arg(Tos)–Cys(MBzl)–OMe (V) was obtained.

Figure 3 illustrates the synthesis of protected pentapeptide IX with sequence [Glu[7] to Thr[11]]. First, Z–Arg(Tos)–OH·CHA[16] was condensed with HCl·H–Thr–OEt in the presence of HOBt by DCC.[17] Z–Arg(Tos)–Thr–OEt (VI) thus obtained was hydrogenated catalytically and then coupled with Z–Ala–ONSu[15] to give Z–Ala–Arg(Tos)–Thr–OEt (VII). The protecting group was removed by catalytic hydrogenation and the resulting tripeptide ethyl ester was condensed with Z–Leu–ONSu[15] to give Z–Leu–Ala–Arg(Tos)–Thr–OEt (VIII). The Z group was cleaved by catalytic hydrogenation and the tetrapeptide ethyl ester thus obtained was coupled with Z–Glu(OBu^t)–ONSu[18] to yield Z–Glu(OBu^t)–Leu–Ala–Arg(Tos)–Thr–OEt (IX).

As shown in Fig. 4, protected pentapeptide XII with sequence [Leu[12] to Gly[16]] was constructed by stepwise elongation using Z–Leu–Gly–OEt[19] as starting material. The Z group was removed by catalytic hydrogenation, and the resulting peptide ester was coupled with Z–Arg(Tos)–OH[16] in the presence of HOBt by DCC. The protected tripeptide ester (X) was hydrogenated catalytically, the peptide chain being elongated by stepwise acylation with Z–Lys(Boc)–ONp[20] and Z–Leu–ONSu.[15] The pentapeptide derivative, Z–Leu–Lys(Boc)–Arg(Tos)–Leu–Gly–OEt (XII), was thus obtained.

Construction of the sequence [Lys[1] to Gly[16]] was carried out as illustrated in Fig. 5. Namely, the pentapeptide ethyl ester (IX) was converted to the corresponding hydrazide (XIII) by usual hydrazinolysis. The hydrazide (XIII) was converted to the corresponding azide by the method of Honzl and Rudinger[21] and coupled directly with pentapeptide ethyl ester, which was obtained by removal of the Z group of pentapeptide ethyl ester (XII) by catalytic hydrogenation. The decapeptide derivative (XIV) thus obtained was then hydrogenated over a palladium-catalyst in DMF and the resulting decapeptide ester was coupled with hexapeptide acid (XV) in the presence of HOBt by DCC[17] prepared from the corresponding methyl ester (V) by saponification. However, the resulting material could not be easily purified. Therefore, the decapeptide derivative (XIV) was hydrogenated over a palladium-catalyst in methanol, the catalyst was filtered off and the filtrate was mixed with DMF. Only the methanol in the mixture was evaporated under reduced pressure, and the remaining solution was mixed with hexapeptide acid (XV), and treated under the same conditions as those described above. Thus, a protected hexadecapeptide (XVI) corresponding to positions 1—16 of human lysozyme, Boc–Lys(Boc)–Val–Phe–Glu(OBu^t)–Arg(Tos)–Cys(MBzl)–Glu(OBu^t)–Leu–Ala–Arg(Tos)–Thr–Leu–Lys(Boc)–Arg(Tos)–Leu–Gly–OEt, could be prepared in pure form. Investigations toward a semisynthesis of the enzyme by coupling its natural fragment with synthetic peptides given in this and the following[22] papers are in progress.

Experimental

All melting points were measured by the capillary method and are uncorrected. Thin layer chromatography was performed on silica gel G (Merck) using the following solvent systems (volume ratios); $CHCl_3$: MeOH: AcOH (95: 5: 3), AcOEt: benzene (1: 1), 1-butanol: AcOH: H_2O (4: 1: 1), and $CHCl_3$: MeOH : AcOH : H_2O (10 : 10 : 1 : 10, lower phase). Peptide derivatives were hydrolyzed in 6 M HCl with phenol in sealed tubes at 105 °C for 24 or 48 h, and the hydrolysates were analyzed in a Hitachi KLA-5 analyzer by the method of Moore *et al.*[23] The purity of the peptide derivatives synthesized was confirmed by thin layer chromatography and by the ratio of their constituent amino acids measured in acid hydrolysates by amino acid analysis. Optical

rotations of the peptide derivatives synthesized were measured with a Perkin-Elmer Model 241 polarimeter. The chemicals used in this paper were of reagent grade and were used without further purification. All the amino acids used except glycine were of the L-configuration.

Z-Val-Phe-Glu(OBu^t)-OMe (I). Z-Phe-Glu(OBu^t)-OMe[14] (14.7 g, 29.5 mmol) was dissolved in MeOH (400 ml), and hydrogenated at atmospheric pressure over 5% palladium-charcoal catalyst. The catalyst was filtered off and the filtrate was concentrated to an oil *in vacuo*. The oil was dissolved with Z-Val-ONSu[15] (10.3 g, 29.5 mmol) in $CHCl_3$ (200 ml). The solution was stirred overnight at room temperature and mixed with *N,N*-dimethyl-1,3-propanediamine (3 ml). After 1 h the solution was diluted with $CHCl_3$ and then washed successively with 0.1 M HCl, 5% aqueous $NaHCO_3$ and H_2O. The washed solution was dried over Na_2SO_4, and the dried solution was concentrated to a solid *in vacuo*. The solid was collected with $CHCl_3$ and ether, and then recrystallized from $CHCl_3$ and hexane; wt 15.5 g (85.6%), mp 175—178 °C, $[\alpha]_D^{25}$ −40.6° (*c* 0.6, MeOH).

Found: C, 64.14; H, 7.29; N, 7.06%. Calcd for $C_{32}H_{43}O_8N_3$: C, 64.30; H, 7.25; N, 7.03%.

Boc-Lys(Boc)-Val-Phe-Glu(OBu^t)-OMe (II). Compound I (11.9 g, 19.9 mmol) was dissolved in MeOH (350 ml), and hydrogenated at atmospheric pressure over 5% palladium-charcoal catalyst. The catalyst was filtered off and the filtrate was concentrated to an oil *in vacuo*. The oil was dissolved with Boc-Lys(Boc)-ONSu[12] (8.85 g, 19.9 mmol) in DMF (100 ml) under cooling. The solution was stirred at room temperature for 2 days, and then concentrated to a syrup *in vacuo*. The syrup was dissolved in $CHCl_3$ and washed successively with 0.1 M HCl, 5% aqueous $NaHCO_3$ and H_2O. The washed solution was dried over Na_2SO_4 and then concentrated *in vacuo* to an oil, which was repeatedly crystallized from EtOH and ether; wt 10.3 g (65.3%), mp 168—170 °C, $[\alpha]_D^{25}$ −43.5° (*c* 1.0, MeOH).

Found: C, 60.63; H, 8.30; N, 8.83%. Calcd for $C_{40}H_{65}O_{11}N_5$: C, 60.66; H, 8.27; N, 8.85%.

Boc-Lys(Boc)-Val-Phe-Glu(OBu^t)-N_2H_3 (III). Compound II (7.93 g, 10.0 mmol) was dissolved in DMF (56 ml) and mixed with 90% hydrazine hydrate (5.6 ml). The mixture was stirred at room temperature overnight, and concentrated *in vacuo* to a gelatinous residue, which was triturated with H_2O; wt 7.87 g (99.2%), mp 188.5—191 °C.

Found: C, 59.04; H, 8.32; N, 12.35%. Calcd for $C_{39}H_{65}O_{10}N_7$: C, 59.14; H, 8.27; N, 12.38%.

Boc-Arg(Tos)-Cys(MBzl)-OMe (IV). Boc-Arg(Tos)-OH[16] (4.14 g, 9.88 mmol) was added to a solution of HCl·H-Cys(MBzl)-OMe (3.50 g, 12.0 mmol) and TEA (1.68 ml) in DMF (50 ml), and cooled to −10 °C—−20 °C. HOBt (1.50 g, 11.1 mmol) and DCC (2.30 g, 11.2 mmol) were added to the cooled mixture. The mixture was stirred at the same temperature for 1 h and at 0 °C for 2 days. The precipitate formed was filtered off and the filtrate was concentrated to an oily residue *in vacuo*. The residue was dissolved in AcOEt and the solution was washed successively with 0.2 M HCl, 5% aqueous $NaHCO_3$ and H_2O. The washed solution was dried over Na_2SO_4 and then concentrated to an oil *in vacuo*. The oil was purified on a column of silica gel using a solvent mixture of AcOEt and benzene (1: 1, v/v). A syrupy material was obtained; wt 5.85 g.

Boc-Lys(Boc)-Val-Phe-Glu(OBu^t)-Arg(Tos)-Cys(MBzl)-OMe (V). Compound III (2.38 g, 3.00 mmol) was dissolved in DMF (17 ml), cooled below −20 °C and mixed with 4.60 M HCl in dioxane (2.50 ml). The solution was mixed with isopentyl nitrite (0.37 g, 3.15 mmol) and stirred at the same temperature for 45 min, the solution was then mixed with a solution in DMF (10 ml) of the material prepared by treatment of compound IV (2.55 g, *ca.* 3.9 mmol) with trifluoracetic acid (10 ml) at 0 °C for 60 min. The solution was made slightly basic by adding TEA below −30 °C, and stirred at 0 °C for a day. The precipitate formed was filtered off and the filtrate was concentrated to half its volume *in vacuo*, and then diluted with $CHCl_3$ under cooling. The diluted solution was washed successively with 0.3 M HCl, 5% aqueous $NaHCO_3$ and H_2O, and dried over Na_2SO_4 and concentrated to an oil *in vacuo*. The oil was solidified in AcOEt and hexane; wt 3.83 g. The crude product was recrystallized from MeOH; wt 2.65 g (66.6%), mp 217—218 °C (dec), $[\alpha]_D^{20}$ −23.0° (*c* 1.0, DMF). Amino acid ratio in the acid hydrolysate: Glu, 1.00 (1); Cys, 0.49 (1); Val, 0.89 (1); Phe 1.00 (1); Lys, 0.91 (1); Arg, 1.06 (1).

Found: C, 57.15; H, 7.28; N, 10.24; S, 4.71%. Calcd for $C_{64}H_{96}O_{16}N_{10}S_2\cdot H_2O$: C, 57.21; H, 7.35; N, 10.43; S, 4.77%.

Z-Arg(Tos)-Thr-OEt (VI). A solution of HCl·H-Thr-OEt (14.7 g, 80.0 mmol) in tetrahydrofuran (100 ml) was added to a suspension of Z-Arg(Tos)-OH·CHA[16] (42.0 g, 75.0 mmol) in tetrahydrofuran (300 ml). The resulting solution was mixed with HOBt (10.8 g, 80.0 mmol), cooled to −10 °C and then mixed with a solution of DCC (16.5 g, 80.0 mmol) in tetrahydrofuran (100 ml). The mixture was stirred at −10 °C for 1 h and then overnight at room temperature. The precipitate thus formed was filtered off and the filtrate was concentrated to a syrup *in vacuo*. The syrup was dissolved in AcOEt and washed successively with 0.5 M HCl, 5% aqueous $NaHCO_3$ and H_2O. It was then dried over Na_2SO_4 and concentrated to an oily residue *in vacuo*. The oily residue was purified on a column of silica gel using a mixture of AcOEt and benzene (1: 1, v/v). The purified material was solidified in $CHCl_3$ and ether; wt 37.0 g (83.5%), mp 64—66 °C, $[\alpha]_D^{24}$ −6.4° (*c* 1.1, MeOH).

Found: C, 54.39; H, 6.42; N, 11.60; S, 5.21%. Calcd for $C_{27}H_{37}O_8N_5S$: C, 54.81; H, 6.30; N, 11.84; S, 5.40%.

Z-Ala-Arg(Tos)-Thr-OEt (VII). Compound VI (30.0 g, 50.0 mmol) was dissolved in MeOH (800 ml) and hydrogenated at atmospheric pressure over 5% palladium-charcoal catalyst. The catalyst was filtered off and the filtrate was concentrated to an oil under reduced pressure. The oil was dissolved with Z-Ala-ONSu[15] (19.2 g, 60.0 mmol) in DMF (50 ml). The mixture was stirred at room temperature for a day, and concentrated to a residue under reduced pressure. The residue was dissolved in AcOEt, and washed successively with 0.5 M HCl, 5% aqueous $NaHCO_3$ and H_2O, and dried over Na_2SO_4. The dried solution was concentrated *in vacuo* to a syrup, which was solidified in a mixture of $CHCl_3$ and ether. The crude product was reprecipitated from a mixture of $CHCl_3$, MeOH, and ether; wt 26.5 g (80.2%), mp 66 °C (sintered), $[\alpha]_D^{24}$ −25.1° (*c* 0.9, MeOH).

Found: C, 54.10; H, 6.52; N, 12.74; S, 5.23%. Calcd for $C_{30}H_{42}O_9N_6S$: C, 54.37; H, 6.39; N, 12.68; S, 4.83%.

Z-Leu-Ala-Arg(Tos)-Thr-OEt (VIII). Compound VII (19.9 g, 30.0 mmol) was dissolved in MeOH (500 ml) and hydrogenated at atmospheric pressure over 5% palladium-charcoal catalyst. The catalyst was filtered off and the filtrate was concentrated to an oil under reduced pressure. The oil was dissolved with Z-Leu-ONSu[15] (13.0 g, 36.0 mmol) in DMF (65 ml). The solution was stirred at room temperature for 24 h, and then concentrated to a residue under reduced pressure. The residue was dissolved in AcOEt and washed successively with 0.7 M HCl, 5% aqueous $NaHCO_3$ and H_2O. The solution was dried over Na_2SO_4, and concentrated to a syrupy residue under reduced pressure. The residue was precipitated repeatedly with $CHCl_3$ and ether; wt 19.3 g

(82.6%), mp 80—85 °C, $[\alpha]_D^{24}$ −32.6° (*c* 1.0, MeOH).

Found: C, 54.44; H, 6.95; N, 12.48; S, 4.01%. Calcd for $C_{36}H_{53}O_{10}N_7S$: C, 54.46; H, 6.98; N, 12.35; S, 4.12%.

Z-Glu(OBu^t)-Leu-Ala-Arg(Tos)-Thr-OEt (IX). Compound VIII (15.5 g, 20.0 mmol) was dissolved in MeOH (350 ml) and hydrogenated at atmospheric pressure over 5% palladium-charcoal catalyst. The catalyst was filtered off and the filtrate was concentrated to a foaming residue *in vacuo*. The residue was dissolved with Z-Glu(OBu^t)-ONSu[18] (10.7 g, 24.0 mmol) in DMF (40 ml). The solution was stirred at room temperature for a day, and then concentrated to a residue *in vacuo*. The residue was dissolved in AcOEt, and washed successively with 0.5 M HCl, 5% aqueous $NaHCO_3$ and H_2O. The washed solution was dried over Na_2SO_4 and then concentrated *in vacuo* to a syrup, which was precipitated from a mixture of AcOEt, EtOH, and ether. The crude product was reprecipitated from the same solvent mixture; wt 13.3 g (69.3%), mp 151—153 °C, $[\alpha]_D^{24}$ −37.6° (*c* 0.9, MeOH). Amino acid ratio in the acid hydrolysate: Thr, 0.89 (1); Glu, 1.02 (1); Ala, 1.00 (1); Leu, 1.00 (1); Arg, 1.07 (1).

Found: C, 55.73; H, 7.18; N, 11.30; S, 3.02%. Calcd for $C_{45}H_{68}O_{13}M_8S\cdot 1/2H_2O$: C, 55.71; H, 7.17; N, 11.55; S, 3.33%.

Z-Arg(Tos)-Leu-Gly-OEt (X). Z-Leu-Gly-OEt[19] (17.5 g, 50.0 mmol) was dissolved in MeOH (500 ml) and hydrogenated at atmospheric pressure over 5% palladium-charcoal catalyst. The catalyst was filtered off and the filtrate was concentrated to a gelatinous solid *in vacuo*. The solid was dissolved with Z-Arg(Tos)-OH obtained from Z-Arg(Tos)-OH·CHA[10] (28.1 g, 50.0 mmol), and HOBt (8.1 g, 60.0 mmol) in tetrahydrofuran (400 ml) at 0 °C. The solution was cooled to −10 °C and mixed with a solution of DCC (12.4 g, 60.0 mmol) in tetrahydrofuran (150 ml). The mixture was stirred at −10 °C for 4 h and overnight at room temperature. The precipitate formed was filtered off and the filtrate was concentrated to a syrup *in vacuo*. The syrup was dissolved in AcOEt, and washed successively with 0.5 M HCl, 5% aqueous $NaHCO_3$ and H_2O. The washed solution was dried over Na_2SO_4, and concentrated to a syrup *in vacuo*. The syrup was purified by chromatography on a column of silica gel using a mixture of AcOEt and benzene (1: 1, v/v). The main fractions were collected and concentrated to a syrupy residue *in vacuo*. The residue was repeatedly precipitated from $CHCl_3$ and ether; wt 25.0 g (75.8%), mp 65—68 °C, $[\alpha]_D^{24}$ −24.8° (*c* 1.0, MeOH).

Found: C, 55.06; H, 6.62; N, 12.35; S, 4.77%. Calcd for $C_{31}H_{44}O_8N_6S\cdot H_2O$: C, 54.87; H, 6.83; N, 12.38; S, 4.84%.

Z-Lys(Boc)-Arg(Tos)-Leu-Gly-OEt (XI). Compound X (19.8 g, 30.0 mmol) was dissolved in MeOH (500 ml) and hydrogenated at atmospheric pressure over 5% palladium-charcoal catalyst. The catalyst was filtered off and the filtrate was concentrated to a syrup *in vacuo*. The syrup was dissolved with Z-Lys(Boc)-ONp[20] (16.5 g, 33.0 mmol) in DMF (100 ml). The solution was stirred at room temperature for a day and concentrated *in vacuo* to a syrupy residue, which was dissolved in AcOEt. The solution was washed successively with 1 M citric acid, 5% aqueous $NaHCO_3$ and H_2O, dried over Na_2SO_4, and concentrated *in vacuo* to a gelatinous solid, which was collected with a mixture of AcOEt, EtOH, and hexane. The crude material was recrystallized from the same mixture; wt 23.5 g (88.4%), mp 147—149 °C, $[\alpha]_D^{24}$ −20.4° (*c*, 1.0, DMF).

Found: C, 55.87; H, 7.24; N, 12.41; S, 3.52%. Calcd for $C_{42}H_{64}O_{11}N_8S\cdot 1/2H_2O$: C, 56.17; H, 7.30; N, 12.48; S, 3.60%.

Z-Leu-Lys(Boc)-Arg(Tos)-Leu-Gly-OEt (XII). Compound XI (17.8 g, 20.0 mmol) was dissolved in MeOH (500 ml) and hydrogenated at atmospheric pressure over 5% palladium-charcoal catalyst. The catalyst was filtered off and the filtrate was concentrated to a syrup *in vacuo*. The syrup was dissolved with Z-Leu-ONSu[15] (8.4 g, 22.0 mmol) in DMF (50 ml). The solution was stirred at room temperature for 36 h and concentrated to a syrup *in vacuo*. The syrup was dissolved in AcOEt, and washed successively with 5% aqueous $NaHCO_3$ and H_2O. The washed solution was dried over Na_2SO_4, and concentrated to a solid *in vacuo*. The solid was dissolved in a mixture of AcOEt and EtOH and precipitated by adding a mixture of ether and hexane. The crude product was reprecipitated from a mixture of EtOH, hexane and ether; wt 16.0 g (80.8%), mp 159—161 °C, $[\alpha]_D^{27}$ −18.8° (*c* 1.0, DMF). Amino acid ratio in the acid hydrolysate: Gly, 1.00 (1); Leu, 1.96 (2); Lys, 1.00 (1); Arg, 0.98 (1).

Found: C, 56.32; H, 7.44; N, 12.32; S, 3.13%. Calcd for $C_{48}H_{75}O_{12}N_9S\cdot H_2O$: C, 56.50; H, 7.61; N, 12.36; S, 3.19%.

Z-Glu(OBu^t)-Leu-Ala-Arg(Tos)-Thr-N_2H_3 (XIII). Compound IX (19.2 g, 20.0 mmol) was dissolved in a mixture of EtOH (200 ml) and DMF (50 ml) by gentle heating. The solution was cooled to 0—5 °C and mixed with 90% hydrazine hydrate (60 ml). The mixture was stirred at room temperature for a day and concentrated to a residue *in vacuo*. The residue was collected with ether, and reprecipitated from EtOH and ether. The crude material was redissolved in a mixture of DMF and EtOH, and reprecipitated by adding a mixture of hexane and ether; wt 16.9 g (89.9%), mp 125—128 °C, $[\alpha]_D^{24}$ −22.0° (*c* 1.0, DMF).

Found: C, 53.80; H, 7.19; N, 14.84; S, 3.32%. Calcd for $C_{43}H_{66}O_{12}N_{10}S\cdot H_2O$: C, 53.51; H, 7.19; N, 14.51; S, 3.38%.

Z-Glu(OBu^t)-Leu-Ala-Arg(Tos)-Thr-Leu-Lys(Boc)-Arg(Tos)-Leu-Gly-OEt (XIV). Compound XII (3.00 g, 2.94 mmol) was dissolved in MeOH (300 ml) and hydrogenated over 5% palladium-charcoal catalyst in a water bath at 35 °C. The catalyst was filtered off and the filtrate was mixed with DMF (5 ml). Only the methanol in the filtrate was evaporated off under reduced pressure and the remaining solution was left to stand in a refrigerator. Meanwhile, compound XIII (3.13 g, 3.24 mmol) was dissolved in DMF (12 ml) by gentle heating, cooled to −20 °C——30 °C, and mixed with 3 M HCl in dioxane (6 ml) and isopentyl nitrite (0.48 ml, 3.7 mmol). The solution was stirred at the same temperature for 45 min and mixed with the solution obtained above and TEA (2.5 ml). The mixture was stirred at 0 °C for 10 days in a refrigerator. The precipitate formed was filtered off and the filtrate was concentrated to a syrup *in vacuo*. The syrup was triturated in cold 0.2 M HCl (250 ml), filtered and washed well with H_2O. The crude material was repeatedly crystallized from MeOH; wt 1.89 g (35.7%), mp 224—226 °C, $[\alpha]_D^{24}$ −20.3° (*c* 0.50, dimethyl sulfoxide). Amino acid ratio in the acid hydrolysate: Glu, 1.05 (1); Leu, 3.01 (3); Ala, 0.95 (1); Arg, 1.94 (2); Thr, 1.02 (1); Lys, 1.03 (1); Gly, 1.00 (1).

Found: C, 54.55; H, 7.48; N, 13.19; S, 3.69%. Calcd for $C_{83}H_{131}O_{22}N_{17}S_2\cdot 2H_2O$: C, 54.80; H, 7.48; N, 13.09; S, 3.52%.

Boc-Lys(Boc)-Val-Phe-Glu(OBu^t)-Arg(Tos)-Cys(MBzl)-OH (XV). Compound V (5.29 g, 3.94 mmol) was dissolved in a mixture of MeOH (100 ml) and DMF (20 ml), mixed with 1 M NaOH (8 ml) in an ice-bath under cooling and stirred at room temperature for 3 h. The solution was made neutral by adding 1 M HCl and concentrated to a syrupy residue under reduced pressure. The residue was triturated in cold 0.2 M HCl (300 ml), filtered and washed well with H_2O. The crude material was repeatedly crystallized from MeOH and ether; wt 4.27 g (81.5%), mp 234—236

°C, $[\alpha]_D^{24}$ −13.6° (*c* 0.51, dimethyl sulfoxide).

Found: C, 56.83; H, 7.29; N, 10.45; S, 4.95%. Calcd for $C_{63}H_{94}O_{16}N_{10}S_2 \cdot H_2O$: C, 56.91; H, 7.28; N, 10.54; S, 4.81%.

Boc-Lys(Boc)-Val-Phe-Glu(OBu^t)-Arg(Tos)-Cys(MBzl)-Glu(OBu^t)-Leu-Ala-Arg(Tos)-Thr-Leu-Lys(Boc)-Arg(Tos)-Leu-Gly-OEt (XVI). Compound XIV (0.89 g, 0.49 mmol) was dissolved in MeOH (200 ml) and hydrogenated over 5% palladium-charcoal catalyst at 35 °C for 3 h in a water-bath. The catalyst was filtered off and the filtrate was mixed with DMF (4 ml). Only the methanol in the filtrate was evaporated *in vacuo*. The remaining solution was mixed with HOBt (0.14 g, 1.0 mmol) and compound XV (0.79 g, 0.59 mmol), cooled below −20 °C and mixed with DCC (0.15 g, 0.7 mmol). The mixture was stirred at −20 °C for 50 min and at room temperature for 2 days. The gelatinous mixture formed during the course of reaction was made clear by adding a small volume of dimethyl sulfoxide. The precipitate formed was filtered off and the filtrate was concentrated to a residue under reduced pressure. The residue was precipitated from MeOH and ether; wt 0.85 g. The crude product was reprecipitated from acetonitrile and MeOH; wt 0.57 g (39.6%), mp 244 °C (dec), $[\alpha]_D^{24}$ −22.0° (*c* 0.49, dimethyl sulfoxide). Amino acid ratio in the acid hydrolysate: Lys, 1.85 (2); Val, 0.88 (1); Phe, 0.95 (1); Glu, 2.23 (2); Arg, 3.00 (3); Cys, not determined; Leu, 3.11 (3); Ala, 1.00 (1); Thr, 1.10 (1); Gly, 0.97 (1).

Found: C, 56.02; H, 7.42; N, 12.80; S, 4.31%. Calcd for $C_{138}H_{217}O_{35}N_{27}S_4$: C, 56.33; H, 7.43; N, 12.85; S, 4.35%.

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13) The abbreviations used are those recommended by IUPAC-IUB: *J. Biol. Chem.*, **247**, 977 (1972). Additional abbreviations: MBzl, *p*-methoxybenzyl; CHA, cyclohexylamine; HOBt, 1-hydroxybenzotriazole; DCC, dicyclohexylcarbodiimide: DMF, *N,N*-dimethylformamide; TEA, triethylamine.
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