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Synthesis of the Dodecapeptide Amide corresponding to the Entire Amino Acid Sequence of Granuliberin-R, a New Frog Skin Peptide from *Rana rugosa*[1,2]

Terumi Nakajima, Tadashi Yasuhara, Yuko Hirai,[3a] Chieko Kitada, Masahiko Fujino,[3b] Masaharu Takeyama, Kaname Koyama, and Haruaki Yajima[3c]

Institute of Pharmaceutical Sciences, Hiroshima University,[3a] Chemical Research Laboratory, Takeda Chemical Ind. Ltd.[3b] and Faculty of Pharmaceutical Sciences, Kyoto University[3c]

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Amino acid sequence of a new frog skin peptide named granuliberin-R, H–Phe–Gly–Phe–Leu–Pro–Ile–Tyr–Arg–Arg–Pro–Ala–Ser–NH_2, was assessed by two conventional ways of synthesis; the one by the methanesulphonic acid procedure and the other by the catalytic hydrogenation procedure at the final deprotection steps.

Keywords——granuliberin-R, a frog skin peptide; des-amide-granuliberin-R; methanesulfonic acid, as a deprotecting reagent; Arg(*p*-methoxybenzenesulfonyl); alkaline hydrolysis of Arg-Pro bond; mast cell degranulating activity

A new frog skin peptide, named granuliberin-R, with the potent mast cell degranulating activity, was isolated from *Rana rugosa* (Japanese name: *Tsuchigaeru*) and its entire amino acid sequence was disclosed as the linear dodecapeptide amide (I).[4]

Two methods were employed for the synthesis of the dodecapeptide amide, which was hitherto unknown in the invertebrates; the one by the methanesulfonic acid (MSA) procedure[5] and the other confirmatively by the hydrogenation procedure.

This paper presents a detailed account of the synthetic part performed by the MSA procedure as illustrated in Fig. 1. The latter synthesis will be reported together with observations on its structure-function relationship in a separated paper (by M.F.).

Amino acid derivatives bearing protecting groups removable by MSA were employed, *i. e.*, Arg(MBS) and Ser(Bzl). The former derivative is the one newly introduced by Nishimura and Fujino[6] and its protecting group is known to be cleaved smoothly by MSA. The TFA labile Z(OMe) group[7] served as the temporal protection of the α-amino function of intermediates. Five peptide fragments were selected as the building blocks to construct the entire amino acid sequence of granuliberin-R, *i.e.*, A (position 11—12), B (8—10), C (6—7), D (3—5) and E (1—2). Among these, fragments B and D were selected by the reason that the *C*-terminal Pro-peptides can be condensed without risk of racemization.

For the synthesis of the fragment A, Z(OMe)-Ser-OH was converted to Z(OMe)-Ser(Bzl)-OH according to Sugano and Miyoshi[8] and subsequently to the corresponding amide by the

1) Preliminary communication of this paper has appeared in *Chem. Pharm. Bull.* (Tokyo), **25**, 2473 (1977).
2) Amino acids, peptides and their derivatives are of the L-configuration: Following abbreviations were used. Z=benzyloxycarbonyl, Z(OMe)=*p*-methoxybenzyloxycarbonyl, Bzl=benzyl, MBS=*p*-methoxybenzenesulfonyl, DCC=dicyclohexylcarbodiimide, TFA=trifluoroacetic acid.
3) Location: *a) Kasumi, Hiroshima, 734, Japan; b) Yodogawa-ku, Osaka, 532, Japan; c) Sakyo-ku, Kyoto, 606, Japan.*
4) T. Nakajima and T. Yasuhara, *Chem. Pharm. Bull.* (Tokyo), **25**, 2464 (1977).
5) H. Yajima, Y. Kiso, H. Ogawa, N. Fujii, and H. Irie, *Chem. Pharm. Bull.* (Tokyo), **23**, 1164 (1975).
6) O. Nishimura and M. Fujino, *Chem. Pharm. Bull.* (Tokyo), **24**, 1568 (1976).
7) F. Weygand and K. Hunger, *Chem. Ber.*, **95**, 1 (1962).
8) H. Sugano and M. Miyoshi, *J. Org. Chem.*, **41**, 2352 (1976).

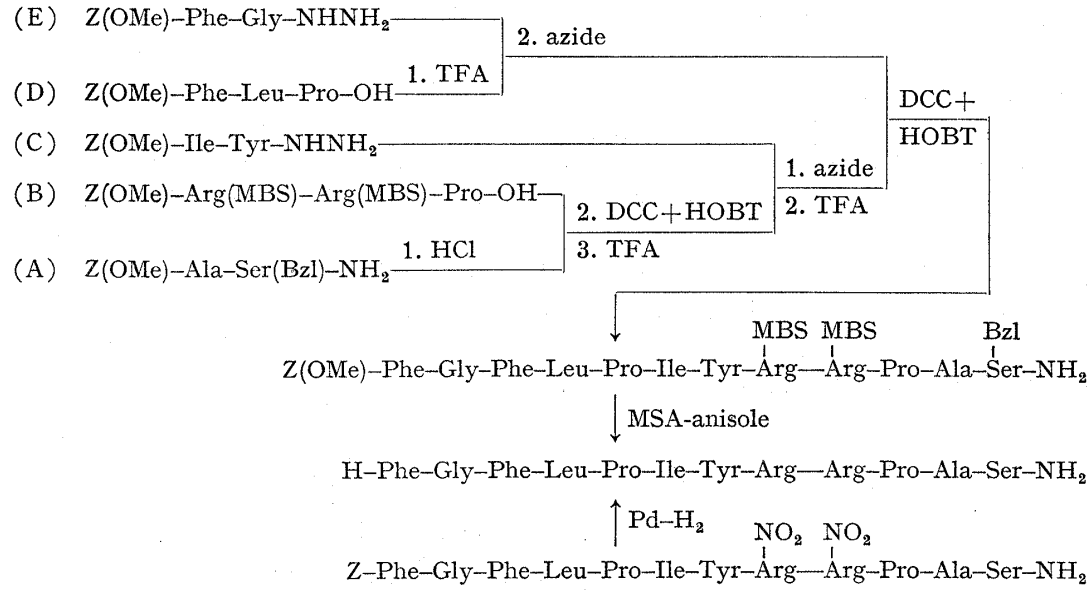

Fig. 1. Synthetic Route to Granuliberin-R

mixed anhydride procedure.[9] This method was further applied to condense Z(OMe)-Ala-OH and the TFA-treated sample of Z(OMe)-Ser(Bzl)-NH_2 to afford the fragment A, Z(OMe)-Ala-Ser(Bzl)-NH_2.

The tripeptide unit, Arg-Arg-Pro, occurs in nature as a partial sequence of adrenocorticotropin[10] and neurotensin.[11] Previously, Arg(NO_2)[12] or Arg(Tos)[13] served to prepared this peptide unit. In our present synthesis, Z(OMe)-Arg(MBS)-OH was employed as shown in Fig. 2.

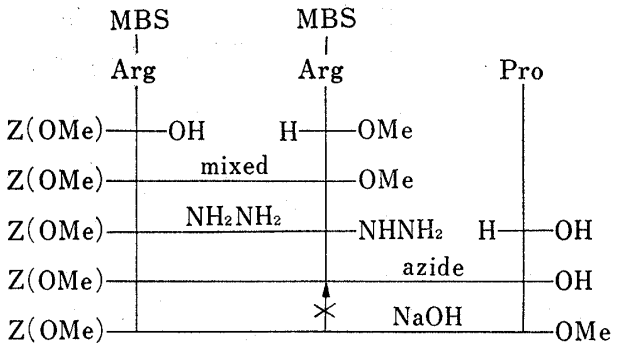

Fig. 2. Synthetic Scheme of the Fragment B, Z(OMe)-Arg(MBS)-Arg(MBS)-Pro-OH

This was condensed with H-Arg(MBS)-OMe by the mixed anhydride procedure to give Z(OMe)-Arg(MBS)-Arg(MBS)-OMe, which after conversion to the corresponding hydrazide, was further condensed with the triethylammonium salt of Pro *via* the azide procedure.[14] Prior to this synthesis, we attempted to synthesize this tripeptide unit by the alkaline saponification of Z(OMe)-Arg(MBS)-Arg(MBS)-Pro-OMe. However, it afforded heterogeneous products, which on acid hydrolysis gave a poor recovery of Pro. This result suggests that a certain hydrolysis of Arg-Pro bond accompanied during the saponification of the methyl ester by sodium hydroxide. 2,4-Dinitrophenyl ester procedure[15] was also tried to synthesize this tripeptide unit stepwisely starting with Pro. However the products were difficult to purify by contamination of 2,4-dinitrophenol. Thus the scheme we employed above seems to be a

9) T. Wieland and H. Bernhard, *Ann. Chem.*, **572**, 190 (1951); R.A. Boissonnas, *Helv. Chim. Acta*, **34**, 874 (1951); J.R. Vaughan, Jr., *J. Am. Chem. Soc.*, **73**, 3547 (1951).
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11) R. Carraway and S.E. Leeman, *J. Biol. Chem.*, **248**, 6854 (1973); *ibid.*, **250**, 1907, 1912 (1973).
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15) J.A. Harrington, P.J. Hextall, G.W. Kenner, and J.M. Turner, *J. Chem. Soc.*, **1957**, 1407; M. Bodanszky and M.A. Ondetti, *Chem. and Ind.*, **1966**, 26.

suitable method of choice to synthesize this particular peptide unit bearing the guanidino function.

Three dipeptide hydrazides, Z(OMe)-Ile-Tyr-$NHNH_2$ (C), Z(OMe)-Phe-Leu-$NHNH_2$ and Z(OMe)-Phe-Gly-$NHNH_2$ (E), were prepared by the DCC condensation[16] of respective Z(OMe)-amino acids and amino acid methyl esters followed by the usual hydrazine treatment of the resulting protected dipeptide esters. Among these, Z(OMe)-Phe-Leu-$NHNH_2$ was further condensed with the triethyl ammonium salt of Pro to afford Z(OMe)-Phe-Leu-Pro-OH (D). This tripeptide unit was alternatively prepared stepwisely by the *p*-nitrophenyl ester procedure[17] starting with Pro. This, after treatment with TFA, was condensed with Z(OMe)-Phe-Gly-$NHNH_2$ (E) *via* the azide procedure to afford the N-terminal pentapeptide, Z(OMe)-Phe-Gly-Phe-Leu-Pro-OH, as shown in Fig. 3.

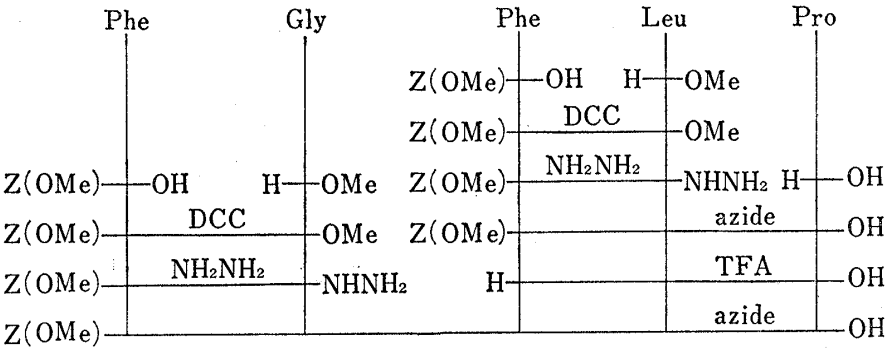

Fig. 3. Synthetic Scheme of the N-Terminal Pentapeptide, Z(OMe)-Phe-Gly-Phe-Leu-Pro-OH

The C-terminal portion of granuliberin-R, Z(OMe)-Ile-Tyr-Arg(MBS)-Arg(MBS)-Pro-Ala-Ser(Bzl)-NH_2, was prepared by assembling three peptide fragments, (A), (B) and (C), according to the scheme illustrated in Fig. 1. Deprotection of Z(OMe)-Ala-Ser(Bzl)-NH_2 can be achieved by the usual TFA treatment. However, it was necessary to convert to the corresponding hydrochloride prior to the next coupling reaction. Thus, the fragment A was treated with 4.2N HCl-dioxane and the resulting product, after neutraization with triethylamine, was condensed with Z(OMe)-Arg(NBS)-Arg(MBS)-Pro-OH (B) by means of the DCC plus 1-hydroxy-benzotriazole (HOBT) procedure.[18] The resulting protected pentapeptide, Z(OMe)-Arg(MBS)-Arg(MBS)-Pro-Ala-Ser(Bzl)-NH_2, was purified by batchwise washing with 5% citric acid and 5% sodium carbonate and water followed by recrystallization from methanol and ethyl acetate. Such batchwise washing and recrystallization procedures were applied for the purification of protected products in the latter steps of the synthesis also. Next, the protected pentapeptide amide, after the usual TFA treatment, was allowed to condense with Z(OMe)-Ile-Tyr-$NHNH_2$ (C) by the azide procedure to give the above mentioned protected heptapeptide amide. This was then treated with TFA and the deprotected product was isolated as a free base by precipitation with ether and 5% sodium carbonate. This free base was then submitted to condensation with the N-terminal pentapeptide obtained above by the DCC plus HOBT procedure. Thus this coupling reaction could be performed smoothly in the absence of the TFA triethylammonium salt. The protected dodecapeptide amide, Z(OMe)-Phe-Gly-Phe-Leu-Pro-Ile-Tyr-Arg(MBS)-Arg(NBS)-Pro-Ala-Ser(Bzl)-NH_2, was isolated in an analytically pure form by batchwise washing followed by recrystallization from ethanol.

16) J.C. Sheehan and G.W. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955).
17) M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959).
18) W. König and R. Geiger, *Chem. Ber.*, **106**, 3626 (1973).

In order to remove all protecting groups, the protected dodecapeptide was exposed to MSA in the presence of anisole at room temperature for 60 min. The deprotected product, precipitated by ether, was converted to the corresponding acetate by Amberlite CG-4B and then purified by column chromatography on CM-cellulose, which was eluted by the gradient elution with 0.2 M ammonium acetate buffer at pH 6.9. A single peak with a small shoulder was observed, when examined the eluates by absorbancy at 275 mμ. For desalting, the product obtained from the main peak was passed through a column or Sephadex G-15 using 3% acetic acid. The dodecapeptide amide thus purified exhibited a single spot on thin-layer chromatography and gave, on acid hydrolysis and aminopeptidase (AP-M)[19] digestion, amino acid ratios predicted by theory.

Alternatively, the identical dodecapeptide amide was obtained, as will be reported in a separate paper, after hydrogenolysis of Z-Phe-Gly-Phe-Leu-Pro-Ile-Tyr-Arg(NO_2)-Arg(NO_2)-Pro-Ala-Ser-NH_2, which was prepared by the DCC plus N-hydroxynorbornenedicarboximide condensation[20] of three peptide subunits, the N-terminal pentapeptide, Z-Phe-Gly-Phe-Leu-Pro-OH, the middle pentapeptide, Boc-Ile-Tyr-Arg(NO_2)-Arg(NO_2)-Pro-OH and the C-terminal dipeptide, H-Ala-Ser-NH_2 successively.

Identity of the synthetic peptides obtained by two methods with the natural peptide was chromatographically assessed. Furthermore, the degranulating activity of both of the synthetic peptides in rat peritoneal mast cells was found equivalent to that of the natural source. Thus, the new MSA procedure could offer an example for the synthesis of biologically active peptides.

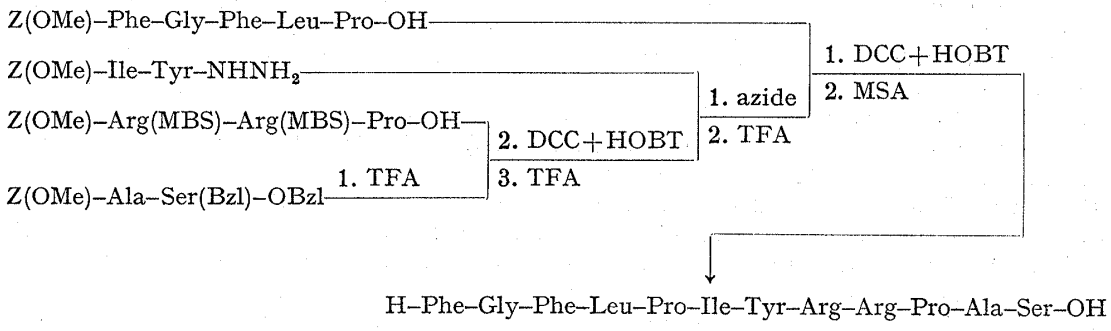

Fig. 4. Synthetic Route to Des-amide Granuliberin-R

In connection with this synthesis, we wish to add the synthesis of des-amide granuliberin-R. Available three peptide fragments were successively condensed with the C-terminal dipeptide ester, H-Ala-Ser(Bzl)-OBzl, as shown in Fig. 4. Deprotection of the resulting protected dodecapeptide, Z(OMe)-Phe-Gly-Phe-Leu-Pro-Ile-Tyr-Arg(MBS)-Arg(MBS)-Pro-Ala-Ser(Bzl)-OBzl, by MSA and subsequent purification were carried out in essentially the same manner as described in the synthesis of granuliberin-R. It seems interesting to note that this dodecapeptide lacking the C-terminal amide exhibited only the activity of the one 5th of that of natural granuliberin-R.

Experimental

General experimental methods employed are essentially the same as those described in the Part LXII.[21] 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–AcOH–pyridine–H_2O (30:6:20:24).

19) G. Pfleiderer and G.P. Celliers, *Biochem. Z.*, **339**, 196 (1963). AP-M (Lot No. 210520) was purchased from the Protein Res. Foundation, Osaka.
20) M. Fijino, S. Kobayashi, M. Obayashi, T. Fukuda, S. Shinagawa, and O. Nishimura, *Chem. Pharm. Bull.* (Tokyo), **22**, 1867 (1974).
21) H. Ogawa, M. Kubota, and H. Yajima, *Chem. Pharm. Bull.* (Tokyo), **24**, 2428 (1976).

(A) **Synthesis of Granuliberin-R**

Z(OMe)-Ser(Bzl)-OH·CHA Salt——According to Sugano and Miyoshi,[8] NaH (2.64 g, 2.2 equiv.) was added to a solution of Z(OMe)–Ser–OH (13.50 g) in DMF (100 ml) under cooling with ice. After evolution of H_2 gas ceased, benzyl bromide (8.9 ml, 1.5 equiv.) was added and the mixture was stirred at room temperature for 60 min (prolonged reaction increased the benzyl ester formation). After neutralization with AcOH, the solution was condensed *in vacuo* and the residue was dissolved in 5% NH_4OH, which was washed with ether (see below). The aqueous phase was acidified with citric acid and the resulting precipitate was extracted with AcOEt, which was washed with 5% citric acid and H_2O–NaCl, dried over Na_2SO_4 and then evaporated. The residue was dissolved in MeOH (30 ml) and cyclohexylamine (CHA, 6 ml) was added. The crystalline mass formed by addition of ether was recrystallized from MeOH and ether; yield 7.75 g (34%), mp 134—137°, $[\alpha]_D^{19}$ +19.7° (c=1.1, MeOH). *Anal.* Calcd. for $C_{19}H_{21}NO_6 \cdot C_6H_{13}N$: C, 65.48; H, 7.47; N, 6.11. Found: C, 65.60; H, 7.57; N, 6.25. (lit.[22] Z(OMe)–Ser(Bzl)–OH, mp 72—73°, $[\alpha]_D$ +16.2° in EtOH).

The above ether washing was evaporated and the residue in MeOH (20 ml) was treated with 1 N NaOH (9.4 ml) at room temperature for 30 min. The solution was neutralized with AcOH and condensed. The residue was treated as stated above to give an additional CHA salt; yield 2.75 g (12%), mp 132—135°, $[\alpha]_D^{19}$ +18.7° (c=0.9, MeOH).

Z(OMe)-Ser(Bzl)-NH$_2$——Under cooling with ice–NaCl, isobutyl chloroformate (3.0 ml) was added to a solution of Z(OMe)–Ser(Bzl)–OH (7.60 g, prepared from the CHA salt as usual) and Et_3N (3.2 ml) in tetrahydrofuran (THF, 100 ml). The solution was stirred for 20 min and then added into 28% NH_4OH (8.0 ml). The mixture was stirred in an ice-bath for 60 min, the solvent was evaporated and the residue was extracted with AcOEt, which was washed with 5% $NaHCO_3$ and H_2O–NaCl, dried over Na_2SO_4 and then evaporated. The residue was recrystallized from THF and ether; yield 5.90 g (82%), mp 120—123°, $[\alpha]_D^{26}$ +34.1° (c=0.7, MeOH). Rf_1 0.70. *Anal.* Calcd. for $C_{19}H_{22}N_2O_5$: C, 63.67; H, 6.19; N, 7.82. Found: C, 63.45; H, 6.19; N, 7.80.

Z(OMe)-Ala-Ser(Bzl)-NH$_2$——Z(OMe)-Ser(Bzl)-NH_2 (5.0 g) was treated with TFA (10 ml) in the presence of anisole (6 ml) in an ice-bath for 60 min and the excess TFA was removed by evaporation. The residue was washed with *n*-hexane and dissolved in THF (30 ml). To this ice-chilled solution, Et_3N (2 ml) and the mixed anhydride (prepared from 4.56 g of Z(OMe)–Ala–OH with 2.5 ml of Et_3N and 2.6 ml of isobutyl chloroformate) in THF (40 ml) were added and the mixture was stirred in an ice-bath for 2 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, 5% $NaHCO_3$ and H_2O and then recrystallized from THF and AcOEt; yield 4.15 g (69%), mp 182—185°, $[\alpha]_D^{22}$ +3.1° (c=0.6, DMF). Rf_1 0.47. *Anal.* Calcd. for $C_{22}H_{29}N_3O_6$: C, 61.52; H, 6.34; N, 9.79. Found: C, 61.74; H, 6.28; N, 9.84.

Z(OMe)-Arg(MBS)-OH·CHA Salt——According to Nishimura and Fujino,[6] freshly prepared *p*-methoxybenzenesulfonyl chloride (111.6 g) in acetone (350 ml) was added dropwise to an ice-chilled solution of Z(OMe)–Arg–OH (60.9 g) in 4 N NaOH (270 ml) and acetone (1200 ml) during a period of 60 min. After stirring in an ice-bath for 2 hr and at room temperature for 2 hr, the mixture was neutralized with citric acid and then condensed *in vacuo*. The product was purified according to the above instruction and then converted to the corresponding CHA salt, which was recrystallized twice from MeOH and acetonitrile; yield 41.3 g (38%), mp 110—114°, $[\alpha]_D^{25}$ +25.1° (c=0.6, MeOH). *Anal.* Calcd. for $C_{22}H_{28}N_4O_8S \cdot C_6H_{13}N$: C, 55.24; H, 6.96; N, 11.51. Found: C, 55.17; H, 6.81; N, 11.61.

Z(OMe)-Arg(MBS)-Arg(MBS)-OMe——Under cooling with ice, an etheral solution of diazomethane was added dropwise to a solution of Z(OMe)–Arg(MBS)–OH (derived from 16.30 g of the CHA salt as usual) in MeOH (100 ml) and the yellow color of the solution was maintained for 30 min. The solution, after addition of a few drops of AcOH, was evaporated and the residue was triturated with ether to afford an amorphous powder; yield 12.15 g (87%), Rf_1 0.70. Z(OMe)–Arg(MBS)–OMe thus obtained (4.44 g) was treated with TFA-anisole (8 ml—5 ml) as stated above and the excess TFA was removed by evaporation. The residue was washed with *n*-hexane and then treated with ether. The resulting powder was collected by filtration, washed with ether, dried over KOH pellets *in vacuo* for 3 hr and then dissolved in DMF (35 ml). To this ice-chilled solution, the mixed anhydride of Z(OMe)–Arg(MBS)–OH (prepared from 5.14 g of the CHA salt with 1.3 ml of isobutyl chloroformate) in THF (40 ml) was added. The mixture was stirred in an ice-bath for 3 hr. The solvent was evaporated and the residue was dissolved in a mixture of AcOEt and *n*-BuOH (5: 1 v/v), which was washed with 5% citric acid, 3% $NaHCO_3$ and H_2O–NaCl, dried over Na_2SO_4 and then evaporated. The residue was triturated with ether and recrystallized from THF and ether; yield 7.11 g (98%), mp 111—115°, $[\alpha]_D^{26}$ +20.7° (c=0.3, DMF), Rf_1 0.57. *Anal.* Calcd. for $C_{36}H_{48}N_8O_{12}S_2$: C, 50.93; H, 5.70; N, 13.20; S, 7.55. Found: C, 51.05; H, 5.62; N, 12.60; S, 7.30.

Z(OMe)-Arg(MBS)-Arg(MBS)-NHNH$_2$——To a solution of Z(OMe)–Arg(MBS)–Arg(MBS)–OMe (3.70 g) in MeOH (35 ml) and THF (15 ml), 80% hydrazine hydrate (3.0 ml) was added. The solution, after standing overnight, was evaporated and the residue was treated with H_2O. The resulting powder was washed with H_2O and then precipitated from DMF with AcOEt; yield 2.95 g (80%), mp 100—103°, $[\alpha]_D^{20}$ −6.1° (c=1.0,

22) F. Weygand and E. Nintz, *Z. Naturforsch., B*, **20**, 429 (1965).

DMF), Rf_1 0.51. *Anal.* Calcd. for $C_{35}H_{48}N_{10}O_{11}S_2$: C, 49.51; H, 5.70; N, 16.50; S, 7.55. Found: C, 49.21; H, 5.63; N, 16.33; S, 7.42.

Z(OMe)-Arg(MBS)-Arg(MBS)-Pro-OH——The azide (prepared from 3.0 g of Z(OMe)–Arg(MBS)–Arg(MBS)–$NHNH_2$ with 1.42 ml of 5.96 N HCl–DMF, 0.57 ml of isoamylnitrite and 1.17 ml of Et_3N) in DMF (15 ml) was added to an ice-chilled solution of H–Pro–OH (0.81 g) and Et_3N (1.46 ml) in H_2O–DMF (5 ml—10 ml) and the mixture was stirred at 4° for 48 hr. The solvent was evaporated and the residue was dissolved in 3% NH_4OH, which was washed with AcOEt. The aqueous phase was acidified with citric acid and the resulting oily precipitate was extracted with AcOEt and *n*-BuOH (3: 1 v/v), which after washing with 5% citric acid and H_2O–NaCl, was dried over Na_2SO_4 and then evaporated. The residue was triturated with ether and recrystallized from EtOH and ether; yield 2.0 g (61%), mp 124—126°, $[\alpha]_D^{24}$ −15.6° (c=0.4, DMF). Rf_1 0.21, Rf_2 0.36. Amino acid ratios in 6 N HCl hydrolysate: Arg 1.94, Pro 1.00. (average recovery 80%). *Anal.* Calcd. for $C_{40}H_{53}N_9O_{13}S_2$: C, 51.54; H, 5.73; N, 13.53. Found: C, 51.28; H, 5.56; N, 13.36.

Z(OMe)-Ile-Tyr-OMe——DCC (5.40 g) was added to a solution of Z(OMe)-Ile–OH (7.20 g) and H–Tyr–OMe (prepared from 9.0 g of the hydrochloride with 5.6 ml of Et_3N) in DMF (150 ml) and the mixture was stirred at room temperature for 48 hr. The solution was filtered, the filtrate was condensed *in vacuo* and 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 MeOH and ether; yield 8.0 g (71%), mp 147—148°, $[\alpha]_D^{24}$ −2.4° (c=0.4, DMF), Rf_1 0.85. *Anal.* Calcd. for $C_{25}H_{32}N_2O_7$: C, 63.54; H, 6.83; N, 5.93. Found: C, 63.77; H, 6.87; N, 6.15.

Z(OMe)-Ile-Tyr-$NHNH_2$——To a solution of Z(OMe)–Ile–Tyr–OMe (8.0 g) in MeOH (120 ml), 80% hydrazine hydrate (10 ml) was added. The gelatinous mass formed on standing overnight was collected by filtration and precipitated from DMF with MeOH; yield 7.40 g (93%), mp 221—222°, $[\alpha]_D^{25}$ −23.9° (c=0.8, DMF), Rf_1 0.58. *Anal.* Calcd. for $C_{24}H_{32}N_4O_6$: C, 61.00; H, 6.83; N, 11.86. Found: C, 61.08; H, 6.92; N, 11.63.

Z(OMe)-Phe-Leu-OMe——DCC (4.75 g) was added to a solution of Z(OMe)–Phe–OH (6.83 g) and H–Leu–OMe (prepared from 7.23 g of the hydrochloride with 5.6 ml of Et_3N) in DMF–THF (70 ml—70 ml) and the mixture was stirred at room temperature for 24 hr. The solution was filtered, the filtrate was condensed *in vacuo* and the residue was extracted with AcOEt, which was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O–NaCl, dried over Na_2SO_4 and then evaporated. The residue was triturated with *n*-hexane and recrystallized from THF and ether; yield 6.60 g (69%), mp 109—110°, $[\alpha]_D^{20}$ −14.3° (c=0.9, DMF). Rf_1 0.92. *Anal.* Calcd. for $C_{25}H_{32}N_2O_6$: C, 65.77; H, 7.07; N, 6.14. Found: C, 65.90; H, 7.01; N, 6.05.

Z(OMe)-Phe-Leu-$NHNH_2$——To a solution of Z(OMe)–Phe–Leu–OMe (3.80 g) in MeOH (70 ml), 80% hydrazine hydrate (5.0 ml) was added. The crystalline mass formed on standing overnight was collected by filtration and recrystallized from EtOH; yield 3.09 g (81%), mp 155—159°, $[\alpha]_D^{20}$ −15.6° (c=1.0, DMF), Rf_1 0.60. *Anal.* Calcd. for $C_{24}H_{32}N_4O_5$: C, 63.14; H, 7.07; N, 12.27. Found: C, 63.40; H, 7.09; N, 12.32.

Z(OMe)-Phe-Leu-Pro-OH——a) The azide (prepared from 3.09 g of Z(OMe)–Phe–Leu–$NHNH_2$ with 2.8 ml of 5.96 N HCl–DMF, 1.36 ml of isoamylnitrite and 2.3 ml of Et_3N) in DMF (30 ml) was added to a solution of H–Pro–OH (1.57 g) and Et_3N (2.8 ml) in H_2O (10 ml). The mixture was stirred at 4° for 48 hr and the solvent was evaporated. The residue was dissolved in 3% NH_4OH, which was washed with AcOEt. The aqueous phase was acidified with citric acid and the resulting oily precipitate was extracted with AcOEt, which was washed with H_2O–NaCl, dried over Na_2SO_4 and then evaporated. The residue was triturated with ether and recrystallized from AcOEt and ether; yield 3.02 g (82%), mp 80—83°, $[\alpha]_D^{20}$ −47.1° (c=1.0, DMF), Rf_1 0.57, Rf_2 0.71. Amino acid ratios in 6 N HCl hydrolysate: Phe 1.01, Leu 1.00, Pro 0.99 (average recovery 96%). *Anal.* Calcd. for $C_{29}H_{37}N_3O_7$: C, 64.54; H, 6.91; N, 7.79. Found: C, 64.47; H, 6.88; N, 7.70.

b) Z(OMe)–Leu-ONP (15.0 g) dissolved in THF (60 ml) was added to a solution of H–Pro–OH (8.3 g) and Et_3N 10.9 ml) in H_2O (40 ml) and the mixture was stirred at room temperature for 24 hr. The solvent was evaporated and the residue was dissolved in H_2O. The aqueous phase after washing with ether, was acidified with citric acid. The resulting precipitate was extracted with AcOEt, which was washed with 10% citric acid and H_2O–NaCl, dried over Na_2SO_4 and then evaporated to give an oil; yield 12.2 g (87%), Rf_1 0.61. Z(OMe)–Leu–Pro–OH thus obtained (11.20 g) was treated with TFA (25 ml) in the presence of anisole (6 ml) in an ice-bath for 45 min and *n*-hexane was added. The resulting oily precipitate was washed with ether, dried over KOH pellets *in vacuo* for 3 hr and then dissolved in DMF (150 ml). To this solution, Et_3N (8.7 ml) and Z(OMe)–Phe–ONP (12.80 g) were added. The mixture was stirred at room temperature for 24 hr. The solvent was evaporated and the residue was dissolved in 3% NH_4OH, which after washing with AcOEt, was acidified with citric acid. The resulting precipitate was extracted with AcOEt. The extract was washed with 10% citric acid and H_2O–NaCl, dried over Na_2SO_4 and then evaporated. The residue was triturated with ether and recrystallized from AcOEt; yield 10.03 g (65%), mp 118—121°, $[\alpha]_D^{26}$ −58.1° (c=0.8, DMF), Rf_2 0.71. Amino acid ratios in 6 N HCl hydrolysate: Phe 1.05, Leu 1.00, Pro 1.00 (average recovery 91%). *Anal.* Found: C, 64.37; H, 6.92; N, 7.83.

Z(OMe)-Phe-Gly-OMe——DCC (7.0 g) was added to a solution of Z(OMe)–Phe–OH (10.0 g) and H–Gly–OMe (prepared from 7.0 g of the hydrochloride with 4.5 ml of Et_3N) in DMF–THF (70 ml—60 ml) and the mixture was stirred at room temperature for 48 hr. The solution was filtered, the filtrate was condensed *in vacuo* and the residue was dissolved in AcOEt, which was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O–NaCl, dried over Na_2SO_4 and then evaporated. The residue was triturated with ether and recrystallized

from MeOH and ether; yield 8.88 g (74%), mp 112—113°, $[\alpha]_D^{20}$ −27.7° (c=1.0, DMF), Rf_1 0.79. *Anal.* Calcd. for $C_{21}H_{24}N_2O_6$: C, 62.99; H, 6.04; N, 7.00. Found: C, 63.04; H, 5.80; N, 7.03.

Z(OMe)-Phe-Gly-NHNH$_2$——To a solution of Z(OMe)–Phe–Gly–OMe (8.88 g) in MeOH (110 ml), 80% hydrazine hydrate (14 ml) was added and the solution was kept on standing overnight. The solvent was evaporated. Treatment of the residue with H_2O afforded a powder, which was recrystallized from EtOH; yield 5.62 g (64%), mp 138—140°, $[\alpha]_D^{25}$ −20.7° (c=1.1, DMF), Rf_1 0.50. *Anal.* Calcd. for $C_{20}H_{24}N_4O_5$: C, 59.99; H, 6.04; N, 13.99. Found: C, 60.24; H, 5.95; N, 14.02.

Z(OMe)-Phe-Gly-Phe-Leu-Pro-OH——Z(OMe)–Phe–Leu–Pro–OH (1.98 g) was treated with TFA (5 ml) in the presence of anisole (3 ml) as usual and dry ether was added. The resulting powder was collected by filtration, dried over KOH pellets *in vacuo* for 3 hr and then dissolved in DMF (15 ml). To this ice-chilled solution, Et_3N (0.5 ml) and the azide (prepared from 1.66 g of Z(OMe)–Phe–Gly–NHNH$_2$ with 1.7 ml of 5.96 N HCl–DMF, 0.8 ml of isoamylnitrite and 2.0 ml of Et_3N) in DMF (15 ml) were added and the mixture was stirred at 4° for 48 hr. The solvent was evaporated and the residue was dissolved in 3% NH_4OH. The solution after washing with AcOEt, was acidified with citric acid. The resulting precipitate was extracted with AcOEt. The extract was washed with H_2O–NaCl, dried over Na_2SO_4 and then evaporated. The residue was triturated with ether and recrystallized from MeOH and ether; yield 1.74 g (59%), mp 223—226°, $[\alpha]_D^{20}$ −42.5° (c=1.0, DMF). Rf_1 0.55. Amino acid ratios in 6 N HCl hydrolysate: Phe 2.03, Gly 1.00, Leu 1.00, Pro 1.05 (average recovery 91%). *Anal.* Calcd. for $C_{40}H_{49}N_5O_9$: C, 64.58; H, 6.64; N, 9.42. Found: C, 64.31; H, 6.37; N, 9.28.

Z(OMe)-Arg(MBS)-Arg(MBS)-Pro-Ala-Ser(Bzl)-NH$_2$——Z(OMe)–Ala–Ser(Bzl)–NH$_2$ (1.29 g) was treated with 4.2 N HCl–dioxane (14 ml) in an ice-bath for 60 min and the excess dioxane was removed by evaporation. The residue was washed with *n*-hexane, dried over KOH pellets *in vacuo* for 3 hr and then dissolved in DMF (20 ml). The solution was neutralized with Et_3N (0.4 ml). To this ice-chilled solution, Z(OMe)–Arg(MBS)–Arg(MBS)–Pro–OH (1.85 g), HOBT (0.34 g) and DCC (0.48 g) were successively added. The mixture was stirred at room temperature for 24 hr, filtered and the filtrate was condensed *in vacuo*. 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 recrystallized from MeOH and AcOEt; yield 2.05 g (87%). mp 131—135°, $[\alpha]_D^{23}$ −15.4° (c=0.5, DMF). Rf_1 0.54. Amino acid ratios in 6 N HCl hydrolysate: Arg 1.91, Pro 1.00, Ala 1.06, Ser 0.84 (average recovery 63%). *Anal.* Calcd. for $C_{53}H_{70}N_{12}O_{15}S_2\cdot H_2O$: C, 53.16; H, 6.06; N, 14.04. Found: C, 53.10; H, 6.19; N, 14.19.

Z(OMe)-Ile-Tyr-Arg(MBS)-Arg(MBS)-Pro-Ala-Ser(Bzl)-NH$_2$——The above protected pentapeptide amide (1.88 g) was treated with TFA (4 ml)–anisole (1.5 ml) as usual and dry ether was added. The resulting powder was collected by filtration, dried over KOH pellets *in vacuo* for 3 hr and then dissolved in DMF (12 ml) containing Et_3N (0.23 ml). To this ice-chilled solution, the azide (prepared from 1.0 g of Z(OMe)–Ile–Tyr–NHNH$_2$ with 0.93 ml of 5.96 N HCl–DMF, 0.44 ml of isoamylnitrite and 1.1 ml of Et_3N) in DMF (10 ml) was added and the mixture was stirred at 4° for 20 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 recrystallized from MeOH and AcOEt; yield 1.72 g (74%), mp 137—142°, $[\alpha]_D^{25}$ −17.4° (c=0.2, DMF). Amino acid ratios in 6 N HCl (in the presence of phenol) hydrolysate: Ile 1.09, Tyr 1.00, Arg 1.96, Pro 0.93, Ala 1.31, Ser 1.02, (average recovery 99%). *Anal.* Calcd. for $C_{68}H_{90}N_{14}O_{18}S_2\cdot H_2O$: C, 55.42; H, 6.29; N, 13.30. Found: C, 55.37; H, 6.23; N, 12.79.

Z(OMe)-Phe-Gly-Phe-Leu-Pro-Ile-Tyr-Arg(MBS)-Arg(MBS)-Pro-Ala-Ser(Bzl)-NH$_2$——The above protected heptapeptide amide (1.71 g) was treated with TFA (4 ml)–anisole (2 ml) as usual and the excess TFA was removed by evaporation. The residue was treated with ether and 5% Na_2CO_3. The resulting powder was washed with H_2O, dried over P_2O_5 *in vacuo* for 3 hr and then dissolved in DMF (20 ml). To this solution, Z(OMe)–Phe–Gly–Phe–Leu–Pro–OH (1.34 g), HOBT (0.3 g) and DCC (0.41 g) were added successively. The mixture was stirred at room temperature for 15 hr and filtered. The filtrate was condensed and the residue was treated with ether and 5% citric acid. The resulting powder was washed batchwisely as stated above and recrystallized from MeOH and AcOEt and from EtOH; yield 2.04 g (86%). mp 149—153°, Rf_1 0.54, $[\alpha]_D^{22}$ −40.0° (c=0.2, DMF). Amino acid ratios in 6 N HCl (in the presence of phenol) hydrolysate: Phe 2.10, Gly 0.99, Leu 1.00, Pro 2.00, Ile 0.98, Tyr 0.91, Arg 1.94, Ala 0.95, Ser 0.75 (average recovery 91%). *Anal.* Calcd. for $C_{99}H_{129}N_{19}O_{23}S_2\cdot 1.5H_2O$: C, 58.16; H, 6.50; N, 13.02. Found: C, 58.12; H, 6.45; N, 13.02.

H-Phe-Gly-Phe-Leu-Pro-Ile-Tyr-Arg-Arg-Pro-Ala-Ser-NH$_2$——The protected dodecapeptide amide (0.50 g) was treated with MSA (4.0 ml) in the presence of anisole (0.13 ml) at room temperature for 60 min and dry ether was added. The oily residue was dissolved in H_2O (10 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, after washing with ether, was lyophilized. The resulting white powder was dissolved in a small amount of H_2O and the solution was applied to a column of CM-cellulose (2.5×19 cm), which was eluted with 0.2 M $AcONH_4$ (1500 ml) at pH 6.9 through a mixing flask containing 0.01 M $AcONH_4$ (500 ml). Individual fractions (11 ml each) were collected and the absorbancy at 275 mμ was determined. A single peak with some shoulder was detected. Fractions corresponding to the main peak (Fig. 5-a, F-1, tube No. 50—60) were collected and the solvent was removed by lyophilization. The residue was then dissolved in a small amount of 3% AcOH and the solution was applied to a column of Sephadex G-15 (3.5×130 cm), which was eluted

with the same solvent. Individual fractions (9 ml each) were collected and the absorbancy at 275 mμ was determined. Fractions corresponding to the symmetrical peak (Fig. 5-b, tube No. 50—68) were collected and the solvent was removed by lyophilization to give a fluffy white powder; yield 129 mg (32%). $[\alpha]_D^{25}$ −91.6° (c=0.1, 1 N AcOH). Rf_3 0.78. Amino acid ratios in 6N HCl and AP-M digest (number in parenthesis): Phe 1.97 (1.97), Gly 1.13 (1.00), Leu 1.00 (1.02), Pro 1.72 (1.94), Ile 1.01 (1.03), Tyr 0.99 (1.00), Arg 1.92 (2.03), Ala 1.12 (0.98), Ser 0.80 (0.93). average recovery 99% (92%). *Anal.* Calcd. for $C_{69}H_{103}N_{19}O_{14}\cdot 2CH_3COOH\cdot 4H_2O$: C, 54.29; H, 7.43; N, 16.48. Found: C, 54.40; H, 7.07; N, 16.37.

Thin-layer chromatographically identical peptide was obtained after catalytic hydrogenation of Z-Phe-Gly-Phe-Leu-Pro-Ile-Tyr-Arg-(NO_2)-Arg(NO_2)-Pro-Ala-Ser-NH_2 (synthetic detail will be reported in future): $[\alpha]_D^{23}$ −87.6° (c=0.6, 1 N AcOH). Amino acid ratios in an acid hydrolysate: Phe 1.96, Gly 1.00 Leu 1.00, Pro 2.00, Ile 0.88, Tyr 0.84, Arg 2.20, Ala 0.96, Ser 0.94 (average recovery 77%).

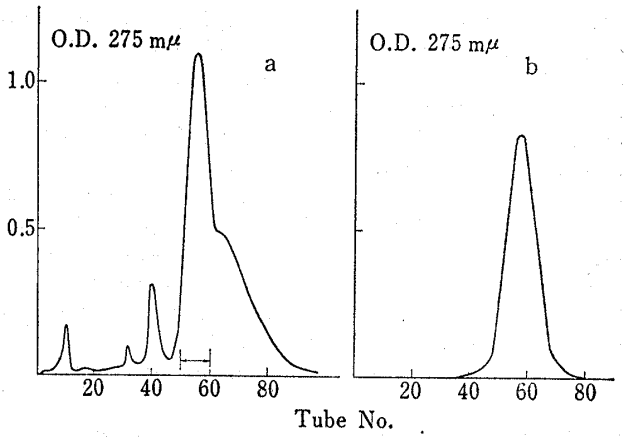

Fig. 5-a. Pxrification of Synthetic Granuliberin-R on CM-Cellxlose

Column : 2.5×19 cm.
Fraction: 11 ml.
Elution : linear gradient 0.2 M NH_4OAc, pH 6.9, mixing flask 0.01 M NH_4OAc (500 ml).

Fig. 5-b. Desalting of Synthetic Granxliberin-R on Sephadex G-15

Column : 3.5×130 cm.
Fraction: 9 ml.
Elution : 3% AcOH.

Comparison of the Synthetic Peptide with Natural Granuliberin-R——An aliqout of the synthetic (two methods) and the natural peptides (*ca.* 1 μg each) was dansylated by the method described previously.[23] These dansylated peptides showed the same *Rf* value (0.08) on thin-layer co-chromatography in the solvent system of *n*-BuOH–AcOH–H_2O (4:1:5). The natural and the synthetic peptides (1 μg each) were digested with chymotrypsin (5 μl of a stock solution; 1 mg of enzyme/ml, 0.1M triethylamine–bicarbonate buffer, pH 8.5) or digested with trypsin (5 μl of a stock solution; 1 mg of enzyme/ml, 0.1 M triethylamine–bicarbonate buffer, pH 8.5) at 35° overnight. These chymotryptic and tryptic digests were dansylated and co-chromatographed in the same solvent system mentioned above. Both chymotryptic and tryptic fragments derived from the synthetic peptides coincided with these of the natural peptide respectively. The minimum concentration of the synthetic peptides which revealed the mast cell degranulating activity was approximately the same with that obtained from the natural peptide.[4]

(B) Synthesis of Desamide-Granuliberin-R

Z(OMe)-Ser(Bzl)-OBzl——To a solution of Z(OMe)–Ser–OH (32.31 g) in DMF (150 ml), NaH (7.92 g) and benzyl bromide (31.36 ml, 2.2 equiv.) were added and the mixture was stirred at room temperature for 3 hr. The solution, after neutralization with AcOH, was condensed *in vacuo* and the residue was dissolved in AcOEt. The organic phase was washed with 5% NH_4OH (from the aqueous phase, Z(OMe)–Ser(Bzl)–OH can be isolated as stated above), 5% citric acid and H_2O, dried over Na_2SO_4 and then evaporated. The oily residue was triturated with *n*-hexane and the resulting powder was recrystallized from AcOEt and ether; yield 15.62 g (29%), mp 50—52°, $[\alpha]_D^{26}$ −8.9° (c=0.7, DMF), Rf_1 0.97. *Anal.* Calcd. for $C_{26}H_{27}NO_6$: C, 69.47; H, 6.05; N, 3.12. Found: C, 69.72; H, 6.07; N, 3.17.

Z(OMe)-Ala-Ser(Bzl)-OBzl——Z(OMe)–Ser(Bzl)–OBzl (15.62 g) was treated with TFA (30 ml)–anisole (11 ml) in an ice-bath for 45 min and the excess TFA was removed by evaporation. The residue was dissolved in AcOEt. The extract was washed with 5% $NaHCO_3$ and H_2O–NaCl, dried over Na_2SO_4 and then filtered. The filtrate was combined with Z(OMe)–Ala–OH (9.68 g) in THF (100 ml) and DCC (7.88 g) was added. The mixture was stirred at room temperature for 48 hr, then filtered, the filtrate was condensed. The residue was triturated with ether and the resulting powder, after washing batchwisely with 5% citric acid, 5% $NaHCO_3$ and H_2O, was recrystallized from AcOEt and ether; yield 11.57 g (64%); mp 126—128°, $[\alpha]_D^{26}$ +0.6° (c=0.3, DMF), Rf_1 0.92. *Anal.* Calcd. for $C_{29}H_{32}N_2O_7$: C, 66.91; H, 6.20; N, 5.38. Found: C, 66.61; H, 6.50; N, 5.43.

Z(OMe)-Arg(MBS)-Arg(MBS)-Pro-Ala-Ser(Bzl)-OBzl——Z(OMe)–Ala-Ser(Bzl)–OBzl (1.51 g) was treated with TFA (3 ml)–anisole (0.8 ml) as usual and the excess TFA was removed by evaporation. The residue was basified with a saturated solution of Na_2CO_3 and then extracted with AcOEt (10 ml). The extract was washed with NaCl–H_2O, dried over Na_2SO_4 and then filtered. To this filtrate, Z(OMe)–Arg(MBS)–Arg(MBS)–Pro–OH (1.80 g), HOBT (0.38 g) and DCC (0.52 g) were successively added and the mixture was stirred at room temperature for 48 hr. The solution was filtered, the filtrate was washed with 10% citric acid, 5% Na_2-

23) Z. Tamura, T. Nakajima, T. Nakayama, J.J. Pisano, and U. Udenfriend, *Anal. Biochem.*, **52**, 595 (1973).

CO_3 and H_2O–NaCl, dried over Na_2SO_4 and then evaporated. The residue was dissolved in a small amount of the solvent consisting of $CHCl_3$–MeOH (10: 1) and the solution was applied to a column of silica (3×20 cm), which was eluted with the same solvent. Fractions containing the substance of Rf_1 0.52 were collected and the solvent was evaporated. The residue was treated with H_2O and the resulting powder was recrystallized from THF and ether; yield 1.31 g (54%), mp 101—102°, $[\alpha]_D^{17}$ −12.8° (c=0.3, DMF). Rf_1 0.52. Amino acid ratios in 6 N HCl hydrolysate: Arg 2.00, Pro 1.01, Ala 1.12, Ser 0.87 (average recovery 93%). *Anal.* Calcd. for $C_{60}H_{75}N_{11}O_{16}S_2$: C, 56.72; H, 5.95; N, 12.13. Found: C, 56.58; H, 5.87; N, 11.85.

Z(OMe)-Ile-Tyr-Arg(MBS)-Arg(MBS)-Pro-Ala-Ser(Bzl)-OBzl——The above protected pentapeptide ester (1.31 g) was treated with TFA (2.6 ml)–anisole (0.6 ml) as usual and dry ether was added. The resulting powder was collected by filtration, dried over KOH pellets *in vacuo* for 3 hr and then dissolved in DMF (5 ml). To this ice-chilled solution, Et_3N (0.42 ml) and the azide (prepared from 0.98 g of Z(OMe)–Ile–Tyr–$NHNH_2$ as mentioned above) were added. The product was purified by batchwise washing as stated above followed by recrystallization from THF and AcOEt; yield 1.25 g (79%), mp 117—118°, $[\alpha]_D^{20}$ −25.9° (c=0.4, DMF). Rf_1 0.52. Amino acid ratios in 6 N HCl hydrolysate: Ile 1.17, Tyr 0.56, Arg 2.00, Pro 1.12, Ala 1.16, Ser 0.65 (average recovery 91%).[24] *Anal.* Calcd. for $C_{75}H_{95}N_{13}O_{19}S_2 \cdot H_2O$: C, 57.56; H, 6.25; N, 11.64. Found: C, 57.58; H, 6.29; N, 11.32.

Z(OMe)-Phe-Gly-Phe-Leu-Pro-Ile-Tyr-Arg(MBS)-Arg(MBS)-Pro-Ala-Ser(Bzl)-OBzl——The above protected heptapeptide ester (1.21 g) was treated with TFA (2.4 ml)–anisole (0.6 ml) as usual and dry ether was added. The resulting powder was converted to the corresponding hydrochloride by 4.21 N HCl–dioxane (0.24 ml) and the hydrochloride was precipitated by ether as a powder, which was dissolved in DMF (10 ml). To this solution, Et_3N (0.11 ml), Z(OMe)–Phe–Gly–Phe–Leu–Pro–OH (1.16 g), HOBT (0.26 g) and DCC (0.35 g) were successively added and the mixture was stirred at room temperature for 48 hr. The solvent was filtered, the filtrate was condensed *in vacuo* and the residue was treated with ether and 10% citric acid. The resulting powder was purified by batchwise washing as stated above and then recrystallized twice from THF and AcOEt; yield 1.03 g (63%), mp 136—138°, $[\alpha]_D^{21}$ −74.3° (c=0.2, DMF). Rf_1 0.64. Amino acid ratios in 6 N HCl hydrolysate: Phe 2.25, Gly 1.14, Leu 1.12, Pro 2.17, Ile 1.10, Tyr 0.54, Arg 1.93, Ala 1.00, Ser 0.76 (average recovery 90%). *Anal.* Calcd. for $C_{106}H_{134}N_{18}O_{24}S_2$: C, 60.38; H, 6.41; N, 11.96. Found: C, 60.17; H, 6.20; N, 11.75.

H-Phe-Gly-Phe-Leu-Pro-Ile-Tyr-Arg-Arg-Pro-Ala-Ser-OH——The above protected dodecapeptide ester (0.42 g) was treated with MSA (3 ml) in the presence of anisole (0.12 ml) at room temperature for 50 min. Dry ether was added and the resulting gummy precipitate was dissolved in H_2O (6 ml). The aqueous phase, after washing with ether, 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 to give a fluffy powder; yield 320 mg (97%.) This powder (155 mg) was dissolved in a small amount of H_2O and the solution was applied to a column of CM-cellulose (3.5×13 cm), which was eluted with pH 6.9, 0.2 M $AcONH_4$ (1000 ml) through a mixing flask containing pH 6.9, 0.01 M $AcONH_4$ (500 ml). Individual fractions (9 ml each) were collected and the absorbancy at 275 mμ was determined. A single peak with some shoulder was detected. Fractions corresponding to the main peak (tube No. 25—55) were combined and the solvent was removed by lyophilization. For desalting, the resulting powder was then applied to a column of Sephadex G-15 (3.5×130 cm), which was eluted with 3% AcOH. Individual fractions (10 ml each) were collected and absorbancy at 275 mμ was determined. Fractions corresponding to the main peak (tube No. 45—58) were collected and the solvent was removed by lyophilization to give a fluffy white powder; yield 80 mg (53.5%), $[\alpha]_D^{27}$ −65.0° (c= 0.2, 1 N AcOH). Rf_3 0.64. Amino acid ratios in 4 N MSA hydrolysate and AP-M digest (number in parenthesis): Phe 2.18 (1.94), Gly 1.00 (1.00), Leu 1.03 (0.99), Pro 2.12 (1.81), Ile 1.02 (1.01), Tyr 1.08 (0.97), Arg 1.96 (2.01), Ala 0.93 (1.00), Ser 0.78 (0.96) average recovery 88% (85%). *Anal.* Calcd. for $C_{69}H_{102}N_{18}O_{15} \cdot 2CH_3COOH \cdot 6H_2O$: C, 53.08; H, 7.44; N, 15.26. Found: C, 52.77; H, 6.88; N, 15.49.

24) Without addition of phenol in acid hydrolysis, Tyr recovery of protected peptides is usually low: B. Iselin, *Helv. Chim. Acta*, **45**, 1510 (1962).