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Studies on Peptides. LXV.[1,2] Conventional Synthesis of a New Hypothalamic Peptide, Bovine Neurotensin

Kouki Kitagawa, Tadashi Akita,[3a] Tomio Segawa, Masumi Nakano[3b] Nobutaka Fujii, and Haruaki Yajima[3c]

Faculty of Pharmaceutical Sciences, Tokushima University,[3a] Institute of Pharmaceutical Sciences, School of Medicine, Hiroshima University[3b] and Faculty of Pharmaceutical Sciences, Kyoto University[3c]

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A detailed account of the synthesis of bovine neurotensin was presented. This tridecapeptide was synthesized in a conventional manner, after deprotecting three different protecting groups employed (Tos, Z and Bzl) by trifluoromethanesulphonic acid.

Main synthetic strategy of desired peptides is determined by the choice of a reagent capable to remove various protecting groups employed at the final stage of peptide synthesis. Currently, we have been interested in evaluating the usefulness of various sulphonic acids as deprotecting reagents[4,5] and at present, as model peptides, syntheses of tuftsin[6] and somatostatin[7] were performed by means of trifluoromethanesulphonic acid (TFMSA). The peptide containing Arg(Tos) and Lys(Z) was deprotected in the former instance and the peptide containing Lys(Z) and Cys(MBzl) in the latter, though certain degree of decomposition of the Cys residue was observed at elevated temperature. As an additional example, we wish to report the synthesis of a peptide named neurotensin, for which two different protecting groups at the Glu and Arg residues are required respectively.

Isolation in a minute amount and subsequent structural elucidation of neurotensin from bovine hypothalami were succeeded by Carraway and Leeman in 1975.[8] It seems worthwhile to note that the C-terminal dipeptide unit, Ile–Leu, is identical with that of xenopsin isolated from frog skins of *Xenopus laevis*.[9] Solid phase synthesis of neurotensin was performed by the same authors,[10] in which two steps of deprotection were performed, *i.e.*, the hydrogen bromide treatment in TFA for the cleavage of the peptide from the resin and at the same time, removal of two type protecting groups, the Bzl group from Glu and Tyr and the Z group from Lys. Catalytic hydrogenation was next performed to remove the nitro group from Arg.

1) Communication of this paper has appeared in *Chem. Pharm. Bull.* (Tokyo), **23**, 3299 (1975); Part LXIV: *ibid.*, **24**, 2447 (1976).
2) Amino acids, peptides and their derivatives mentioned in this communication are of the L-configuration. Abbreviations used are those recommended by IUPAC-IUB Commission on Biochemical Nomenclature: *Biochem.*, **5**, 2485 (1966), *ibid.*, **6**, 362 (1967), *ibid.*, **11**, 1726 (1972). Z=benzyloxycarbonyl, Z(OMe)=*p*-methoxybenzyloxycarbonyl, Tos=tosyl, OBzl=benzyl ester, ONP=*p*-nitrophenyl ester, OPCP=pentachlorophenyl ester, TFA=trifluoroacetic acid, DMF=dimethylformamide, Pyr=pyroglutamyl, MBzl=*p*-methoxybenzyl.
3) Location: *a*) *Sho-machi, Tokushima*, *b*) *Kasumi-cho, Hiroshima*, *c*) *Sakyo-ku, Kyoto*.
4) H. Yajima, N. Fujii, H. Ogawa, and H. Kawatani, *J. C. S. Chem. Commun.*, **1974**, 108.
5) H. Yajima, Y. Kiso, H. Ogawa, N. Fujii, and H. Irie, *Chem. Pharm. Bull.* (Tokyo), **23**, 1164 (1975).
6) H. Yajima, H. Ogawa, H. Watanabe, N. Fujii, M. Kurobe, and S. Miyamoto, *Chem. Pharm. Bull.* (Tokyo), **23**, 371 (1975).
7) N. Fujii and H. Yajima, *Chem. Pharm. Bull.* (Tokyo), **23**, 1596 (1975).
8) R. Carraway and S.E. Leeman, *J. Biol. Chem.*, **248**, 6854 (1973), *ibid.*, **250**, 1907 (1975).
9) K. Araki, S. Tachibana, M. Uchiyama, T. Nakajima, and T. Yasuhara, *Chem. Pharm. Bull.* (Tokyo), **21**, 2801 (1973).
10) R. Carraway and S.E. Leeman, *J. Biol. Chem.*, **250**, 1912 (1973).

From chemical interest mentioned above, we employed Arg(Tos), instead of Arg(NO_2), since in our preliminary experiments,[4] removal of the NO_2 group was found more difficult than that of the Tos group. The TFA labile Z(OMe) group[11] was employed for temporary protection of the α-amino function of necessary intermediates, since side-chain protecting groups employed survive mostly intact under limited TFA treatments, *i.e.*, the Tos, Z and Bzl groups in Arg, Lys and Glu respectively.

Synthetic scheme of the tridecapeptide corresponding to the entire amino acid sequence of neurotensin (I) is illustrated in Fig. 1, in which the C-terminal octapeptide, Z(OMe)–Lys(Z)–Pro–Arg(Tos)–Arg(Tos)–Pro–Tyr–Ile–Leu–OH, served as an amino component. For the synthesis of this amino component, two alternate routes were employed as shown in Fig. 2. In one route, the protected octapeptide was synthesized by uniting four dipeptide units, of these, Z(OMe)–Pro–Tyr–$NHNH_2$[12] and Z(OMe)–Lys(Z)–Pro–OH[13] are the known compounds. First, Z(OMe)–Ile–Leu–OH and Z(OMe)–Arg(Tos)–Arg(Tos)–OMe were prepared by the

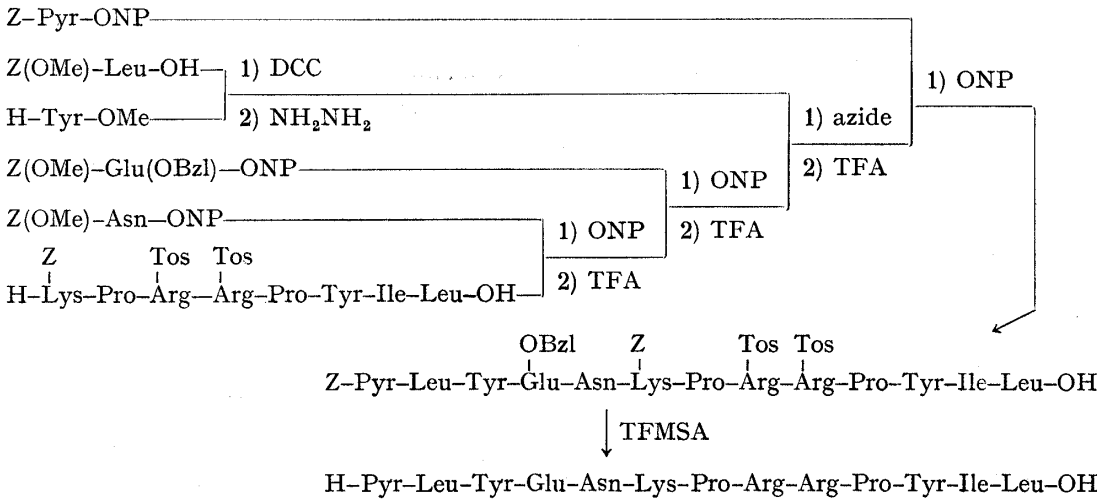

Fig. 1. Synthetic Route to Neurotensin

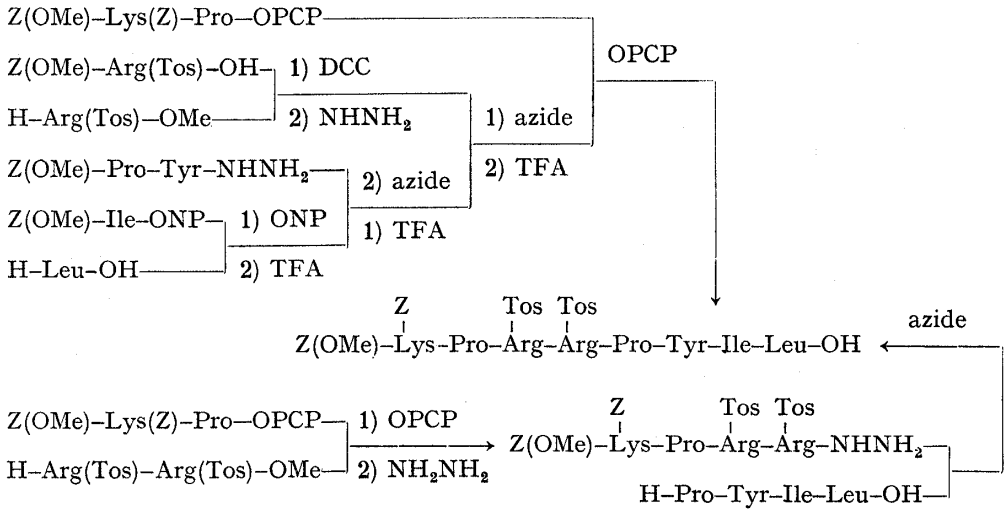

Fig. 2. Synthetic Scheme of the C-Terminal Protected Octapeptide Z(OMe)–(neurotensin 6—13)–OH

11) F. Weygand and K. Hunger, *Chem. Ber.*, **95**, 1 (1962), H. Yajima, F. Tamura, and Y. Kiso, *Chem. Pharm. Bull.* (Tokyo), **21**, 380 (1973).
12) H. Watanabe, H. Ogawa, and H. Yajima, *Chem. Pharm. Bull.* (Tokyo), **23**, 375 (1975).
13) H. Yajima and K. Kitagawa, *Chem. Pharm. Bull.* (Tokyo), **21**, 682 (1973); H. Yajima, K. Kitagawa, and T. Segawa, *ibid.*, **21**, 2500 (1975).

p-nitrophenyl ester[14] and DCC procedures[15] respectively. The latter was converted to the corresponding hydrazide, Z(OMe)–Arg(Tos)–Arg(Tos)–$NHNH_2$, in the usual manner. Z(OMe)–Ile–Leu–OH, after deprotection with TFA, was condensed with Z(OMe)–Pro–Tyr–$NHNH_2$ by the azide procedure[16] to give Z(OMe)–Pro–Tyr–Ile–Leu–OH. Again the azide procedure was employed to introduce the dipeptide unit, Arg(Tos)–Arg(Tos). Since the protected hexapeptide as well as the protected tetrapeptide possess the free carboxyl function, these peptides were separated from the rearrangement products of the respective azides by the extraction procedure from alkaline solutions. Next, the protected hexapeptide, after similar TFA treatment, was condensed with Z(OMe)–Lys(Z)–Pro–OH by the pentachlorophenyl trichloroacetate procedure[17] as performed in the synthesis of substance P,[13] the structure of which was determined by Leeman, *et al.*[18] Precipitation procedure was sufficient enough to purify the desired protected octapeptide.

In an alternate route, Z(OMe)–Lys(Z)–Pro–Arg(Tos)–Arg(Tos)–$NHNH_2$ was first prepared by condensation of Z(OMe)–Lys(Z)–Pro–OPCP[13] and the TFA treated sample of Z(OMe)–Arg(Tos)–Arg Tos)–OMe followed by treatment of the resulting Z(OMe)–Lys(Z)–Pro–Arg(Tos)–Arg(Tos)–OMe with hydrazine hydrate in the usual manner. Thus, the protected octapeptide was prepared by the azide condensation of two tetrapeptide units. However, in this route, purification of the desired compound was achieved by column chromatography on silica using the solvent system of chloroform–methanol–water (8: 3: 1). Starting with the tetrapeptide, the yield of the protected octapeptide was 53% in the former step and 46% in the latter. Thus, the former step can be judged as preferable to the latter.

Starting with the TFA treated sample of the above octapeptide, Z(OMe)–Asn–OH, Z(OMe)–Glu(OBzl)–OH and Z–Pyr–OH were introduced stepwisely by the *p*-nitrophenyl ester procedure and the dipeptide unit, Leu–Tyr, by the azide procedure. Thus the chain elongation from the octapeptide to the tridecapeptide was also performed by taking care of minimizing racemization. Batchwise washing and precipitation procedures was employed for purification of all intermediates, as well as the final protected tridecapeptide, Z–Pyr–Leu–Tyr–Glu(OBzl)–Asn–Lys(Z)–Pro–Arg(Tos)–Arg(Tos)–Pro–Tyr–Ile–Leu–OH. Purity of these protected peptides was confirmed by three criteria; thin-layer chromatography, acid hydrolysis and elemental analysis.

Among protecting groups attached at the protected tridecapeptide, the Tos group seems to be the most difficult one to remove by TFMSA as mentioned above. Our preliminary experiments showed that treatment at 40° for 60 minutes was required for this purpose and under these conditions, the Z and Bzl groups were deblocked completely.[4] The protected tridecapeptide was, therefore treated with TFMSA at 40° and the progress of the reaction was monitored by the positive Sakaguchi test on thin-layer chromatography. The reaction time, 60 minutes, was selected for this deblocking step. The deblocked product, after conversion to the corresponding acetate by Amberlite IR-4B, was purified by column chromatography on Sephadex G-10 and subsequently by partition chromatography on Sephadex G-25.[19] To elute the desired compound, 5% acetic acid was employed in the former step and the solvent system of *n*-butanol–acetic acid–water (4: 1: 5) in the latter step. In the latter chromatography, measurement of the UV absorbancy at 275 mμ revealed the presence of two minor front peaks and one major peak. The product obtained in the latter main peak exhibited a single spot on thin-layer chromatography possessing the nearly identical *Rf* value with that reported by Carraway and Leeman[8,10] in neurotensin from the natural source.

14) M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959).
15) J.C. Sheehan and G.P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955).
16) J. Honzl and J. Rudinger, *Coll. Czech. Chem. Commun.*, **26**, 2333 (1961).
17) M. Fujino and C. Hatanaka, *Chem. Pharm. Bull.* (Tokyo), **16**, 929 (1968).
18) M.M. Changm, S.E. Leeman, and H.D. Niall, *Nature New Biol.*, **232**, 86 (1971).
19) D. Yamashiro, *Nature*, **201**, 76 (1964).

In order to assess the L-configuration of the constituent amino acids of synthetic neurotensin, as well as to confirm the removal of protecting groups from the protected tridecapeptide by TFMSA, the peptide obtained was exposed to the actions of chymotrypsin plus aminopeptidase (AP–M).[20] Since the N-terminal portion of the peptide is the pyroglutamic acid, we expected the recovery of amino acids attached at the carboxyl site of the Tyr residue (position 3) in this hydrolysate. Unexpectedly, two moles of Leu, instead of one, were detected. The recovery of Glu was solely one equivalent. The result suggested that AP–M alone we employed has an ability to digest N-terminal Pyr–peptides. Indeed, the result obtained after digestion by this enzyme alone was identical with that obtained above by combination of two enzymes. Satisfactory recoveries of Lys, Glu and Arg were obtained indicating that various protecting groups attached at these amino acid residues were removed completely by TFMSA under conditions mentioned abov , as far as this main component is concerned. Fortunately, with an aid of this powerful aminopeptidase, it was thus possible to assess the L-configuration of every constituent amino acid of our synthetic neurotensin and confirm the complete removal of protecting groups.

When two other minor products isolated in the above chromatography were exposed to the action of AP–M, the recoveries of Arg and Glu were somewhat low (both around 55—66%) indicating that these fractions were presumably resulted by the incomplete removal of the protecting groups, though these were not further examined. Recently, Nishimura and Fujino[21] reported that the *p*-methoxybenzenesulfonyl group attached at the N^G of Arg can be cleaved by methanesulphonic acid[5] in more mild conditions. Application of this new derivative may open the improved synthesis of neurotensin.

Exact physiological property of neurotensin is still unknown. As far as its contractile activity is concerned, the activity of synthetic peptide in isolated rat uterus was in order of bradykinin>neurotensin>substance P. However, in guinea-pig ileum, the relative potency of neurotensin was about 0.014 of our synthetic substance P. Further physiological evaluation of this new hypothalamic principle are waited in future investigations.

Experimental

General experimental methods employed here are essentially the same as those described in the Part LXII[22] of this series. Thin layer chromatography was performed on silica gel (Kieselgel, G, Merck). *Rf* values refer to the following solvent systems: Rf_1 $CHCl_3$–MeOH–H_2O (8: 3: 1), Rf_2 $CHCl_3$–MeOH–AcOH (9: 1: 0.5), Rf_3 $CHCl_3$–MeOH–H_2O (45: 10: 1), Rf_4 *n*-BuOH–pyridine–AcOH–H_2O (15: 10: 3: 12).

Z(OMe)-Ile-Leu-OH——Z(OMe)-Ile-ONP[23] (41.64 g) dissolved in AcOEt (150 ml) was added to a solution of H-Leu-OH (20.0 g) and Et_3N (35 ml) in H_2O (150 ml) and the mixture was stirred at room temperature for 48 hr. The aqueous phase was separated, washed with ether and then acidified with citric acid. 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 *n*-hexane and then recrystallized from AcOEt and *n*-hexane; yield 23.45 g (58%), mp 138—140°, $[\alpha]_D^{22}$ −20.0° (c=1.1, MeOH), Rf_1 0.63. *Anal.* Calcd. for $C_{21}H_{32}O_6N_2$: C, 61.74; H, 7.90; N, 6.86. Found: C, 61.48; H, 8.02; N, 6.75.

Z(OMe)-Pro-Tyr-Ile-Leu-OH——Z(OMe)-Ile-Leu-OH (4.68 g) was treated with TFA (9 ml) in the presence of anisole (2.3 ml) in an ice-bath for 45 min. The excess TFA was evaporated *in vacuo* and the residue, after washing with *n*-hexane, was treated with ether. The resulting powder was collected by filtration, dried over KOH pellets *in vacuo* for 3 hr and then dissolved in DMF (40 ml) containing Et_3N (1.5 ml). This solution was kept in an ice-bath until the azide was prepared. To a solution of Z(OMe)-Pro-Tyr-$NHNH_2$ (6.84 g) in DMF (40 ml), 3.13 N HCl–DMF (9.6 ml) and isoamyl nitrite (2.4 ml) were added under cooling with ice-NaCl. After stirring for 5 min, when the hydrazine test became negative, the solution was neutralized with Et_3N (4.2 ml) and then combined with the above solution containing the dipeptide. The mixture was stirred

20) G. Pfleiderer and P.G. Celliers, *Biochem. Z.*, **339**, 186 (1963). AP–M was purchased from the Protein Research Foundation (Osaka), Lot No. 191226.
21) O. Nishimura and M. Fujino, *Abstract of the 9th Annual Meeting of the Japan Pharmaceutical Association*, Vol. 2, 1975 p. 2.
22) H. Ogawa, M. Kubota, and H. Yajima, *Chem. Pharm. Bull.* (Tokyo), **24**, 2428 (1976).
23) F. Wandesande, *Bull. Soc. Chim. Belg.*, **78**, 385 (1969).

at 4° for 48 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 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 then recrystallized from MeOH and ether; yield 6.96 g (95%), mp 123—125°, $[\alpha]_D^{22}$ −55.6° (c=0.8, MeOH). Rf_1 0.38, Rf_2 0.34. Amino acid ratios in 3 N Tos-OH hydrolysate: Pro 1.00, Tyr 1.08, Ile 1.00, Leu 1.02 (average recovery 86%). *Anal.* Calcd. for $C_{35}H_{48}O_9N_4$: C, 62.85; H, 7.23; N, 8.37. Found: C, 62.60; H, 7.29; N, 8.30.

Z(OMe)-Arg(Tos)-Arg(Tos)-OMe——DCC (8.40 g) was added to a mixture of Z(OMe)-Arg(Tos)-OH (14.78 g) and H-Arg(Tos)-OMe (prepared from 11.37 g of the hydrochloride with 4.2 ml of Et_3N) in DMF (150 ml) and the solution, after stirring at room temperature for 24 hr, was filtered. The filtrate was condensed *in vacuo* and the residue was treated with ice-cold 5% citric acid and ether. The resulting oily precipitate turned to the solid. This was washed batchwisely with 5% citric acid, 5% sodium carbonate and H_2O and then recrystallized from EtOH and ether; yield 22.30 g (91%), mp 108—101°, $[\alpha]_D^{22}$ +1.0° (c=4.4, DMF), Rf_1 0.68. *Anal.* Calcd. for $C_{36}H_{48}O_1N_{80}S_2\cdot 2H_2O$: C, 50.69; H, 6.15; N, 13.14. Found: C, 50.69; H, 6.18; N, 12.98.

Z(OMe)-Arg(Tos)-Arg(Tos)-NHNH$_2$——To a solution of Z(OMe)-Arg(Tos)-Arg(Tos)-OMe (4.90 g) in MeOH (50 ml), 80% hydrazine hydrate (3.0 ml) was added and the solution was kept on standing overnight. The solvent was evaporated and the residue was triturated with ether. The resulting powder was washed with H_2O and then recrystallized from EtOH and ether; yield 4.30 g (88%), mp 113—118°, $[\alpha]$– +1.2° (c=0.8, MeOH), Rf_1 0.63. *Anal.* Calcd. for $C_{35}H_{48}O_9N_{10}S_2$: C, 51.45; H, 5.92; N, 17.14. Found: C, 51.65; H, 6.20; N, 16.88.

Z(OMe)-Lys(Z)-Pro-Arg(Tos)-Arg(Tos)-OMe——Z(OMe)-Arg(Tos)-Arg(Tos)-OMe (8.17 g) was treated with TFA (16 ml) in the presence of anisole (4.0 ml) in an ice-bath for 45 min. The excess TFA was evaporated and the residue was treated with ether. The resulting powder was collected by filtration, dried over KOH pellets *in vacuo* and then dissolved in DMF (100 ml). Et_3N (3.15 ml) and Z(OMe)-Lys(Z)-Pro-OPCP (9.85 g) were combined and the mixture was stirred at room temperature for 24 hr. The solvent was evaporated and the residue was extracted with AcOEt, which was washed with 5% citric acid, 5% sodium carbonate and H_2O–NaCl, dried over sodium sulfate and then evaporated. The residue was dissolved in $CHCl_3$ and the solution was applied to a column of silica (2.8×14 cm), which was eluted with $CHCl_3$–MeOH (95: 5). Fractions containing the substance of Rf_1 0.81 were combined and the solvent was removed by evaporation. Treatment of the residue with ether afforded a fine powder, which was washed with H_2O and then recrystallized from MeOH and ether; yield 10.13 g (86%), mp 95—101°, $[\alpha]_D^{22}$ −23.6° (c=0.8, MeOH). Rf_1 0.81, Rf_2 0.37. Amino acid ratios in an acid hydrolysate: Lys 0.81, Pro 1.00, Arg 1.99 (average recovery 89%). *Anal.* Calcd. for $C_{55}H_{73}N_{11}O_{14}S_2\cdot 2H_2O$: C, 54.48; H, 6.40; N, 12.70. Found: C, 54.53; H, 6.42; N, 12.40.

Z(OMe)-Lys(Z)-Pro-Arg(Tos)-Arg(Tos)-NHNH$_2$——To a solution of Z(OMe)-Lys(Z)-Pro-Arg(Tos)-Arg-(Tos)-OMe (10.13 g) in MeOH (100 ml), 80% hydrazine hydrate (5.0 ml) was added and the solution was kept on standing overnight. The solvent was evaporated and the residue was treated with ether. The resulting powder was washed with ether and H_2O and then recrystallized from MeOH and ether; yield 7.53 g (74%), mp 108—111°, $[\alpha]_D^{23}$ −17.7° (c=0.6, MeOH). Rf_1 0.72, Rf_2 0.23. *Anal.* Calcd. for $C_{54}H_{73}O_{13}N_{13}S_2$: C, 55.13; H, 6.25; N, 15.48. Found: C, 55.00; H, 6.36; N, 15.18.

Z(OMe)-Arg(Tos)-Arg(Tos)-Pro-Tyr-Ile-Leu-OH——Z(OMe)-Pro-Tyr-Ile-Leu-OH (1.00 g) was treated with TFA (2.0 ml) in the presence of anisole (0.5 ml) in an ice-bath for 45 min. The excess TFA was removed by evaporation and the residue was treated with ether. The resulting powder was collected by filtration, dried over KOH pellets *in vacuo* and then dissolved in DMF (20 ml) containing Et_3N (0.21 ml). To this solution, the azide (prepared from 1.65 g of Z(OMe)-Arg(Tos)-Arg(Tos)-$NHNH_2$ with 1.3 ml of 3.13 N HCl–DMF, 0.34 ml of isoamylnitrite, 0.56 ml of Et_3N) in DMF (50 ml) was added. The mixture was stirred at 4° for 48 hr and the solvent was evaporated. The residue was dissolved in 5% NH_4OH, which after washing with AcOEt, was acidified with citric acid and the resulting oily precipitate was extracted with AcOEt. The organic phase was washed with 10% citric acid and H_2O–NaCl, dried over sodium sulfate and then evaporated. The residue was dissolved in a small amount of the solvent consisting of $CHCl_3$–MeOH–H_2O (8: 3: 1) and the solution was applied to a column of silica (2.7×15 cm), which was eluted with the same solvent system. Fractions containing the substance of Rf_1 0.56 were combined and the solvent was evaporated. Treatment of the residue with H_2O afforded a fine powder, which was recrystallized from MeOH and AcOEt; yield 1.25 g (65%), mp 160—164°, $[\alpha]_D^{23}$ −19.7° (c=1.3, MeOH). Amino acid ratios in an acid hydrolysate: Arg 2.28, Pro 1.18, Tyr 0.52, Ile 1.00, Leu 0.94 (average recovery 83%). *Anal.* Calcd. for $C_{61}H_{84}O_{15}N_{12}S_2\cdot 3H_2O$: C, 54.52; H, 6.75; N, 12.51. Found: C, 54.54; H, 6.39; N, 12.84.

Z(OMe)-Lys(Z)-Pro-Arg(Tos)-Arg(Tos)-Pro-Tyr-Ile-Leu-OH——a (2+6). Z(OMe)-Arg(Tos)-Arg(Tos)-Pro-Tyr-Ile-Leu-OH (1.13 g) was treated with TFA (2.3 ml) in the presence of anisole (0.6 ml) in an ice-bath for 45 min. The excess TFA was removed by evaporation and the residue was treated with ether. The resulting powder was dried over KOH pellets *in vacuo* for 3 hr and then dissolved in DMF (20 ml). Et_3N (0.38 ml) and Z(OMe)-Lys(Z)-Pro-OPCP (0.79 g) were added and the mixture was stirred at room temperature for 48 hr. The solvent was evaporated and the residue was treated with ether. The resulting powder was washed with 5% citric acid and H_2O and then recrystallized from MeOH and AcOEt; yield 1.20 g (81%); mp

149—153°, $[\alpha]_D^{22}$ −42.5° (c=1.1, MeOH), Rf_1 0.49. Amino acid ratios in an acid hydrolysate: Lys 0.92, Pro 2.07, Arg 2.19, Tyr 0.53, Ile 1.00, Leu 0.91 (average recovery 87%). *Anal.* Calcd. for $C_{80}H_{109}O_{19}N_{15}S_2 \cdot 3H_2O$: C, 56.41; H, 6.80; N, 12.33. Found: C, 56.45; H, 6.78; N, 12.49. b (4+4). Isoamylnitrite (0.8 ml) was added to an ice-cold solution of Z(OMe)-Lys(Z)-Pro-Arg(Tos)-Arg(Tos)-$NHNH_2$ (7.06 g) in DMF (50 ml) and 3.13 N HCl–DMF (4.0 ml). After stirring for 5 min, when the hydrazine test became negative, the solution was neutralized with Et_3N (1.68 ml). This solution was added to a solution of H-Pro-Tyr-Ile-Leu-OH (prepared from 3.34 g of the Z(OMe)-derivative by treatment with TFA as stated above followed by neutralization with 0.7 ml of Et_3N) in DMF (20 ml). The mixture was stirred at 4° for 48 hr and the solvent, after addition of a few drops of AcOH, was evaporated. Treatment of the residue with *n*-hexane and then ether afforded a fine powder, which was washed with 5% citric acid and H_2O and then dissolved in a small amount of the solvent system consisting of $CHCl_3$–MeOH–H_2O (8: 3: 1). The desired product was isolated as stated above; yield 3.77 g (46%). Amino acid ratios in 3 N Tos-OH hydrolysate: Lys 1.15, Pro 1.69; Arg not det. Tyr 1.05, Ile 1.00, Leu 0.94 (average recovery 82%).

Z(OMe)-Asn-Lys(Z)-Pro-Arg(Tos)-Arg(Tos)-Pro-Tyr-Ile-Leu-OH——Z(OMe)-Lys(Z)-Pro-Arg(Tos)-Arg(Tos)-Pro-Tyr-Ile-Leu-OH (1.20 g) was treated with TFA (2.5 ml) in the presence of anisole (0.6 ml) in an ice-bath for 45 min. The TFA salt formed by addition of dry ether was collected by filtration, dried over KOH pellets *in vacuo* and then dissolved in DMF (20 ml). Et_3N (0.31 ml) and Z(OMe)-Asn-ONP[24] (0.31 g) were added and the mixture was stirred at room temperature for 24 hr. The solvent was evaporated and the residue was treated with ether. The resulting powder was washed with 5% citric acid and H_2O and then precipitated from MeOH with AcOEt; yield 1.10 g (85%), mp 142—145°, $[\alpha]_D^{23}$ −43.5° (c=1.0, MeOH), Rf_1 0.55. Amino acid ratios in an acid hydrolysate: Asp 0.74, Lys 0.92, Pro 1.87, Arg 2.38, Tyr 0.58, Ile 1.00, Leu 0.92 (average recovery 83%). *Anal.* Calcd. for $C_{84}H_{115}O_{21}N_{17}S_2 \cdot 5H_2O$: C, 54.44; H, 6.80; N, 12.85. Found: C, 54.02; H, 6.68; N, 12.54.

Z(OMe)-Glu(OBzl)-Asn-Lys(Z)-Pro-Arg(Tos)-Arg(Tos)-Pro-Tyr-Ile-Leu-OH——The above protected nonapeptide (1.76 g) was treated with TFA (4 ml) in the presence of anisole (0.9 ml) in an ice-bath for 45 min, when dry ether was added. The resulting TFA salt formed by addition of dry ether was collected by filtration, dried over KOH pellets *in vacuo* and then dissolved in DMF (25 ml). Et_3N (0.42 ml) and Z(OMe)-Glu(OBzl)-ONP[25] (0.63 g) were added and the mixture was stirred at room temperature for 24 hr. The solvent was evaporated and the oily residue was treated with ether. The resulting powder was washed with 10% citric acid and H_2O and purified further by precipitation twice from MeOH with AcOEt; yield 1.42 g (72%), mp 125—130°, $[\alpha]_D^{23}$ −38.6° (c=0.8, MeOH), Rf_1 0.52. Amino acid ratios in an acid hydrolysate: Glu 1.05, Asp 0.95, Lys 0.98, Pro 2.26, Arg 2.71, Tyr 0.62, Ile 1.00, Leu 0.96 (average recovery 87%). *Anal.* Calcd. for $C_{26}H_{128}O_{24}N_{18}S_2 \cdot 3H_2O$: C, 56.62; H, 6.63; N, 12.38. Found: C, 56.31; H, 6.57; N, 12.69.

Z(OMe)-Leu-Tyr-$NHNH_2$——DCC (22.70 g) was added to a solution of Z(OMe)-Leu-OH (29.53 g) and H-Tyr-OMe (prepared from 23.17 g of the hydrochloride with 14 ml of Et_3N) in DMF AcOEt (50—300 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% sodium carbonate, 10% citric acid and H_2O, dried over sodium sulfate and then evaporated. The resulting oil (Rf_1 0.93) was dissolved in MeOH (300 ml) and 80% hydrazine hydrate (50 ml) was added. The crystalline solid formed on standing overnight, was collected by filtration and then recrystallized from MeOH; yield 39.50 g (84%), mp 179—182°, $[\alpha]_D^{23}$ −29.7° (c=0.9, MeOH). Rf_1 0.57. *Anal.* Calcd. for $C_{24}H_{32}O_6N_4 \cdot 1/2H_2O$: C, 59.85; H, 6.90; N, 11.63. Found: C, 60.21; H, 7.00; N, 11.65.

Z(OMe)-Leu-Tyr-Glu(OBzl)-Asn-Lys(Z)-Pro-Arg(Tos)-Arg(Tos)-Pro-Tyr-Ile-Leu-OH——The above protected decapeptide (1.00 g) was treated with TFA (2.0 ml) in the presence of anisole (0.5 ml) in an ice-bath for 45 min, when dry ether was added. The resulting powder was isolated, dried as stated above and then dissolved in DMF (20 ml) containing Et_3N (0.07 ml). To this solution, the azide (prepared from 0.47 g of Z(OMe)-Leu-Tyr-$NHNH_2$ with 1.0 ml of 2.02 N HCl–DMF, 0.15 ml of isoamyl nitrite and 0.28 ml of Et_3N) in DMF (20 ml) was added. The mixture was stirred at 4° for 48 hr and then the solvent, after addition of a few drops of AcOH, was evaporated. Treatment of the residue with ether afforded a fine powder, which was washed batchwisely with 5% citric acid and H_2O and precipitated twice from MeOH with AcOEt; yield 0.85 g (75%), mp 145—148°, $[\alpha]_D^{23}$ −38.9° (c=0.9, MeOH), Rf_1 0.45, Rf_3 0.21. Amino acid ratios in an acid hydrolysate: Leu 1.97, Tyr 1.20, Asp 0.95, Glu 1.09, Lys 1.04, Arg 2.12, Pro 2.03, Ile 1.00 (average recovery 88%). *Anal.* Calcd. for $C_{111}H_{148}O_{27}N_{20}S_2 \cdot 3H_2O$: C, 57.65; H, 6.71; N, 12.11. Found: C, 57.48; H, 6.76; N, 12.26.

Z-Pyr-Leu-Tyr-Glu(OBzl)-Asn-Lys(Z)-Pro-Arg(Tos)-Arg(Tos)-Pro-Tyr-Ile-Leu-OH——The above protected dodecapeptide (2.04 g) was treated with TFA (4.5 ml) as usual in the presence of anisole (1.0 ml) in an ice-bath for 45 min and then 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 this solution, Et_3N (0.39 ml) and Z-Pyr-ONP[26] (0.42 g) were added and the mixture was stirred at room temperature for 24 hr. The solvent was evaporated and the

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residue was treated with ether and then AcOEt. The resulting powder was washed with 10% citric acid and H_2O and then precipitated twice from MeOH and AcOEt; yield 2.07 g (98%), mp 148—150°, $[\alpha]_D^{23}$ −8.2° (c= 0.5, DMF), Rf_1 0.65, Rf_3 0.37. Amino acid ratios in an acid hydrolysate: Glu 2.27, Leu 2.07, Tyr 1.76, Asp 1.00, Lys 1.24, Pro 2.22, Arg 2.38, Ile 1.00 (average recovery 90%). *Anal.* Calcd. for $C_{115}H_{151}O_{28}N_{21}S_2 \cdot 4H_2O$: C, 57.26; H, 6.64; N, 12.20. Found: C, 56.82; H, 6.74; N, 12.37.

H-Pyr-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH——The above protected tridecapeptide (136 mg) was immersed well in anisole (0.5 ml) and TFMSA (1.5 ml) was added and the solution was stirred at 40° for 60 min, meanwhile the Sakaguchi test of the solution became positive. *n*-Hexane was added and the resulting oily precipitate was washed with ether, dried over KOH pellets *in vacuo* for 30 min and then dissolved in 5% AcOH (50 ml), which was treated with Amberlite IR-4B (acetate form, approximately 3 g) for 45 min. The resin was removed by filtration and the filtrate was lyophilized. The residue was dissolved in a small amount of 10% AcOH and the solution was applied to a column of Sephadex G-10 (2.0×142 cm), which was eluted with the same solvent. Individual fractions (4 ml each) were collected and absorbancy at 275 mμ was determined. Fractions corresponding to the front main peak (tube No. 30—70) were combined and the solvent was removed by lyophilization to give a fluffy powder; yield 59 mg (deblocking step 55%). This sample (55 mg) was dissolved in a small amount of the upper phase of *n*-BuOH–AcOH–H_2O (4:1:5) and the solution was applied to a column of Sephadex G-25 (2×140 cm) previously equilibrated with the lower phase of the above solvent system. The column was then eluted with the upper phase of the above solvent system. Individual fractions (4 ml each) were collected and absorbancy at 275 mμ was determined. Two minor front peaks and one major peak (tube No. 41—60) were detected. Fractions of this major peak were combined and the solvent was evaporated. Lyophilization of the residue gave a fluffy white powder; yield 32 mg (purification step 58%). $[\alpha]_D^{23}$ −65.6° (c=0.5, H_2O), Rf_4 0.66 (lit.[8] 0.68). Amino acid ratios in an acid hydrolysate: Glu 1.92, Leu 2.09, Tyr 1.86, Asp 0.95, Lys 1.04, Pro 2.18, Arg 2.25, Ile 1.00 (average recovery 87%). Amino acid ratios in AP-M digest (peptide 0.429 mg/AP-M 2U); Leu 1.59, Tyr 1.92, Glu 0.97, Asn 1.15, Lys 0.99, Pro 2.00, Arg 1.96, Ile 1.02 (average recovery 85%). *Anal.* Calcd. for $C_{78}H_{121}O_{20}N_{21} \cdot 3CH_3COOH \cdot 6H_2O$: C, 51.44; H, 7.45; N, 15.00. Found: C, 51.57; H, 7.71; N, 14.48.

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