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Studies on Peptides. XXXVIII.[1,2] Structure-Activity Correlations in Substance P

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The undecapeptide amide, H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2, corresponding to the entire amino acid sequence of substance P, a bovine hypothalamic peptide, was synthesized by the conventional method. This and C-terminal tetra, penta, hexa, hepta and octapeptide amides were submitted for biological assays. Synthetic substance P caused considerable decrease in blood pressure and heart rate in anesthetized rat, but other shorter chain peptides were less active. When contractility on isolated guinea-pig ileum was examined, the heptapeptide amide exhibited much higher activity than synthetic substance P.

Distribution of a substance (powder P) in various tissues, especially brain and intestine, which is capable to lower arterial blood pressure and stimulate the contraction of isolated intestinal tissues, was noted by von Euler and Gaddum[4] in 1931. Its responces, including effects on the central and local nervous systems,[5] were thought to be different from those of acetylcholine. Recently, identity between substance P and the sialogogic peptide found by Leeman and Hammerschlag[6] in extracts of bovine brain and hypothalami, was suggested[7] and finally established by Chang and Leeman[8] in 1970, after isolation of this active principle in pure form.

In 1971, the amino acid sequence of bovine substance P (I) was determined by Chang, *et al.*[9] and its solid phase synthesis has been reported by the same group of investigators.[10] We have investigated the relationship between chain length and activity of this important hypothalamic principle by synthesizing the undecapeptide amide (I) using the systematic chain elongation procedure as shown in Chart 1. The intermediates with various chain length were also submitted for biological assays.

The C-terminal tetrapeptide amide, Z(OMe)-Phe-Gly-Leu-Met-NH_2, was obtained in a stepwise manner or by condensation of Z(OMe)-Phe-Gly-OH and H-Leu-Met-NH_2[11] with

1) Part XXXVII: H. Yajima, K. Kitagawa, and M. Kurobe, *Chem. Pharm. Bull.* (Tokyo), **21**, 2566 (1973); The preliminary communication of this paper has appeared in *Chem. Pharm. Bull.* (Tokyo), **21**, 682 (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 of Biochemistry Nomenclature in July 1965 and July 1966; *Biochemistry*, **5**, 2485 (1966), *ibid.*, **6**, 362 (1967). Z=benzyloxycarbonyl; Z(OMe)=*p*-methoxybenzyloxycarbonyl.
3) Location: *a) Sakyo-ku, Kyoto; b) 1-2-3, Kasumi, Hiroshima.*
4) U.S. von Euler and J.H. Gaddum, *J. Physiol.* (London), **72**, 74 (1931).
5) F. Lembeck and G. Zetler, *Int. Rev. Neurobiol.*, **4**, 159 (1962); C.A. Baile and H. Neinardi, *Brit. J. Pharmacol. Chemother.*, **30**, 302 (1967).
6) S.E. Leeman and R. Hammerschlag, *Endocrinology*, **81**, 803 (1967).
7) F. Lembeck and K. Starke, *Naunyn-Schmiedebergs Arch. Pharmakol. Exp. Pathol.*, **259**, 375 (1968).
8) M.M. Chang and S.E. Leeman, *J. Biol. Chem.*, **245**, 4784 (1970).
9) M.M. Chang, S.E. Leeman, and H.D. Niall, *Nature New Biol.*, **232**, 86 (1971).
10) G.W. Tregear, H.D. Niall, J.T. Potts, Jun., S.E. Leeman, and M.M. Chang, *Nature New Biol.*, **232**, 87 (1971).
11) K. Lübke, E. Schröder, R. Schmieden, and H. Gibian, *Ann. Chem.*, **679**, 195 (1964).

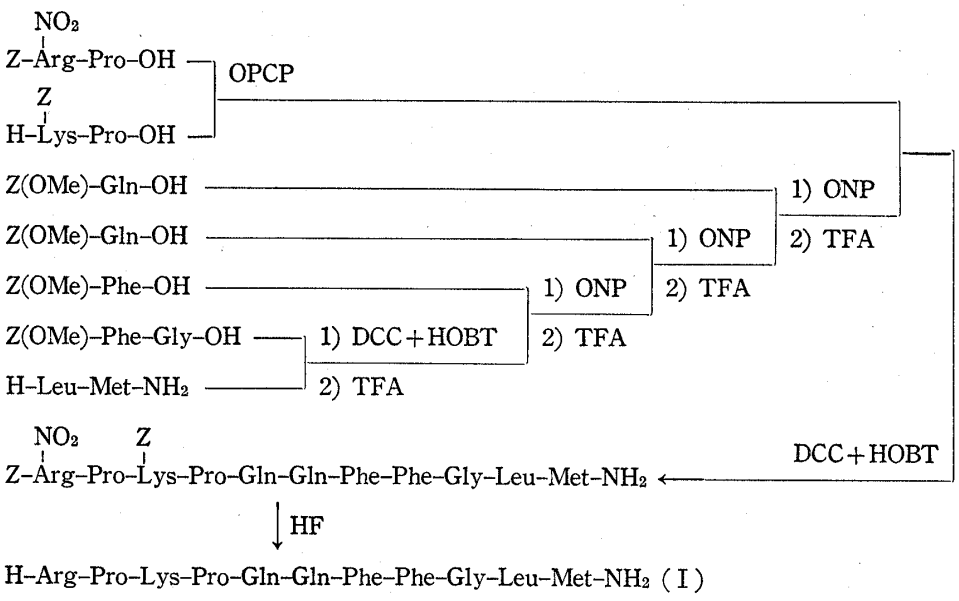

Chart 1. Synthetic Route to Substance P

ONP=*p*-nitrophenyl ester
OPCP=pentachlorophenyl ester

dicyclohexylcarbodiimide (DCC) in the presence of N-hydroxybenztriazole (HOBT).[12] The latter reagent suppresses the formation of acylurea, which is a known side reaction of DCC coupling reaction. Combination of trifluoroacetic acid (TFA) treatment[13] and the *p*-nitrophenyl ester (ONP) procedure[14] was applied to the elongation of the tetrapeptide amide in a stepwise manner for the synthesis of the protected heptapeptide amide, Z(OMe)-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2. Purification of this amide was achieved by washing the crude product with base and acid followed by precipitation from dimethylformamide (DMF) with ethyl acetate. By applying the similar purification procedure, the intermediates, Z(OMe)-Phe-Phe-Gly-Leu-Met-NH_2 and Z(OMe)-Gln-Phe-Phe-Gly-Leu-Met-NH_2, were also obtained in analytically pure form.

As an alternate route to the synthesis of the above protected heptapeptide amide, either condensation of Z(OMe)-Gln-Gln-Phe-Phe-Gly-OH with H-Leu-Met-NH_2 or coupling reaction of Z(OMe)-Gln-Gln-Phe-$NHNH_2$ with H-Phe-Gly-Leu-Met-NH_2 was considered. However these two routes were judged as impractical owing to less solubility of the above protected pentapeptide and tripeptide methyl ester derivatives in DMF.

For the synthesis of the N-terminal tetrapeptide, Z-Arg(NO_2)-Pro-Lys(Z)-Pro-OH, the pentachlorophenyl trichloroacetate procedure[15] was applied to condense Z-Arg(NO_2)-Pro-OH[16] and H-Lys(Z)-Pro-OH derived from Z(OMe)-Lys(Z)-Pro-OH, which was prepared by the reaction of the 2,4-5-trichlorophenyl ester[17] of Z(OMe)-Lys(Z)-OH[13,18] with H-Pro-OH in the presence of triethylamine.

For the final coupling reaction, the protected heptapeptide amide obtained above was treated with TFA in the presence of anisole. The TFA salt of the heptapeptide amide was converted to the corresponding hydrochloride and subsequently neutralized with triethylamine.

12) W. König and R. Geiger, *Chem. Ber.*, **103**, 788 (1970).
13) F. Weygand and K. Hunger, *Chem. Ber.*, **95**, 1 (1962).
14) M. Bodanszky, *Chem. Ind.*, **1955**, 1517; M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959).
15) M. Fujino and C. Hatanaka, *Chem. Pharm. Bull.* (Tokyo), **16**, 929 (1968).
16) R.A. Boissonnas, St. Guttmann, and P.A. Jaquenoud, *Helv. Chim. Acta*, **43**, 1349 (1960).
17) R.L. Huguenin, *Helv. Chim. Acta*, **47**, 1934 (1964).
18) H. Yajima, F. Tamura, Y. Kiso, and M. Kurobe, *Chem. Pharm. Bull.* (Tokyo), **21**, 1380 (1973).

The product was then condensed with Z-Arg(NO_2)-Pro-Lys(Z)-Pro-OH by DCC plus HOBT to give the fully protected undecapeptide amide, Z-Arg(NO_2)-Pro-Lys(Z)-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2, which was purified by batchwise washing with base and acid followed by precipitation from DMF with ethyl acetate. This was then treated with hydrogen fluoride[19] in the presence of anisole and Met at 0° for 30 minutes to remove all protecting groups. Addition of scavengers, anisole and Met, are known to be effective in preventing possible alkylation of the Met residue during this deblocking procedure. The deblocked undecapeptide amide was purified by column chromatography on CM-cellulose using the pH 6.9, 0.1 M ammonium acetate buffer. The synthetic peptide amide thus purified was homogeneous on thin-layer chromatography (TLC) and its purity was further assessed by elemental and amino acid analyses. By owing to lack of the prolidase-like activity of aminopeptidase (AP-M),[20] which we have employed, complete digestion of the synthetic undecapeptide amide could not be achieved.

Biological activity of the synthetic undecapeptide amide was measured by Dr. M. Otsuka of Tokyo Medical and Dental University in comparison with the solid phase synthetic peptide of known activity.[10] When contractility on isolated guinea-pig ileum was examined, our synthetic peptide was active as the standard sample or even more potent. Detail account of these results will be published by Otsuka, *et al.*

In order to examine the relationship between chain-length and activity, we have prepared the nonadecapeptide amide, H-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2, after coupling Z(OMe)-Lys(Z)-Pro-OH with H-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2 by DCC plus HOBT followed by deblocking with hydrogen fluoride as described above. This and other deblocked intermediates were submitted for biological assay to compare with our synthetic substance P. Deblocked peptides were not particularly purified, however considering the enough purity of the corresponding blocked materials and the satisfactory recovery of the constituent amino acids in each acid hydrolysate, it can be judged that each sample possesses adequate purity for the biological assay. When effects on isolated intestinal tissues were compared on weight basis, the hexapeptide amide H-Gln-Phe-Phe-Gly-Leu-Met-NH_2 and the heptapeptide amide, H-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2, exhibited much higher activity than that of the synthetic substance P as shown in Table I. An analogous situation has been found in physalaemin[21] and eledoisin,[22,23] polypeptides that are known to have similar biological activity to substance P.

Table I. Contractility of Synthetic Peptides on Isolated Guinea-pig Ileum

Peptides	Relative potency
TFA·H–Phe–Gly–Leu–Met–NH_2	1/80—1/40
TFA·H–Phe–Phe–Gly–Leu–Met–NH_2	1/40—1/20
TFA·H–Gln–Phe–Phe–Gly–Leu–Met–NH_2	2
TFA·H–Gln–Gln–Phe–Phe–Gly–Leu–Met–NH_2	2—4
AcOH·H–Lys–Pro–Gln–Gln–Phe–Phe–Gly–Leu–Met–NH_2	1/2—1
Synthetic substance P (I) (acetate form)	1

Injection of our synthetic substance P intravenously into anesthetized rat showed considerable decrease in blood pressure and heart rate which lasted 3 to 5 minutes, while other short chain peptides in Table I exhibited transient and slight hypotension followed by a slight

19) S. Sakakibara and T. Shimonishi, *Bull. Chem. Soc. Japan*, **38**, 1412 (1965).
20) E.C. Jorgensen, G.C. Windridge, and W. Patton, *J. Med. Chem.*, **12**, 733 (1969).
21) V. Erspamer, A. Anastasi, G. Bertaccini, and J.M. Cei, *Experientia*, **20**, 489 (1964).
22) V. Erspamer and A. Anastasi, *Experientia*, **18**, 58 (1962).
23) K. Lübke, R. Hempel, and E. Schröder, *Acta Chim. Hung.*, **44**, 131 (1965).

increase of blood pressure which returned to normal level within 20 seconds. The heart rate also decreased by these short chain peptides but it returned to normal value in 20 seconds and no clear relationship between dosage and response has been found in this assay system. Further precise biological evaluation of synthetic peptides will be required in future.

Experimental

General experimental methods employed are essentially the same as described in the Part XXII[24] of this series. TLC was performed on silica gel (Kieselgel G, Merck). *Rf* values refer to the following solvent systems; Rf_1 $CHCl_3$–MeOH–H_2O (40: 15: 5), Rf_2 $CHCl_3$–MeOH–AcOH (9: 1: 0.5), Rf_3 and Rf_4 *n*-butanol–pyridine–AcOH–H_2O (30: 20: 6: 24) and (4: 1: 1: 2).

Z(OMe)-Amino Acid Active Esters——The *p*-nitrophenyl and 2,4,5-trichlorophenyl (TCP) esters of following Z(OMe)-amino acids were prepared according to the procedure of Bodanszky[14] and Huguenin[17] for the preparation of corresponding Z-derivatives respectively.

Z(OMe)-Gly-ONP (Recrystallized from AcOEt): mp 82—83°. *Anal.* Calcd. for $C_{17}H_{16}O_7N_2$: C, 56.66; H, 4.48; N, 7.78. Found: C, 56.92; H, 4.65; N, 7.63.

Z(OMe)-Phe-ONP (Recrystallized from AcOEt and ether): mp 90—91°. *Anal.* Calcd. for $C_{24}H_{22}O_7N_2$: C, 63.99; H, 4.92; N, 6.22. Found: C, 64.22; H, 5.01; N, 6.21.

Z(OMe)-Leu-ONP (Recrystallized from Ethanol and Petroleum Ether): mp 61—63°, *Anal.* Calcd. for $C_{21}H_{24}O_7N_2$: C, 60.57; H, 5.81; N, 6.73. Found: C, 60.58; H, 5.99; N, 6.62.

Z(OMe)-Gln-ONP (Recrystallized from Tetrahydrofuran and Ether): mp 154—157°. *Anal.* Calcd. for $C_{20}H_{21}O_8N_3$: C, 55.68; H, 4.91; N, 9.74. Found: C, 56.27; H, 5.08; N, 9.93.

Z(OMe)-Lys(Z)-OTCP (Recrystallized from AcOEt): mp 83—84°. *Anal.* Calcd. for $C_{29}H_{29}O_7N_2Cl_3$: C, 55.82; H, 4.69; N, 4.49. Found: C, 56.10; H, 4.63; N, 4.60.

Z(OMe)-Met-NH_2——A mixed anhydride prepared in the usual manner from Z(OMe)-Met-OH (31.33 g) in THF (200 ml) with Et_3N (13.8 ml) and ethyl chloroformate (10.8 ml), was poured into 28% NH_4OH (26 ml) with vigorous stirring, which was continued in an ice-bath for 2 hr. The solvent was evaporated and the resulting crystalline solid was washed bachwisely with 5% Na_2CO_3 and H_2O and then recrystallized from MeOH; yield 19.44 g (62%), mp 146—150°. $[\alpha]_D^{27}$ −4.93° (c=1.0, DMF), Rf_1 0.55. *Anal.* Calcd. for $C_{14}H_{20}O_4N_2S$: C, 53.82; H, 6.45; N, 8.96. Found: C, 54.04; H, 6.49; N, 8.80.

Z(OMe)-Leu-Met-NH_2——In the presence of anisole (7 ml), Z(OMe)-Met-NH_2 (15.60 g) was treated with TFA (20 ml) at room temperature for 45 min. Dry ether (stored over $FeCO_3$) was added and the resulting oily precipitate, after washing with fresh ether, was dried over KOH pellets *in vacuo* overnight and then dissolved in DMF (120 ml). To this solution, Et_3N (13.8 ml) and Z(OMe)-Leu-ONP (20.80 g) were added and the mixture was stirred at room temperature for 48 hr. The solvent was evaporated and the resulting solid was washed bachwisely with ether, 10% citric acid and H_2O and then recrystallized from MeOH; yield 12.97 g (61%), mp 197—200°, $[\alpha]_D^{25}$ −23.7° (c=0.6, DMF), Rf_1 0.68. *Anal.* Calcd. for $C_{20}H_{31}O_5N_3S$: C, 56.45; H, 7.34; N, 9.88. Found: C, 56.18; H, 7.45; N, 9.91.

Z(OMe)-Phe-Gly-OMe——In the usual manner, DCC (12.38 g) was added to a solution of Z(OMe)-Phe-OH(19.76 g) and H-Gly-OMe (prepared from 7.53 g of the hydrochloride with 8.4 ml of Et_3N) in DMF (150 ml) and the solution was stirred at room temperature for 48 hr. After filtration, the filtrate was condensed and the residue was dissolved in AcOEt, which after washing with 5% Na_2CO_3, 10% citric acid and H_2O, was dried over Na_2SO_4 and then evaporated. The crystalline solid formed by addition of ether to the residue was recrystallized from AcOEt; yield 14.20 g (61%), mp 113—115°, $[\alpha]_D^{26}$ −16.1° (c=0.6, DMF), Rf_1 0.75. *Anal.* Calcd. for $C_{21}H_{24}O_6N_2$: C, 62.99; H, 6.04; N, 7.00. Found: C, 62.89; H, 6.11; N, 7.01.

Z(OMe)-Phe-Gly-OH——Z(OMe)-Phe-Gly-OMe (4.00 g) in MeOH (20 ml) was treated with 1N NaOH (13 ml) at room temperature for 45 min. The solution was neutralized with AcOH and the solvent was evaporated. The residue was dissolved in 5% Na_2CO_3, which was washed with AcOEt. The aqueous phase was acidified with citric acid and the resulting product was extracted into AcOEt, which was washed with H_2O-NaCl, dried over Na_2SO_4 and then evaporated. Addition of ether to the residue afforded the solid, which was recrystallized from AcOEt; yield 2.82 g (74%), mp 125—128°, $[\alpha]_D^{24}$ −31.2° (c=0.7, DMF), Rf_1 0.27. *Anal.* Calcd. for $C_{20}H_{22}O_6N_2$: C, 62.16; H, 5.74; N, 7.25. Found: C, 62.22; H, 5.81; N, 7.21.

Z(OMe)-Gly-Leu-Met-NH_2——In the presence of anisole (3.9 ml), Z(OMe)-Leu-Met-NH_2 (7.80 g) was treated with TFA (16 ml) at room temperature for 45 min. An oily precipitate formed by addition of dry ether (stored over $FeCO_3$) was dried over KOH pellets *in vacuo* overnight and then dissolved in DMF (30 ml). To this solution, Et_3N (5.2 ml) and Z(OMe)-Gly-ONP (8.50 g) were added and the mixture was stirred at room temperature for 48 hr. After evaporation of the solvent, ether was added to the residue and the resulting solid was washed batchwisely with 5% Na_2CO_3, 10% citric acid and H_2O and then recrystallized from

24) H. Yajima, Y. Okada, H. Kawatani, and N. Mizokami, *Chem. Pharm. Bull.* (Tokyo), **17**, 1229 (1969).

MeOH; Yield 5.30 g (61%), mp 158—159°, $[\alpha]_D^{27}$ −26.6° (c=0.8, DMF). Rf_1 0.80. *Anal.* Calcd. for $C_{22}H_{34}O_6N_4S$: C, 54.75; H, 7.10; N, 11.61. Found: C, 55.05; H, 6.94; N, 11.73.

Z(OMe)-Phe-Gly-Leu-Met-NH_2——a) In the presence of anisole (2.4 ml), Z(OMe)-Gly-Leu-Met-NH_2 (4.83 g) was treated with TFA (9.7 ml) at room temperature for 45 min. Dry ether was added to give an oily precipitate, which after drying over KOH pellets *in vacuo* overnight, was dissolved in DMF (40 ml). To this solution, Et_3N (2.8 ml) and Z(OMe)-Phe-ONP (4.50 g) were added and the mixture was stirred at room temperature for 48 hr. After evaporation of the solvent, the solid product formed by addition of ether was washed batchwisely with 5% Na_2SO_3, 10% citric acid and H_2O, and then recrystallized from MeOH and AcOEt, yield 2.50 g (40%), mp 208—209°, $[\alpha]_D^{27}$ −33.3° (c=0.7, DMF). Rf_1 0.77. *Anal.* Calcd. for $C_{31}H_{43}O_7N_5S$: C, 59.12; H, 6.88; N, 11.12. Found: C, 58.90; H, 6.74; N, 11.25.

b) The TFA salt of H-Leu-Met-NH_2 (prepared from 4.90 g of the Z(OMe)- derivative by treatment of 10 ml of TFA in the presence of 2.5 ml of anisole as stated above) was once dissolved in 1N HCl (11.6 ml) and the solvent was evaporated under high vacuum. The residue, after drying KOH pellets *in vacuo* overnight, was dissolved in DMF (30 ml). To this solution, Et_3N (3.2 ml), HOBT (2.35 g) Z(OMe)-Phe-Gly-OH (4.50 g) and DCC (4.80 g) were added and the mixture was stirred at room temperature for 48 hr. After filtration of the reaction mixture, the product was isolated as stated above; yield 6.00 g (83%), mp 205—206°, Rf_1 0.77.

Z(OMe)-Phe-Phe-Gly-Leu-Met-NH_2——In the presence of anisole (2.4 ml), Z(OMe)-Phe-Gly-Leu-Met-NH_2 (4.75 g) was treated with TFA (9.5 ml) for 45 min as stated above. The white solid precipitate formed by addition of dry ether, was collected by filtration, dried over KOH pellets *in vacuo* (a part of the sample was submitted for the biological assay. Rf_1 0.53 amino acid ratios in an acid hydrolysate $Phe_{0.97}$ $Gly_{1.00}$ $Leu_{1.00}$ $Met_{0.70}$ average recovery 94.2%). It was then dissolved in DMF (50 ml). To this solution, Et_3N (2.1 ml) and Z(OMe)-Phe-ONP (3.40 g) were added and the mixture was stirred at room temperature for 48 hr. During this period, the product began to precipitate as gellatinus mass. The solvent was evaporated and the resulting solid was washed batchwisely with AcOEt, 5% Na_2CO_3, 10% citric acid and H_2O. It was recrystallized from DMF and AcOEt; yield 4.75 g (81%), mp 224—225°, $[\alpha]_D^{27}$ −37.6° (c=0.6, DMF). Rf_1 1.0, Rf_2 0.32. *Anal.* Calcd. for $C_{40}H_{52}O_8N_6S$: C, 61.83; H, 6.75; N, 10.82. Found: C, 61.54; H, 6.66: N, 10.75.

Z(OMe)-Gln-Phe-Phe-Gly-Leu-Met-NH_2——In a similar manner as stated above, Z(OMe)-Phe-Phe-Gly-Leu-Met-NH_2 (4.75 g) was treated with TFA (9.5 ml) in the presence of anisole (2.4 ml). Addition of petroleum ether gave an oily precipitate, which was solidified by washing with ether. The TFA salt thus obtained was dried over KOH pellets *in vacuo* overnight (a part of the sample was submitted for the biological assay. Rf_1 0.58 amino acid ratios in an acid hydrolysate $Phe_{2.06}$ $Gly_{1.00}$ $Leu_{0.99}$ $Met_{0.75}$ average recovery 82.6%). It was dissolved in DMF (50 ml). To this solution, Et_3N (2.5 ml) and Z(OMe)-Gln-ONP (2.63 g) were added and the mixture was stirred at room temperature for 48 hr. After evaporation of the solvent, the resulting gellatinus mass was washed batchwisely with AcOEt, 5% Na_2CO_3, 10% citric acid and H_2O and then precipitated from DMF with AcOEt, yield 3.25 g (59%), mp 242—243°, $[\alpha]_D^{24}$ −18.9° (c=1.0, dimethyl sulfoxide; DMSO). Rf_1 0.60. *Anal.* Calcd. for $C_{45}H_{60}O_{10}N_8S$: C, 59.71; H, 6.68; N, 12.38. Found: C, 59.70; H, 6.90; N, 12.15.

Z(OMe)-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2——In the usual manner, Z(OMe)-Gln-Phe-Phe-Gly-Leu-Met-NH_2 (3.25 g) was treated with TFA (6.5 ml) in the presence of anisole (1.7 ml) for 45 min. The TFA salt was precipitated by addition of petroleum ether and then washed with ether to form the solid, which after drying over KOH pellets *in vacuo* (Rf_4 0.50 amino acid ratios in an hydrolysate $Gln_{1.02}$ $Phe_{2.01}$ $Gly_{0.96}$ $Leu_{1.00}$ $Met_{0.71}$ average recovery 87.1%), was then dissolved in DMF (40 ml). To this solution, Et_3N (1.1 ml) and Z(OMe)-Gln-ONP (1.56 g) were added and the mixture was stirred at room temperature for 48 hr. The thick mass formed during the reaction. The solvent was evaporated and the solid product formed by addition of AcOEt and H_2O to the residue, was washed batchwisely with 5% Na_2CO_3; 10% citric acid and H_2O and then precipitated from DMF with AcOEt; yield 2.58 g (68%), mp 257—259°, $[\alpha]_D^{24}$ −17.9° (c=0.6, DMSO), Rf_1 0.60. *Anal.* Calcd. for $C_{50}H_{68}O_{12}N_{10}S \cdot H_2O$: C, 57.12; H, 6.71; N, 13.33. Found: C, 56.93; H, 6.60; N, 13.51.

Z-Arg(NO_2)-Pro-OH——Z-Arg(NO_2)-Pro-OH (10.60 g) was dissolved in a mixture of dioxane (60 ml) and tetrahydrofuran (THF) (60 ml). Under cooling with ice, 2,4-dinitrophenol[25] (11.05 g) and DCC (6.19 g) were added and the mixture was stirred for 4 hr. The solution was filtered and the filtrate containing the dinitrophenyl ester (Rf_1 0.70) was combined with a solution of H-Pro-OH (11.51 g) and Et_3N (13.9 ml) in 60% aqueous dioxane (70 ml). After the solution was stirred at room temperature for 20 hr, the solvent was evaporated and the residure was dissolved in 1N Na_2CO_3. The aqueous phase, after washing with AcOEt, was acidified with 5N HCl and the resulting precipitate was extracted with AcOEt, which was washed with H_2O-NaCl, dried over Na_2SO_4 and then evaporated. The solid residue was recrystallized from MeOH; yield 10.80 g (80%), mp 115—117°, $[\alpha]_D^{23}$ −20.4° (c=0.1, DMF). (lit.[16] mp 119°, $[\alpha]_D^{22}$ −26.5° in DMF). Rf_1 0.32. *Anal.* Calcd. for $C_{19}H_{26}O_7N_6$: C, 50.66; H, 5.82; N, 18.66. Found: C, 50.57; H, 5.77; N, 18.39.

Z-Arg(NO_2)-Pro-OPCP——Pentachlorophenyl trichloroacetate (0.91 g) was added to a solution of Z-Arg(NO_2)-Pro-OH (0.90 g) and Et_3N (0.3 ml) in DMF (5 ml). The solution was stirred at room temperature

25) M. Bodanszky and M.A. Ondetti, *Chem. Ind.*, **1966**, 26.

for 5 hr, and the solvent was evaporated. The crystalline solid formed by addition of H_2O, was recrystallized from EtOH; yield 1.10 g (82%), mp 125—127°. Rf_1 0.72. *Anal.* Calcd. for $C_{25}H_{25}O_7N_6Cl_5$: C, 42.97; H, 3.61; N, 12.03. Found: C, 42.67; H, 3.64; N, 12.17.

Z(OMe)-Lys(Z)-Pro-OH——Z(OMe)-Lys(Z)-OPCP (31.19 g) in AcOEt (200 ml) was added to a solution of H-Pro-OH (11.40 g) and Et_3N (20.7 ml) in H_2O (100 ml) and the mixture was stirred at room temperature for 48 hr. The aqueous phase was separated, washed with fresh AcOEt and then acidified with citric acid. The oily precipitate was extracted with AcOEt, which after washing with H_2O-NaCl, dried over Na_2SO_4 and then evaporated to give an oily residue; yield 17.98 g (74%), Rf_1 0.56.

Z-Arg(NO_2)-Pro-Lys(Z)-Pro-OH——In the presence of anisole (3.7 ml), Z(OMe)-Lys(Z)-Pro-OH (7.30 g) was treated with TFA (15 ml) at room temperature for 45 min, when petroleum ether was added to give an oily precipitate. After petroleum ether was removed by decantation, the residue was washed with ether, dried over KOH pellets *in vacuo* overnight and then dissolved in DMF (50 ml). To this solution, Z-Arg (NO_2)-Pro-OPCP (9.45 g) and Et_3N (5.7 ml) were added and the mixture was stirred at room temperature for 48 hr. After evaporation of the solvent, the residue was dissolved in H_2O containing a small amount of Et_3N. The aqueous phase, after washing with AcOEt, was acidified with 5% citric acid and the resulting precipitate 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 gave the solid, which was washed throughly with ether and then recrystallized with EtOH and ether; yield 4.40 g (41%), mp 101—103°, $[\alpha]_D^{20}$ −63.0° (c=0.8, MeOH). Rf_1 0.56. (stained by $Ce(SO_4)_2$.[26]) *Anal.* Calcd. for $C_{38}H_{51}O_{11}N_9$: C, 56.35; H, 6.35; N, 15.56. Found: C, 56.15; H, 6.48; N, 15.58.

Z(OMe)-Lys(Z)-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2——Z(OMe)-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2 (0.76 g) was treated in the usual manner with TFA (1.5 ml) in the presence of anisole (0.4 ml) for 60 min. The oily precipitate formed by addition of petroleum ether turned to the solid by treatment with ether (stored over Fe_2CO_3), which was dried over KOH pellets *in vacuo* (Rf_4 0.32 amino acid ratios in an acid hydrolysate. $Gln_{2.18}$ $Phe_{2.07}$ $Gly_{1.00}$ $Leu_{0.99}$ $Met_{0.66}$ average recovery 87.5%). This was then dissolved in 1N HCl (1.73 ml) and the solution was lyophilized. The hydrochloride thus obtained was dissolved in DMF (20 ml) and Et_3N (0.15 ml) was added. To this solution, Z(OMe)-Lys(Z)-Pro-OH (0.77 g), HOBT (0.13 g) and DCC (0.20 g) were combined successively and the mixture was stirred at room temperature for 48 hr. The solution, after filtration, was condensed *in vacuo*. The solid formed by addition of AcOEt to the residue was washed batchwisely with 5% Na_2CO_3, 10% citric acid and H_2O and then recrystallized from DMF and AcOEt; yield 0.35 g (35%), mp 248° (dec.). $[\alpha]_D^{26}$ −37.0° (c=1.1, DMF). Rf_1 0.50, *Anal.* Calcd. for $C_{69}H_{93}O_{16}N_{13}S\cdot H_2O$: C, 58.74; H, 6.78; N, 12.90. Found: C, 58.71; H, 6.92; N, 12.94.

H-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2——Z(OMe)-Lys(Z)-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2 (0.25 g) was treated with HF (approximately 10 ml) in the presence of anisole (0.3 ml) and H-Met-OH (15 mg) in an ice-bath for 30 min. The HF was removed *in vacuo* and the residue, after drying over KOH pellets *in vacuo* overnight, was dissolved in H_2O, which was filtered with an aid of filter cell. The filtrate was then treated with Amberlite IR-4B (acetate cycle) (approximately 5 g) for 30 min. The solution was filtered and the filtrate was washed with AcOEt and once lyophilized. The product was again dissolved in H_2O (20 ml) and a few drop of mercaptoethanol was added and the solution was kept on standing overnight. This was then applied to a column of CM-cellulose (Whatman CM 23, 13.0×35 cm), which was eluted first with H_2O (450 ml) and then with the following pH 6.9 ammonium acetate buffers: 0.01M (200 ml), 0.025M (200 ml), 0.05M (200 ml) and 0.075M (200 ml). Individual fractions, 15 ml each, were collected and absorbancy at 260 mμ was determined. A peak present in the 0.05M eluate was detected and the contents of these tubes (54 to 70) were combined. The solvent was evaporated and the residue lyophilized to constant weight to give fluffy powder; yield 0.15 g (69%) $[\alpha]_D^{24}$ −58.9° (c=0.4, 5% AcOH). Rf_3 0.82. Amino acid ratios in an acid hydrolysate: $Lys_{0.98}$ $Pro_{0.88}$ $Glu_{2.19}$ $Phe_{2.36}$ $Gly_{1.00}$ $Leu_{1.04}$ $Met_{0.94}$ (average recovery 91%). *Anal.* Calcd. for $C_{52}H_{79}O_{11}N_{13}S\cdot 2CH_3COOH\cdot 2H_2O$: C, 53.79; H, 7.34; N, 14.56. Found: C, 53.54; H, 7.12; N, 15.07.

Z-Arg(NO_2)-Pro-Lys(Z)-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2——The hydrochloride of H-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2 (prepared from 0.76 g of the Z(OMe)-derivative as described above) was dissolved in DMF (25 ml) and Et_3N (0.25 ml) was added. To this solution, Z-Arg(NO_2)-Pro-Lys(Z)-Pro-OH (0.59 g), HOBT (0.16 g) and DCC (0.24 g) were added consecutively and the mixture was stirred at room temperature for 48 hr. The solution was filtered and the filtrate was condensed *in vacuo*. The powder formed by addition of AcOEt to the residue was washed bachwisely with 5% Na_2CO_3, 10% citric acid and H_2O and then recrystallized twice from DMF and AcOEt, yield 0.82 g (67%), mp 224—225°, $[\alpha]_D^{26}$ −37.0° (c=1.0, DMF). Rf_1 0.50. Amino acid ratios in acid hydrolysate: $Arg_{0.76}$ $Lys_{1.09}$ $Pro_{1.56}$ $Glu_{2.15}$ $Phe_{2.02}$ $Gly_{1.02}$ $Leu_{1.00}$ $Met_{0.90}$ (average recovery 90%). *Anal.* Calcd. for $C_{79}H_{109}O_{19}N_{19}S\cdot 2H_2O$: C, 55.91; H, 6.71; N, 15.68. Found: C, 55.53; H, 6.67; N, 15.44.

H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2——Z-Arg(NO_2)-Pro-Lys(Z)-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2 (0.50 g) was treated with HF (approximately 15 ml) in the presence of anisole (0.8 ml)

26) H. Yajima, F. Tamura, and Y. Kiso, *Chem. Pharm. Bull.* (Tokyo), **18**, 2574 (1970).

and H-Met-OH(50 mg) in an ice bath for 30 min. After evaporation of HF, the residue was kept over KOH pellets *in vacuo* overnight and the dissolved in H_2O, which after washing with AcOEt, was lyophilized. The product was again dissolved in H_2O (100 ml) and a few drop of mercaptoethanol was added. This solution was applied to a column of CM-cellulose (Whatmann CM 23, 3.5×18.0 cm), which was eluted first with H_2O (400 ml) and then with the following pH 6.9 ammonium acetate buffers: 0.05M (300 ml), 0.075M (300 ml), 0.1M (500 ml) and 0.125M (250 ml). Individual fractions, 18 ml each, were collected and absorbancy at 260 mμ was determined. A peak present in the 0.1M eluate was detected and the contents of these tubes 70 to 92 were combined. The solvent was removed first by evaporation and finally by lyophilization to constant weight to give fluffy powder; yield 0.17 g (36%), $[\alpha]_D^{27}$ −76.0° (c=0.4, 5% AcOH). Rf_3 0.55 (lit.[27] Rf_3 0.60), Rf_4 0.34. Amino acid ratios in an acid hydrolysate $Arg_{1.03}$ $Lys_{0.83}$ $Pro_{2.07}$ $Glu_{2.08}$ $Phe_{2.30}$ $Gly_{0.90}$ $Leu_{1.00}$ $Met_{0.90}$ (average recovery 96%), *Anal.* Calcd. for $C_{63}H_{98}O_{13}N_{18}S \cdot 3CH_3OCOH \cdot 4H_2O$: C, 51.80, H, 7.43; N, 15.76. Found: C, 51.75; H, 7.25; N, 15.66.

Z(OMe)-Gln-Phe-OMe——Z(OMe)-Gln-ONP (25.88 g) was added to a solution of H-Phe-OMe (prepared from 12.94 g of the hydrochloride and 16.6 ml of Et_3N) in DMF (200 ml) and the mixture was stirred at room temperature for 48 hr. After evaporation of the solvent, the residue was treated with ether to form a solid, which after washing batchwisely with 5% Na_2CO_3, 10% citric acid and H_2O, was recrystallized from MeOH and dioxane; yield 22.70 g (80%), mp 188—189°, $[\alpha]_D^{25}$ +2.95° (c=0.7, DMSO). Rf_1 1.0, Rf_2 0.53. *Anal.* Calcd. for $C_{24}H_{29}O_7N_3$: C, 61.13; H, 6.20; N, 8.91. Found: C, 60.98; H, 6.13; N, 8.95.

Z(OMe)-Gln-Gln-Phe-OMe——The solid TFA salt of H-Gln-Phe-OMe (prepared from 20.50 g of Z(OMe)-Gln-Phe-OMe by treatment with 40 ml of TFA in the presence of 10 ml of anisole for 45 min and then precipitation with dry ether) was dissolved in DMF (120 ml). To this solution, Et_3N (11.9 ml) and Z(OMe)-Gln-ONP (18.55 g) were added. After stirring for 48 hr ,the solution was condensed *in vacuo*. The resulting solid, after washing with ether, 5% Na_2CO_3, 10% citric acid and H_2O, was recrystallized from DMF and AcOEt; yield 20.58 g (80%), mp 246—247°, Rf_1 0.60. $[\alpha]_D^{25}$ +2.98° (c=1.0, DMSO). *Anal.* Calcd. for $C_{29}H_{37}O_9N_5$: C, 58.08; H, 6.22; N, 11.68. Found: C, 58.00; H, 6.17; N, 11.55.

Z-Phe-Phe-Gly-OMe——In the usual manner, Z-Phe-Gly-OMe (15.0 g) was hydrogenated over a Pd catalyst in a mixture of 1N HCl (40 ml) and THF (100 ml) and the solution was filtered. The filtrate was evaporated and the residue was dissolved in dioxane (50 ml). To this solution, Et_3N (5.5 ml) and a mixed anhydride (prepared from 12.0 g of Z-Phe-OH with 5.5 ml of Et_3N and 4.3 ml of ethyl chloroformate in 60 ml of THF) were combined. After stirring for 2 hr, the solution was condensed, and the forming solid was washed batchwisely with 5% Na_2CO_3, 10% citric acid and H_2O and recrystallized from MeOH; yield 8.2 g (41%), mp 182—184°, $[\alpha]_D^{32}$ −22.7° (c=0.6, DMF). Rf_1 0.90. *Anal.* Calcd. for $C_{29}H_{31}O_6N_3 \cdot 1/2H_2O$: C, 66.14; H, 6.12; N, 7.98. Found: C, 66.23; H, 5.93; N, 8.01.

Z-Gln-Phe-Phe-Gly-OMe——In the usual manner, Z-Phe-Phe-Gly-OMe (8.0 g) was hydrogenated over a Pd catalyst in THF (50 ml) containing 1N HCl (20 ml). After filtration, the filtrate was condensed and the residue was dissolved in DMF (20 ml). To this solution, Et_3N (2.2 ml) and a mixed anhydride (prepared from 4.32 g of Z-Gln-OH with 2.2 ml of Et_3N and 1.7 ml of ethyl chloroformate in 40 ml of THF) were added. After stirring for 2 hr, the solution was condensed and the residue was treated with ether. The resulting solid was washed with 5% Na_2CO_3, 10% citric acid and H_2O and then recrystallized from dioxane and MeOH; yield 2.95 g (30%), mp 240—243°, $[\alpha]_D^{28}$ −30.8° (c=1.0, DMF). Rf_2 0.88. *Anal.* Calcd. for $C_{34}H_{39}O_8N_5 \cdot 1/2H_2O$: C, 62.37; H, 6.16; N, 10.70. Found: C, 62.49; H, 6.13; N, 10.54.

Z(OMe)-Gln-Gln-Phe-Phe-Gly-OMe——In the usual manner, Z-Gln-Phe-Phe-Gly-OMe (8.0 g) in a mixture of THF (70 ml) and 1N HCl (12.4 ml) was hydrogenated over a Pd catalyst. The catalyst was removed by filtration and the filtrate was condensed. The residue was dissolved in DMF (40 ml) and Et_3N (1.7 ml) was added. To this solution, a mixed anhydride (prepared from 3.84 g of Z(OMe)-Gln-OH with 1.7 ml of Et_3N and 1.3 ml of ethyl chloroformate) in THF (35 ml). After stirring for 2 hr, the solution was condensed *in vacuo* and the residue formed by addition of ether, was washed with 5% Na_2CO_3, 10% citric acid and H_2O, and then precipitated from DMF with MeOH; yield 8.60 g (86%), mp 250—251°, $[\alpha]_D^{33}$ −23.5° (c=0.7, DMF). Rf_1 1.0, Rf_2 0.86. *Anal.* Calcd. for $C_{40}H_{49}O_{11}N_7$: C, 59.76; H, 6.14; N, 12.19. Found: C, 60.02; H, 6.05; N, 11.93.

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