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Studies on Secretin. I. Synthesis of Completely Protected Secretin[1,2]

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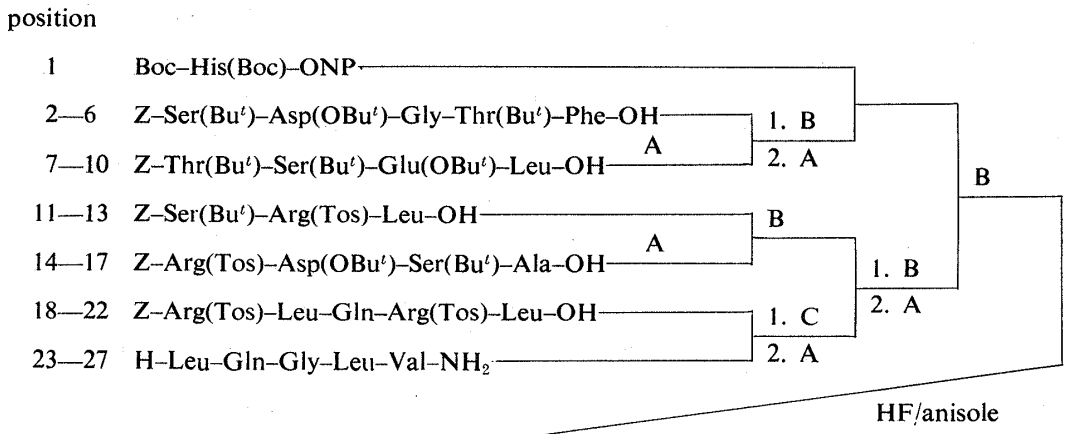
In order to establish a new process for large-scale production of highly purified secretin, a synthesis based on a new strategy was investigated. A maximal protection method applying the HF deprotecting procedure in the final stage was employed. Six small protected fragments (2—6, 7—10, 11—13, 14—17, 18—22, 23—27) were each prepared by stepwise chain elongation. Thereafter, three protected fragments (1—10, 11—17, 18—27) were synthesized from them, and assembled to prepare the protected secretin. The optical purity of the product after each fragment condensation was checked by high performance liquid chromatography. Completely protected secretin having a high degree of chemical and optical homogeneity was obtained.

Keywords——secretin synthesis; completely protected secretin; fragment condensation; optical purity; HPLC

The intestinal hormone secretin was isolated in pure form by Jorpes and Mutt,[3] who also determined its entire amino acid sequence consisting of 27 amino acids.[4] Secretin has many pharmacological actions. The primary actions of this peptide appear to be on the release of water and bicarbonate from the pancreas and on the inhibition of gastrin-induced gastric acid release.[5] Clinically secretin has been used not only as a diagnostic agent of pancreatic disease but also as a potentially effective therapeutic agent for duodenal ulcer,[6] and furthermore a new trial on the use of this peptide to treat patients with acute upper gastrointestinal bleeding is in progress.[7] For these clinical purposes, natural secretin extracted from the porcine upper small intestine has mostly been employed. Since there have been some difficulties in obtaining a large quantity of natural secretin, many groups of investigators have synthesized secretin by either conventional solution methods (stepwise approaches[8] or fragment condensations[9]) or solid phase methods,[10] with verification of its physicochemical properties and biological activities. However, from a manufacturing viewpoint, problems may be involved in the reported synthetic methods because of insufficient checking of the purity or poor adaptability to large-scale production. Thus, we developed a new strategy for the synthesis of secretin and consequently obtained a chemically and optically pure secretin which had the same physicochemical properties and biological activities as natural secretin. We wish to report this synthesis in three consecutive papers. In this paper, the synthesis of the protected secretin is presented.

We undertook the synthesis of secretin employing the following strategy:

1) A maximal protection method[11] applying the hydrogen fluoride (HF) deprotecting procedure in the final stage was selected. 2) Three protected fragments (S1—10, S11—17, S18—27), which were similar in molecular weight, were assembled successively to provide the protected heptacosapeptide corresponding to protected secretin. This procedure is suitable for use on a manufacturing scale because it decreases the number of condensations involving the



(A: H_2/Pd, B: DCC/HOSu, C: MA)

Fig. 1. Synthetic Route to Secretin

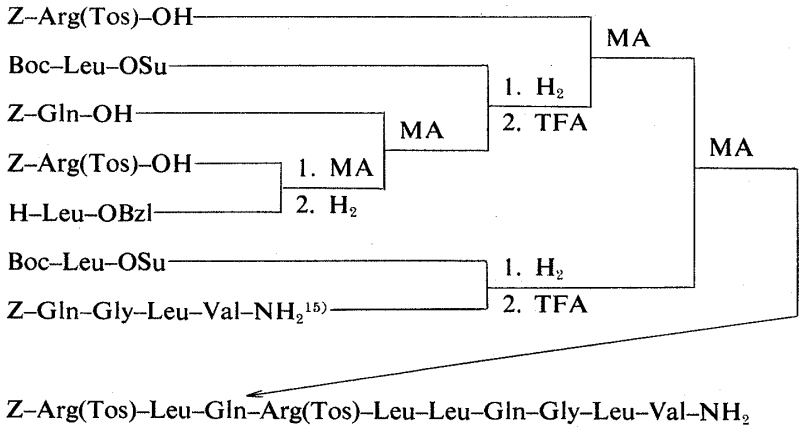


Fig. 2. Synthetic Scheme for the Protected Decapeptide, Z-S18—27-NH_2 (**3**)

more valuable large protected peptides. 3) The optical purity of the product obtained after each fragment condensation was checked by high performance liquid chromatography (HPLC) to ascertain whether it could be used in the following step. The optical purity was checked without any prior deprotecting process for the sake of convenience in manufacture.

An outline of the strategy is presented in Fig. 1. The following side-chain-protected amino acids were used for the synthesis: His(Boc), Ser(Bu^t), Asp(OBu^t), Thr(Bu^t), Glu(OBu^t) and Arg(Tos). Bu^t protection was used for ω-carboxy and hydroxy groups of amino acids since the Asp(OBzl)–Gly unit is known to be susceptible to succinimide formation.[12] For α-amino-protection, the Z group was employed except in the cases of His(Boc), Leu^{19} and Leu^{23} where the Boc group was applied. The synthesis of each protected peptide was carried out as follows.

In order to prepare the C-terminal decapeptide, Z–Arg(Tos)–Leu–Gln–Arg(Tos)–Leu–Leu–Gln–Gly–Leu–Val–NH_2 [Z-S18—27-NH_2 (**3**)], coupling of Z–Arg(Tos)–Leu–Gln–OH with H–Arg(Tos)–Leu–Leu–Gln–Gly–Leu–Val–NH_2 was first tried by the DCC plus HOSu[13] or HOBt[14] method. Yields of **3** obtained by these methods were very low, though the couplings were carried out at the same position (position 20) that Wünsch *et al.*[15] and Jäger *et al.*[9c] adopted. In their syntheses, guanidino groups of Arg^{18} and Arg^{21} were protected by bis-Z and by protonation with HBr, respectively. In ours, both Arg^{18} and Arg^{21} were pro-

tected by Tos groups. Therefore, the very low yields of **3** may be due to the steric hindrance of the Arg(Tos)[21] residue.

Our next attempt at the synthesis of the fragment (**3**) was conducted by changing the coupling position from 20 to 22 as shown in Fig. 2. The two fragments, Z–Arg(Tos)–Leu–Gln–Arg(Tos)–Leu–OH (**2**) and Boc–Leu–Gln–Gly–Leu–Val–NH_2 (**1**) were synthesized by stepwise chain elongation starting with H–Leu–OBzl and H–Val–NH_2, respectively.

Z–Gln–Arg(Tos)–Leu–OH and Z–Gln–Gly–Leu–Val–NH_2[16] each have a Gln residue as the N-terminus. It was reported by several authors that peptides having α-amino-free Gln at the N-terminus frequently cyclize to yield pyroglutamyl peptides in alkaline or weakly acidic solution.[17] In order to suppress this undesirable reaction, a novel procedure in which Z–Gln–Arg(Tos)–Leu–OH and Z–Gln–Gly–Leu–Val–NH_2 were hydrogenated in the presence of equimolar Boc–Leu–OSu to yield Boc–Leu–Gln–Arg(Tos)–Leu–OH and Boc–Leu–Gln–Gly–Leu–Val–NH_2 (**1**), respectively, was developed. In this procedure the coupling reaction starts as soon as the hydrogenolysis of Z-group proceeds and so the peptide with a free amino group exists only for a short time. This procedure may also serve to shorten the synthetic process.

In order to prepare the protected decapeptide (**3**), the fragment (**2**) was condensed with the α-deprotected peptide of (**1**) by the DCC–HOSu (additive) procedure. Since it is known that racemization may occur during fragment condensation,[18] the optical purity of the product was checked in this case. In order to check the optical purity of the product by HPLC, the product and its diastereoisomer [D-Leu[22]]Z-S18—27-NH_2 prepared in the same manner[19] were subjected to HPLC. The separation of the diastereoisomer from Z-S18—27-NH_2 (**3**) was satisfactory and the content of the isomer in the reaction product was determined to be about 10%, as was also the case in the coupling by the DCC–HOBt (additive) procedure. However, [D-Leu[22]]Z-S18—27-NH_2 in the product amounted to only about 5% in the case of coupling by the mixed anhydride method performed at −25 °C. In addition, this isomer was found to be removable from the product by boiling in MeOH or column chromatography on silica gel with $CHCl_3$–MeOH–H_2O (80 : 20 : 1) as an eluent. Consequently, the fragment (**3**) was synthesized by condensation of **2** with **1** by the mixed anhydride method at −25 °C, followed by purification with MeOH. The optical purity of the protected decapeptide thus obtained was determined to be more than 99% by HPLC as shown in Fig. 3. The middle heptapeptide, Z–Ser(Bu[t])–Arg(Tos)–Leu–Arg(Tos)–Asp(OBu[t])–Ser(Bu[t])–Ala–OH [Z-S11—17-OH (**6**)], was prepared according to Fig. 4. Z–Arg(Tos)–Asp(OBu[t])–Ser(Bu[t])–Ala–OH (**4**) was syn-

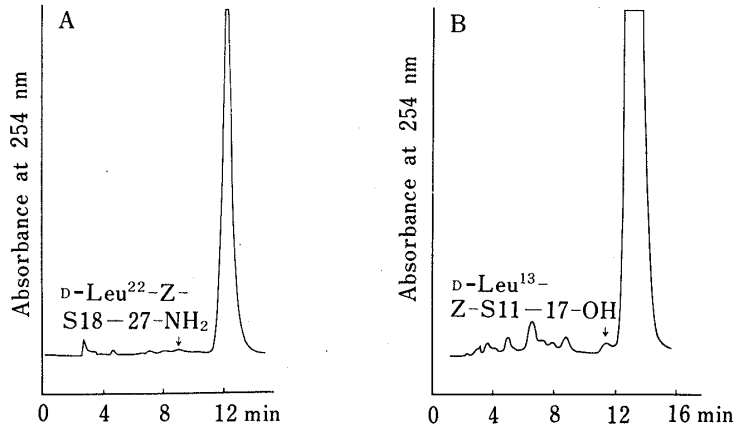


Fig. 3. HPLC of the Protected Peptides, Z-S18—27-NH_2 (**3**) A and Z-S11—17-OH (**6**) B

Column; (A), (B): Nucleosil $10C_{18}$ (4.6 × 250 mm).
Eluent; (A) MeOH/H_2O/AcOH (80/20/1). (B) MeOH/H_2O/AcOH (80/20/0.5).

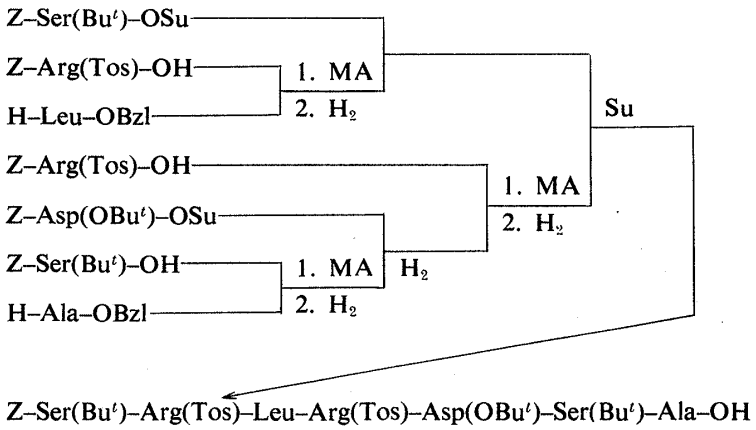


Fig. 4. Synthetic Scheme for the Protected Heptapeptide, Z-S11—17-OH (**6**)

thesized by stepwise chain elongation starting with H–Ala–OBzl.

The specific rotation ($[\alpha]_D^{20}$) of H–Ser(Bu^t)–Ala–OH prepared by hydrogenolysis of Z–Ser(Bu^t)–Ala–OBzl in aqueous MeOH was $-11°$ ($c=2$, H_2O). However, after hydrogenolysis in neat MeOH, the specific rotation of the peptide was $-17°$ ($c=2$, H_2O) and this value was almost identical with that of H–Ser(Bu^t)–Ala–OH, $[\alpha]_D^{20}$ $-16.5°$ ($c=4$, H_2O), reported by Wünsch *et al.*[20] who had obtained it by hydrogenolysis of Z–Ser(Bu^t)–Ala–OH in aqueous MeOH. Both dipeptides having different specific rotation exhibited a single spot with the same *Rf* value on thin layer chromatography (TLC) with the solvent system, isoamyl alcohol/pyridine/water (7 : 7 : 6), reported by Wünsch *et al.*[20] However, when a new solvent system, isopropanol/HCOOH/water (20 : 1 : 5), was used, the by-product in the dipeptide having the specific rotation of $-17°$ was detected as a ninhydrin-negative and Cl_2–KI–starch reagent-positive spot on TLC. Although the reason why this by-product was formed is not clear, we decided to carry out the hydrogenolysis of Z–Ser(Bu^t)–Ala–OBzl in aqueous MeOH to prepare H–Ser(Bu^t)–Ala–OH.

Z–Ser(Bu^t)–Arg(Tos)–Leu–OH (**5**) was prepared without particular difficulty starting with H–Leu–OBzl. The protected heptapeptide (**6**) was synthesized by coupling the fragment (**5**) with the N^{α}-deprotected product of **4** by the HOSu active ester method without isolation of the corresponding active ester. In order to check the optical purity of the product, the aforementioned HPLC procedure was used. The content of the diastereoisomer [D-Leu^{13}]Z-S11—17-OH in the product was less than 1%, as shown in Fig. 3.

When the HOBt active ester method was employed, the isomer [D-Leu^{13}]Z-S11—17-OH in the product amounted to about 38%. This observation is compatible with the report of König and Geiger, who found that considerable racemization occurred during fragment condensation by the HOBt active ester method.[21]

The N-terminal decapeptide, Boc–His(Boc)–Ser(Bu^t)–Asp(OBu^t)–Gly–Thr(Bu^t)–Phe–Thr(Bu^t)–Ser(Bu^t)–Glu(OBu^t)–Leu–OH [Boc-S1—10-OH (**10**)] was prepared according to Fig. 5.

The fragments, Z–Ser(Bu^t)–Asp(OBu^t)–Gly–Thr(Bu^t)–Phe–OH (**8**) and Z–Thr(Bu^t)–Ser(Bu^t)–Glu(OBu^t)–Leu–OH (**7**), were prepared without particular difficulty by stepwise chain elongation starting with H–Phe–OBzl and H–Leu–OBzl, respectively. The fragment (**8**) was condensed with the N^{α}-deprotected product of **7** by the HOSu active ester method to yield Z–Ser(Bu^t)–Asp(OBu^t)–Gly–Thr(Bu^t)–Phe–Thr(Bu^t)–Ser(Bu^t)–Glu(OBu^t)–Leu–OH [Z-S2—10-OH (**9**)]. The optical purity of the product was checked by HPLC, and the content of the isomer [D-Phe^6]Z-S2—10-OH in the product was determined to be less than 1%, as shown in Fig. 6.

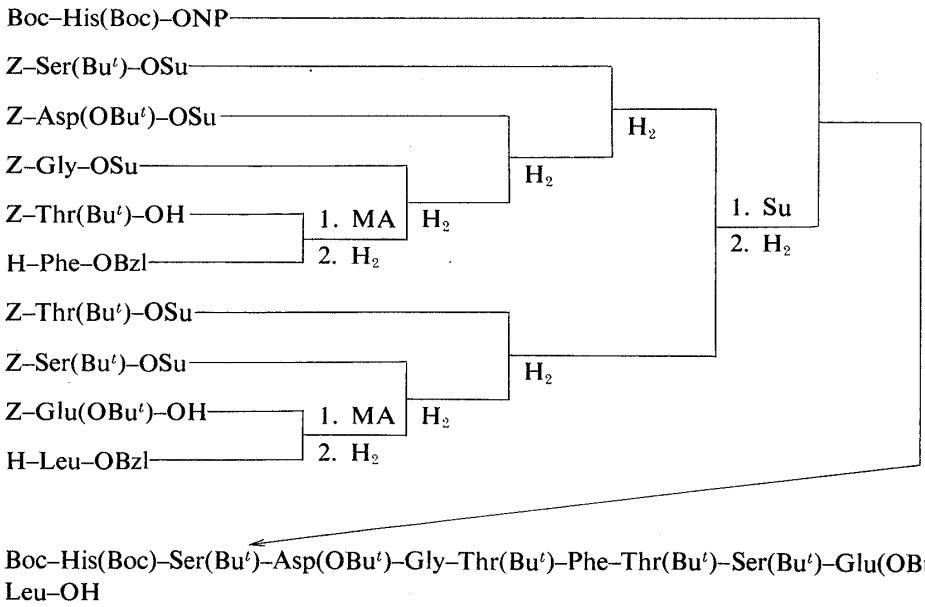


Fig. 5. Synthetic Scheme for the Protected Decapeptide, Boc-S1—10-OH (**10**)

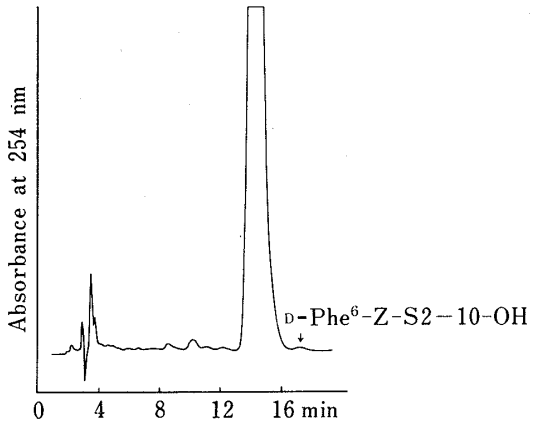


Fig. 6. HPLC of the Protected Peptide, Z-S2—10-OH (**9**)

Column; Nucleosil $10C_{18}$ (4.6×250mm). Eluent; MeOH/H_2O/AcOH (80/20/0.5).

Beyerman *et al.* reported that Boc, Z and Tos groups were preferable for the protection of the imidazole group of His[22] and that Boc–His(Tos) was superior in stability to Z–His(Z).[23] However, a comparison of stability between the Tos group and the Boc group has not been reported. Therefore, we examined the stability of these two groups to HOSu and found that the Boc group was cleaved gradually but was more stable than the Tos group. For this reason, we incorporated Boc–His(Boc) by the Np active ester method in the last step of the synthesis of **10**.

As mentioned above, optically almost pure peptides Z-S11—17-OH and Z-S2—10-OH were obtained by fragment condensations using the HOSu active ester method. This active ester method might be widely applicable to other fragment condensations, like the DCC–HOSu (additive) method.

In order to prepare protected secretin, Boc–His(Boc)–Ser(Bu^t)–Asp(OBu^t)–Gly–Thr(Bu^t)–Phe–Thr(Bu^t)–Ser(Bu^t)–Glu(OBu^t)–Leu–Ser(Bu^t)–Arg(Tos)–Leu–Arg(Tos)–Asp(OBu^t)–Ser(Bu^t)–Ala–Arg(Tos)–Leu–Gln–Arg(Tos)–Leu–Leu–Gln–Gly–Leu–Val–NH_2 [Boc-S1—27-NH_2 (**12**)], the three fragments were assembled according to Fig. 1. Z-S11—17-OH (**6**) was coupled with the N^{α}-deprotected peptide of Z-S18—27-NH_2 (**3**) by the DCC–HOSu (additive) method to yield Z-S11—27-NH_2 (**11**). The optical purity of the

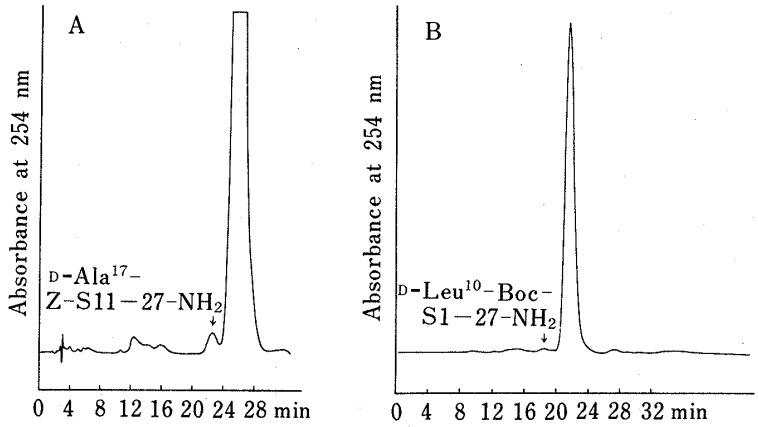


Fig. 7. HPLC of the Protected Peptides, Z-S11—27-NH_2 (**11**) A and Boc-S1—27-NH_2 (**12**) B

Column; (A) Nucleosil $10C_{18}$ (4.6 × 250 mm). (B) Nucleosil 50-5 (4.6 × 250 mm). Eluent; (A) MeOH/H_2O (80/20) with 0.01 M *d*-camphorsulfornic acid. (B) MeOH/$CHCl_3$/H_2O (6/94/1) with 1% *d*-camphorsulfonic acid.

product was checked by the HPLC procedure and the content of the isomer [D-Ala^{17}]Z-S11—27-NH_2 in the product was determined to be less than 1%, as shown in Fig. 7. The same result was also obtained in the case of the coupling by the DCC–HOBt (additive) method.

In order to obtain protected secretin (**12**), Boc-S1—10-OH (**10**) was condensed with the N^{α}-deprotected peptide of Z-S11—27-NH_2 (11) by the DCC–HOSu (additive) method and the crude product was purified by column chromatography on silica gel. The content of the isomer [D-Leu^{10}]Boc-S1—27-NH_2 in the product was less than 1%, as shown in Fig. 7. The same result was also obtained in the case of the coupling by the DCC–HOBt (additive) method.

The homogeneity of the protected secretin thus obtained was assessed by TLC, HPLC, amino acid analysis, and elemental analysis. The results indicated that we had obtained completely protected secretin having a high degree of chemical and optical homogeneity. The steps of final deprotection and purification are reported in the following paper.

Experimental

Melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. Optical rotations were measured with a JASCO DIP-4 polarimeter. Acid hydrolysis was performed in 6 N HCl at 110 °C for 22 h. The amino acid composition of acid hydrolysate was determined with a Hitachi KLA-5 amino acid analyzer and values are uncorrected for amino acid destruction. TLC were performed on silica gel (Kieselgel 60 F254, Merck). Evaporations of solvents were carried out *in vacuo* at 40—50 °C. A mixed anhydride was prepared according to Wieland[24] and Boissonnas.[25] Ethyl chloroformate (1 mol eq) was added at about −20 °C to a solution of a carboxy component and *N*-methylmorpholine[26] (1 mol eq) in THF or DMF. The mixture was stirred for 5 min and a solution of an amine component was added. The protecting groups Z and Bzl were cleaved by catalytic hydrogenation. A solution of the protected peptide was stirred with 10% Pd–C in a current of H_2 at room temperature, then the catalyst was removed by filtration and the solvent was evaporated off.

Boc–Leu–Gln–Gly–Leu–Val–NH_2 [Boc-S23—27-NH_2 (1)]——Z–Gln–Gly–Leu–Val–NH_2[16] (54.8 g, 0.1 mol) and Boc–Leu–OSu (37.2 g, 0.11 mol) were dissolved in CF_3CH_2OH (600 ml) and 10% Pd–C (3 g) was added. The mixture was stirred at room temperature in a current of H_2 for 4 h and *N*-(2-aminoethyl)piperazine (10 ml) was added. After 1 h, Pd–C was filtered off and the filtrate was concentrated to dryness. The resulting powder was suspended in MeOH, and this suspension was stirred at 60 °C then poured into water. The precipitate was filtered and washed with water; yield 50 g (80%), mp 236—238 °C (dec.), $[\alpha]_D^{20}$ −18.9° (c = 2, DMF), *Rf* 0.52 ($CHCl_3$–MeOH, 4 : 1). These data were consistent with those of an authentic sample.[15]

Z–Arg(Tos)–Leu–OBzl (Z-S21—22-OBzl)——A solution of H–Leu–OBzl·TosOH (39.4 g, 0.1 mol) and *N*-methylmorpholine (11 ml, 0.1 mol) in DMF (200 ml) was added to a mixed anhydride prepared from Z–Arg(Tos)–OH

(46.2 g, 0.1 mol) in THF (500 ml). The mixture was stirred at 0 °C for 4 h and the solvent was evaporated off. The residue was dissolved in AcOEt and the solution was washed with 0.1 N HCl, 0.1 N $NaHCO_3$ and water. After evaporation of AcOEt, the product was triturated with ether to give a precipitate; yield 58 g (87%), mp 142—143.5 °C (dec.), $[\alpha]_D^{24}$ −20.8° (c=2, MeOH), *Rf* 0.59 ($CHCl_3$–MeOH, 9:1). *Anal.* Calcd for $C_{34}H_{43}N_5O_7S$: C, 61.34; H, 6.51; N, 10.52. Found: C, 61.58; H, 6.58; N, 10.45.

H–Arg(Tos)–Leu–OH (H-S21—22-OH)——Z-S21—22-OBzl (55 g, 82.6 mmol) was hydrogenated in a mixture of MeOH (800 ml) and water (100 ml) and the product was triturated with ether to give a precipitate; yield 36.5 g (quantitative), mp 137—145 °C (dec.), $[\alpha]_D^{25}$ +9.4° (c=2, MeOH), *Rf* 0.73 (*n*-BuOH–AcOH–H_2O, 4:1:5), *Anal.* Calcd for $C_{19}H_{31}N_5O_5S \cdot H_2O$: C, 49.66; H, 7.23; N, 15.24. Found: C, 49.84; H, 7.17; N, 15.16.

Z–Gln–Arg(Tos)–Leu–OH (Z-S20—22-OH)——A solution of H-S21—22-OH (16.3 g, 37 mmol) and *N*-methylmorpholine (4.06 ml, 37 mmol) in DMF (100 ml) was added to a mixed anhydride prepared from Z–Gln–OH (11.4 g, 40.7 mmol) in DMF (100 ml). The mixture was stirred at 0 °C for 3 h and diluted with 0.1 N citric acid. The resulting powder was filtered off and precipitated from MeOH–AcOEt–ether; yield 18.7 g (72%), mp 127.5—130 °C (dec.), $[\alpha]_D^{25}$ −19.5° (c=2, MeOH), *Rf* 0.62 (*n*-BuOH–AcOH–H_2O, 4:1:1). *Anal.* Calcd for $C_{32}H_{46}N_7O_9S$: C, 54.53; H, 6.57; N, 13.91. Found: C, 54.13; H, 6.47; N, 13.65.

Boc–Leu–Gln–Arg(Tos)–Leu–OH (Boc-S19—22-OH)——Z-S20—22-OH (16.9 g, 24 mmol), Boc–Leu–OSu (8.7 g, 26.4 mmol) and *N*-methylmorpholine (2.64 ml, 24 mmol) were dissolved in *N*-methylpyrrolidone (235 ml) and 10% Pd–C (3 g) was added. The mixture was stirred at room temperature in a current of H_2 for 5 h and *N*-(2-aminoethyl)piperazine (1 ml) was added. After 1 h, MeOH (100 ml) was added, Pd–C was filtered off and the filtrate was concentrated. AcOEt and 0.1 N citric acid were added and the AcOEt layer was washed with water. The solvent was evaporated off and the residue was triturated with ether to give a precipitate; yield 15.6 g (83%), mp 138—141 °C (dec.), $[\alpha]_D^{25}$ −30.6° (c=2, MeOH), *Rf* 0.38 ($CHCl_3$–MeOH, 7:3). *Anal.* Calcd for $C_{35}H_{59}N_8O_{10}S \cdot H_2O$: C, 52.41; H, 7.67; N, 13.97. Found: C, 52.49; H, 7.45; N, 13.43.

Z-Arg(Tos)–Leu–Gln–Arg(Tos)–Leu–OH [Z-S18—22-OH (2)]——Boc-S19—22-OH (14.1 g, 18 mmol) was treated with TFA–CH_2Cl_2–anisole (50 ml–50 ml–5 ml) for 60 min. The mixture was concentrated and ether was added. The precipitate was filtered, dried over NaOH pellets and dissolved in DMF (100 ml) with *N*-methylmorpholine (4 ml, 36 mmol). This solution was added to a mixed anhydride prepared from Z–Arg(Tos)–OH (8.3 g, 18 mmol) in DMF (100 ml). The mixture was stirred at 0 °C for 4 h, stored at 4 °C overnight and poured into 0.1 N citric acid. The precipitate was filtered off and washed with water; yield 19.4 g (94%), mp 169—173 °C (dec.), $[\alpha]_D^{25}$ −26.0° (c=2, MeOH), *Rf* 0.23 ($CHCl_3$–MeOH–AcOH, 85:10:5). *Anal.* Calcd for $C_{51}H_{76}N_{12}O_{13}S \cdot H_2O$: C, 53.39; H, 6.85; N, 14.65. Found: C, 53.56; H, 6.75; N, 14.72.

Z-Arg(Tos)–Leu–Gln–Arg(Tos)–Leu–Leu–Gln–Gly–Leu–Val–NH_2 [Z-S18—27-NH_2 (3)]——Boc-S23—27-NH_2 (2.26 g, 3.6 mmol) was treated with TFA–CH_2Cl_2–anisole (10 ml–10 ml–1 ml) for 60 min. The mixture was concentrated and ether was added. The precipitate was filtered off, dried over NaOH pellets and dissolved in DMF (25 ml) with *N*-methylmorpholine (3.9 ml, 3.6 mmol) in DMF (75 ml). This solution was added at a temperature up to −25 °C to a mixed anhydride prepared from Z-S18—22-OH (4.05 g, 3.6 mmol). The mixture was stirred at −25 to −20 °C for 2 h, and at −8 °C for 2 h, then stored at 4 °C overnight. The solution was concentrated to about 20 ml and water was added. The resulting powder was filtered off, and suspended in MeOH and the suspension was refluxed with stirring for 2 h. After standing overnight at room temperature, the precipitate was collected by filtration; yield 3.28 g (56%), mp 238—248 °C (dec.), $[\alpha]_D^{28}$ −31.5° (c=2, AcOH), *Rf* 0.41 ($CHCl_3$–MeOH–AcOH, 80:15:5). *Anal.* Calcd for $C_{75}H_{117}N_{19}O_{18}S_2 \cdot 2H_2O$: C, 53.90; H, 7.05; N, 15.47. Found: C, 53.88; H, 7.14; N, 15.78.

Z–Ser(Bu^t)–Ala–OBzl (Z-S16—17-OBzl)——A solution of H–Ala–OBzl·TosOH (70.3 g, 0.2 mol) and triethylamine (27.8 ml, 0.2 mol) in THF (300 ml) was added to a mixed anhydride prepared from Z–Ser(Bu^t)–OH (59.0 g, 0.2 mol) in THF (700 ml). The mixture was stirred at 0 °C for 3 h and the solvent was evaporated off. The residue was dissolved in AcOEt and the solution was washed with 0.1 N citric acid, 0.1 N $NaHCO_3$ and water. After evaporation of AcOEt, the product was triturated with petroleum ether to give a precipitate; yield 84 g (92%), mp 78—81 °C, $[\alpha]_D^{25}$ −17.9° (c=2, MeOH), *Rf* 0.82 (AcOEt). *Anal.* Calcd for $C_{25}H_{32}N_2O_6$: C, 65.77; H, 7.07; N, 6.14. Found: C, 65.85; H, 7.05; N, 6.27.

H–Ser(Bu^t)–Ala–OH (H-S16—17-OH)——Z-S16—17-OBzl (82 g, 0.18 mol) was hydrogenated in a mixture of MeOH (500 ml) and water (100 ml) and the product was triturated with ether to give crystals; yield 43.5 g (97%), mp 106 °C (dec.), $[\alpha]_D^{25}$ −11.5° (c=2, H_2O), *Rf* 0.4 (isoamyl alcohol–pyridine–H_2O, 7:7:6). *Anal.* Calcd for $C_{10}H_{20}N_2O_4 \cdot H_2O$: C, 47.98; H, 8.86; N, 11.17. Found: C, 48.05; H, 9.16; N, 11.15.

Z–Arg(Tos)–Asp(OBu^t)–Ser(Bu^t)–Ala–OH [Z-S14—17-OH (4)]——A solution of H–Asp(OBu^t)–Ser(Bu^t)–Ala–OH[20] (15.5 g, 38.5 mmol) and *N*-methylmorpholine (4.3 ml, 38.5 mmol) in DMF (290 ml) was added to a mixed anhydride prepared from Z–Arg(Tos)–OH (16.2 g, 35 mmol) in DMF (290 ml). The mixture was stirred at 0 °C for 3 h and the solvent was evaporated off. The residue was dissolved in AcOEt and the solution was washed with 0.1 N citric acid and water. After evaporation of AcOEt, the product was triturated with ether to give a precipitate; yield 26.6 g (90%), $[\alpha]_D^{26}$ −9.9° (c=2, MeOH), *Rf* 0.51 ($CHCl_3$–MeOH, 4:1). *Anal.* Calcd for $C_{39}H_{57}N_7O_{12}S$: C, 55.24; H, 6.78; N, 11.56. Found: C, 55.08; H, 7.10; N, 11.56.

Z–Ser(Bu^t)–Arg(Tos)–Leu–OH [Z-S11—13-OH (5)]——A solution of Z–Ser(Bu^t)OSu (31.5 g, 80.4 mmol) in

DMF (100 ml) was added to a solution of H-S12—13-OH (35.5 g, 80.4 mmol) and triethylamine (11.2 ml, 80.4 mmol) in DMF (200 ml). The mixture was stirred at 0 °C for 6 h and stored at 4 °C overnight. The solvent was evaporated off, the residue was dissolved in AcOEt and the solution was washed with 0.1 N citric acid and water. After evaporation of AcOEt, the product was triturated with ether to give a powder, which was precipitated from MeOH–$CHCl_3$–AcOEt; yield 41.9 g (73%), mp 175—177.5 °C (dec.), $[\alpha]_D^{27}$ −10.9° (c=2, MeOH), Rf 0.61 ($CHCl_3$–MeOH, 3:1). *Anal.* Calcd for $C_{34}H_{50}N_6O_8S \cdot H_2O$: C, 56.65; H, 7.27; N, 11.66. Found: C, 56.62; H, 7.09; N, 11.62.

H–Arg(Tos)–Asp(OBu^t)–Ser(Bu^t)–Ala–OH (H-S14—17-OH)——Z-S14—17-OH (25 g, 29.5 mmol) was hydrogenated in 80% CF_3CH_2OH and the product was triturated with ether to give a precipitate; yield 21.0 g, (99%), Rf 0.56 (n-BuOH–AcOH–H_2O, 4:1:5). *Anal.* Calcd for $C_{31}H_{51}N_7O_{10}S \cdot H_2O$: C, 50.87; H, 7.29; N, 13.39. Found: C, 50.68; H, 7.31; N, 12.98.

Z–Ser(Bu^t)–Arg(Tos)–Leu–Arg(Tos)–Asp(OBu^t)–Ser(Bu^t)–Ala–OH [Z-S11—17-OH (6)]——Z-S11—13-OH (20.3 g, 28.2 mmol) and HOSu (3.57 g, 31 mmol) were dissolved in DMF (90 ml) and DCC (5.8 g, 28.2 mmol) was added at −5 °C. The solution was stirred at −5 °C for 3 h and stored at 4 °C overnight. To this solution, a suspension of H-S14—17-OH (20 g, 28 mmol) in DMF (110 ml) with N-methylmorpholine (3.1 ml, 28 mmol) was added. The mixture was stirred at 0 °C for 6 h and stored at 4 °C overnight. The precipitate was filtered off, the filtrate was concentrated and 0.1 N citric acid was added. The resulting precipitate was filtered off, washed with water and dissolved in CF_3CH_2OH–MeOH–$CHCl_3$. After most of the solvent had been evaporated off, the product was triturated with AcOEt to give a powder; yield 29.1 g (73%), mp 166—171 °C (dec.), $[\alpha]_D^{22}$ −11.6° (c=2, CF_3CH_2OH), Rf 0.57 ($CHCl_3$–MeOH, 7:2). *Anal.* Calcd for $C_{65}H_{99}N_{13}O_{18}S_2$: C, 55.18; H, 7.05; N, 12.87. Found: C, 54.96; H, 7.21; N, 12.75.

Z–Glu(OBu^t)–Leu–OBzl (Z-S9—10-OBzl)——A solution of H–Leu–OBzl·TosOH (152.9 g, 0.39 mol) and N-methylmorpholine (42.9 ml, 0.39 mol) in CH_2Cl_2 (700 ml) was added to a mixed anhydride prepared from Z–Glu(OBu^t)–OH (131.4 g, 0.39 mol) in THF (600 ml). The mixture was stirred at −10 °C for 2 h and the solvent was evaporated off. The residue was dissolved in AcOEt and the solution was washed with 0.1 N $NaHCO_3$, 0.1 N citric acid and water. After evaporation of the AcOEt, the product was triturated with petroleum ether to give a precipitate; yield 180 g (86%), mp 85—87 °C, Rf 0.53 (isopropyl ether). *Anal.* Calcd for $C_{30}H_{40}N_2O_7$: C, 66.64; H, 7.46; N, 5.18. Found: C, 66.57; H, 7.40; N, 5.08.

H–Glu(OBu^t)–Leu–OH (H-S9—10-OH)——Z-S9—10-OBzl (180 g, 0.33 mol) was hydrogenated in MeOH (1.5 l) and the product was triturated with ether to give a precipitate; yield 61 g (58%), mp 188—192 °C (dec.), $[\alpha]_D^{29}$ −8.4° (c=2, AcOH), Rf 0.7 (n-BuOH–AcOH–H_2O, 4:1:5). *Anal.* Calcd for $C_{15}H_{28}N_2O_5$: C, 56.94; H, 8.92; N, 8.85. Found: C, 56.97; H, 9.17; N, 8.97.

Z–Ser(Bu^t)–Glu(OBu^t)–Leu–OH (Z-S8—10-OH)——A solution of Z–Ser(Bu^t)–OSu (98 g, 0.25 mol) in DMF (100 mol) was added to a solution of H-S9—10-OH (60 g, 0.19 mol) and N-methylmorpholine (20.9 ml, 0.19 mol) in DMF (500 ml). The mixture was stirred at 0 °C for 5 h and stored at 4 °C overnight. The solvent was evaporated off, the residue was dissolved in AcOEt and the solution was washed with 0.1 N citric acid and water. After evaporation of the AcOEt, the product was triturated with ether–petroleum ether to give a precipitate; yield 74 g (66%), mp 99—101 °C, $[\alpha]_D^{25}$ −16.6° (c=2, MeOH), Rf 0.5 (AcOEt). *Anal.* Calcd for $C_{30}H_{47}N_3O_9 \cdot HCON(CH_3)_2$: C, 59.44; H, 8.16; N, 8.40. Found: C, 59.19; H, 8.17; N, 8.24.

H–Ser(Bu^t)–Glu(OBu^t)–Leu–OH (H-S8—10-OH)——Z-S8—10-OH (70 g, 0.118 mol) was hydrogenated in MeOH (600 ml) and the product was triturated with ether to give a precipitate; yield 52 g (96%), mp 156—160 °C (dec.), $[\alpha]_D^{27}$ −9.9° (c=2, AcOH), Rf 0.58 (n-BuOH–AcOH–H_2O, 4:1:5). *Anal.* Calcd for $C_{22}H_{41}N_3O_7 \cdot 1/2H_2O$: C, 56.39; H, 9.04; N, 8.97. Found: C, 56.03; H, 9.39; N, 9.01.

Z–Thr(Bu^t)–Ser(Bu^t)–Glu(OBu^t)–Leu–OH [Z-S7—10-OH (7)]——H-S8—10-OH (51.0 g, 0.111 mol) was suspended in DMF (500 ml) with N-methylmorpholine (12.2 ml, 0.111 mol). Then a solution of Z–Thr(Bu^t)–OSu (52.4 g, 0.129 mol) in DMF (150 ml) was added. The mixture was stirred at 0 °C for 5 h and stored at 4 °C overnight. The solvent was evaporated off, the residue was dissolved in AcOEt and the solution was washed with 0.1 N citric acid and water. After evaporation of the AcOEt, the product was precipitated from ether–petroleum ether; yield 69 g (84%), mp 85—92 °C, $[\alpha]_D^{28}$ −5.0° (c=1, MeOH), Rf 0.37 ($CHCl_3$–MeOH, 20:1). *Anal.* Calcd for $C_{38}H_{62}N_4O_{11} \cdot 1/2H_2O$: C, 60.05; H, 8.36; N, 7.37. Found: C, 60.13; H, 8.62; N, 7.80.

H–Thr(Bu^t)–Ser(Bu^t)–Glu(OBu^t)–Leu–OH (H-S7—10-OH)——Z-S7—10-OH (30 g, 40 mmol) was hydrogenated in MeOH (600 ml) and the product was triturated with ether to give a precipitate; yield 17.0 g (70%), mp 214—218 °C (dec.), $[\alpha]_D^{28}$ −18.7° (c=2, CF_3CH_2OH), Rf 0.77 (n-BuOH–AcOH–H_2O, 4:1:5). *Anal.* Calcd for $C_{30}H_{56}N_4O_9$: C, 58.42; H, 9.15; N, 9.08. Found: C, 58.20; H, 9.35; N, 9.07.

Z–Thr(Bu^t)–Phe–OBzl (Z-S5—6-OBzl)——A solution of H–Phe–OBzl·TosOH (85.4 g, 0.2 mol) and N-methylmorpholine (22 ml, 0.2 mol) in CH_2Cl_2 (180 ml) was added to a mixed anhydride prepared from Z–Thr(Bu^t)–OH (61.8 g, 0.2 mol) in THF (400 ml). The mixture was stirred at 0 °C for 4 h and the solvent was evaporated off. The residue was dissolved in AcOEt and the solution was washed with 0.1 N $NaHCO_3$, 0.1 N citric acid and water. After evaporation of the AcOEt, the product was obtained as an oil; yield 109 g (99%), Rf 0.5 ($CHCl_3$). *Anal.* Calcd for $C_{32}H_{38}N_2O_6 \cdot H_2O$: C, 68.06; H, 7.14; N, 4.96. Found: C, 68.45; H, 6.89; N, 4.85.

H–Thr(Bu^t)–Phe–OH (H-S5—6-OH)——Z-S5—6-OBzl (108 g, 0.2 mol) was hydrogenated in MeOH (700 ml)

and the product was triturated with water to give a precipitate; yield 44 g (70%), mp 104—108 °C, $[\alpha]_D^{25}$ +46.7° ($c=1$, 80% AcOH), *Rf* 0.54 (*n*-BuOH–AcOH–H_2O: 4:1:5). *Anal.* Calcd for $C_{17}H_{26}N_2O_4 \cdot H_2O$: C, 59.97; H, 8.29; N, 8.23. Found: C, 60.40; H, 8.57; N, 8.35.

Z–Ser(Bu^t)–Asp(OBu^t)–Gly–Thr(Bu^t)–Phe–Thr(Bu^t)–Ser(Bu^t)–Glu(OBu^t)–Leu–OH [Z-S2—10-OH (9)]——Z–Ser(Bu^t)–Asp(OBu^t)–Gly–Thr(Bu^t)–Phe–OH[27] (18.1 g, 21.9 mmol) and HOSu (2.52 g, 21.9 mmol) were dissolved in DMF (120 ml) and DCC (4.51 g, 21.9 mmol) was added at −5 °C. The solution was stirred at −5 °C for 3 h and stored at 4 °C overnight, then a suspension of H-S7—10-OH (13.5 g, 21.9 mmol) in a mixture of DMF (80 ml) and *N*-methylpyrrolidone (20 ml) with *N*-methylmorpholine (2.65 ml, 24 mmol) was added. The mixture was stirred at 0 °C for 2 h and stored at 4 °C overnight. The precipitate was removed by filtration and the filtrate was poured into 0.1 N citric acid. The resulting precipitate was filtered off and washed with water and ether; yield 27 g (87%), mp 242 °C (dec.), $[\alpha]_D^{24}$ +7.1° ($c=1.6$, $CHCl_3$–MeOH, 2:1), *Rf* 0.38 ($CHCl_3$–MeOH, 9:1). *Anal.* Calcd for $C_{72}H_{115}N_9O_{20}$: C, 60.60; H, 8.14; N, 8.84. Found: C, 60.59; H, 8.39; N, 9.09.

H–Ser(Bu^t)–Asp(OBu^t)–Gly–Thr(Bu^t)–Phe–Thr(Bu^t)–Ser(Bu^t)–Glu(OBu^t)–Leu–OH (H-S2—10-OH)——Z-S2—10-OH (15.0 g, 10.5 mmol) was hydrogenated in 80% CF_3CH_2OH and the product was triturated with ether to give a precipitate; yield 13.4 g (98%), $[\alpha]_D^{23}$ −14.9° ($c=1$, CF_3CH_2OH), *Rf* 0.38 ($CHCl_3$–MeOH, 4:1).

Boc–His(Boc)–Ser(Bu^t)–Asp(OBu^t)–Gly–Thr(Bu^t)–Phe–Thr(Bu^t)–Ser(Bu^t)–Glu(OBu^t)–Leu–OH [Boc-S1—10-OH (10)]——Boc–His(Boc)–ONp (12.2 g, 25.5 mmol) was added to a solution of H-S2—10-OH (11.0 g, 8.51 mmol) and *N*-methylmorpholine (0.94 ml, 8.51 mmol) in *N*-methylpyrolidone (220 ml). The mixture was stirred at 40 °C for 44 h and poured into water containing AcOH and ether. The solid material was filtered off and precipitated from CF_3CH_2OH–MeOH; yield 12.1 g (87%), mp 234 °C (dec.), $[\alpha]_D^{25}$ −0.78° ($c=2$, CF_3CH_2OH), *Rf* 0.43 ($CHCl_3$–MeOH, 10:1). *Anal.* Calcd for $C_{80}H_{132}N_{12}O_{23}$: C, 58.95; H, 8.16; N, 10.31. Found: C, 58.76; H, 8.37; N, 10.18.

Z–Ser(Bu^t)–Arg(Tos)–Leu–Arg(Tos)–Asp(OBu^t)–Ser(Bu^t)–Ala–Arg(Tos)–Leu–Gln–Arg(Tos)–Leu–Leu–Gln–Gly–Leu–Val–NH$_2$ [Z-S11—27-NH$_2$ (11)]——Z-S18—27-NH_2 (12 g, 7.33 mmol) was hydrogenated in 80% AcOH and the resulting amine acetate was converted to the HCl salt as usual. This amine hydrochloride (11 g, 7.2 mmol), Z-S11—17-OH (10.12 g, 7.2 mmol) and HOSu (1.15 g, 10 mmol) were dissolved in DMF (130 ml) with *N*-methylmorpholine (0.80 ml, 7.2 mmol). DCC (1.62 g, 7.86 mmol) was added at −5 °C and the mixture was stirred at −5 °C for 3 h then stored at 4 °C for 3 d. The solvent was evaporated off and the residue was triturated with 0.1 N $NaHCO_3$. The solid material was filterd off and precipitated from MeOH–$CHCl_3$–ether; yield 13.4 g (65%), mp 236 °C (dec.), $[\alpha]_D^{27}$ −10.5° ($c=1$, CF_3CH_2OH), *Rf* 0.65 ($CHCl_3$–MeOH, 3:1). *Anal.* Caccd for $C_{132}H_{208}N_{32}O_{33}S_4$: C, 54.67; H, 7.23; N, 15.45. Found: C, 54.19; H, 7.39; N, 15.30.

Boc–His(Boc)–Ser(Bu^t)–Asp(OBu^t)–Gly–Thr(Bu^t)–Phe–Thr(Bu^t)–Ser(Bu^t)–Glu(OBu^t)–Leu–Ser(Bu^t)–Arg(Tos)–Leu–Arg(Tos)–Asp(OBu^t)–Ser(Bu^t)–Ala–Arg(Tos)–Leu–Gln–Arg(Tos)–Leu–Leu–Gln–Gly–Leu–Val–NH$_2$ [Boc-S1—27-NH$_2$ (12)]——Z-S11—27-NH_2 (5.22 g, 1.8 mmol) was hydrogenated in 80% AcOH and the resulting amine acetate was converted to the HCl salt as usual. This amine hydrochloride (5 g, 1.78 mmol), Boc-S1—10-OH (3.49 g, 2.14 mmol) and HOSu (345 mg, 3 mmol) were dissolved in a mixture of DMF (85 ml) and *N*-methylpyrrolidone (85 ml) with *N*-methylmorpholine (0.21 ml, 1.87 mmol). DCC (529 mg, 2.57 mmol) was added and the mixture was stirred at 4 °C for 2 d, then at room temperature for 3 d. The solution was poured into a mixture of water and ether. The precipitate was filtered off and purified by column chromatography on silica gel (5.5 i.d. × 20 cm; $CHCl_3$–MeOH, 6:1); yield 4.0 g (53%), mp 251 °C (dec.), $[\alpha]_D^{23}$ −7.4° ($c=1$, CF_3CH_2OH), *Rf* 0.57 ($CHCl_3$–MeOH, 4:1). *Anal.* Calcd for $C_{204}H_{332}N_{44}O_{53}S_4 \cdot 5H_2O$: C, 54.83; H, 7.71; N, 13.79. Found: C, 54.90; H, 7.73; N, 13.74. Amino acid ratios in a 6 N-HCl hydrolysate: His 1.03, Arg 4.05, Asp 1.94. Thr 1.88, Ser 3.85, Glu 2.92, Gly 1.99, Ala 1.01, Val 0.98, Leu 6.10, Phe 1.00 (average recovery 93%).

References and Notes

1) A part of this work was presented at the 102nd Annual Meeting of the Pharmaceutical Society of Japan, Osaka, April 1982 and the 21st Symposium on Peptide Chemistry, Tsukuba, October 1983.
2) Amino acids, peptides and their derivatives mentioned in this report are of the L-configuration. The following abbreviations are used: Z = benzyloxycarbonyl, Boc = *tert*-butoxycarbonyl, Bzl = benzyl, Bu^t = *tert*-butyl, Tos = *p*-toluenesulfonyl, TFA = trifluoroacetic acid, DCC = *N*,*N*′-dicyclohexylcarbodiimide, HOSu = *N*-hydroxysuccinimide, Su = *N*-hydroxysuccinimide ester, HOBt = *N*-hydroxybenzotriazole, Np = *p*-nitrophenyl, DMF = dimethylformamide, THF = tetrahydrofuran.
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