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Studies on Peptides. XL.[1,2] Synthesis of the Protected Dodecapeptide corresponding to Positions 1 to 12 of the Basic Trypsin Inhibitor from Bovine Pancreas (Kunitz and Northrop)

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The protected dodecapeptide, Z–Arg(NO_2)–Pro–Asp(OBzl)–Phe–Cys(Bzl)–Leu–Glu(OBzl)–Pro–Pro–Tyr–Thr–Gly–OH, corresponding to positions 1 to 12 of the basic trypsin inhibitor from bovine pancreas (Kunitz and Northrop) was synthesized. The synthesis was achieved by condensation of three peptide subunits, Z–Arg(NO_2)–Pro–OH, Z(OMe)–Asp(OBzl)–Phe–Cys(Bzl)–Leu–Glu(OBzl)–Pro–OH and H–Pro–Tyr–Thr–Gly–OH with pentachlorophenyl trichloroacetate.

Recently four groups of investigators have disclosed the primary structure of the basic trypsin inhibitor (BTI, Kunitz and Northrop) and the kallikrein inactivator from bovine origin. Kassell and Laskowski[4] elucidated the complete sequence of BTI from bovine pancreas as shown in Fig. 1. Independently and simultaneously, Anderer and Hornle[5] determined the structure of the kallikrein inactivator from bovine lung. It was shown that these two polypeptides are identical in sequence with the exception of one amide group at the Asp residue in position 50.

H–Arg–Pro–Asp–Phe–Cys[5]–Leu–Glu–Pro–Pro–Tyr[10]–Thr–Gly–Pro–Cys[15]–Lys–Ala–Arg–Ile–Ile–Arg[20]–
Tyr–Phe–Tyr–Asn–Ala[25]–Lys–Ala–Gly–Leu–Cys[30]–Gln–Thr–Phe–Val–Tyr[35]–Gly–Gly–Cys–Arg–Ala[40]–
Lys–Arg–Asn–Asn–Phe[45]–Lys–Ser–Ala–Glu–Asp[50]–Cys–Met–Arg–Thr–Cys[55]–Gly–Gly–Ala–OH

Fig. 1. Structure of the Basic Trypsin Inhibitor

S-S bridge: 5—55, 14—38, 30—51

Chauvet, *et al.*[6] and Dolouha, *et al.*[7] published independently the linear structure of the trypsin inhibitor from bovine pancreas. These authors placed Asp instead of Asn at position 50. The difference of Asp and Asn at position 50 seems to be due to the different

1) Part XXXIX: Y. Kiso, Y. Kai, and H. Yajima, *Chem. Pharm. Bull.* (Tokyo), **21**, 2507 (1973).
2) Amino acids, peptides and their derivatives mentioned in this communication are of the L-configuration. Abbreviations used are those recommended by IUPAC-IUB Commission on Biochemical Nomenclature: *Biochemistry*, **5**, 2485 (1966); *ibid.*, **6**, 362 (1967), *ibid.*, **11**, 1726 (1972). Boc=*tert*-butoxycarbonyl, Z=benzyloxycarbonyl, Z(OMe)=*p*-methoxybenzyloxycarbonyl, OBzl=benzyl ester, ONP=*p*-nitrophenyl ester, OPCP=pentachlorophenyl ester.
3) Location: *Sakyo-ku, Kyoto.*
4) B. Kassell and M. Laskowski Sr., *Biochem. Biophys. Res. Commun.*, **17**, 792 (1962); B. Kassell, M. Radicevic, M.J. Ansfield, and M. Laskowski Sr., *ibid.*, **18**, 255 (1965); B. Kassell and M. Laskowski Sr., *ibid.*, **20**, 463 (1965).
5) *a*) F.A. Anderer and S. Hörnle, *Z. Naturforsch.*, **20b**, 457 (1965); F.A. Anderer, *ibid.*, **20b**, 462, 499 (1965); *b*) F.A. Anderer and S. Hörnle, *J. Biol. Chem.*, **241**, 1568 (1966).
6) J. Chauvet, G. Nouvel, and R. Acher, *Biochim. Biophys. Acta*, **92**, 200 (1964); *idem, ibid.*, **115**, 121, 130 (1966); R. Acher and J. Chauvet, *Bull. Soc. Chim. France*, 3954 (1967).
7) V. Dlouha, J. Neuwirthova, B. Meloun, and F. Sörm, *Coll. Czeck. Chem. Commun.*, **30**, 1705 (1965), **32**, 131 (1967); V. Dlouha, D. Pospisilovia, B. Meloun, and F. Sörm, *ibid.*, **30**, 1311 (1965), **33**, 1363 (1968).

procedure employed for the preparation of inhibitors from different bovine organs.[8,9] Later the same inhibitor was also isolated from various bovine organs such as kidney and parotid gland.[10]

Thus accumulated structural investigations of this basic protease inhibitor seem to have established the primary sequence of this protein. Anderer [5b] and others[11] demonstrated that reduction of all three disulfide linkages of the molecule in 8 moles urea lead to its inactivation and on oxidation, the specific activity could be completely restored. Because of these properties, we have selected to synthesize this protein.

Recently Izumiya and his associates[12] reported the solid phase synthesis of BTI with approximately 30% biological activity. They adopted the stepwise addition procedure of Boc-amino acids on the polymer support introduced by Merrifield.[13] We have engaged in the synthesis of BTI by the method different from that of these authors. The entire linear amino acid sequence of BTI was subdivided into 5 fragments with Gly as terminus to avoid the racemization during the condensation of these fragments as shown in Fig. 2. As we will report in the later series of this synthesis, we have assembled these peptide subunits on polymer support. After deblocking of all protecting groups and subsequent oxidation followed by affinity-chromatographic purification, a substance was obtained which exhibited 82% activity of the native inhibitor. However its purity and yield are not fully satisfactory to our criteria of chemical synthesis. An alternate, improved synthesis of BTI therefore is being studied. In this report, we wish to report our initial approach to the synthesis of a peptide with high biological activity.

(I) positions 1–12, Z-(BTI 1–12)-OH

NO_2 OBzl Bzl OBzl

Z–Arg–Pro–Asp–Phe–Cys–Leu–Glu–Pro–Pro–Tyr–Thr–Gly–OH

(II) positions 13–28, Z(OMe)-(BTI 13–28)-OH

Bzl Z Tos Tos Z

Z(OMe)–Pro–Cys–Lys–Ala–Arg–Ile–Ile–Arg–Tyr–Phe–Tyr–Asn–Ala–Lys–Ala–Gly–OH

(III) positions 29–37, Z(OMe)-(BTI 29–37)-OH

Bzl

Z(OMe)–Leu–Cys–Gln–Thr–Phe–Val–Tyr–Gly–Gly–OH

(IV) positions 38–56, Z(OMe)-(BTI 38–56)-OH

Bzl Tos Z Tos Z OBzl OBzl Bzl Tos Bzl

Z(OMe)–Cys–Arg–Ala–Lys–Arg–Asn–Asn–Phe–Lys–Ser–Ala–Glu—Asp—Cys–Met–Arg–Thr–Cys–Gly–OH

(V) positions 57–58, Z(OMe)-(BTI 57–58)-OH

Z(OMe)–Gly–Ala–OH

Fig. 2. Synthetic Subunits of BTI

The synthetic data of the protected dodecapeptide (I), Z–Arg(NO_2)–Pro–Asp(OBzl)–Phe–Cys(Bzl)–Leu–Glu(OBzl)–Pro–Pro–Tyr–Thr–Gly–OH, corresponding to positions 1 to 12 of BTI are presented. The benzyl ester was applied for the protection of the ω-carboxyl group of Asp and Glu and the nitro group was introduced for the protection of the guanidino group of Arg. The sulfhydryl group of Cys was masked by the benzyl group. These protecting

8) M. Laskowski Sr., *Ann. New York Acad. Sci.*, **146**, 374 (1968).
9) F.A. Anderer and S. Hörnle, *ibid.*, **146**, 381 (1968).
10) E. Werle and W. Appel, *Naturwiss.*, **45**, 60 (1968); E. Werle, *Z. Physiol. Chem.*, **338**, 228 (1964); H. Kraut and N. Bhargava, *ibid.*, **338**, 231 (1964).
11) J. Chauvet and R. Acher, *Bull. Soc. Chim. Biol.*, **48**, 1248 (1966); D. Pospisilova, B. Meloun, I. Fric, and F. Sorm, *Coll. Czeck. Chem. Commun.*, **32**, 4108 (1967).
12) K. Noda, S. Terada, N. Mitsuyasu, M. Waki, T. Kato, and N. Izumiya, *Naturwiss.*, **58**, 147 (1971).
13) R.B. Merrifield, *J. Am. Chem. Soc.*, **85**, 2149 (1963).

groups are known to be cleaved by treatment with hydrogen fluoride.[14] For the synthesis of peptides containing Cys, the Z(OMe) group removable by trifluoroacetic acid (TFA)[15] was used extensively as the temporary protection of the α-amino group. The procedure for the preparation of *p*-methoxybenzyl mixed carbonate[16] made it possible to supply enough quantity of Z(OMe) amino acids required.

The protected dodecapeptide (I) was synthesized by assembling three subunits as shown in Fig. 3. For the preparation of the N-terminal protected dipeptide, Z–Arg(NO_2)–Pro–OH (1-a), Z–Arg(NO_2)–OH was converted to the corresponding dinitrophenyl ester,[17] which without isolation, was allowed to react with H–Pro–OH in the presence of triethylamine as described previously.[18a] Physical constants of the resulting Z–Arg(NO_2)–Pro–OH, agreed well with those of literature.[18b] This was then converted to the corresponding pentachlorophenyl ester by pentachlorophenyl trichloroacetate.[19]

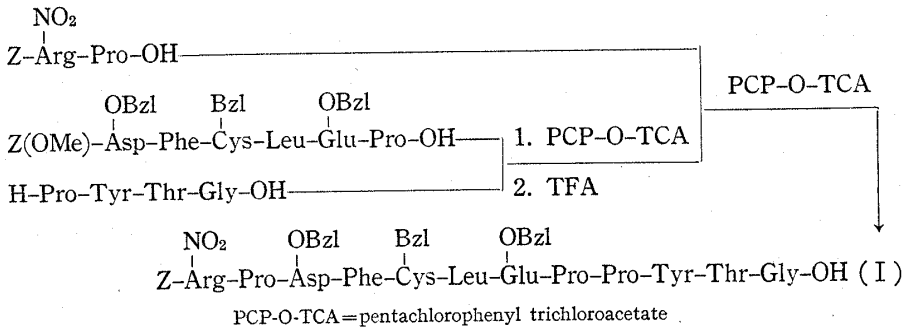

Fig. 3. Synthetic Route to the Protected Dodecapeptide (I, positions 1 to 12), Z–(BTI 1—12)–OH

Synthetic outline of the protected hexapeptide, Z(OMe)–Asp(OBzl)–Phe–Cys(Bzl)–Leu–Glu(OBzl)–Pro–OH (1-b, positions 3 to 8), is illustrated in Fig. 4. This peptide was prepared in the stepwise manner starting from H–Pro–OH. Z(OMe)–Glu(OBzl)–Pro–OH was obtained by the reaction of Z(OMe)–Glu(OBzl)–ONP with the triethylammonium salt of H–Pro–OH. Attempt to crystallize this protected dipeptide has been unsuccessful. This was then treated with trifluoroacetic acid in the usual manner and the resulting H–Glu(OBzl)–Pro–OH was condensed with Z(OMe)–Leu–OH by the *p*-nitrophenyl ester procedure.[20] Crystallization of the resulting protected tripeptide, Z(OMe)–Leu–Glu(OBzl)–Pro–OH, has also been unsuccessful. Combination of the TFA treatment and the ONP method was applied to elongate the peptide chain to the hexapeptide stage. The protected tetra and pentapeptides, Z(OMe)–Cys(Bzl)–Leu–Glu(OBzl)–Pro–OH and Z(OMe)–Phe–Cys(Bzl)–Leu–Glu(OBzl)–Pro–OH, were purified by the extraction procedure and both were isolated as amorphous powder. The protected hexapeptide (I-b), after purification by the batchwise washing procedure, was obtained in analytically pure form.

Synthetic route to the C-terminal tetrapeptide, H–Pro–Tyr–Thr–Gly–OH (I-c, positions 9 to 12), is illustrated in Fig. 5. Z–Thr–OPCP was allowed to react with H–Gly–OH in the presence of triethylamine to give Z–Thr–Gly–OH, which after hydrogenation, was condensed

14) S. Sakakibara and Y. Shimonishi, *Bull. Chem. Soc. Japan*, **38**, 1412 (1965).
15) F. Weygand and K. Hunger, *Chem. Ber.*, **95**, 1 (1962).
16) H. Yajima, F. Tamura, and Y. Kiso, *Chem. Pharm. Bull.* (Tokyo), **18**, 2574 (1970).
17) M. Bodanszky and M.A. Ondetti, *Chem. Ind.*, **1966**, 26.
18) *a*) H. Yajima, K. Kitagawa, and T. Segawa, *Chem. Pharm. Bull.* (Tokyo), **21**, 2500 (1973); *b*) R.A. Boissonnas, St. Guttmann, and P.A. Jaquenoud, *Helv. Chim. Acta*, **43**, 1349 (1960).
19) M. Fujino and C. Hatanaka, *Chem. Pharm. Bull.* (Tokyo), **16**, 929 (1968).
20) M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959).

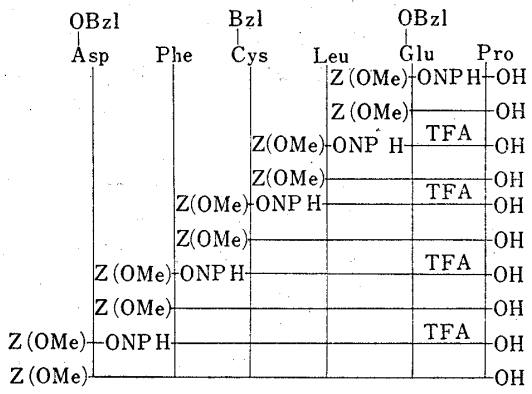

Fig. 4. Synthetic Route to the Protected Hexapeptide (I-b, positions 3 to 8)

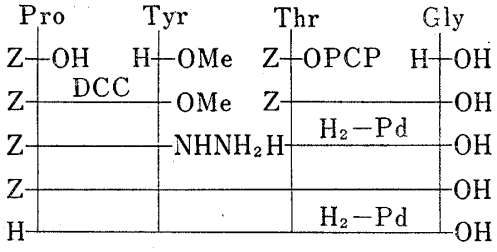

Fig. 5. Synthetic Route to the Tetrapeptide (I-c, positions 9 to 12)

with Z–Pro–Tyr–$NHNH_2$[21] by the modified azide procedure.[22] The resulting Z–Pro–Tyr–Thr–Gly–OH was subsequently hydrogenated to give the free tetrapeptide in crystalline form.

To assemble these peptide subunits thus obtained, the protected hexapeptide (I-b) was first converted to the corresponding PCP ester by pentachlorophenyl trichloroacetate and the resulting active ester, after purification by column chromatography on silica gel, was allowed to react with the C-terminal tetrapeptide (I-c) as illustrated in Fig. 3. Column chromatographic purification on silica was also effective in isolating the analytically pure protected decapeptide, Z(OMe)–Asp(OBzl)–Phe–Cys(Bzl)–Leu–Glu(OBzl)–Pro–Pro–Tyr–Thr–Gly–OH. This, after treatment with TFA, was condensed with Z–Arg(NO_2)–Pro–OH by the same PCP procedure and the desired protected dodecapeptide (I) was purified by the batchwise procedure. Homogeneity of the synthetic I was assessed by thin layer chromatography and elemental and amino acid analyses.

Experimental

General experimental methods employed are essentially the same as described in the Part XXII[23] of this series. Thin-layer chromatography was performed on silica gel (Kiselgel G, Merck). *Rf.* values refer to the following solvent stysems: Rf_1 $CHCl_3$–MeOH–H_2O (40: 15: 5), Rf_2 *n*-BuOH–AcOH–pyridine–H_2O (4: 1: 1: 2), Rf_3 $CHCl_3$–MeOH–AcOH (9: 1: 0.5).

Acylamino Acid Active Esters——The *p*-nitrophenyl and pentachlorophenyl esters of the following acylamino acids were prepared according to Bodanszky, *et al.*[20] and Kovacs, *et al.*[24] respectively.

Z–Thr–OPCP (recrystallized from tetrahydrofuran and EtOH): mp 169—171°, $[\alpha]_D^{26}$ −11.5° (c=0.3, DMF). *Anal.* Calcd. for $C_{18}H_{14}O_5NCl_5$: C, 43.10; H, 2.81; N, 2.79. Found: C, 42.84; H, 2.86; N, 2.90.

Z(OMe)–Glu(OBzl)–ONP (recrystallized from EtOH and petroleum ether): mp 120—122°. *Anal.* Calcd. for $C_{27}H_{26}O_9N_2$: C, 62.06; H, 5.02; N, 5.36. Found: C, 62.02; H, 5.14; N, 5.58.

Z(OMe)–Asp(OBzl)–ONP (recrystallized from EtOH and petroleum ether): mp 92—94°. *Anal.* Calcd. for $C_{26}H_{24}O_9N_2$: C, 61.41; H, 4.76; N, 5.51. Found: C, 61.39; H, 4.75; N, 5.50.

Z(OMe)–Phe–ONP (recrystallized from EtOH and petroleum ether): mp 89—91°. (lit.[25] mp 92—93°). *Anal.* Calcd. for $C_{24}H_{22}O_7N_2$: C, 63.99; H, 4.92; N, 6.22. Found: C, 64.27; H, 4.90; N, 6.28.

Z–Arg(NO_2)–Pro–OPCP——This active ester was prepared according to Fujino and Hatanaka[19] as described previously.[18a] mp 125—127° (lit.[18a] mp 125—127°). *Anal.* Calcd. for $C_{25}H_{25}O_7N_6Cl_5$: C, 42.97; H, 3.61; N, 12.03. Found: C, 42.71; H, 3.82; N, 11.88.

Z(OMe)–Glu(OBzl)–Pro–OH——Z(OMe)–Glu(OBzl)–ONP (31.40 g) in dioxane (250 ml) was added to a solution of H–Pro–OH (34.54 g) and triethylamine (42 ml) in H_2O (100 ml) and the solution was stirred at room temperature for 5 hr. The solvent was evaporated and the residue was dissolved in H_2O, which was washed with ether and then acidified with 10% citric acid. The resulting precipitate was extracted with

21) H. Yajima, Y. Kinomura, T. Oshima, and Y. Okada, *Chem. Pharm. Bull.* (Tokyo), **15**, 1922 (1967).
22) J. Honzl and J. Rudinger, *Coll. Czech. Chem. Commun.*, **26**, 2333 (1961).
23) H. Yajima, Y. Okada, H. Kawatani, and N. Mizokami, *Chem. Pharm. Bull.* (Tokyo), **17**, 1229 (1969).
24) J. Kovacs, M.Q. Ceprini, C.A. Dupraz, and G.N. Schmit, *J. Org. Chem.*, **32**, 3696 (1957).
25) E. Klieger, *Ann. Chem.*, **724**, 204 (1969).

AcOEt, which after washing with H_2O–NaCl, was evaporated to give an oily product; yield 30.05 g (*ca.* 100%). Rf_1 0.34.

Z(OMe)-Leu-Glu(OBzl)-Pro-OH——Z(OMe)–Glu(OBzl)–Pro–OH (30.05 g) was treated with TFA (22 ml) in the presence of anisole (15 ml) at 0° for 60 min. Dry ether was added to give an oily precipitate. Ether was removed by decantation and repetition of this operation gave the amorphous powder (Rf_2 0.83), which was dissolved in H_2O (50 ml) containing triethylamine (16.8 ml). To this solution, Z(OMe)–Leu–ONP (25.0 g) in dioxane (200 ml) was added and the mixture was stirred at room temperature for 5 hr. After evaporation of the solvent, H_2O was added. The aqueous phase was washed with ether and then acidified with 10% citric acid. The resulting oil was extracted with AcOEt, which after washing with H_2O–NaCl, was evaporated to give an oily product; yield 35.70 g (93%), Rf_3 0.54.

H-Leu-Glu(OBzl)-Pro-OH——Z(OMe)–Leu–Glu(OBzl)–Pro–OH (35.70 g) was treated with TFA (23 ml) in the presence of anisole (10 ml) at 0° for 60 min. Dry ether was added to form an oily precipitate, which turned to the solid by trituration with ether; yield 25.05 g (83%), Rf_2 0.85. *Anal.* Calcd. for $C_{23}H_{33}O_6N_3 \cdot CF_3COOH \cdot H_2O$: C, 51.81; H, 6.26; N, 7.25. Found: C, 51.94; H, 6.28; N, 7.48.

Z(OMe)-Cys(Bzl)-Leu-Glu(OBzl)-Pro-OH——Z(OMe)–Cys(Bzl)–ONP (24.83 g) in dioxane (350 ml) was added to a solution of H–Leu–Glu(OBzl)–Pro–OH·TFA salt (22.46 g) and triethylamine (11.2 ml) in 40% aqueous dioxane (350 ml), and the solution was stirred at room temperature for 8 hr. After evaporation of the solvent, the residue was dissolved in H_2O, which was washed with AcOEt. The aqueous phase was acidified with 10% citric acid and the resulting oil was extracted with AcOEt, which after washing with H_2O–NaCl, was evaporated to give an oily residue. Treatment of the residue with ether and petroleum ether afforded an amorphous powder; yield 32.20 g (75%), $[\alpha]_D^{28}$ −39.4° (c=0.5, DMF). Rf_3 0.59. *Anal.* Calcd. for $C_{42}H_{52}O_{10}N_4S \cdot H_2O$: C, 61.30; H, 6.61; N, 6.81. Found: C, 61.30; H, 6.60; N, 6.59.

H-Cys(Bzl)-Leu-Glu(OBzl)-Pro-OH——Z(OMe)–Cys(Bzl)–Leu–Glu(OBzl)–Pro–OH (28.80 g) was treated with TFA (15 ml) in the presence of anisole (6 ml) at 0° for 60 min. The powder formed by addition of dry ether was collected by filtration and dried over KOH pellets *in vacuo*; yield 25.0 g (96%), $[\alpha]_D^{27}$ −86.8° (c= 0.3, DMF). Rf_3 0.16. *Anal.* Calcd. for $C_{33}H_{44}O_7N_4S \cdot CF_3COOH \cdot H_2O$: C, 54.39; H. 6.13; N, 7.25. Found: C, 54.34; H, 6.39; N, 7.62.

Z(OMe)-Phe-Cys(Bzl)-Leu-Glu(OBzl)-Pro-OH——Z(OMe)–Phe–ONP (13.51 g) in dioxane (100 ml) was added to a solution of H–Cys(Bzl)–Leu–Glu(OBzl)–Pro–OH·TFA salt (22.64 g) and triethylamine (8.4 ml) in dioxane (250 ml) and the solution was stirred at room temperature for 24 hr. After evaporation of the solvent, H_2O was added to the residue. The aqueous phase was washed with ether and then acidified with 10% citric acid. The resulting oil was extracted with AcOEt, which after washing with H_2O–NaCl, was dried over Na_2SO_4 and then evaporated. Trituration of the residue with ether gave the solid, which was precipitated from AcOEt with ether; yield 19.10 g (65%), mp 103—106°, $[\alpha]_D^{25}$ −34.9° (c=0.6, DMF). Rf_3 0.42. *Anal.* Calcd. for $C_{51}H_{61}O_{11}N_5S \cdot H_2O$: C, 63.14; H, 6.55; N, 7.22. Found: C, 63.05; H, 6.40; N, 7.30.

H-Phe-Cys(Bzl)-Leu-Glu(OBzl)-Pro-OH——In the usual manner, the above Z(OMe)-pentapeptide (13.60 g) was treated with TFA (30 ml) in the presence of anisole (7 ml) at 0° for 60 min. Dry ether was added and the resulting solid was collected by filtration; yield 11.32 g (96%), mp 190—193°, $[\alpha]_D^{24}$ −32.1° (c=0.3, DMF). Rf_3 0.33. *Anal.* Calcd. for $C_{42}H_{53}O_8N_5S \cdot CF_3COOH \cdot 3H_2O$: C, 59.91; H, 7.06; N, 8.32. Found: C, 60.04; H, 6.80; N, 8.23.

Z(OMe)-Asp(OBzl)-Phe-Cys(Bzl)-Leu-Glu(OBzl)-Pro-OH——Z(OMe)–Asp(OBzl)–ONP (7.63 g) was added to a solution of the TFA salt of H–Phe–Cys(Bzl)–Leu–Glu(OBzl)–Pro–OH (11.79 g) and triethylamine (1.9 ml) in DMF (100 ml) and the mixture was stirred at room temperature for 8 hr. After evaporation of the solvent, ether and 10% citric acid were added to the residue. The resulting solid was collected, washed with H_2O and ether and recrystallized from tetrahydrofuran and AcOEt; yield 12.0 g (74%), mp 125—129°, $[\alpha]_D^{26}$ −33.1° (c=0.2, DMF). Rf_3 0.55. *Anal.* Calcd. for $C_{62}H_{72}O_{14}N_6S \cdot 1/2H_2O$: C, 63.84; H, 6.31; N, 7.21. Found: C, 63.56; H, 6.31; N, 7.22.

Z(OMe)-Asp(OBzl)-Phe-Cys(Bzl)-Leu-Glu(OBzl)-Pro-OPCP——To a solution of Z(OMe)–Asp(OBzl)–Phe–Cys(Bzl)–Leu–Glu(OBzl)–Pro–OH (8.16 g) in DMF (60 ml) containing triethylamine (1 ml), pentachlorophenyl trichloroacetate (3.29 g) was added. After the solution was stirred at room temperature for 3 hr, H_2O was added. The resulting precipitate was collected by filtration and recrystallized from tetrahydrofuran and EtOH; yield 8.37 g (85%), mp 180—186°. *Anal.* Calcd. for $C_{68}H_{71}O_{14}N_6SCl_5$: C, 58.10; H, 5.09; N, 5.98. Found: C, 58.21; H, 5.35; N, 6.26.

Z-Thr-Gly-OH——*Z*–Thr–OPCP (70.22 g) in a mixture of tetrahydrofuran (300 ml) and dioxane (150 ml) was added to a solution of H–Gly–OH (18.77 g) and triethylamine (35 ml) in H_2O (300 ml) and the solution was stirred at room temperature for 72 hr. The solvent was evaporated and the residue was dissolved in H_2O, which after washing with ether, was acidified with 5 N HCl. The resulting oil was extracted with AcOEt, which was washed with H_2O–NaCl, dried over Na_2SO_4 and then evaporated. Trituration of the residue with ether afforded the solid, which was recrystallized from AcOEt and ether; yield 35.0 g (81%), mp 149—151°, $[\alpha]_D^{25}$ −33.0° (c=0.2, DMF). *Anal.* Calcd. for $C_{14}H_{18}O_6N_2$: C, 54.19; H, 5.85; N, 9.02. Found: C, 54.31; H, 6.08; N, 9.02.

H-Thr-Gly-OH——Z-Thr-Gly-OH (33.0 g) in MeOH (600 ml) containing AcOH (5 ml) was hydrogenated over a Pd catalyst in the usual manner. The product was recrystallized from H_2O and MeOH; yield 16.90 g (90%), mp 269° (decomp.), $[\alpha]_D^{20}$ +58.0° (c=0.1, H_2O). (lit.[26] mp 244—246°, $[\alpha]_D^{28}$ +53.7° in H_2O). *Anal.* Calcd. for $C_{16}H_{12}O_4N_2$: C, 40.90; H, 6.87; N, 15.90. Found: C, 40.79; H, 6.73; N, 15.83.

Z-Pro-Tyr-Thr-Gly-OH——Z-Pro-Tyr-$NHNH_2$[21] (8.52 g) was dissolved in 0.5 N HCl–DMF (80 ml) and under cooling with ice-NaCl, isoamylnitrite (2.7 ml) was added. After 15 min, the solution was neutralized with triethylamine and then combined with a solution of H-Thr-Gly-OH (1.76 g) and triethylamine (2.8 ml) in H_2O (30 ml). The mixture was stirred at 4° for 72 hr. After evaporation of the solvent, the residue was dissolved in 10% Na_2CO_3, which was washed with AcOEt and then acidified with 1 N HCl. The resulting oil was extracted with AcOEt. The organic phase was washed with H_2O–NaCl, dried over Na_2SO_4 and then evaporated. Addition of the ether to the residue gave the solid, which was recrystallized from AcOEt; yield 5.14 g (90%), mp 168—170°, $[\alpha]_D^{24}$ −58.1° (c=0.1, DMF). *Anal.* Calcd. for $C_{28}H_{34}O_9N_4$: C, 58.94; H, 6.01; N, 9.82. Found: C, 58.82; H, 5.92; N, 9.56.

H-Pro-Tyr-Thr-Gly-OH——Z-Pro-Tyr-Thr-Gly-OH (17.12 g) in MeOH (450 ml) containing AcOH (5 ml) was hydrogenated in the usual manner. The product was crystallized from H_2O–EtOH, yield 12.68 g (93%), mp 242° (decomp.), $[\alpha]_D^{24}$ −16.5° (c=0.2, H_2O). *Anal.* Calcd. for $C_{20}H_{28}O_7N_4 \cdot 2H_2O$; C, 52.86; H, 6.65; N, 12.33. Found: C, 52.62; H, 6.82; N, 12.50.

Z(OMe)-Asp(OBzl)-Phe-Cys(Bzl)-Leu-Glu(OBzl)-Pro-Pro-Tyr-Thr-Gly-OH——Z(OMe)-Asp(OBzl)-Phe-Cys(Bzl)-Leu-Glu(OBzl)-Pro-OPCP (7.03 g) was added to a solution of H-Pro-Tyr-Thr-Gly-OH (6.98 g) in DMF (80 ml) and H_2O (8 ml) containing triethylamine (2.2 ml) and the solution was stirred at room temperature for 48 hr. The solvent was evaporated and 2% citric acid was added to the residue. The resulting solid was washed with H_2O and ether, recrystallized once from tetrahydrofuran and AcOEt and then further purified by column chromatography on silica gel using $CHCl_3$ as eluent; yield 4.97 g (61%), mp 155—160°, $[\alpha]_D^{27}$ −87.6° (c=0.1, DMF). Rf_1 0.38. *Anal.* Calcd. for $C_{82}H_{98}O_{20}N_{10}S \cdot 5H_2O$: C, 59.12; H, 6.53; N, 8.41. Found: C, 58.90; H, 6.02; N, 8.77.

H-Asp(OBzl)-Phe-Cys(Bzl)-Leu-Glu(OBzl)-Pro-Pro-Tyr-Thr-Gly-OH——Z(OMe)-Asp(OBzl)-Phe-Cys-(Bzl)-Leu-Glu(OBzl)-Pro-Pro-Tyr-Thr-Gly-OH (3.0 g) was treated with TFA (4 ml) in the presence of anisole (1.2 ml) at 0° for 60 min. Dry ether was added and the resulting solid was collected by filtration; yield 2.98 g (96%), mp 146—150°, $[\alpha]_D^{23}$ −96.5° (c=0.1, DMF). Rf_2 0.84. *Anal.* Calcd. for $C_{73}H_{90}O_{17}N_{10}S \cdot CF_3COOH \cdot 8H_2O$: C, 53.94; H, 6.45; N, 8.39. Found: C, 53.59; H, 5.96; N, 8.82.

Z-Arg(NO_2)-Pro-Asp(OBzl)-Phe-Cys(Bzl)-Leu-Glu(OBzl)-Pro-Pro-Tyr-Thr-Gly-OH (I)——Z-Arg(NO_2)-Pro-OPCP (1.40 g) was added to a solution of H-Asp(OBzl)-Phe-Cys(Bzl)-Leu-Glu(OBzl)-Pro-Pro-Tyr-Thr-Gly-OH (2.98 g) in pyridine (8 ml) containing triethylamine (0.3 ml) and the solution was stirred at room temperature for 18 hr. The solvent was evaporated and ether was added to the residue. The resulting solid was washed batchwisely with ether, 0.5 N HCl and AcOEt and then recrystallized from tetrahydrofuran and AcOEt; yield 2.20 g (63%), mp 148—155°, $[\alpha]_D^{25}$ −71.5° (c=0.5, DMF). Rf_3 0.30. Amino acid ratios in an acid hydrolysate $Arg_{0.77}$ $Pro_{2.80}$ $Asp_{1.04}$ $Phe_{1.04}$ $Leu_{1.09}$ $Glu_{1.10}$ $Tyr_{0.78}$ $Thr_{0.91}$ $Gly_{1.00}$ (average recovery 96%). *Anal.* Calcd. for $C_{92}H_{114}O_{23}N_{16}S \cdot 6H_2O$: C, 56.60; H, 6.50; N, 11.48. Found: C, 56.38; H, 6.42; N, 12.07.

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