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Studies on Peptides. LXVI.[1,2] Synthesis of the Protected Heptadecapeptide Amide related to the C-Terminal Portion of Porcine Cholecystokinin-Pancreozymin (CCK-PZ)

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The heptadecapeptide amide corresponding to the C-terminal portion of porcine cholecystokinin-pancreozymin (CCK-PZ) was synthesized in the protected, but unsulfated form.

In the histrical background, the gallbladder contracting principle (cholecystokinin) and the pancreatic enzyme expulsing principle (pancreozymin) were thought to be the different substances, until Mutt and Jorpes[4] concluded in 1964, after careful isolation studies, that these biological activities are elicited by a single polypeptide designated tentatively as cholecystokinin-pancreozymin (CCK–PZ). Existence of the former principle in porcine intestinal mucosa was discovered by Ivy and Oldberg[5] as early as 1928 and the latter by Harper and Raper[6] in 1943.

The complete amino acid sequence of CCK–PZ was disclosed by Mutt and Jorpes[7] in 1971. It has been shown that the C-terminal portion is identical with that of gastrin[8] and consequently with caerulein[9] and phyllocaerulein[10] and the Tyr residue at position 27 is esterified with sulfuric acid. For structural confirmation, the N-terminal octapeptide and the C-terminal dodecapeptide amide were synthesized by Bodanszky, *et al.*[11] and Ondetti, *et al.*[12] Through these synthetic studies, it was found that the C-terminal sequence has all the biological properties of CCK–PZ. However, its total synthesis has remained to be accomplished to date. Radioimmunoassay of CCK–PZ has also been encountered the difficulty, because of the unavailability of pure CCK–PZ from natural sources.

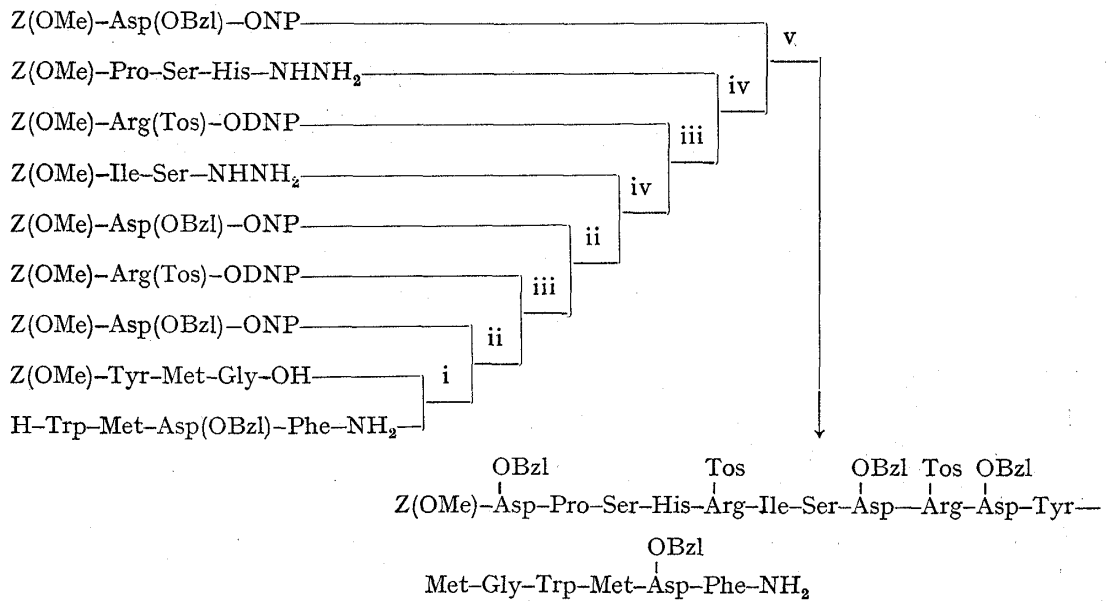
We wish to report initially in the three consecutive papers the synthesis of the tritriacontapeptide corresponding to the entire amino acid sequence of the unsulfated form of CCK–PZ, termed as [27-Tyr]-CCK–PZ, iodination of which seems to proceed much easier than genuine CCK–PZ.

1) Preliminal communication of this paper has appeared in *Chem. Pharm. Bull.* (Tokyo), **24**, 1110 (1976); Part LXV: *ibid.*, **24**, 2692 (1976).
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: *Biochem.*, **5**, 2485 (1966), *ibid.*, **6**, 362 (1967), *ibid.*, **11**, 1726 (1972). Z=benzyloxycarbonyl, Z(OMe)=*p*-methoxybenzyloxycarbonyl, Tos=tosyl, OBzl=benzyl, ester, ONP=*p*-nitrophenyl ester, ODNP=2,4-dinitrophenyl ester, OPCP=pentachlorophenyl ester, TFA=trifluoroacetic acid, DMF=dimethylformamide, DCC=dicyclohexylcarbodiimide.
3) Location: *Sakyo-ku, Kyoto, 606, Japan.*
4) E. Jorpes, V. Mutt and K. Toczko, *Acta Chem. Scand.*, **18**, 2408 (1964).
5) A.C. Ivy and E. Oldberg, *Am. J. Physiol.*, **86**, 599 (1928).
6) A.A. Harper and M.S. Raper, *J. Physiol.*, **102**, 115 (1943).
7) V. Mutt and J.E. Jorpes, *Biochem. Biophys. Res. Commun.*, **26**, 392 (1967); *idem*, Eur. J. Biochem., **6**, 156 (1968); *idem*, *Biochem. J.*, **125**, 57p (1971).
8) H. Gregory, P.M. Hardy, D.S. Jones, G.W. Kenner and R.C. Sheppard, *Nature*, **204**, 931 (1964).
9) A. Anastasi, V. Erspamer and R. Endean, *Experientia*, **23**, 699 (1967).
10) L. Bernardi, G. Bosisio, R. De Castiglione and O. Goffredo, *Experientia*, **25**, 7 (1969).
11) M. Bodanszky, N. Chaturvedi, D. Hudson and M. Itoh, *J. Org. Chem.*, **37**, 2303 (1972).
12) M.A. Ondetti, J. Pluscec, E.F. Sabo, J.T. Sheehan, and N. Williams, *J. Am. Chem. Soc.*, **92**, 195 (1970); J. Pluscec, J.T. Sheehan, E.F. Sabo, N. Williams, O. Kocy and M.A. Ondetti, *J. Med. Chem.*, **13**, 349 (1970).

As strategies in synthesizing such a peptide containing Met and Lys, the method we employed in the synthesis of motilin,[13] one of the intestinal polypeptides, is in our opinion, the most rational approach, since the catalytic hydrogenation of the α-amino protecting Z group can not be used, because of the presence of Met, which poisons the catalyst. In this synthesis, the TFA labile Z(OMe) group[14] played also a major role for the temporary protection of the α-amino function in all intermediates. Amino acid derivatives bearing protecting groups removable by hydrogen fluoride[15] were employed, *i.e.*, Asp(OBzl), Lys(Z) and Arg(Tos) and these protecting groups are known to survive under limited TFA treatments.

We have further demonstrated that this approach could be extended to the synthesis of peptides containing the acid sensitive Trp residue. As an example, synthesis of porcine gastric inhibitory polypeptide (GIP)[16] possessing three Trp residues was achieved successfully, in which destruction of Trp was minimized by the use of anisole containing 2% ethanedithiol[17] during various TFA treatments. Oxidation of the Met residue was also minimized by performing every reaction under the nitrogen atmosphere and ether stored over ferrous sulfate was used, when necessary. Thus experiments described herein is our second example of synthesizing a complex peptide possessing Met, Lys and Trp.

In the first section, synthesis of the heptadecapeptide amide corresponding to positions 17 through 33 of unsulfated CCK-PZ is described. The chain length of this C-terminal portion of CCK-PZ is equivalent to the entire amino acid sequence of gastrin[8] mentioned above. Scheme we employed in the synthesis of the heptadecapeptide amide is illustrated in Fig. 1. Within this sequence, four residues of Asp are present. Though, it is known that Asp-containing peptide fragments can be condensed by the azide procedure, if fragments are prepared



i=a) OPCP, b) TFA. ii=a) ONP, b) TFA. iii=a) ODNP, b) TFA. iv=a) azide, b) TFA. v=ONP.

Fig. 1. Synthetic Route to the Protected Heptadecapeptide Amide Z(OMe)-(CCK-PZ 17—33)-NH_2

13) H. Yajima, Y. Kai, and H. Kawatani, *J.C.S., Chem. Commun.*, **1975**, 159; *idem.*, *Chem. Pharm Bull.* (Tokyo), **23**, 2339 (1975); Y. Kai, H. Kawatani, H. Yajima, and Z. Itoh, *ibid.*, **23**, 2346 (1975).
14) F. Weygand and K. Hunger, *Chem. Ber.*, **95**, 1 (1962), H. Yajima, F. Tamura, and Y. Kiso, *Chem. Pharm. Bull.* (Tokyo), **18**, 2574 (1970).
15) S. Sakakibara, Y. Shimonishi, Y. Kishida, H. Okada, and H. Sugihara, *Bull. Chem. Soc. Japan*, **40**, 2164 (1967).
16) H. Yajima, H. Ogawa, M. Kubota, T. Tobe, M. Fujimura, K. Henmi, K. Torizuka, H. Adachi, H. Imura and T. Taminato, *J. Am. Chem. Soc.*, **97**, 5593 (1975).
17) J.J. Sharp, A.B. Robinson, and M.D. Kamen, *J. Am. Chem. Soc.*, **95**, 6097 (1973).

starting with substituted hydrazines, as seen in the synthesis of Ondetti *et al.*,[12] we prefered in the present synthesis to introduce these residues stepwisely. Thus relatively small four peptide fragments were selected as building blocks to construct the C-terminal portion of CCK-PZ, *i.e.*, Z(OMe)-Trp-Met-Asp(OBzl)-Phe-NH_2 (position 30—33), Z(OMe)-Tyr-Met-Gly-OH (27—29), Z(OMe)-Ile-Ser-$NHNH_2$ (22—23) and Z(OMe)-Pro-Ser-His-$NHNH_2$ (18—20).

The C-terminal tetrapeptide amide, Trp-Met-Asp-Phe, is the unit used also previously in the synthesis of gastrin.[18] We have alternatively synthesized this unit by using the Z(OMe) protecting groups as illustrated in Fig. 2. Starting with H-Phe-NH_2, three residues, Z(OMe)-Asp(OBzl)-OH, Z(OMe)-Met-OH and Z(OMe)-Trp-OH, were introduced stepwisely by the DCC,[19] trichlorophenyl ester[20] and *p*-nitrophenyl ester[21] procedures respectively. The resulting tetrapeptide amide, Z(OMe)-Trp-Met-Asp(OBzl)-Phe-NH_2, served after treatment with TFA in the presence of anisole containing 2% ethanedithiol as stated above, as an amino component for the next chain elongation reaction.

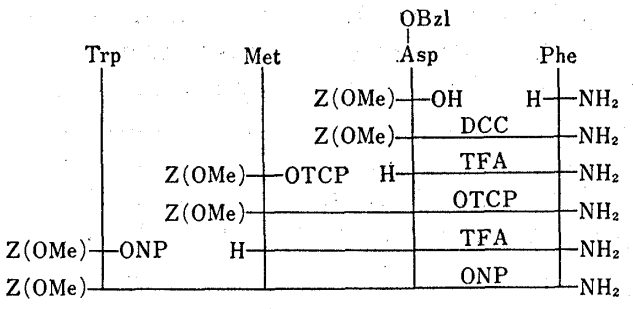


Fig. 2. Synthetic Scheme of the Protected Tetrapeptide Amide, Z(OMe)-(CCK-PZ 30—33)-NH_2

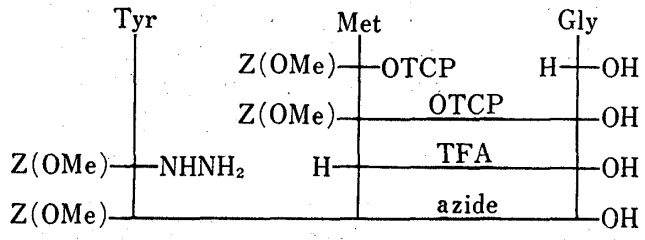


Fig. 3. Synthetic Scheme of the Protected Tripeptide, Z(OMe)-(CCK-PZ 27—29)-OH

For the synthesis of Z(OMe)-Tyr-Met-Gly-OH, the trichlorophenyl ester of Z(OMe)-Met-OH prepared above was allowed to react with the triethylammonium salt of Gly and the resulting Z(OMe)-Met-Gly-OH, after treatment with TFA, was condensed with Z(OMe)-Tyr-$NHNH_2$ by the azide procedure[22] as shown in Fig. 3.

Z(OMe)-Ile-Ser-$NHNH_2$ and Z(OMe)-Pro-Ser-$NHNH_2$ were prepared by the DCC procedure starting with the same methyl ester followed by exposure the resulting respective dipeptide esters to hydrazine hydrate. The latter was further condensed with H-His-OMe by the azide procedure and the resulting Z(OMe)-Pro-Ser-His-OMe was similarly exposed to hydrazine hydrate to afford Z(OMe)-Pro-Ser-His-$NHNH_2$ as shown in Fig. 4.

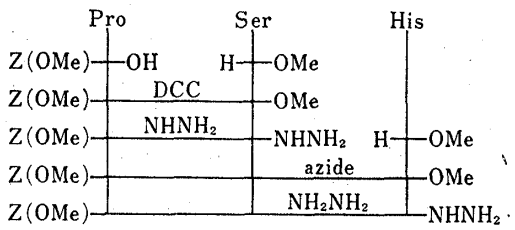


Fig. 4. Synthetic Scheme of the Protected Tripeptide Hydrazide, Z(OMe)-(18—20)-$NHNH_2$

Thus, the chain elongation in the present synthesis was performed with relatively small fragments prepared above and available amino acid derivatives, Z(OMe)-Asp(OBzl)-OH and Z(OMe)-Arg(Tos)-OH. First, H-Trp-Met-Asp(OBzl)-Phe-NH_2 prepared above was con-

18) J.C. Anderson, M.A. Barton, R.A. Gregory, P.M. Hardy, G.W. Kenner, J.K. MacLeod, J. Preston, R.C. Sheppard, and J.C. Morley, *Nature*, **204**, 933 (1964).
19) J.C. Sheehan and G.P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955).
20) R.L. Huguemin, *Helv. Chim. Acta*, **47**, 1934 (1964).
21) M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959).
22) J. Honzl and J. Rudinger, *Coll. Czech. Chem. Commun.*, **26**, 2333 (1961).

densed with Z(OMe)–Tyr–Met–Gly–OH by means of pentachlorophenyl trichloroacetate[23] according to the route illustrated in Fig. 1. This fragment condensation reaction proceeding through the corresponding pentachlorophenyl active ester was accelerated by 1 N hydroxybenzotriazole (HOBT)[24] to bring up the yield in 71%. Next, the *p*-nitrophenyl and 2,4-dinitrophenyl ester[25] procedures were employed to introduce three residues of Z(OMe)–Asp(OBzl)–OH and two residues of Z(OMe)–Arg(Tos)–OH respectively. In the latter instance, formation of the active ester was confirmed by thin-layer chromatography and this, without isolation, was submitted to the respective condensation reactions. In addition, the azide procedure was employed to introduce two peptide fragments, Ile–Ser and Pro–Ser–His units, to minimize racemization.

All of protected peptide amides we handled here are less soluble in ethyl acetate. Therefore, purification was performed by batchwise washing, rather than extraction, with dilute citric acid followed by precipitation from DMF with ethyl acetate or methanol. Since relatively small acylating components were employed, these purification procedures were sufficient enough to isolate chromatographically and analytically pure intermediates. Purity of the protected heptadecapeptide amide, Z(OMe)–Asp(OBzl)–Pro–Ser–His–Arg(Tos)–Ile–Ser–Asp-(OBzl)–Arg(Tos)–Asp(OBzl)–Tyr–Met–Gly–Trp–Met–Asp(OBzl)–Phe–NH_2, was further assessed by amino acid analysis. The content of Trp can be estimated by hydrolysis with 3N Tos-OH.[26] However, in the short column of an analysor, the Trp peak was overlapped with that of Arg(Tos), when two or more Arg(Tos) residues were present, since complete hydrolysis of Arg(Tos) to Arg was difficult to achieve with 3N Tos–OH hydrolysis. Ther fore, the usual hydrolysis with 6N hydrochloric acid was also performed to determine the Arg content.

The protected heptadecapeptide amide of established purity served as an amino component for the synthesis of [27-Tyr]–CCK–PZ, as we will describe in the latter paper. For biological testing, a part of the sample was deblocked by hydrogen fluoride. Chain-length activity correlationship of our synthetic peptides will be summarized in the latter paper also.

Experimental

General experimental methods employed here are essentially the same as those described in the Part LXII[27] of this series. Thin-layer chromatography was performed on silica gel (Kieselgel G, Merck). *Rf* values refer to the following solvent systems: Rf_1 $CHCl_3$–MeOH–H_2O (8: 3: 1), Rf_2 *n*-BuOH–AcOH–pyridine–H_2O (4: 1: 1: 2).

Z(OMe)-Asp(OBzl)-Phe-NH_2——DCC (14.83 g) was added to a mixture of Z(OMe)-Asp(OBzl)-OH (23.22 g) and H-Phe-NH_2 (11.82 g) in DMF (150 ml) and the solution was stirred at room temperature for 24 hr. After filtration, the filtrate was condensed *in vacuo* and the residue was treated with ether. The resulting powder was washed batchwisely with 5% citric acid, 5% sodium carbonate and H_2O and precipitated from DMF with AcOEt; yield 20.16 g (63%), mp 180—182°, $[\alpha]_D^{19}$ −18.6° (c=0.3, DMF), Rf_1 0.40. *Anal.* Calcd. for $C_{29}H_{31}N_3O_7$: C, 65.27; H, 5.85; N, 7.87. Found: C, 65.18; H, 5.89; N, 7.85.

Z(OMe)-Met-Asp(OBzl)-Phe-NH_2——Z(OMe)-Asp(OBzl)-Phe-NH_2 (16.0 g) was treated with TFA (22 ml) in the presence of anisole (9.7 ml) in an ice-bath for 45 min and then dry ether was added. The resulting powder was collected by filtration, dried over KOH pellets *in vacuo* and then dissolved in DMF (100 ml). Et_3N (8.4 ml) and Z(OMe)-Met-OTCP (17.74 g) were added and the mixture was stirred at room temperature for 24 hr. The solvent was evaporated and the residue was triturated with AcOEt. The resulting powder was washed batchwisely with 5% citric acid, H_2O and ether and then precipitated from DMF with AcOEt; yield 14.96 g (75%), mp 197—199°, $[\alpha]_D^{19}$ −10.1° (c=0.2, DMF) Rf_1 0.83 *Anal.* Calcd. for $C_{34}H_{40}N_4O_8S$: C, 61.43; H, 6.07; N, 8.43. Found: C, 61.59; H, 6.07; N, 8.49.

Z(OMe)-Trp-Met-Asp(OBzl)-Phe-NH_2——Z(OMe)-Met-Asp(OBzl)-Phe-NH_2 (3.32 g) was treated with TFA (3.8 ml) in the presence of anisole (1.2 ml) in an ice-bath for 45 min and then dry ether was added. The resulting powder was collected by filtration, dried over KOH pellets *in vacuo* and then dissolved in DMF (80

23) M. Fujino and C. Hatanaka, *Chem. Pharm. Bull.* (Tokyo), **16**, 929 (1968).
24) W. König and R. Geiger, *Chem. Ber.*, **106**, 3626 (1973).
25) M. Bodanszky and M.A. Ondetti, *Chem. Ind.*, **1966**, 26.
26) T.Y. Liu and Y.H. Chang, *J. Biol. Chem.*, **246**, 2842 (1971).
27) H. Ogawa, M. Kubota, and H. Yajima, *Chem. Pharm. Bull.* (Tokyo), **24** 2428 (1976).

ml), to which Et_3N (1.4 ml) and Z(OMe)-Trp-ONP (2.94 g) were combined. The mixture was stirred at room temperature for 24 hr and the solvent was evaporated *in vacuo*. The residue was treated with AcOEt and the resulting powder, after washing with 5% citric acid and H_2O as stated above, was precipitated from DMF with AcOEt; yield 3.02 g (71%), mp 188—190°, $[\alpha]_D^{18}$ −20.3° (c=0.6, DMF), Rf_1 0.69. *Anal.* Calcd. for $C_{45}H_{50}O_9N_6S$: C, 63.51; H, 5.92; N, 9.88. Found: C, 63.42, H, 5.78; N, 9.87.

Z(OMe)-Met-Gly-OH——Z(OMe)-Met-OTCP (24.64 g) in THF (100 ml) was added to a solution of H-Gly-OH (5.63 g) and Et_3N (16.8 ml) in H_2O (50 ml) and the mixture was stirred at room temperature for 24 hr. The solvent was evaporated the residue was dissolved in H_2O, which after washing with AcOEt, was acidified with citric acid. The resulting oily precipitate was extracted with AcOEt, which was washed with H_2O-NaCl, dried over sodium sulfate and then evaporated. Treatment of the residue with ether afforded the solid, which was recrystallized from AcOEt with petroleum ether; yield 16.48 g (89%), mp 125—127°, $[\alpha]_D^{19}$ +8.1° (c=0.3, DMF), Rf_1 0.38. *Anal.* Calcd. for $C_{16}H_{22}O_6N_2S$: C, 51.88; H, 5.99; N, 7.56. Found: C, 51.73; H, 5.70; N, 7.52.

Z(OMe)-Tyr-Met-Gly-OH——Z(OMe)-Met-Gly-OH (22.18 g) was treated with TFA (45 ml) in the presence of anisole (19.4 ml) in an ice-bath for 60 min and dry ether was added. The resulting oily precipitate was dried over KOH pellets *in vacuo* and then dissolved in ice-cold DMF (80 ml) containing Et_3N (25.1 ml). To a solution of Z(OMe)-Tyr-$NHNH_2$ (17.90 g) in DMF (80 ml), 3.13 N HCl-DMF (38.2 ml) and isoamylnitrite (8.0 ml) were added under cooling with ice-NaCl. After stirring for 5 min, the solution was neutralized with Et_3N (16.7 ml) and then combined with the above solution containing H-Met-Gly-OH. The mixture was stirred at 4° for 48 hr and then the solvent was evaporated. The residue was dissolved in H_2O, which after washing with AcOEt, was acidified with citric acid. The resulting oily precipitate was extracted with AcOEt, which was washed with 5% citric acid and H_2O-NaCl, dried over sodium sulfate and then evaporated. The residue was triturated with petroleum ether and recrystallized from AcOEt and petroleum ether; yield 16.48 g (62%), mp 108—110°, $[\alpha]_D^{18}$ −2.9° (c=0.5, MeOH), Rf_1 0.25. *Anal.* Calcd. for $C_{25}H_{31}O_8N_3S\cdot 1/2H_2O$: C, 55.34; H, 5.94; N, 7.75. Found: C, 55.49; H, 5.82; N, 7.59.

Z(OMe)-Tyr-Met-Gly-Trp-Met-Asp(OBzl)-Phe-NH_2——Pentachlorophenyl trichloroacetate (PCP-O-TCA) (3.62 g) was added to a solution containing Z(OMe)-Tyr-Met-Gly-OH (4.26 g) and Et_3N (1.23 ml) in DMF (50 ml) and the mixture was stirred at room temperature for 2 hr, while the starting material disappeared on thin layer chromatography and a new spot of Rf_1 1.0 was detected. The solvent was evaporated and the residue was treated with H_2O. The resulting powder was precipitated from tetrahydrofuran (THF) with petroleum ether (yield 5.30 g) and used, without further purification, to the following coupling reaction. Z-(OMe)-Trp-Met-Asp(OBzl)-Phe-NH_2 (10.0 g) was treated with TFA (13.4 ml) in the presence of anisole containing 2% ethanedithiol (19 ml) in an ice-bath for 50 min and then dry ether was added. The resulting precipritate was collected by filtration, dried over KOH pellets *in vacuo* and dissolved in DMF (50 ml). To this solution, Et_3N (3.3 ml), HOBT (1.59 g) and the above pentachlorophenyl ester (11.10 g) were added and the mixture was stirred at room temperature for 18 hr. The solvent was evaporated and the residue was treated with AcOEt. The resulting powder was washed batchwisely with 5% citric acid and H_2O and then precipitated from DMF with MeOH; yield 10.12 g (71%), mp 215—217°, $[\alpha]_D^{19}$ −12.3° (c=0.4, DMF), Rf_1 0.68. Amino acid ratios in 3 N Tos-OH hydrolysate: Tyr 0.90, Met 1.93, Gly 1.01, Trp 0.90, Asp 1.05, Phe 1.00 (average recovery 88%). *Anal.* Calcd. for $C_{61}H_{71}O_{13}N_9S_2\cdot H_2O$: C, 60.03; H, 6.03; N, 10.33. Found: C, 60.23; H, 6.18; N, 10.25.

Z(OMe)-Asp(OBzl)-Tyr-Met-Gly-Trp-Met-Asp(OBzl)-Phe-NH_2——The above protected heptapeptide amide (10.12 g) was treated as stated above with TFA (12.8 ml) in the presence of anisole containing 2% ethanedithiol (18.2 ml) in an ice-bath for 50 min, when dry ether was added. The resulting powder was dried over KOH pellets as usual and then dissolved in DMF (100 ml), to which Et_3N (2.4 ml), HOBT (1.14 g) and Z(OMe)-Asp(OBzl)-ONP (6.42 g) were added and the mixture was stirred at room temperature for 18 hr. The solvent was evaporated, the residue was treated with AcOEt and the resulting powder was purified as stated above by batchwise washing followed by precipitation from DMF with MeOH; yield 10.28 g (87%), mp 221—223°, $[\alpha]_D^{19}$ −8.7° (c=0.6, DMF), Rf_1 0.68. Amino acid ratios in 3N Tos-OH hydrolysate: Asp 2.05, Tyr 0.81, Met 1.98, Gly 1.00, Trp 0.87, Phe 1.00 (average recovery 84%). *Anal.* Calcd. for $C_{72}H_{82}O_{16}N_{10}S_2$: C, 61.43; H, 5.87; N, 9.95. Found: C, 61.45; H, 5.81; N, 9.69.

Z(OMe)-Arg(Tos)-Asp(OBzl)-Tyr-Met-Gly-Trp-Met-Asp(OBzl)-Phe-NH_2——The above protected octapeptide amide (10.28 g) was treated with TFA (13.9 ml) in the presence of anisole (19.7 ml) containing 2% ethanedithiol as stated above. The resutling TFA salt was dissolved in DMF (50 ml), to which Et_3N (2.0 ml) was added. Z(OMe)-Arg(Tos)-OH (10.79 g) and 2,4-dinitrophenol (4.43 g) were dissolved in THF (50 ml) and DCC (4.96 g) was added. The solution, after stirring at room temperature for 3 hr, was filtered, the filtrate was combined with the above solution containing the octapeptide amide and the mixture was stirred at room temperature for 18 hr. The solvent was evaporated and the residue was treated with AcOEt. The resulting powder was purified as stated above by batchwise washing followed by precipitation from DMF with MeOH; yield 10.70 g (86%), mp 204—206°, $[\alpha]_D^{19}$ −12.6° (c=0.4, DMF), Rf_1 0.68. Amino acid ratios in an acid hydrolysate: Arg 0.92, Asp 2.04, Tyr 0.75, Met 1.89, Gly 1.00, Phe 0.91 (average recovery 88%). *Anal.* Calcd. for $C_{85}H_{100}O_{19}N_{14}S_3$: C, 59.42; H, 5.87; N, 11.42. Found: C, 59.60; H, 6.08; N, 11.28.

Z(OMe)-Asp(OBzl)-Arg(Tos)-Asp(OBzl)-Tyr-Met-Gly-Trp-Met-Asp(OBzl)-Phe-NH_2——The above protected nonapeptide amide (10.70 g) was treated with TFA (11.9 ml) in the presence of anisole (16.9 ml) con-

taining 2% ethanedithiol and the resulting TFA salt isolated as stated above was dissolved in DMF (100 ml), to which Et_3N (1.7 ml), HOBT (0.84 g) and Z(OMe)-Asp(OBzl)-ONP (6.36 g) were added. The mixture was stirred at room temperature for 18 hr and the solvent was evaporated. The product was isolated as stated above by batchwise washing followed by precipitation from DMF with MeOH; yield 9.85 g (82%), mp 213—216°, $[\alpha]_D^{19}$ −9.8° (c=0.4, DMF), Rf_1 0.70. Amino acid ratios in an acid hydrolysate: Asp 3.04, Arg 1.11, Tyr 0.76, Met 1.79, Gly 1.00, Phe 0.96 (average recovery 92%). *Anal.* Calcd. for $C_{96}H_{111}O_{22}N_{15}S_3 \cdot 2H_2O$: C, 58.85; H, 5.92; N, 10.72. Found: C, 58.68; H, 5.74; N, 10.60.

Z(OMe)-Ile-Ser-OMe——In the usual manner, Z(OMe)-Ile-OH (29.53 g) and H-Ser-OMe (prepared from 15.56 g of the hydrochloride with 13.9 ml of Et_3N) in DMF (150 ml) was condensed with DCC (22.70 g). After reaction at room temperature overnight, the solution was filtered, the filtrate was condensed and the residue was recrystallized twice from AcOEt; yield 30.87 g (78%), mp 155—158°, $[\alpha]_D^{18}$ −2.4° (c=0.3, DMF), Rf_1 0.77. *Anal.* Calcd. for $C_{19}H_{28}O_7N_2$: C, 57.56; H, 7.12; N, 7.07. Found: C, 57.27; H, 7.07; N, 6.99.

Z(OMe)-Ile-Ser-NHNH$_2$——To a solution of Z(OMe)-Ile-Ser-OMe (15.0 g) in MeOH (200 ml), 80% hydrazine hydrate (10.6 ml) was added. The solid mass formed on standing overnight was recrystallized from MeOH; yield 11.99 g (80%), mp 234—236°, $[\alpha]_D^{18}$ +8.6° (c=0.5, DMF), Rf_1 0.50. *Anal.* Calcd. for $C_{18}H_{28}O_6N_4S$: C, 54.53; H, 7.12; N, 14.13. Found: C, 54.31; H, 6.89; N, 13.85.

Z(OMe)-Ile-Ser-Asp(OBzl)-Arg(Tos)-Asp(OBzl)-Tyr-Met-Gly-Trp-Met-Asp(OBzl)-Phe-NH$_2$——The above protected decapeptide amide (9.85 g) was treated with TFA (11.7 ml) in the presence of anisole (17 ml) containing 2% ethanedithiol and the resulting TFA salt, isolated as stated above, was dissolved in DMF (70 ml) containing Et_3N (1.6 ml). Under cooling with ice-NaCl, isoamylnitrite (1.64 ml) was added to a solution of Z(OMe)-Ile-Ser-NHNH$_2$ (4.06 g) in DMF (40 ml) and 2.03N HCl-DMF (12.1 ml). The solution, after stirring for 5 min, was neutralized with Et_3N (3.4 ml) and then combined with the above solution containing the decapeptide amide. The mixture was stirred at 4° for 48 hr and the solvent was evaporated. The product was isolated as stated above by batchwise washing followed by precipitation from DMF with MeOH; yield 7.83 g (72%), mp 220—223°, $[\alpha]_D^{19}$ −9.8° (c=0.2, DMF), Rf_1 0.68. Amino acid ratios in an acid hydrolysate: Ile 1.07, Ser 0.90, Asp 3.12, Arg 0.96, Tyr 0.72, Met 2.08, Gly 1.00, Phe 1.10 (average recovery 84%). *Anal.* Calcd. for $C_{105}H_{127}O_{25}N_{17}S_3 \cdot 3H_2O$: C, 57.91; H, 6.16; N, 10.94. Found: C, 58.17; H, 6.04; N, 10.73.

Z(OMe)-Arg(Tos)-Ile-Ser-Asp(OBzl)-Arg(Tos)-Asp(OBzl)-Tyr-Met-Gly-Trp-Met-Asp(OBzl)-Phe-NH$_2$——The above protected dodecapeptide amide (6.36 g) was treated with TFA (6.8 ml) in the presence of anisole (6.5 ml) containing 2% ethanedithiol and the resulting TFA salt, isolated as stated above, was dissolved in DMF (70 ml) containing Et_3N (0.84 ml). To this solution, Z(OMe)-Arg(Tos)-ODNP (prepared from 4.42 g of Z(OMe)-Arg(Tos-OH, 1.93 g of 2,4-dinitrophenol and 2.16 g of DCC as mentioned above) in THF (50 ml) was combined and the mixture was stirred at room temperature for 18 hr. The solvent was evaporated and the product was isolated as stated above by batchwise washing followed by precipitation from DMF with MeOH; yield 6.04 g (83%), mp 220—225°, $[\alpha]_D^{19}$ −7.1° (c=0.5, DMF), Rf_1 0.68. Amino acid ratios in an acid hydrolysate: Arg 2.14, IIe 1.02, Ser 0.96, Asp 2.96, Tyr 0.78, Met 1.77, Gly 1.00, Phe 0.92 (average recovery 90%). *Anal.* Calcd. for $C_{118}H_{145}O_{28}N_{21}S_4 \cdot 4H_2O$: C, 56.56; H, 6.11; N, 11.34. Found: C, 56.29; H, 6.05; N, 11.47.

Z(OMe)-Pro-Ser-NHNH$_2$——DCC (12.40 g) was added to a mixture of Z(OMe)-Pro-OH (12.46 g) and H-Ser-OMe (prepared from 9.36 g of the hydrochloride with 8.4 ml of Et_3N) in AcOEt-DMF (150 ml—100 ml) and the solution, after stirring for 18 hr, was filtered. The filtrate was condensed *in vacuo* and the residue was extracted with AcOEt, which was washed with 5% citric acid, 5% sodium carbonate and H_2O-NaCl, dried over sodium sulfate and then evaporated. The residue was dissolved in MeOH (200 ml) and 80% hydrazine hydrate (14 ml) was added. The solid mass formed on standing overnight was collected by filtration and recrystallized from MeOH; yield 10.08 g (53%), mp 178—179°, $[\alpha]_D^{18}$ +13.3° (c=0.3, DMF), Rf_1 0.60. *Anal.* Calcd. for $C_{17}H_{24}O_6N_4 \cdot H_2O$: C, 51.25; H, 6.58; N, 14.06. Found: C, 51.40; H, 6.34; N, 14.14.

Z(OMe)-Pro-Ser-His-NHNH$_2$——Z(OMe)-Pro-Ser-NHNH$_2$ (3.0 g) was dissolved in DMF (30 ml) and 2.03N HCl-DMF (9.41 ml) was added. Under cooling with ice-NaCl, isoamylnitrite (1.38 ml) was added dropwisely and the mixture, after stirring for 5 min, was neutralized with Et_3N (2.66 ml). This solution was then combined with a solution of H-His-OMe (prepared from 1.91 g of the hydrochloride with 3.32 ml of Et_3N) in 50% aqueous DMF (40 ml) and the mixture was stirred at 4° for 48 hr. The solvent was evaporated and the residue was treated with ether. The resulting powder was collected by filtration and then dissolved in MeOH (30 ml), to which 80% hydrazine hydrate (2.2 ml) was added. After stirring overnight, the solution was condensed and the residue was treated with ether. The resulting powder was washed with EtOH and then recrystallized twice from MeOH and ether; yield 2.49 g (61%), mp 180—185°, $[\alpha]_D^{18}$ −6.8° (c=0.3, DMF), Rf_1 0.50. *Anal.* Calcd. for $C_{23}H_{31}O_7N_7 \cdot H_2O$: C, 51.58; H, 6.21; N, 18.31. Found: C, 51.23; H, 6.04; N, 18.08.

Z(OMe)-Pro-Ser-His-Arg(Tos)-Ile-Ser-Asp(OBzl)-Arg(Tos)-Asp(OBzl)-Tyr-Met-Gly-Trp-Met-Asp(OBzl)-Phe-NH$_2$——Z(OMe)-Arg(Tos)-Ile-Ser-Asp(OBzl)-Arg(Tos)-Asp(OBzl)-Tyr-Met Gly-Trp-Met-Asp(OBzl)-Phe-NH$_2$ (5.18 g) was treated with TFA (8.1 ml) in the presence of anisole (6.9 ml) containing 2% ethanedithiol in an ice-bath for 50 min as stated above and the resulting TFA salt was dissolved in DMF (80 ml) containing Et_3N (0.6 ml). Isoamylnitrite (0.68 ml) was added to an ice-cold solution of Z(OMe)-Pro-Ser-His-NHNH$_2$ (2.21 g) in DMF (22 ml) and 2.03N HCl-DMF (7.56 ml). The solution, after stirring for 5 min, was neutralized with Et_3N (2.15 ml) and then combined with the above solution containing tridecapeptide amide. The mixture was stirred at 4° for 48 hr and the solvent was evaporated. Treatment of the residue with AcOEt afford-

ed the solid, which was washed batchwisely with 5% citric acid and H_2O and then precipitated from DMF with AcOEt; yield 4.52 g (77%), mp 215—218°, $[\alpha]_D^{18}$ −11.2° (c=0.3, DMF), Rf_1 0.50. Amino acid ratios in an acid hydrolysate: Pro 1.08, Ser 2.13, His 1.10, Asp 2.85, Arg 2.09, Ile 1.06, Tyr 0.73, Met 1.82, Gly 1.00, Phe 0.90 (average recovery 95%). *Anal.* Calcd. for $C_{132}H_{164}O_{32}N_{26}S_4 \cdot 5H_2O$: C, 55.72; H, 6.16; N, 12.80. Found: C, 55.58; H, 6.11; N, 12.50.

Z(OMe)-Asp(OBzl)-Pro-Ser-His-Arg(Tos)-Ile-Ser-Asp(OBzl)-Arg(Tos)-Asp(OBzl)-Tyr-Met-Gly-Trp-Met-Asp(OBzl)-Phe-NH_2——The above protected hexadecapeptide amide (4.50 g) was treated with TFA (6.1 ml) in the presence of anisole (4.3 ml) containing 2% ethanedithiol in an ice-bath for 50 min. The resulting TFA, salt, isolated as stated above, was dissolved in DMF (45 ml), to which Et_3N (0.56 ml), HOBT (0.22 g) and Z(OMe)-Asp(OBzl)-ONP (1.63 g) were added. The mixture was stirred at room temperature for 18 hr and the solvent was evaporated *in vacuo*. The product was purified as stated above by batchwise washing followed by precipitation from DMF with MeOH; yield 4.05 g (85%), mp 213—215°, $[\alpha]_D^{18}$ −12.8° (c=0.2, DMF), Rf_1 0.51. Amino acid ratios in an acid hydrolysate: Asp 3.94, Pro 1.04, Ser 2.09, His 1.16, Arg 2.15, Ile 1.00, Tyr 0.77, Met 1.62, Gly 0.95, Phe 0.87 (average recorery 90%). Amino acid ratios in 3N Tos-OH hydrolysate: Asp 4.24, Pro 1.10, Ser 1.87, His 1.09, Ile 0.91, Tyr 0.92, Met 1.80, Gly 1.24, Arg 0.22, Arg(Tos) +Trp (*ca.* 2.85 calcd. as Arg(Tos), overlapped) (average recovery 89%). *Anal.* Calcd. for $C_{143}H_{175}O_{35}N_{27}S_4 \cdot 6H_2O$: C, 55.97; H, 6.14; N, 12.32. Found: C, 55.91; H, 6.21; N, 12.14.

In order to prepare a sample for bioassay, the above protected heptadecapeptide amide (86 mg) was treated with HF (approximately 5 ml) in the presence of anisole (1 ml) containing 2% ethanedithiol and skatole (50 mg) in an ice-bath for 60 min. The excess HF was removed by evaporation at 0° and dry ether was added. The resulting gummy precipitate was dissolved in H_2O (5 ml), which was treated with Amberlite CG-4B (acetate form, approximately 3 g) for 30 min. The resin was removed by filtration and the filtrate was lyophilized. A fluffy powder obtained was dissolved in a small amount of 5% AcOH and the solution was applied to a column of Sephadex G-15 (1.5×140 cm), which was eluted with the same solvent. Individual fractions (4 ml each) were collected and absorbancy at 280 mμ was determined. Fractions corresponding to the front main peak (tube No. 38—53) were combined and the solvent was removed by lyophilization to give a white fluffy powder; yield 40 mg (65%), Rf_2 0.39. Amino acid ratios in 3N Tos-OH hydrolysate: Asp 3.86, Pro 1.24, Ser 2.00, His 1.09, Arg 2.14, Ile 0.88, Tyr 0.86, Met 1.72, Gly 1.07, Trp 0.73, Phe 0.88 (average recovery 87%).

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