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Studies on Peptides. XLI.[1,2] Synthesis of the Protected Hexadecapeptide Corresponding to Positions 13 to 28 of the Basic Trypsin Inhibitor from Bovine Pancreas (Kunitz and Northrop)

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The protected hexadecapeptide, Z(OMe)–Pro–Cys(Bzl)–Lys(Z)–Ala–Arg(Tos)–Ile–Ile–Arg(Tos)–Tyr–Phe–Tyr–Asn–Ala–Lys(Z)–Ala–Gly–OH, corresponding to positions 13 through 28 of the basic trypsin inhibitor (Kunitz and Northrop) was synthesized by the successive azide coupling of four peptide subunits, H–Asn–Ala–Lys(Z)–Ala–Gly–OH, Z(OMe)–Tyr–Phe–Tyr–$NHNH_2$, Z(OMe)–Ala–Arg(Tos)–Ile–Ile–Arg(Tos)–$NHNH_2$ and Z(OMe)–Pro–Cys(Bzl)–Lys(Z)–$NHNH_2$.

Following to the synthesis of the protected dodecapeptide (I, positions 1 through 12) related to the N-terminal portion of the basic trypsin inhibitor (BTI),[1] we wish to report the synthesis of the protected hexadecapeptide corresponding to positions 13 through 28 of this protease inhibitor.

Synthesis of this hexadecapeptide sequence, Pro–Cys–Lys–Ala–Arg–Ile–Ile–Arg–Tyr–Phe–Tyr–Asn–Ala–Lys–Ala–Gly, requires the protecting group for the ε-amino function of the Lys residues which are located at the 3rd position from both ends of the peptide chain. The Z group removable by hydrogen fluoride[4] was applied for this purpose. Combination of the ε-Z and α-Z(OMe) groups allows us to elongate the peptide chain, since the latter can be cleaved by trifluoroacetic acid (TFA),[5] while the former is mostly unaffected under limited conditions.[6] Thus synthesis of peptides containing both Lys(Z) and Cys(Bzl) is possible. In addition of these protecting groups, the Tos group removable also by hydrogen fluoride was applied for the protection of the guanidino function of Arg,[7] since the azide procedure of Arg(Tos)-peptides, instead of Arg(NO_2)-peptides, is known to be useful for the condensation of peptide fragments.[8]

Bearing these protecting groups removable all by hydrogen fluoride, the protected hexadecapeptide, Z(OMe)–Pro–Cys(Bzl)–Lys(Z)–Ala–Arg(Tos)–Ile–Ile–Arg(Tos)–Tyr–Phe–Tyr–Asn–Ala–Lys(Z)–Ala–Gly–OH (II), was prepared by assembling four fragments: Z(OMe)–Pro–Cys(Bzl)–Lys(Z)–$NHNH_2$ (II-a, position 13—15), Z(OMe)–Ala–Arg(Tos)–Ile–Ile–Arg(Tos)–$NHNH_2$ (II-b, position 16—20), Z(OMe)–Tyr–Phe–Tyr–$NHNH_2$ (II-c, positions 21—23) and H–Asn–Ala–Lys(Z)–Ala–Gly–OH (II-d, position 24—28) as shown in Fig. 1.

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1) Part XL, H. Yajima and Y. Kiso, *Chem. Pharm. Bull.* (Tokyo), **22**, 1061 (1974).

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: *Biochemistry*, **5** 2485 (1966); *ibid.*, **6**, 362 (1967), *ibid.*, **11**, 1726 (1972). Boc=*tert*-butoxycarbonyl, Z = benzyloxycarbonyl, Z(OMe) = *p*-methoxybenzyloxycarbonyl, Tos = tosyl, Bzl = benzyl, ONP=*p*-nitrophenyl ester.

3) Location: *Sakyo-ku, Kyoto.*

4) S. Sakakibara and Y. Shimonishi, *Bull. Chem. Soc. Japan*, **38**, 1412 (1965).

5) F. Weygand and K. Hunger, *Chem. Ber.*, **95**, 1 (1962).

6) A. Marglin and R.B. Merrifield, *J. Am. Chem. Soc.*, **88**, 5051 (1966); A. Yaron and S.F. Schlossman, *Biochemistry*, **7**, 2673 (1968).

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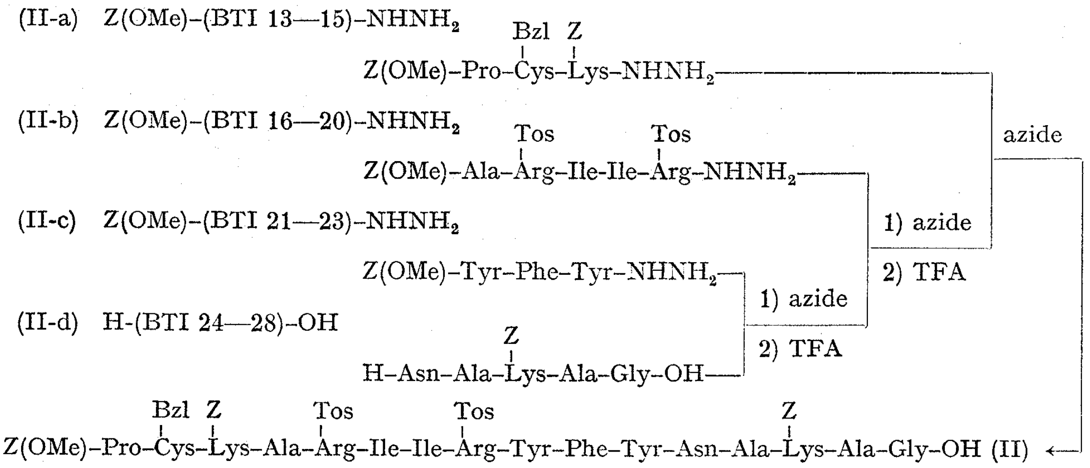

Fig. 1. Synthetic Route to the Protected Hexadecapeptide Corresponding to Positions 13 to 28 of BTI (II), Z(OMe)-(BTI 13—28)-OH

The pentapeptide, H–Asn–Ala–Lys(Z)–Ala–Gly–OH (II-d), was prepared as illustrated in Fig. 2. Z(OMe)–Lys(Z)–OH[9] was condensed by dicyclohexylcarbodiimide (DCC) with H–Ala–Gly–OMe derived from Z–Ala–Gly–OMe[10] by catalytic hydrogenation. The resulting protected tripeptide ester was then saponified to afford Z(OMe)–Lys(Z)–Ala–Gly–OH. The N-terminal dipeptide, Z(OMe)–Asn–Ala–OMe, was prepared by two alternate ways: one by the mixed anhydride procedure and the other by triphenylphosphite in the presence of imidazole. Presumably owing to the dehydration reaction of the β-amide bond of Asn,[11] the former gave poor yield of the desired dipeptide ester, however the latter gave much better yield than the former as pointed out by Mittin, *et al.*[12] No CN absorption band in infrared (IR) was detected in both of purified products and the values of elemental analysis agreed with those of theory. The dipeptide ester was then converted to the corresponding hydrazide, which after conversion to the azide by the procedure of Honzl and Rudinger,[13] was condensed with H–Lys(Z)–Ala–Gly–OH derived from the above Z(OMe)–derivative by the careful TFA treatment. Homogeneity of the resulting protected pentapeptide, Z(OMe)–Asn–Ala–Lys(Z)–Ala–Gly–OH, was established by elemental and amino acid analysis. This was then treated with TFA and the resulting partially protected pentapeptide, II-d H-(BTI 24—28)-OH, was obtained, after neutralization with triethylamine, as amorphous powder.

Prior to this coupling reaction, stability of the ε-Z group toward the action of TFA was examined by thin-layer chromatography, since it is known that TFA cleaves the Z group after long exposure.[6,14] When Z(OMe)–Lys(Z)–OH was exposed to the action of TFA in an ice-bath for 40 to 45 min, formation of Lys was negligible. Such conditions were settled for the selective removal of the Z(OMe) group from Lys(Z)–peptides.

The synthesis of the protected tripeptide hydrazide, Z(OMe)–Tyr–Phe–Tyr–$NHNH_2$ (II-c), was performed in a stepwise manner starting from H–Tyr–OMe as illustrated in Fig. 3. Z–Phe–OH was condensed with H–Tyr–OMe by DCC without protection of the phenolic hydroxyl group of the amino component. The protected dipeptide ester, Z–Phe–Tyr–OMe, after hydrogenation, was coupled to Z(OMe)–Tyr–OH by DCC to give Z(OMe)–Tyr–Phe–Tyr–OMe, which without purification, was converted to the corresponding hydrazide in the usual

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11) M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959); P.G. Katsoyannis and K. Suzuki, *ibid.*, **83**, 4057 (1961).
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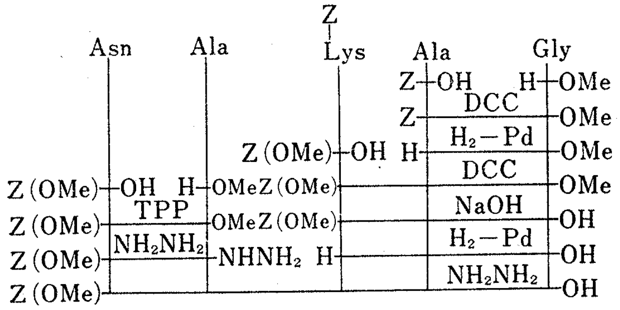

Fig. 2. Synthetic Route to the Pentapeptide II-d, H-(BTI 24—28)-OH

TPP=triphenylphosphite

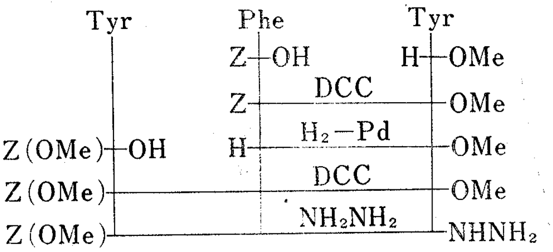

Fig. 3. Synthetic Route to the Protected Tripeptide II-c, Z(OMe)-(21—23)-$NHNH_2$

manner. Recrystallization of the product with ethanol afforded the desired hydrazide in analytically pure form.

The fragment, Z(OMe)–Ala–Arg(Tos)–Ile–Ile–Arg(Tos)–$NHNH_2$ (II-b), was prepared as illustrated in Fig. 4. Since condensation of acyl–Arg(NO_2)–OH by the active ester procedure[15] or by the azide procedure utilizing Arg(NO_2)–peptides,[16] will give rise to some side reaction, the azide procedure of Arg(Tos)–peptides was used for the condensation of this peptide fragment. Successful application of this type of reaction was demonstrated by Otsuka and Inouye as mentioned earlier.[8]

H–Arg(Tos)–OMe was condensed with Z–Ile–OH by DCC to give Z–Ile–Arg(Tos)–OMe as an amorphous powder. The Z group of this dipeptide ester was removed by hydrogen bromide in acetic acid and the resulting H–Ile–Arg(Tos)–OMe was coupled to Z–Ile–OH by DCC. The protected tripeptide ester, Z–Ile–Ile–Arg(Tos)–OMe, thus obtained was a gelatinous compound, which however could be recrystallized from ethyl acetate. This, after hydrogenation, was condensed with Z–Arg(Tos)–OH by the mixed anhydride procedure. Z–Arg(Tos)–Ile–Ile–Arg(Tos)–OMe thus obtained as an amorphous powder, was again submitted to catalytic hydrogenation and the resulting H–Arg(Tos)–Ile–Ile–Arg(Tos)–OMe was condensed with the mixed anhydride of Z(OMe)–Ala–OH. Since most of these Arg(Tos)–peptides are amorphous compounds, it was more advantageous to apply the mixed anhydride procedure rather than the DCC procedure to prepare the desired compounds in pure form. The protected pentapeptide ester, Z(OMe)–Ala–Arg(Tos)–Ile–Ile–Arg(Tos)–OMe obtained in the above coupling reaction, was then converted to the corresponding hydrazide (II-b).

It should be recalled that during the course of structural investigations of BTI, the number of Ile residues present between Arg residues was uncertain, since the usual 24 hr acid hydrolysis gave low recovery of Ile.[17] Acid hydrolysis of our protected pentapeptide hydrazide (II-b) also gave one mole of Ile after 24 hr hydrolysis and nearly two moles were obtained only after 72 hr hydrolysis.

For the synthesis of the N-terminal tripeptide unit, Z(OMe)–Pro–Cys(Bzl)–Lys(Z)–$NHNH_2$ (II-a), the *p*-nitrophenyl ester procedure[18] was applied. Z(OMe)–Cys(Bzl)–ONP was allowed to react with the triethylammonium salt of H–Lys(Z)–OH to give Z(OMe)–Cys(Bzl)–Lys(Z)–OH. This, after the limited treatment with TFA as described above, was condensed with Z(OMe)–Pro–ONP to give Z(OMe)–Pro–Cys(Bzl)–Lys(Z)–OH, which was converted to the corresponding hydrazide (II-a), through its methyl ester as shown in Fig. 5.

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[15] M. Bodanszky and J.T. Sheehan, *Chem. Ind.*, **1960**, 1268.

[16] J.S. Fruton, *Advan. Protein Chem.*, **5**, 1 (1949); A.F. McKay, *Chem. Ber.*, **51**, 301 (1952).

[17] V. Dlouha, D. Pospisilova, B. Meloun, and F. Sŏrm, *Coll. Czech. Chem. Commun.*, **33**, 1363 (1968).

[18] M. Bodanszky, *Nature*, **175**, 685 (1955); *idem*, *Acta Chim. Acad. Sci. Hung.*, **10**, 335 (1956).

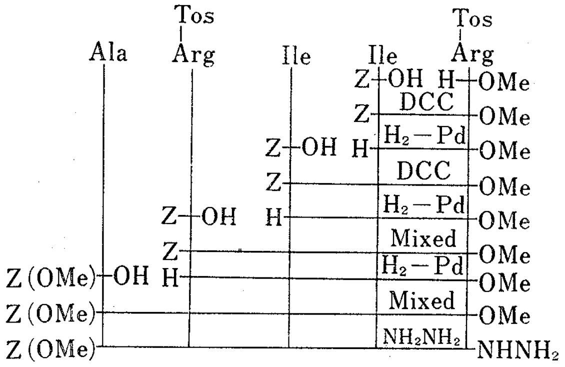

Fig. 4. Synthetic Route to the Protected Pentapeptide Hydrazide II-b, Z(OMe)-(BTI 16—20)-$NHNH_2$

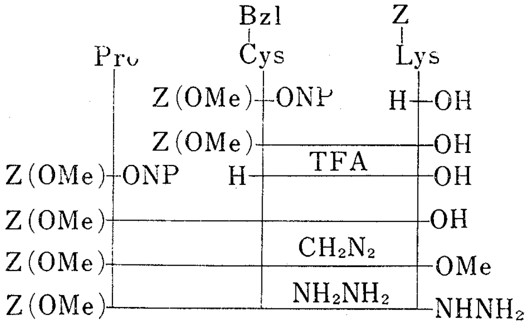

Fig. 5. Synthetic Route to the Protected Tripeptide Hydrazide II-a, Z(OMe)-(BTI 13—15)-$NHNH_2$

Assembling of peptide subunits synthesized above was carried out according to Fig. 1. The partially protected pentapeptide II-d, H-(BTI 24—28)-OH, was allowed to react with II-c, Z(OMe)-(BTI 21—23)-$NHNH_2$, by the procedure of Honzl and Rudinger.[13] The crude product was purified by batchwise washing with citric acid and methanol in order to remove the rearrangement product of the azide and the unreacted amino component. Such batchwise washing procedure was quite effective in purifying the protected octapeptide, Z(OMe)-Tyr-Phe-Tyr-Asn-Ala-Lys(Z)-Ala-Gly-OH, Z(OMe)-(BTI 21—28)-OH, and the peptides listed below. This protected octapeptide, after exposure to the action of TFA, was coupled to the protected pentapeptide hydrazide (II-b), Z(OMe)-(BTI 16—20)-$NHNH_2$ by essentially the same azide procedure. The desired protected tridecapeptide, Z(OMe)-Ala-Arg(Tos)-Ile-Ile-Arg(Tos)-Tyr-Phe-Tyr-Asn-Ala-Lys(Z)-Ala-Gly-OH, Z-(OMe)-(BTI 16—28)-OH, was obtained in chromatographically and analytically pure form, after purification by batchwise washing followed by recrystallization from dimethylformamide (DMF) and methanol. Its homogeneity was confirmed by amino acid analysis. As mentioned above, Ile was recovered in low yield after 24 hr hydrolysis.

The Z(OMe) group of this protected tridecapeptide was removed by TFA and the resulting product was submitted to the final coupling reacton with II-a, Z(OMe)-(BTI 13—15)-$NHNH_2$ by the modified azide procedure. The reaction was performed in dimethylsulfoxide (DMSO), instead of DMF, because of the less solubility in the latter solvent. The rearrangement product of the small tripeptide azide and the unreacted amino component could be removed simply by batchwise washing with hot methanol. The final protected hexadecapeptide (II) Z(OMe)-(BTI 13—28)-OH, was obtained in chromatographically and analytically pure form after reprecipitation from DMF and methanol. The acid hydrolysate contained the constituent amino acids in ratios predicted by theory except for low recovery of Ile as stated above. Thus the homogeneity of the protected hexadecapeptide, (II) with Gly terminus was assessed. As far as the solubility problem is concerned, this sequence, among other synthetic subunits of BTI, represents the least soluble peptide in DMF.

Experimental

General experimental methods employed are essentially the same as described in the Part XXII[19] 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 (40: 15: 5), Rf_2 *n*-BuOH-pyridine-AcOH-H_2O (4: 1: 1: 2).

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

Z-Ala-Gly-OMe——The title compound was prepared by the DCC procedure. mp 92—93°, $[\alpha]^{28}_{D}$ $-24.7°$ ($c=1.1$, MeOH). (lit.[10] mp 98—99°, $[\alpha]_{D}$ $-25°$ in MeOH). Rf_1 0.67. *Anal.* Calcd. for $C_{14}H_{18}O_5N_2$: C, 57.13; H, 6.17; N, 9.52. Found: C, 57.39; H, 5.93; N, 9.57.

Z(OMe)-Lys(Z)-Ala-Gly-OMe——Z-Ala-Gly-OMe (6.60 g) in MeOH (50 ml) was hydrogenated over a Pd catalyst in the presence of 1 N HCl (22.5 ml). The catalyst was removed by filtration and the filtrate was evaporated. The residue was lyophilized. The hydrochloride thus obtained was dissolved in DMF (30 ml). To this ice-cooled solution, triethylamine (3.2 ml), Z(OMe)-Lys(Z)-OH (10.0 g) and DCC (5.20 g) were added consecutively. The mixture was stirred at room temperature overnight and the solvent, after filtration, was evaporated *in vacuo*. The residue was dissolved in AcOEt, which was washed with 10% citric acid, 5% Na_2CO_3 and H_2O, dried over Na_2SO_4 and then evaporated *in vacuo* to give gelatinous mass. The product was recrystallized from MeOH; yield 10.60 g (80%), mp 137—140°, $[\alpha]^{28}_{D}$ $-5.6°$ ($c=0.9$, DMF). Rf_1 0.84. *Anal.* Calcd. for $C_{29}H_{38}O_9N_4$: C, 59.37; H, 6.53; N, 9.55. Found: C, 59.32; H, 6.69; N, 9.68.

Z(OMe)-Lys(Z)-Ala-Gly-OH——To a solution of Z(OMe)-Lys(Z)-Ala-Gly-OMe (17.79 g) in MeOH (180 ml). 1 N NaOH (34 ml) was added and the solution was stirred at room temperature for 50 min. The solution was neutralized with AcOH and the solvent was evaporated *in vacuo*. The residue was dissolved in 10% Na_2CO_3, which was washed with AcOEt. The aqueous phase was acidified with 10% citric acid. The resulting solid was collected by filtration and recrystallized from MeOH and AcOEt; yield 14.06 g (80%), mp 172—173°, $[\alpha]^{28}_{D}$ $-3,2°$ ($c=0.6$, DMF). Rf_1 0.42. *Anal.* Calcd. for $C_{28}H_{36}O_9N_4$: C, 58.73; H, 6.34; N, 9.79. Found: C, 58.46; H, 6.54; N, 9.60.

H-Lys(Z)-Ala-Gly-OH——Under cooling with ice, TFA (36 ml) was added to a mixture of Z(OMe)-Lys(Z)-Ala-Gly-OH (14.0 g) and anisole (6.8 ml). After 40 min, dry ether was added to from white hygroscopic precipitate, which was collected by filtration; yield 14.0 g (100%), mp 152—154°, $[\alpha]^{24}_{D}$ $-5.8°$ ($c=0.5$, 10% AcOH), Rf_1 0.16, Rf_2 0.81.

Z(OMe)-Asn-Ala-OMe——(a) To a ice-cooled solution of H-Ala-OMe·HCl (2.80 g) and triethylamine (2.8 ml) in DMF (20 ml), a mixed anhydride (prepared from 5.92 g of Z(OMe)-Asn-OH and 2.8 ml of triethylamine with 2.0 ml of ethyl chloroformate in 50 ml of dry tetrahydrofuran), was added. After the solution was stirred for 2 hr, the solvent was evaporated *in vacuo* and the residue was dissolved in AcOEt, which was washed with 10% citric acid, 10% Na_2CO_3 and H_2O, dried over Na_2SO_4 and then evaporated. The resulting solid was recrystallized from MeOH; yield 2.29 g (30%), mp 174—176°, $[\alpha]^{29}_{D}$ $-9.6°$ ($c=0.4$, DMF). Rf_1 0.59. *Anal.* Calcd. for $C_{17}H_{23}O_7N_3$: C, 53.53; H, 6.08; N, 11.02. Found: C, 53.47; H, 6.23; N, 10.84.

(b) To a solution of Z(OMe)-Asn-OH (24.0 g), H-Ala-OMe·HCl (11.2 g) and triethylamine (11.2 ml) in DMF (120 ml), imidazole (8.8 g) and triphenylphosphite (40.0 g) were added. After stirring at 40° for 48 hr, the solution was condensed *in vacuo*. To the residue, ether was added to form the solid, which was collected by filtration, washed with H_2O and recrystallized from MeOH and AcOEt; yield 25.01 g (81%), mp 172—174°, $[\alpha]^{29}_{D}$ $-9.8°$ ($c=0.5$, DMF). Mixed mp with the sample obtained in (a) was 172—174°. *Anal.* Calcd. for $C_{17}H_{23}O_7N_3$: C, 53.53; H, 6.08; N, 11.02. Found: C, 53.36; H, 6.02; N, 10.74.

Z(OMe)-Asn-Ala-NHNH$_2$——Z(OMe)-Asn-Ala-OMe (12.0 g) was dissolved in MeOH (300 ml) and 80% hydrazine hydrate (12.0 ml) was added. The solution was kept at room temperature for 15 hr. The resulting solid mass was collected and washed with hot MeOH; yield 10.01 g (83%), mp 210—211°. *Anal.* Calcd. for $C_{16}H_{23}O_6N_5$: C, 50.39; H, 6.08; N, 18.36. Found: C, 50.58; H, 6.15; N, 18.50.

Z(OMe)-Asn-Ala-Lys(Z)-Ala-Gly-OH——The entire operation was carried out in a cold room at 4°. Under cooling with ice-NaCl, Z(OMe)-Asn-Ala-NHNH$_2$ (3.81 g) was dissolved in 0.5 N HCl in DMF (40 ml) and isoamylnitrite (1.34 ml) was added. After stirring for 5 min, the solution was neutralized with triethylamine (3 ml) and then combined with a solution of H-Lys(Z)-Ala-Gly-OH (4.08 g) and triethylamine (2.0 ml) in 20% aqueous DMF (24 ml). After the mixture was stirred for 48 hr, the solvent was evaporated *in vacuo* and to the residue, MeOH was added to form the gelatinous mass, which was collected by filtration, washed with 10% citric acid and H_2O and recrystallized from DMF and MeOH; yield 4.22 g (56%), mp 231—232°, $[\alpha]^{29}_{D}$ $-10.2°$ ($c=0.5$, DMSO), Rf_1 0.22. *Anal.* Calcd. for $C_{35}H_{47}O_{12}N_7$: C, 55.47; H, 6.25; N, 12.94. Found: C, 55.23; H, 6.53; N, 13.01.

H-Asn-Ala-Lys(Z)-Ala-Gly-OH (II-d), H-(BTI 24—28)-OH——Z(OMe)-Asn-Ala-Lys(Z)-Ala-Gly-OH (1.60 g) was treated with TFA (3 ml) in the presence of anisole (0.6 ml) at 0° for 30 min. Dry ether was added and the resulting precipitate was recrystallized from MeOH and ether in the presence of a small amount of triethylamine; yield 1.30 g (95%), mp 175—177°, $[\alpha]^{25}_{D}$ $-18.5°$ ($c=0.1$, DMF), Rf_2 0.46. Amino acid ratios in an acid hydrolysate $Asp_{1.15}$ $Ala_{2.10}$ $Lys_{0.89}$ $Gly_{1.00}$ (average recovery 97%). *Anal.* Calcd. for $C_{26}H_{39}O_9N_7 \cdot 4H_2O$: C, 49.63; H, 6.75; N, 13.97. Found: C, 49.15; H, 6.53; N, 14.02.

Z-Phe-Tyr-OMe——DCC (12.40 g) was added to a mixture of Z-Phe-OH (14.90 g) and H-Tyr-OMe (prepared from 11.50 g of the hydrochloride with 7 ml of triethylamine) in acetonitrile (100 ml). After stirring overnight, the solution was filtered and then evaporated *in vacuo*. The residue was dissolved in AcOEt, which after washing with 1 N HCl and 5% Na_2CO_3, dried over Na_2SO_4 and then evaporated. The resulting oily product was crystallized with ether. It was recrystallized from AcOEt and ether; yield 16.51 g

(69%), mp 139—141°, $[\alpha]_D^{25}$ −15.0° (c=1.2, MeOH), (lit.20) mp 137—139°, lit.21) mp 139—141°, $[\alpha]_D^{32}$ −14.7° in MeOH). Rf_1 0.81. *Anal.* Calcd. for $C_{27}H_{28}O_6N_2$: C, 68.05; H, 5.92; N, 5.88. Found: C, 68.30; H, 6.11; N, 6.18.

Z(OMe)-Tyr-Phe-Tyr-NHNH$_2$ (II-c), Z(OMe)-(BTI 16—20)-NHNH$_2$——Z-Phe-Tyr-OMe (12.0 g) in MeOH (100 ml) was hydrogenated in the usual manner in the presence of 1 N HCl (25 ml). The catalyst was removed by filtration and the filtrate was condensed *in vacuo*. The residue (Rf_1 0.78) was dissolved in DMF (50 ml) and triethylamine (3.5 ml) was added. To this solution, Z(OMe)-Tyr-OH (8.68 g) and DCC (6.0 g) were added. The solution was stirred overnight and then filtered. The filtrate was condensed *in vacuo*. The residue was dissolved in AcOEt, which after washing with 10% citric acid, 5% Na_2CO_3 and H_2O, dried over Na_2SO_4 and then evaporated. The residue was dissolved in MeOH (100 ml) and 80% hydrazine hydrate (15 ml) was added. The crystalline solid formed on standing overnight, was collected by filtration and recrystallized from EtOH, yield 8.09 g (48%), mp 213° (decomp.), $[\alpha]_D^{25}$ −24.4° (c=0.1, DMF). Rf_1 0.66. *Anal.* Calcd. for $C_{36}H_{39}O_8N_5$: C, 64.56; H, 5.87; N, 10.46. Found: C, 64.63; H, 6.03; N, 10.23.

Z-Ile-Arg(Tos)-OMe——To a solution of H-Arg(Tos)-OMe8) (5.20 g) and Z-Ile-OH (3.84 g) in acetonitrile (50 ml), DCC (3.30 g) was added under cooling with ice and the mixture was stirred at room temperature for 24 hr. After filtration, the solvent was evaporated *in vacuo* and the residue was dissolved in AcOEt, which was washed with 1 N HCl, 5% NH_4OH and H_2O, dried over Na_2SO_4 and then evaporated. Ether was added to the residue to form the semi solid, which was washed with ether and dried to give amorphous powders; yield 4.80 g (56%), $[\alpha]_D^{27}$ −2.9° (c=0.7, DMF). Rf_1 0.60. *Anal.* Calcd. for $C_{28}H_{39}O_7N_5S$: C, 57.03; H, 6.67; N, 11.88. Found: C, 56.90; H, 6.90; N, 11.93.

Z-Ile-Ile-Arg(Tos)-OMe——Z-Ile-Arg(Tos)-OMe (5.70 g) in MeOH (50 ml) was hydrogenated over a Pd catalyst in the presence of 1 N HCl (9.7 ml). The catalyst was removed by filtration and the filtrate was condensed *in vacuo*. The residue (Rf_1 0.48) was dissolved in a mixture of acetonitrile (50 ml) and DMF (30 ml). To this solution, triethylamine (1.4 ml), Z-Ile-OH (2.57 g) and DCC (2.40 g) were combined under cooling with ice. The solution was stirred at room temperature overnight and then filtered. The filtrate was condensed *in vacuo* and the residue was dissolved in AcOEt, which was washed with 1 N HCl, 5% NH_4OH and H_2O, dried over Na_2SO_4 and then evaporated. The resulting gelatinous mass was collected by filtration and recrystallized from AcOEt; yield 5.33 g (78%), mp 163—164°, $[\alpha]_D^{26}$ −9.1° (c=0.2, DMF). Rf_1 0.86. *Anal.* Calcd. for $C_{34}H_{50}O_8N_6S$: C, 58.10; H, 7.17; N, 11.96. Found: C, 58.30; H, 7.34; N, 12.20.

H-Ile-Ile-Arg(Tos)-OMe. Hydrochloride——Z-Ile-Ile-Arg(Tos)-OMe (5.33 g) was hydrogenated over a Pd catalyst in a mixture of MeOH (150 ml) and 1 N HCl (8 ml). After filtration, the filtrate was condensed *in vacuo* and the residue was lyophilized to give hygroscopic powders; yield 4.50 g (94%), $[\alpha]_D^{27}$ −27.3° (c=0.1, MeOH), Rf_1 0.57. *Anal.* Calcd. for $C_{26}H_{44}O_6N_6S \cdot HCl \cdot 1.5H_2O$: C, 49.39; H, 7.65; N, 13.29. Found: C, 49.47; H, 7.54; N, 13.27.

Z-Arg(Tos)-Ile-Ile-Arg(Tos)-OMe——To a solution of H-Ile-Ile-Arg(Tos)-OMe (prepared from 4.50 g. of the hydrochloride with 1 ml of triethylamine) in tetrahydrofuran (30 ml), a mixed anhydride (prepared from 3.58 g of Z-Arg(Tos)-OH7) in 50 ml of tetrahydrofuran with 1 ml of triethylamine and 0.7 ml of ethyl chloroformate) was added and the mixture was stirred at 0° for 30 min and at room temperature for 2 hr. The solvent was evaporated *in vacuo* and the residue dissolved in AcOEt, which was washed with 1 N HCl, 10% Na_2CO_3 and H_2O, dried over Na_2SO_4 and evaporated. Ether was added to the residue to give amorphous powder, which was collected by filtration and dried over P_2O_5; yield 6.11 g (86%), $[\alpha]_D^{26}$ −25.9° (c=0.1, DMF). Rf_1 0.77. *Anal.* Calcd. for $C_{47}H_{68}O_{11}N_{10}S_2$: C, 55.72; H, 6.77; N, 13.82. Found: C, 55.77; H, 7.07; N, 13.64.

H-Arg(Tos)-Ile-Ile-Arg(Tos)-OMe•Hydrochloride——Z-Arg (Tos)-Ile-Ile-Arg (Tos)-OMe (6.10 g) in MeOH (40 ml) was hydrogenated in the usual manner in the presence of 1 N HCl (7 ml). The catalyst was removed by filtration and the filtrate was condensed *in vacuo* and the residue was lyophilized to give hygroscopic powder; yield 5.45 g (90%), Rf_1 0.39. *Anal.* Calcd. for $C_{39}H_{62}O_9N_{10}S_2 \cdot HCl \cdot 5.5H_2O$: C, 46.10; H, 7.34; N, 13.75. Found: C, 45.94; H, 7.08; N, 14.07.

Z(OMe)-Ala-Arg(Tos)-Ile-Ile-Arg(Tos)-OMe——To a solution of H-Arg(Tos)-Ile-Ile-Arg(Tos)-OMe (prepared from 5.45 g of the hydrochloride and 1 ml of triethylamine) in DMF (30 ml), a mixed anhydride (prepared from 2.70 g of Z(OMe)-Ala-OH in 40 ml of tetrahydrofuran with 1 ml of triethylamine and 0.67 ml of ethyl chloroformate) was added and the solution was stirred at 0° for 30 min and at room temperature for 2 hr. The solvent was evaporated and the residue was dissolved in AcOEt, which was washed with 10% Na_2CO_3 and H_2O, dried over Na_2SO_4 and then evaporated. The residue was treated with ether to form the solid, which was collected by filtration and recrystallized from MeOH and AcOEt; yield 5.33 g (86%), mp 150°, $[\alpha]_D^{25}$ −10.2° (c=0.2, DMF). Rf_1 0.64. *Anal.* Calcd. for $C_{51}H_{75}O_{13}N_{11}S_2 \cdot H_2O$: C, 54.09; H, 6.85; N, 13.61. Found: C, 54.17; H, 6.90; N, 13.31.

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Z(OMe)-Ala-Arg(Tos)-Ile-Ile-Arg(Tos)-NHNH$_2$ (II-b), Z(OMe)-(BTI 16—20)-NHNH$_2$——Z(OMe)–Ala–Arg(Tos)–Ile–Ile–Arg(Tos)–OMe (5.30 g) was dissolved in MeOH (80 ml). After addition of 80% hydrazine hydrate (2.6 ml), the solution was kept in a refrigerator for 3 days. The resulting mass was collected by filtration and recrystallized from DMF and MeOH; yield 4.17 g (79%), mp 209—211°, $[\alpha]_D^{24}$ $-28.1°$ (c=0.1, DMF). Rf_1 0.55. *Anal.* Calcd. for $C_{50}H_{75}O_{12}N_{13}S_2$: C, 53.89; H, 6.78; N, 16.34. Found: C, 53.84; H, 6.86; N, 16.07.

Amino acid ratios in acid hydrolysates (110°, 6 N HCl).

hr	18	32	72
Ala	1.00	1.00	1.00
Arg	2.00		
Ile	1.17	1.45	1.81

Z(OMe)-Cys(Bzl)-Lys(Z)-OH——Z(OMe)–Cys(Bzl)–ONP (1.02 g) was added to a solution of H–Lys(Z)–OH (0.56 g) and triethylamine (0.56 ml) in 50% aqueous dioxane (40 ml). After the solution was stirred at room temperature overnight, the solvent was evaporated and the residue was dissolved in AcOEt. The organic phase was washed successively with 10% citric acid and H_2O, dried over Na_2SO_4 and then evaporated. Treatment of the residue with ether gave solid which was recrystallized from AcOEt and ether; yield 0.83 g (65%), mp 141—143°, $[\alpha]_D^{24}$ $-27.9°$ (c=0.3, DMF). Rf_1 0.35°. *Anal.* Calcd. for $C_{33}H_{39}O_8N_3S$: C, 62.16; H, 6.17; N, 6.59. Found: C, 61.92; H, 5.99; N, 6.60.

Z(OMe)-Pro-Cys(Bzl)-Lys(Z)-OH——Z(OMe)–Cys(Bzl)–Lys(Z)–OH (0.78 g) was treated with TFA (1.5 ml) in the presence of anisole (0.3 ml) at 0° for 30 min. The solvent was evaporated at 20° *in vacuo.* The residue was dissolved in DMF (10 ml) and triethylamine (0.4 ml) was added. To this solution, Z(OMe)–Pro–ONP (0.95 g, an oily product prepared according to Bodanszky[18)]) was added and the mixture was stirred at room temperature for 48 hr. The solvent was evaporated and the residue, after acidifying with citric acid, was extracted with AcOEt, which was washed with H_2O, dried over Na_2SO_4 and then evaporated. The residue was recrystallized from AcOEt and ether; yield 0.53 g (59%), mp 145—148°, $[\alpha]_D^{24}$ $-42.9°$ (c=0.1, DMF). Rf_1 0.66. *Anal.* Calcd. for $C_{38}H_{46}O_9N_4S$: C, 62.11; H, 6.31; N, 7.63. Found: C, 61.93; H, 6.38; N, 7.52.

Z(OMe)-Pro-Cys(Bzl)-Lys(Z)-OMe——An ethereal solution of diazomethane (prepared from 1.5 g of nitrosomethyl urea) was added to a solution of Z(OMe)–Pro–Cys(Bzl)–Lys(Z)–OH (0.45 g) in MeOH (20 ml). After stirring was continued for 2 hr, the solvent was removed by evaporation. The residue was recrystallized from AcOEt and ether; yield 0.40 g (89%), mp 131—133°, $[\alpha]_D^{23}$ $-61.1°$ (c=0.1, DMF). Rf_1 0.94. *Anal.* Calcd. for $C_{39}H_{48}O_9N_4S$: C, 62.55; H, 6.46; N, 7.48. Found: C, 62.57; H, 6.43; N, 7.51.

Z(OMe)-Pro-Cys(Bzl)-Lys(Z)-NHNH$_2$ (II-a), Z(OMe)-(BTI 13—15)-NHNH$_2$——To a solution of Z(OMe)–Pro–Cys(Bzl)–Lys(Z)–OMe (0.27 g) in MeOH (5 ml), 80% hydrazine hydrate (0.2 ml) was added and the solution was kept on standing overnight. The resulting solid was recrystallized from MeOH; yield 0.22 g (80%), mp 175—177°. *Anal.* Calcd. for $C_{33}H_{48}O_8N_6S$: C, 60.95; H, 6.46; N, 11.22. Found: C, 61.04; H, 6.51; N, 11.19.

Z(OMe)-Tyr-Phe-Tyr-Asn-Ala-Lys(Z)-Ala-Gly-OH——The entire operation was carried out in a cold room at 4°. Under cooling with ice–NaCl, Z(OMe)–Tyr–Phe–Tyr–MHNH$_2$ (3.70 g) was dissolved in 0.3 N HCl–DMF (30 ml) and isoamylnitrite (0.74 ml) was added. After stirring for 5 min, the solution was neutralized with triethylamine (2 ml). This solution was combined with a solution of H–Asn–Ala–Lys(Z)–Ala–Gly–OH (4.12 g) and triethylamine (1.2 ml) in 10% aqueous DMF (20 ml) and the mixture was stirred for 48 hr. The solvent was evaporated *in vacuo* and the residue was triturated with MeOH to give the solid, which was collected by filtration, washed with 10% citric acid, H_2O and MeOH and recrystallized from DMF and MeOH; yield 3.83 g (56%), mp 220° (decomp.), $[\alpha]_D^{20}$ $-32.1°$ (c=0.2, DMF). Rf_1 0.11, Rf_2 0.77. Amino acid ratios in acid hydrolysate $Tyr_{1.02}$ $Phe_{0.97}$ $Asp_{1.16}$ $Ala_{2.01}$ $Lys_{0.96}$ $Gly_{1.00}$ (average recovery 81%, acid hydrolysis of Z–Tyr–peptides gives low recovery of Tyr as mentioned by Iselin[22)] *Anal.* Calcd. for $C_{62}H_{74}O_{17}N_{10} \cdot H_2O$: C, 59.60; H, 6.13; N, 11.21. Found: C, 59.79; H, 6.30; N, 11.35.

Z(OMe)-Ala-Arg(Tos)-Ile-Ile-Arg(Tos)-Tyr-Phe-Tyr-Asn-Ala-Lys(Z)-Ala-Gly-OH——Under cooling with ice, TFA (12 ml) was added to a mixture of Z(OMe)–Tyr–Phe–Tyr–Asn–Ala–Lys(Z)–Ala–Gly–OH (3.83 g) and anisole (2.4 ml). After 1 hr, dry ether was added to form the white precipitate, which was collected by filtration and dried over KOH pellets *in vacuo.* It was then submitted to the next coupling reaction. A solution of Z(OMe)–Ala–Arg(Tos)–Ile–Ile–Arg(Tos)–N_3 (prepared as usual from 3.44 g of the corresponding hydrazide and 0.44 ml of isoamylnitrite in 20 ml of DMF) was added to a solution of H–Tyr–Phe–Tyr–Asn–Ala–Lys(Z)–Ala–Gly–OH (3.28 g) obtained above and triethylamine (1 ml) in 20% aqueous DMF (50 ml). The mixture was stirred at 4° for 48 hr and the solvent was evaporated *in vacuo.* The residue was triturated with MeOH to give the solid, which was collected by filtration, washed with 10% citric acid and H_2O and recrystallized from DMF and MeOH; yield 2.50 g (39%), mp 270° (decomp.), $[\alpha]_D^{23}$ $-45.2°$ (c=0.1, DMF). Rf_2 0.86. Amino acid ratios in an acid hydrolysate (24 hr): $Ala_{3.17}$ $Arg_{1.68}$ $Ile_{1.18}$ $Tyr_{0.96}$ $Phe_{1.00}$

[22)] B. Iselin, *Helv. Chim. Acta,* 45, 1510 (1962).

$Asp_{0.97}$ $Lys_{0.91}$ $Gly_{1.00}$ (average recovery 90%). *Anal.* Calcd. for $C_{103}H_{137}O_{26}N_{21}S_2$: C, 57.55; H, 6.42; N, 13.69. Found: C, 57.29; H, 6.68; N, 13.76.

Z(OMe)-Pro-Cys(Bzl)-Lys(Z)-Ala-Arg(Tos)-Ile-Ile-Arg(Tos)-Tyr-Phe-Tyr-Asn-Ala-Lys(Z)-Ala-Gly-OH (II), Z(OMe)-(BTI 13—28)-OH——In an ice-bath, Z(OMe)–Ala–Arg(Tos)–Ile–Ile–Arg(Tos)–Tyr–Phe–Tyr–Asn–Ala–Lys(*Z*)–Ala–Gly–OH (2.50 g) was treated with TFA (10 ml) in the presence of anisole (0.7 ml) for 45 min. Dry ether was added the resulting precipitate was collected by filtration and dried over KOH pellets *in vacuo.* To a solution of this partially protected tridecapeptide in DMSO (20 ml), triethylamine (0.3 ml) and the azide (prepared from 1.50 g of Z(OMe)–Pro–Cys(Bzl)–Lys(Z)–$NHNH_2$ in 5 ml of DMF with 4 ml of 1 N HCl–DMF, 0.27 ml of isoamylnitrite and 0.56 ml of triethylamine were added. The mixture was stirred at 4° for 48 hr and the solvent was evaporated *in vacuo.* The residue was triturated with MeOH and the resulting solid was collected by filtration, washed with hot MeOH and recrystallized from DMF and MeOH; yield 2.02 g (75%), mp 248° (decomp.), $[\alpha]_D^{26}$ $-87.4°$ (c=0.1, DMF). Rf_2 0.88. Amino acid ratios in an acid hydrolysate (24 hr): $Pro_{1.04}$ $Lys_{2.04}$ $Ala_{3.25}$ $Arg_{2.19}$ $Ile_{1.32}$ $Tyr_{1.11}$ $Phe_{0.89}$ $Asp_{1.02}$ $Gly_{1.00}$ (average recovery 95%). *Anal.* Calcd. for $C_{132}H_{173}O_{31}N_{25}S_3 \cdot 3H_2O$: C, 57.52; H, 6.55; N, 12.71. Found: C, 57.62; H, 6.57; N, 12.94.

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