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Studies on Peptides. LXXVI.[1,2] Synthesis of Kassinin, a New Frog Skin Peptide

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Kassinin, a dodecapeptide amide isolated by Erspamer *et al.* from the skin of *Kassina senegalensis*, was synthesized by deprotection of Z(OMe)–Asp(OBzl)–Val–Pro–Lys(Z)–Ser–Asp(OBzl)–Gln–Phe–Val–Gly–Leu–Met(O)–NH_2 with methanesulphonic acid, followed by reduction of the Met(O) residue to Met with 2-mercaptoethanol. The contractile activity of synthetic kassinin in isolated guinea-pig ileum was 0.4 of that of synthetic substance P.

Keywords——kassinin, a frog skin peptide; tachykinin peptide; deprotection by methanesulphonic acid; sodium perborate oxidation of Met; trichloroethyloxycarbonylhydrazine; smooth muscle contractile activity

Erspamer and Anastasi *et al.*[4] elucidated recently the amino acid sequence of a new frog skin peptide named kassinin (I) from African amphibian (Kassina senegalensis). This peptide was classified as one of the tachykinin peptides in a respect of having the characteristic C-terminal tripeptide end, Gly-Leu-Met-NH_2.

We have synthesized the dodecapeptide amide corresponding to the entire amino acid sequence of kassinin as an example for the synthesis of peptides containing Met by the methanesulphonic acid (MSA) procedure.[5] Considering to prevent alkylation at the sulphur atom of Met at the final deprotection step in the MSA-anisole system,[6] Z(OMe)-Met-OH was converted, as a starting material, to the protected form, Z(OMe)-Met(O)-OH, by oxidation with sodiumperborate.[7] Four peptide fragments were first prepared as building blocks; *i.e.*, A (position 11—12), B (8—10), C (4—5) and D (1—3).

For the synthesis of A, Z(OMe)-Leu-Met(O)-NH_2, the above mentioned sulphoxide was first converted to the corresponding amide, Z(OMe)-Met(O)-NH_2, *via* the mixed anhydride method,[8] which after treatment with TFA,[9] was condensed with Z(OMe)-Leu-OH by the NP method.[10] The fragment B, Z(OMe)-Phe-Val-Gly-$NHNH_2$, was prepared by the azide

1) Part LXXV: H. Yajima, J. Iwai, K. Koyama, M. Nakamura, K. Miyata, and A. Tanaka, *Chem. Pharm. Bnll.* (Tokyo), **26**, 571 (1978).
2) Amino acids, peptides and their derivatives are of the L-configuration. Following abbreviations were used: Z(OMe)=*p*-methoxybenzyloxycarbonyl, Z=benzyloxycarbonyl, Bzl=benzyl, NP=*p*-nitrophenyl, Troc=trichloroethyloxycarbonyl, DMF=dimethylformamide, TFA=trifluoroacetic acid, DMSO=dimethylsulfoxide, NMP=N-methylpyrrolidone, HMPA=hexamethylphosphoramide.
3) Location: *a) Sakyo-ku, Kyoto, 606 Japan*; *b) Kasumi, Hiroshima, 734 Japan.*
4) A. Anastasi, P. Montecucchi, V. Erspamer, and J. Visser, *Exp.*, **33**, 857 (1977); See also G. Bertaccini, *Pharmacol. Review*, **28**, 127 (1976).
5) H. Yajima, Y. Kiso, H. Ogawa, N. Fujii, and H. Irie, *Chem. Pharm. Bull.* (Tokyo), **23**, 1164 (1975).
6) H. Irie, N. Fujii, H. Ogawa, H. Yajima, M. Fujino, and S. Shinagawa, *J.C.S. Chem. Comm.*, **1976**, 922.
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8) Th. Wieland and H. Bernhard, *Ann. Chem.*, **572**, 190 (1951); J.R. Vaughan, *J. Am. Chem. Soc.*, **73**, 3547 (1951).
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condensation[11] of the known dipeptide hydrazide, Z(OMe)-Phe-Val-$NHNH_2$,[12] with Gly, followed by the esterification and hydrazinolysis. The fragment C, Z(OMe)-Lys(Z)-Ser-$NHNH_2$, was prepared by the hydrazine treatment of the known dipeptide ester, Z(OMe)-Lys(Z)-Ser-OMe.[12]

The amide bond formation between Val and Pro by the usual DCC condensation[13] was found difficult to establish, because of the steric hindrance of these two amino acids.[14] Thus, synthesis of the N-terminal tripeptide unit D, Z(OMe)-Asp(OBzl)-Val-Pro-OH, was prepared by the azide coupling of Z(OMe)-Asp(OBzl)-Val-$NHNH_2$ with Pro. The former hydrazide was prepared by condensation of Z(OMe)-Asp(OBzl)-OH and H-Val-NHNH-Troc[12] followed by removal of the Troc group from the resulting Z(OMe)-Asp(OBzl)-Val-NHNH-Troc with Zn.[15]

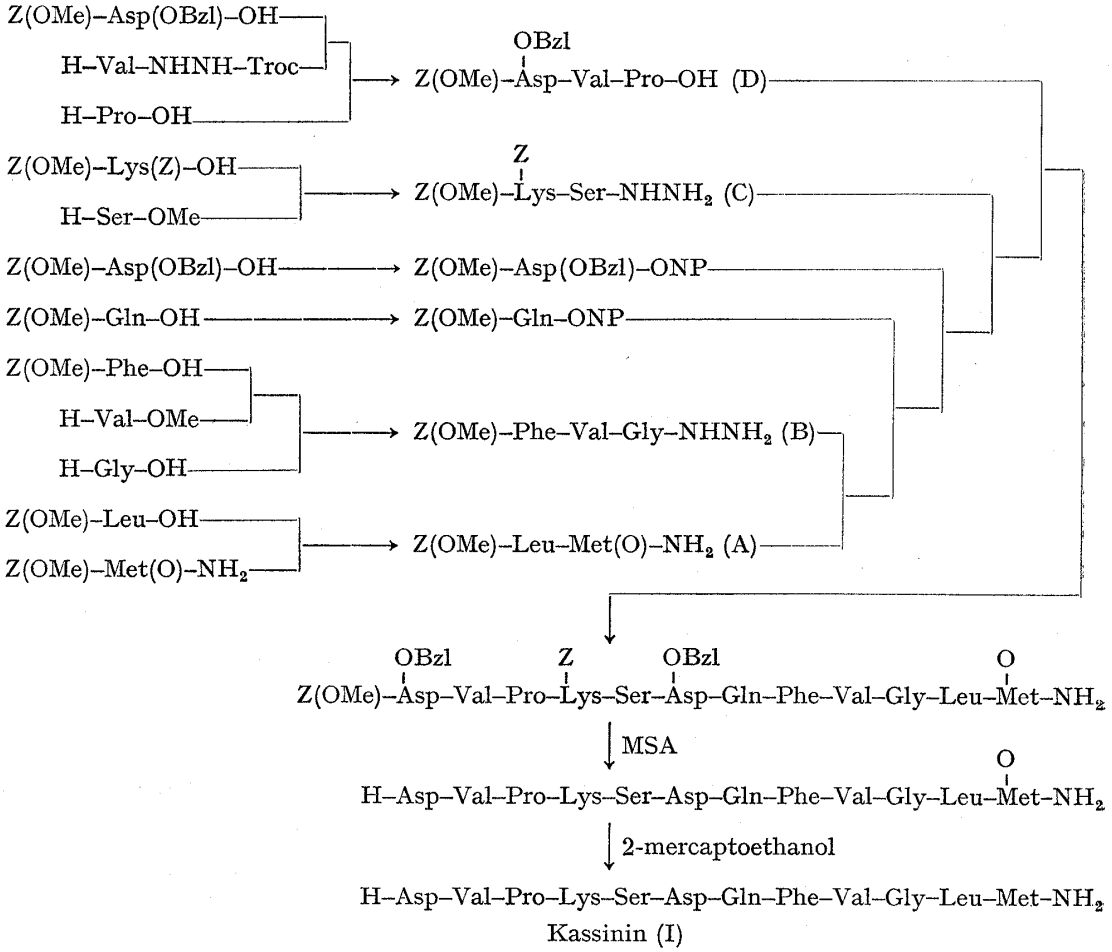

Fig. 1. Synthetic Route to Kassinin

Construction of the entire amino acid sequence of kassinin was then carried out according to the scheme illustrated in Fig. 1. The Z(OMe) group from intermediates was removed by the usual TFA treatment. As the condensation tools, the azide procedure was applied for the fragments B and C, the NP method for Gln and Asp(OBzl) at positions 7 and 6 and the pentachlorophenyl trichloroacetate procedure[16] for the fragment D. All intermediates and

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12) H. Ogawa, M. Kubota, and H. Yajima, *Chem. Pharm. Bull.* (Tokyo), **24**, 2428 (1976).
13) J.C. Sheehan and G.P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955).
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the protected dodecapeptide amide were purified by batchwise washing with 5% citric acid and water followed by repeated precipitation from DMF or DMSO with appropriate solvents, such as methanol and the purity of these protected peptides was assessed by three criteria; thin layer chromatography, elemental analysis and acid hydrolysis. Through this synthesis, it was noted that the solubility of protected peptides in DMF, though they are relatively small peptides, was so poor that coupling reactions, in some instances, had to be performed with an aid of NMP or HMPA.

For deprotection, the above protected dodecapeptide amide was exposed to MSA in the presence of anisole in an ice-bath for 30 minutes and at room temperature for 30 minutes. The deprotected peptide, after precipitation with ether, was converted to the corresponding acetate by Amberlite CG-4B and subsequently treated with dilute ammonia for 30 minutes. This treatment was performed by the reason that the reversible conversion of the N→O shift, if any, could be achieved, as mentioned in the hydrogen fluoride treatment of peptides containing Ser and Thr.[17] Finally, the product, [12-Met(O)]-kassinin, was incubated with 2-mercaptoethanol to reduce the Met(O) residue to Met according to Iselin.[18] Progress of the reduction was monitored by the positive Met test on thin layer chromatography and the desired peptide could be isolated in a homogeneous form by precipitation from 50% acetic acid with ethanol.

When contractile activity in isolated guinea-pig ileum was examined, the relative potency of synthetic kassinin was 0.41±0.01 of that of synthetic substance P.[19]

Experimental

General experimental methods employed are essentially the same as those described in the Part LXII.[12] 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 $CHCl_3$–MeOH–AcOH (9:1:0.5), Rf_3 (*n*-BuOH–pyridine–AcOH–H_2O (1:1:1:1), Rf_4 *n*-BuOH–pyridine–AcOH–H_2O (30:20:6:24).

Z(OMe)-Met(O)-NH_2——A mixed anhydride (prepared from 5.20 g of Z(OMe)–Met(O)–OH with 2.45 ml of Et_3N and 2.50 ml of isobutylchloroformate) in THF (50 ml) was added to an ice-chilled solution of 28% NH_4OH (10 ml) and the mixture was stirred in an ice-bath for 3 hr. The resulting powder was collected by filtration, washed batchwisely with 3% NH_4OH and H_2O and then recrystallized from MeOH and ether; yield 4.55 g (87%), mp 194—196°, $[\alpha]_D^{27}$ +93.8° (c=0.2, MeOH), Rf_1 0.81. *Anal.* Calcd. for $C_{14}H_{20}N_2O_5S$: C, 51.20; H, 6.14; N, 8.53. Found: C, 51.00; H, 6.21; N, 8.54.

Z(OMe)-Leu-Met(O)-NH_2——Z(OMe)–Met(O)–NH_2 (4.55 g) was treated with TFA–anisole (10—5 ml) in an ice-bath for 60 min and dry ether was added. The resulting powder was dried over KOH pellets *in vacuo* for 3 hr and then dissolved in DMF (40 ml), to which Et_3N (3.9 ml) and Z(OMe)–Leu–ONP (5.79 g) were added. The mixture after stirring at room temperature for 48 hr, was condensed and the residue was treated with 5% citric acid and ether. The resulting powder was washed batchwisely with 5% citric acid, 5% Na_2CO_3 and H_2O and then precipitated from DMF with AcOEt; yield 4.99 g (82%). mp 161—163°, $[\alpha]_D^{27}$ −6.5° (c=1.2, DMF). Rf_1 0.72. *Anal.* Calcd. for $C_{20}H_{31}N_3O_6S\cdot 1/2H_2O$: C, 53.31; H, 7.16; N, 9.33. Found: C, 53.31; H, 7.02; N, 9.37.

Z(OMe)-Phe-Val-Gly-OH——The azide (prepared from 6.64 g of Z(OMe)–Phe–Val–$NHNH_2$ with 5.87 ml of 5.96 N HCl–DMF, 2.28 ml of isoamylnitrite and 7.25 ml of Et_3N) in DMF (40 ml) was added to an ice-chilled solution of H–Gly–OH (1.35 g) and Et_3N (2.5 ml) in H_2O (20 ml). The mixture was stirred at 4° for 48 hr, the solvent was evaporated and the residue was treated with 5% citric acid and AcOEt. The resulting powder was washed batchwisely with 10% citric acid and H_2O and then precipitated from DMF with AcOEt; yield 6.22 g (85%), mp 179—182°, $[\alpha]_D^{27}$ −9.1° (c=1.1, DMF), Rf_1 0.48. *Anal.* Calcd. for $C_{25}H_{31}N_3O_7$: C, 61.84; H, 6.44; N, 8.66. Found: C, 61.60; H, 6.31; N, 8.64.

Z(OMe)-Phe-Val-Gly-OMe——An ethereal solution of diazomethane was added to an ice-chilled solution of Z(OMe)–Phe–Val–Gly–OH (5.80 g) in DMF (25 ml) and the yellow color was persisted for 30 min. After addition of a few drops of AcOH, the solvent was evaporated and the residue was treated with MeOH. The resulting powder was then precipitated from DMF with MeOH; yield 5.14 g (87%), mp 189—193°, $[\alpha]_D^{27}$ −13.3°

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18) B. Iselin, *Helv. Chim. Acta*, **44**, 61 (1961).
19) H. Yajima and K. Kitagawa, *Chem. Pharm. Bull.* (Tokyo), **21**, 682 (1973).

(c=0.8, DMF). Rf_2 0.86. *Anal.* Calcd. for $C_{26}H_{33}N_3O_7$: C, 62.51; H, 6.66; N, 8.41. Found: C, 62.54; H, 6.45; N, 8.39.

Z(OMe)-Phe-Val-Gly-NHNH$_2$——To a solution of Z(OMe)–Phe–Val–Gly–OMe (5.14 g) in DMF–MeOH (25—5 ml), 80% hydrazine hydrate (5.2 ml) was added. After standing overnight, the solution was condensed and the residue was treated with MeOH. The resulting mass was recrystallized from MeOH; yield 4.88 g (95%), mp 195—196°, $[\alpha]_D^{27}$ −0.9° (c=1.2, DMF), Rf_1 0.61, Rf_2 0.46. Amino acid ratios in 6 N HCl hydrolysate: Phe 1.01, Val 1.03, Gly 1.00 (recovery 93%). *Anal.* Calcd. for $C_{25}H_{33}N_5O_6$: C, 60.10; H, 6.66; N, 14.02. Found: C, 60.09; H, 6.67; N, 14.00.

Z(OMe)-Phe-Val-Gly-Leu-Met(O)-NH$_2$——Z(OMe)–Leu–Met(O)–NH$_2$ (3.98 g) was treated with TFA-anisole (8—3 ml) in the usual manner and dry ether was added. The resulting powder was dried over KOH pellets *in vacuo* for 3 hr and then dissolved in DMF (30 ml) containing Et_3N (2.7 ml). To this ice-chilled solution, the azide (prepared from 4.50 g of Z(OMe)–Phe–Val–Gly–NHNH$_2$ with 6.06 ml of 3.27 N HCl–DMF, 1.32 ml of isoamylnitrite and 2.73 ml of Et_3N) in DMF (4 ml) was added. The mixture was stirred at 4° for 24 hr, the solvent was evaporated and the residue was treated with ether and 5% citric acid. The resulting powder was washed batchwisely with 5% citric acid and H_2O and then precipitated from DMSO with MeOH; yield 4.97 g (74%), mp 235—239°, $[\alpha]_D^{27}$ −8.4° (c=1.0, DMSO), Rf_1 0.61. Amino acid ratios in 6 N HCl hydrolysate: Phe 1.02, Val 1.03, Gly 1.03, Leu 1.00, Met+Met(O) 0.66 (average recovery 96%). *Anal.* Calcd. for $C_{36}H_{52}N_6O_9S$: C, 58.04; H, 7.04; N, 11.28. Found: C, 58.34; H, 7.00; N, 11.39.

Z(OMe)-Gln-Phe-Val-Gly-Leu-Met(O)-NH$_2$——The above protected pentapeptide amide (4.97 g) was treated with TFA–anisole (15—4 ml) in the usual manner and dry ether was added. The resulting powder isolated as stated above was then dissolved in DMSO–DMF (30—20 ml), to which Et_3N (2.03 ml), HOBT (0.30 g) and Z(OMe)–Gln–ONP (3.45 g) were added. After stirring at room temperature for 24 hr, the solution was condensed and the residue was treated ether and 5% citric acid. The resulting powder was washed batchwisely with 5% citric acid, 5% Na_2CO_3 and H_2O and then precipitated from DMSO with MeOH; yield 5.03 g (86%), mp 244—247°, $[\alpha]_D^{27}$ −18.4°, (c=1.3, DMSO). Rf_1 0.55, Rf_2 0.12. Amino acid ratios in 6 N HCl hydrolysate: Glu 1.01, Phe 1.05, Val 0.88, Gly 1.00, Leu 1.06, Met+Met(O) 0.68 (recovery 93%). *Anal.* Calcd. for $C_{41}H_{60}N_8O_{11}S\cdot H_2O$: C, 55.26; H, 7.01; N, 12.58. Found: C, 55.33; H, 6.91; N, 12.55.

Z(OMe)-Asp(OBzl)-Gln-Phe-Val-Gly-Leu-Met(O)-NH$_2$——The above protected hexapeptide amide (5.03 g) was treated with TFA–anisole (20—5 ml) and the deprotected peptide isolated as stated above was dissolved in DMSO–NMP (20—15 ml), to which Et_3N (1.8 ml), Z(OMe)–Asp(OBzl)–ONP (3.81 g) and HOBT (0.3 g) were added. The mixture, after stirring at room temperature for 24 hr, was condensed and the residue was treated with 5% citric acid and ether. The resulting powder was purified by batchwise washing as stated above followed by precipitation from DMSO with MeOH; yield 5.21 g (84%), mp 222—227°, $[\alpha]_D^{27}$ −16.5° (c=1.1, DMSO), Rf_1 0.57, Rf_3 0.86. Amino acid ratios in 6 N HCl hydrolysate: Asp 1.01, Glu 1.03, Phe 1.00, Val 1.01, Gly 1.02, Leu 1.00, Met+Met(O) 0.84 (average recovery 93%). *Anal.* Calcd. for $C_{52}H_{71}N_9O_{14}S$: C, 57.92; H, 6.64; N, 11.69. Found: C, 57.66; H, 6.62; N, 11.57.

Z(OMe)-Lys(Z)-Ser-NHNH$_2$——To a solution of Z(OMe)–Lys(Z)–Ser–OMe (6.51 g) in MeOH (60 ml), 80% hydrazine hydrate (7.3 ml) was added. The solid mass formed on standing overnight, was collected by filtration and recrystallized from dioxane and MeOH; yield 5.98 g (92%), mp 189—191°, $[\alpha]_D^{27}$ −9.2° (c=1.0, DMF), Rf_1 0.59. *Anal.* Calcd. for $C_{26}H_{35}N_5O_8\cdot 1/4H_2O$: C, 56.77; H, 6.51; N, 12.73. Found: C, 56.80; H, 6.61; N, 12.63.

Z(OMe)-Lys(Z)-Ser-Asp(OBzl)-Gln-Phe-Val-Gly-Leu-Met(O)-NH$_2$——Z(OMe)–Asp(OBzl)–Gln–Phe–Val–Gly–Leu–Met(O)–NH$_2$ (5.21 g) was treated with TFA–anisole (20—5 ml) in the usual manner and the deprotected peptide isolated as stated above was dissolved in HMPA (10 ml). To this ice-chilled solution, Et_3N (0.88 ml) and the azide (prepared from 3.16 g of Z(OMe)–Lys(Z)–Ser–NHNH$_2$ with 3.9 ml of 3.27 N HCl–DMF, 0.85 ml of isoamylnitrite and 1.76 ml of Et_3N) in DMF (20 ml) were added. The mixture was stirred at 4° for 48 hr, the most of the solvent was evaporated *in vacuo* at 45° and the residue was treated with 5% citric acid and ether. The precipitated powder was washed batchwisely as stated above and then precipitated from DMSO with MeOH; yield 5.71 g (83%), mp 238—240°, $[\alpha]_D^{27}$ −8.4° (c=1.0, DMSO), Rf_3 0.76. Amino acid ratios in 6 N HCl hydrolysate: Lys 0.98, Ser 0.77, Asp 1.04, Glu 1.03, Phe 1.00, Val 1.04, Gly 1.03, Leu 1.06, Met+Met(O) 0.74 (average recovery 84%). *Anal.* Calcd. for $C_{69}H_{94}N_{12}O_{19}S\cdot 2.5H_2O$: C, 56.27; H, 6.78; N, 11.41. Found: C, 56.38; H, 6.78, N, 11.58.

Z(OMe)-Asp(OBzl)-Val-NHNH-Troc——Z(OMe)–Val–NHNH–Troc (3.82 g) was treated with TFA–anisole (5 ml—3 ml) as usual and dry ether was added. An oily precipitate was dried over KOH pellets *in vacuo* for 3 hr and then dissolved in DMF (20 ml), to which Et_3N (1.7 ml), HOBT (1.24 g) and Z(OMe)–Asp(OBzl)–ONP (4.54 g) were added. The mixture was stirred at room temperature for 24 hr and the solvent was evaporated. The residue was dissolved in AcOEt, which was washed with 10% citric acid, 5% $NaHCO_3$ and H_2O, dried over Na_2SO_4 and then evaporated. The residue was triturated with ether and recrystallized from AcOEt and ether; yield 3.67 g (67%), mp 128—130°, $[\alpha]_D^{27}$ −34.5° (c=1.5, MeOH), Rf_1 0.87. *Anal.* Calcd. for $C_{28}H_{33}Cl_3N_4O_9$: C, 49.75; H, 4.92; N, 8.29. Found: C, 50.18; H, 4.94; N, 8.26.

Z(OMe)-Asp(OBzl)-Val-NHNH$_2$——Zn dust (approximately 5 g) was added to a solution of Z(OMe)–Asp(OBzl)–Val–NHNH–Troc (3.25 g) in AcOH–DMF (15—20 ml) and the mixture, after stirring at room temperature for 3 hr, was filtered, the filtrate was condensed *in vacuo* and the residue was treated with 10%

EDTA. The gelatinous mass formed on standing in a refrigerator overnight was collected by filtration, washed with 5% $NaHCO_3$ and H_2O and then recrystallized from MeOH; yield 1.78 g (74%). mp 210—212°, $[\alpha]_D^{27}$ −36.5° (c=0.5, DMF), Rf_1 0.63. *Anal.* Calcd. for $C_{25}H_{32}N_4O_7$: C, 59.99; H, 6.44; N, 11.19. Found: C, 59.83; H, 6.39; N, 11.10.

Z(OMe)-Asp(OBzl)-Val-Pro-OH——The azide (prepared from 1.18 g of Z(OMe)–Asp(OBzl)–Val–$NHNH_2$ with 0.84 ml of 5.96 N HCl–DMF, 0.35 ml of isoamylnitrite and 1.05 ml of Et_3N) in DMF (10 ml) was added to an ice-chilled solution of H–Pro–OH (0.54 g) and Et_3N (0.66 ml) in H_2O (5 ml) and the mixture was stirred at 4° for 48 hr. The solvent, after addition of a few drops of AcOH, was evaporated and the residue was dissolved in AcOEt, which was washed with 10% citric acid and H_2O, dried over Na_2SO_4 and then evaporated. The residue was triturated with *n*-hexane and recrystallized from ether and *n*-hexane; yield 1.12 g (81%) mp 61—64°, $[\alpha]_D^{27}$ −50.7° (c=0.7, MeOH), Rf_1 0.57. *Anal.* Calcd. for $C_{30}H_{37}N_3O_9 \cdot 1/2H_2O$: C, 60.80; H, 6.46; N, 7.09. Found: C, 60.89; H, 6.56; N, 7.10.

Z(OMe)-Asp(OBzl)-Val-Pro-Lys(Z)-Ser-Asp(OBzl)-Gln-Phe-Val-Gly-Leu-Met(O)-NH_2——The above protected nonapeptide amide (1.41 g) was treated with TFA (5.0 ml)-anisole (1.5 ml) as usual and dry ether was added. The resulting powder was then dissolved in NMP (2.5 ml)–DMSO (5.0 ml), to which Et_3N (0.14 ml), HOBT (0.15 g) and the PCP ester (prepared from 0.81 g of Z(OMe)–Asp(OBzl)–Val–Pro–OH with 0.74 g of PCP–O–TCA and 0.23 ml of Et_3N according to Fujino *et al.*[16]) in DMF (8 ml) were successively added. The mixture was stirred at room temperature for 48 hr, the solvent was evaporated and the residue was treated with 5% citric acid and ether. The resulting powder was washed batchwisely as stated above and then precipitated from DMF with MeOH; yield 1.10 g (61%), mp 246—248°, $[\alpha]_D^{26}$ −23.2° (c=0.6, DMF). Amino acid ratios in an acid hydrolysate: Asp 1.82, Val 1.81, Pro 0.91, Lys 1.05, Ser 0.71, Glu 0.96, Phe 1.00, Gly 1.01, Leu 1.07, Met 0.51+some Met(O) (recovery 87%). *Anal.* Calcd. for $C_{90}H_{121}N_{15}O_{24}S \cdot 2.5H_2O$: C, 57.68; H, 6.78; N, 11.21. Found: C, 57.62; H, 6.65; N, 11.35.

H-Asp-Val-Pro-Lys-Ser-Asp-Gln-Phe-Val-Gly-Leu-Met-NH_2——The above protected dodecapeptide amide (0.55 g) was treated with MSA (3.0 ml) in the presence of anisole (1.0 ml) in an ice-bath for 30 min and then at room temperature for 30 min and dry ether was added. The resulting oily precipitate was dissolved in H_2O (30 ml), which was treated with Amberlite CG-4B (acetate form, approximately 3 g) for 30 min and then filtered. The filtrate was lyophilized. The resulting powder was dissolved in H_2O (45 ml) and 2 N NH_4OH (15 ml) was added under cooling with ice. After 30 min, the solution was again lyophilized. The product was dissolved in 50% AcOH and the solution, after addition of 2-mercaptoethanol (0.3 ml), was incubated at 45° for 3 days and finally at 80° for 3 hr. A last trace of the starting material was reduced completely by the final treatment. The solvent was evaporated and the residue was precipitated twice from 50% AcOH with EtOH; yield 135 mg (74%), $[\alpha]_D^{27}$ −61.5° (c=0.3, 50% AcOH), Rf_4 0.65 (ninhydrin and Met positive spot). Amino acid ratios in an acid hydrolysate: Asp 1.67, Val 2.03, Pro 1.03, Lys 0.85, Ser 0.68, Glu 0.99, Phe 1.10, Gly 1.05, Leu 1.00 Met 0.82 (average recovery 79%). *Anal.* Calcd. for $C_{59}H_{95}N_{15}O_{18}S \cdot 2CH_3COOH \cdot 6H_2O$: C, 48.42; H, 7.42, N, 13.45. Found: C, 48.42; H, 7.09; N, 13.75.

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