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Studies on Peptides. XLII.[1,2] Synthesis of the Protected Nonapeptide Corresponding to Positions 29 to 37 of the Basic Trypsin Inhibitor from Bovine Pancreas (Kunitz and Northrop)

Haruaki Yajima,[3] Nariakira Mizokami,[3a] Michiko Kiso,[3b] Tooru Jinnouchi[3c] Yoshiyuki Kai, and Yoshiaki Kiso

Faculty of Pharmaceutical Sciences, Kyoto University[3]

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The protected nonapeptide, Z(OMe)–Leu–Cys(Bzl)–Gln–Thr–Phe–Val–Tyr–Gly–Gly–OH, corresponding to positions 29 to 37 of the basic trypsin inhibitor (Kunitz and Northrop), was synthesized by the azide coupling of Z(OMe)–Leu–Cys(Bzl)–Gln–Thr–$NHNH_2$ and H–Phe–Val–Tyr–Gly–Gly–OH.

Following to the synthesis of the protected dodecapeptide, Z–Arg(NO_2)–Pro–Asp(OBzl)–Phe–Cys(Bzl)–Leu–Glu(OBzl)–Pro–Pro–Tyr–Thr–Gly–OH (I. position 1 to 12)[4] and the 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, position 13 to 28),[1] we wish to report the synthesis of the protected nonapeptide (III) corresponding to position 29 through 37 of the basic trypsin inhibitor (BTI).

The nonapeptide, Leu–Cys–Gln–Thr–Phe–Val–Tyr–Gly–Gly, was prepared in a protected form by the coupling reaction of the tetra- and pentapeptide units, (III-a) and (III-b), as shown in Chart 1.

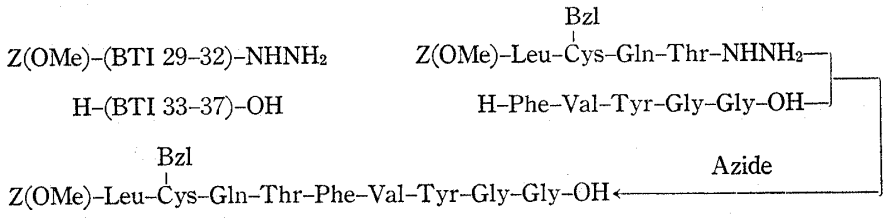

Chart 1. Synthetic Route to the Protected Nonapeptide (III), Z(OMe)–(BTI 29–37)–OH

First, synthetic outline of the C-terminal pentapeptide is described. This was prepared in a stepwise manner starting Z–Gly–Gly–OMe[5] as shown in Chart 2. This Z–dipeptide ester, after hydrogenation, was condensed with Z–Tyr(Bzl)–ONP[6] to give Z–Tyr(Bzl)–Gly–Gly–OMe. The protected tripeptide ester was then hydrogenated and the resulting tripeptide ester, H–Tyr–Gly–Gly–OMe, was condensed with Z–Val–OH by the mixed anhydride procedure[7]

1) Part XLI: H. Yajima, Y. Okada, H. Watanabe, and Y. Kiso, *Chem. Pharm. Bull.* (Tokyo), **22**, 1067 (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). Z=benzyloxycarbonyl, Z(OMe)=*p*-methoxybenzyloxycarbonyl, Bzl=benzyl, ONP=*p*-nitrophenyl ester. Tos=tosyl.
3) Location: *Sakyo-ku, Kyoto*; *a*) Present address: *Takeda Chemical Industries, Ltd.* (Osaka); *b*) *Protein Research Foundation* (Osaka); *c*) *Sumitomo Chemical Industries*.
4) Part XL: H. Yajima and Y. Kiso, *Chem. Pharm. Bull.* (Tokyo), **22**, 1061 (1974).
5) T. Wieland and B. Hainke, *Ann. Chem.*, **599**, 70 (1956); N.F. Albertson and F.C. McKay, *J. Am. Chem. Soc.*, **75**, 5323 (1957).
6) M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959).
7) J.R. Vaughan, Jr. and R.L. Osato, *J. Am. Chem. Soc.*, **73**, 3547 (1951).

to give Z–Val–Tyr–Gly–Gly–OMe, which was subsequently hydrogenated. The resulting tetrapeptide ester was then allowed to react with Z–Phe–ONP to give the fully protected pentapeptide ester, Z–Phe–Val–Tyr–Gly–Gly–OMe. The above protected tetra and pentapeptide esters were purified by recrystallization twice from methanol or combination of methanol and ethyl acetate. Alakaline saponification of this protected pentapeptide ester followed by catalytic hydrogenation afforded the free pentapeptide in crystalline form.

Prior to the synthesis of this pentapeptide, we attempted to elongate the peptide chain without protecting the C-terminal Gly residue. Z–Tyr(Bzl)–Gly–Gly–OH was similarly prepared. However, purification of Z–Val–Tyr–Gly–Gly–OH, prepared by the mixed anhydride procedure, was somewhat technically difficult. Z–Tyr(Bzl)–Gly–Gly–OH was, therefore, converted to the corresponding methyl ester and the latter synthesis was followed as stated above.

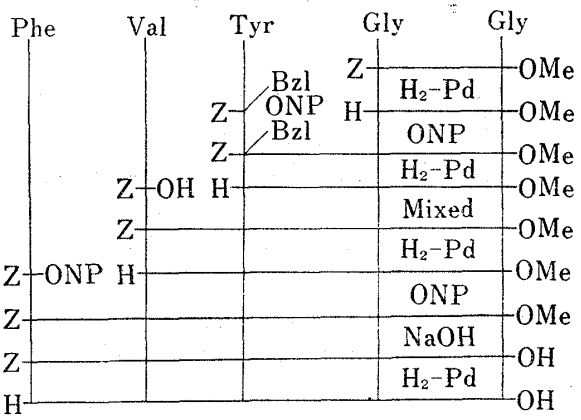

Chart 2. Synthetic Route to the Pentapeptide (III-b) H-(BTI 33—37)-OH

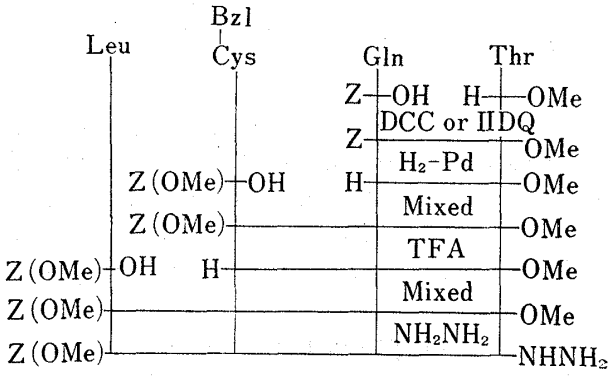

Chart 3. Synthetic Route to the protected Tetrapeptide Hydrazide (III-a) Z(OMe)-(BTI 29—32)-$NHNH_2$

IIDQ=N-isobutoxycarbonyl-2-isobutoxy-1,2-dihydroquinoline

The N-terminal tetrapeptide hydrazide (III-a) was synthesized in a stepwise manner starting H–Thr–OMe as shown in Chart 3. Z–Gln–Thr–OMe was obtained by the dicyclohexylcarbodiimide coupling[8] of Z–Gln–OH with H–Thr–OMe. As described previously, the same dipeptide ester was obtained by N-isobutoxycarbonyl-2-isobutoxy-1,2-dihydroquinoline which was introduced recently as a coupling reagent in peptide synthesis.[9] It was confirmed that the purified product did not exhibit any CN absorption band in infrared (IR) spectra. The Z group of the protected dipeptide ester was catalytically removed in the presence of one equimole of hydrochloric acid. After removing the catalyst by filtration, the filtrate, after neutralization with triethylamine, was submitted to the next coupling with Z(OMe)–Cys(Bzl)–OH by the mixed anhydride procedure. This care suppresses the risk of hydrolysis of the amide group of Gln by acid. Z(OMe)–Cys(Bzl)–Gln–Thr–OMe was then treated with trifluoroacetic acid (TFA) to remove the α-amino protecting group and the resulting H–Cys–(Bzl)–Gln–Thr–OMe was then condensed with Z(OMe)–Leu–OH by the mixed anhydride procedure. The protected tetrapeptide ester, Z(OMe)–Leu–Cys(Bzl)–Gln–Thr–OMe, was converted to the corresponding hydrazide (III-a) in the usual manner.

As shown in Chart 1, the coupling reaction between III-a and III-b was performed according to the azide procedure modified by Honzl and Rudinger.[10] The protected nonapeptide (III) was purified by batchwise washing with acid followed by recrystallization from

8) J.C. Sheehan and C.P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955).
9) Y. Kiso and H. Yajima, *Chem. Commun.*, **1972**, 942; Y. Kiso, Y. Kai, and H. Yajima, *Chem. Pharm. Bull.* (Tokyo), **21**, 2507 (1973).
10) J. Honzl and J. Rudinger, *Coll. Czech. Chem. Commun.*, **26**, 2333 (1961).

dimethylformamide (DMF) and ethyl acetate. Its homogeneity was assessed by thin layer chromatography, amino acid and elemental analyses.

Experimental

General experimental methods employed are essentially the same as described in the Part XXII[11] of this series. Thin-layer chromatography was performed on silica gel (Kiselgel G, Merck). *Rf* values refer to the following solvent systems; Rf_1 $CHCl_3$–MeOH–H_2O (40: 15: 5), Rf_2 *n*-butanol–pyridine–AcOH–H_2O (4: 1: 1: 2).

Z-Tyr(Bzl)-Gly-Gly-OH——To a solution of H-Gly-Gly-OH[12] (4.0 g) and triethylamine (10 ml) in H_2O (100 ml), Z-Tyr(Bzl)-ONP (20.0 g) in tetrahydrofuran (300 ml) was added and the solution was stirred at room temperature for 48 hr. The solvent was evaporated *in vacuo* and the residue was dissolved in H_2O, which, after washing with AcOEt, was acidified with 3 N HCl. The resulting solid was collected, washed with H_2O and recrystallized from AcOEt; yield 13.10 g (84%), mp 148—149°, $[\alpha]_D^{26}$ −17.7° (c=1.1, 50% tetrahydrofuran). *Anal.* Calcd. for $C_{28}H_{29}O_7N_3$: C, 64.73; H, 5.62; N, 8.09. Found: C, 64.44; H, 5.52; N, 8.18.

Z-Tyr(Bzl)-Gly-Gly-OMe——Z-Gly-Gly-OMe[5] (5.61 g) in 90% aqueous tetrahydrofuran (50 ml) containing AcOH (2.3 ml) was hydrogenated in the usual manner. After filtration, the filtrate was combined with a solution of Z-Tyr(Bzl)-ONP[6] (10.39 g) and triethylamine (8.7 ml) in tetrahydrofuran (300 ml) and the solution was stirred overnight. The solvent was evaporated and the residue was dissolved in AcOEt, which was washed 5% Na_2CO_3, 0.5 N HCl and H_2O, dried over Na_2SO_4 and then evaporated. The solid obtained by addition of ether to the residue was recrystallized from AcOEt and ether; yield 7.92 g (75%), mp 161—163°, $[\alpha]_D^{26}$ −18.8° (c=0.9, 50% tetrahydrofuran). *Anal.* Calcd. for $C_{29}H_{31}O_7N_3$: C, 65.28; H, 5.86; N, 7.88. Found: C, 65.61; H, 5.90; N, 8.02.

(b) An ethereal solution of diazomethane was added to an ice-cooled solution of Z-Tyr(Bzl)-Gly-Gly-OH (13.0 g) in MeOH (300 ml) until yellow color persisted for 2.5 hr. The excess diazomethane was decomposed with acetic acid, the solvent was evaporated *in vacuo* and the residue was recrystallized from MeOH and AcOEt; yield 13.0 g (99%), mp 159—160°. Identity of the compounds obtained in a and b was confirmed by mixed mp.

Z-Val-Tyr-Gly-Gly-OMe——Z-Tyr(Bzl)-Gly-Gly-OMe (11.0 g) was dissolved in MeOH (50 ml) with slight warming. After addition of 1 N HCl (6.3 ml) and H_2O (20 ml), hydrogenolysis was performed over a Pd catalyst in the usual manner. The catalyst was removed by filtration and the filtrate was condensed *in vacuo* to give an oily residue; yield 7.0 g (99%), Rf_2 0.46. To a solution of this oily hydrochloride of H-Tyr-Gly-Gly-OMe (1.38 g) and triethylamine (0.56 ml) in 70% aqueous tetrahydrofuran (30 ml), was added a mixed anhydride prepared from Z-Val-OH (1.0 g) in dry tetrahydrofuran (20 ml) with triethylamine (0.56 ml) and ethyl chloroformate (0.39 ml). The solution was stirred in an ice-bath for 30 min and then at room temperature for 2.5 hr. After evaporation of the solvent, the residue was acidified with 5% citric acid to form the solid, which was collected by filtration, washed with 5% $NaHCO_3$ and H_2O and recrystallized twice with MeOH; yield 1.13 g (52%), mp 202—204°, $[\alpha]_D^{26}$ −32.1° (c=0.7, MeOH). *Anal.* Calcd. for $C_{27}H_{34}O_8N_4$: C, 59.77; H, 6.32; N, 10.33. Found: C, 59.54; H, 6.27; N, 10.18.

Z-Phe-Val-Tyr-Gly-Gly-OMe——Z-Val-Tyr-Gly-Gly-OMe (0.40 g) in 80% MeOH containing 1 N HCl (0.74 ml) was hydrogenated over a Pd catalyst in the usual manner. The solvent, after removing the catalyst by filtration, was evaporated and the residue was lyophilized; yield 0.32 g (95%), Rf_2 0.61. The tetrapeptide methyl ester hydrochloride thus obtained was dissolved in DMF (30 ml) containing triethylamine (0.17 ml). Z-Phe-ONP (0.36 g) was added and the solution was stirred at room temperature for 24 hr. After evaporation of the solvent, H_2O was added. The resulting solid was washed with ether and recrystallized twice from MeOH and AcOEt; yield 0.44 g (88%), mp 239—241°, $[\alpha]_D^{30}$ −7.2° (c=0.9, DMF). *Anal.* Calcd. for $C_{36}H_{43}O_9N_5$: C, 62.68; H, 6.28; N, 10.15. Found: C, 62.21; H, 6.3; N, 10.24.

Z-Phe-Val-Tyr-Gly-Gly-OH——To a solution of Z-Phe-Val-Tyr-Gly-Gly-OMe (0.34 g) in dioxane (30 ml), 1 N NaOH (0.1 ml) was added and the solution was stirred at room temperature for 45 min. After addition of a small amount of AcOH, the solvent was evaporated and the residue was acidified with 5% HCl to form the gelatinous mass, which was collected by filtration, washed with H_2O and recrystallized from MeOH and AcOEt; yield 0.26 g (79%), mp 210—212°, $[\alpha]_D^{30}$ −22.9° (c=1.0, DMF). *Anal.* Calcd. for $C_{35}H_{41}O_9N_5 \cdot 1/2H_2O$: C, 61.39; H, 6.27; N, 10.37. Found: C, 61.37; H, 6.16; N, 10.32.

H-Phe-Val-Tyr-Gly-Gly-OH (III-b)——Z-Phe-Val-Tyr-Gly-Gly-OH (5.0 g) in 70% MeOH (140 ml) containing AcOH (0.4 ml) was hydrogenated over a Pd catalyst until the evolution of CO_2 ceased. The solution, after filtration, was condensed and the solid residue was recrystallized from H_2O; yield 3.80 g (95%), mp 245° (decomp.), $[\alpha]_D^{26}$ −30.4° (c=1.3, 50% AcOH). Rf_2 0.48. Amino acid ratios in acid hydrolysate $Phe_{1.00}$ $Val_{1.05}$ $Tyr_{0.96}$ $Gly_{2.05}$ (average recovery 94%). *Anal.* Calcd. for $C_{27}H_{35}O_7N_5$: C, 59.87; H, 6.51; N, 12.93. Found: C, 60.11; H, 6.65; N, 12.73.

11) H. Yajima, Y. Okada, H. Kawatani, and N. Mizokami, *Chem. Pharm. Bull.* (Tokyo), 17, 1229 (1969).
12) J.C. Sheehan and V.S. Frank, *J. Am. Chem. Soc.*, 71, 1856 (1949).

Z-Gln-Thr-OMe——DCC (24.70 g) was added to an ice-chilled mixture of Z-Gln-OH (28.03 g) and H-Thr-OMe (prepared from 16.97 g of the hydrochloride with 13.8 ml of triethylamine) in DMF (280 ml). After stirring at room temperature overnight, the solution was filtered and the filtrate was condensed *in vacuo*. Ether was added and the resulting mass was collected by filtration, washed batchwisely with 5% Na_2CO_3, 5% citric acid and H_2O, and recrystallized twice from MeOH and AcOEt; yield 27.66 g (70%). mp 164—165°, $[\alpha]_D^{26}$ +2.1° (c=0.1, DMF). *Anal.* Calcd. for $C_{13}H_{25}O_7N_3$: C, 54.67; H, 6.37; N, 10.63. Found: C, 54.48; H, 6.22; N, 10.34. Alternate synthesis of Z-Gln-Thr-OMe by N-isobutoxycarbonyl-2-isobutoxy-1,2-dihydroquinoline has been described previously:[9] mp 162—165°, $[\alpha]_D^{26}$ +2.1° in DMF.

Z(OMe)-Cys(Bzl)-Gln-Thr-OMe——Z-Gln-Thr-OMe (11.42 g) in a mixture of tetrahydrofuran (150 ml) and 1 N HCl (28.8 ml) was hydrogenated over a Pd catalyst in the usual manner. The catalyst was removed by filtration. The filtrate was then combined with a mixed anhydride, prepared from Z(OMe)-Cys(Bzl)-OH (11.93 g) in dry tetrahydrofuran (200 ml) with triethylamine (4.4 ml) and ethyl chloroformate (2.8 ml). After the solution was stirred in an ice-bath for 2.5 hr, the solvent was evaporated. Trituration of the residue with petroleum ether gave the solid, which was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O, dried over Na_2SO_4 and then recrystallized from tetrahydrofuran and ether; yield 28.10 g (63%), mp 192—195°, $[\alpha]_D^{24}$ −32.0° (c=0.9, 50% tetrahydrofuran). *Anal.* Calcd. for $C_{29}H_{38}O_9N_4S$: C, 56.30; H, 6.19; N, 9.06. Found: C, 56.05; H, 6.27; N, 9.19.

H-Cys(Bzl)-Gln-Thr-OMe——Z(OMe)-Cys(Bzl)-Gln-Thr-OMe (1.24 g) was treated with TFA (3 ml) in the presence of anisole (1 ml) at room temperature for 1.5 hr. Dry ether was added and the resulting precipitate was collected by filtration and dried over P_2O_5 and KOH pellets *in vacuo*; yield 1.06 g (93%), Rf_2 0.83. *Anal.* Calcd. for $C_{20}H_{30}O_6N_4S \cdot TFA \cdot H_2O$: C, 45.05; H, 5.67; N, 9.55. Found: C, 45.28; H, 5.76; 9.43.

Z(OMe)-Leu-Cys(Bzl)-Gln-Thr-OMe——The tripeptide methyl ester trifluoroacetate obtained as described above (11.74 g) was dissolved in tetrahydrofuran (100 ml) containing triethylamine (2.8 ml). To this solution, was added a mixed anhydride prepared from Z(OMe)-Leu-OH (17.72 g) in dry tetrahydrofuran (200 ml) with triethylamine (8.4 ml) and isobutyl chloroformate (7.9 ml). The solution was stirred in an ice-bath for 30 min and at room temperature for 2.5 hr. After evaporation of the solvent, the residue was acidified with 5% citric acid to form the solid, which was washed batchwisely with 5% $NaHCO_3$ and H_2O and recrystallized twice from tetrahydrofuran and ether; yield 22.51 g (76%), mp 213—216°, $[\alpha]_D^{24}$ −34.2° (c=0.7, DMF). Rf_1 0.63. Amino acid ratios in an acid hydrolysate $Leu_{1.05}$ $Glu_{1.06}$ $Thr_{1.00}$ (average recovery 100%). *Anal.* Calcd. for $C_{35}H_{49}O_{10}N_5S \cdot 1/2H_2O$: C, 56.74; H, 6.79; N, 9.45. Found: C, 56.71; H, 6.74; N, 9.62.

Z(OMe)-Leu-Cys(Bzl)-Gln-Thr-NHNH$_2$ (III-a)——To a solution of Z(OMe)-Leu-Cys(Bzl)-Gln-Thr-OMe (7.32 g) in DMF (50 ml), 80% hydrazine hydrate (2.4 ml) was added. The gelatinous mass formed on standing overnight was collected by filtration and washed with DMF; yield 4.10 g (56%), mp 225—228°, *Anal.* Calcd. for $C_{34}H_{49}O_9N_7S$: C, 55.80; H, 6.75; N, 13.40. Found: C, 55.70; H, 6.90; N, 13.33.

Z(OMe)-Leu-Cys(Bzl)-Gln-Thr-Phe-Val-Tyr-Gly-Gly-OH (III)——To a solution of Z(OMe)-Leu-Cys-(Bzl)-Gln-Thr-NHNH$_2$ (1.46 g) in DMF (40 ml), 1 N HCl-DMF (4 ml) and isoamylnitrite (0.36 ml) were added at −20°. The solution was stirred at that temperature for 10 min and then the pH of the solution was adjusted to 8 with triethylamine (0.6 ml). This solution was then combined with a solution of H-Phe-Val-Tyr-Gly-Gly-OH (1.08 g) and triethylamine (0.84 ml) in 50% DMF (80 ml). After the mixture was stirred at 4° for 24 hr, the solvent was evaporated, the residue was acidified with 5% citric acid and the resulting solid was collected by filtration washed batchwisely with AcOEt and 5% citric acid and recrystallized from MeOH and AcOEt; yield 1.01 g (40%), mp 230—235°, $[\alpha]_D^{27}$ −38.7° (c=0.4, DMF). Rf_2 0.76. Amino acid ratios in an acid hydrolysate $Leu_{1.09}$ $Glu_{1.11}$ $Thr_{0.98}$ $Phe_{0.97}$ $Val_{1.00}$ $Tyr_{0.86}$ $Gly_{2.09}$ (average recovery 89%). *Anal.* Calcd. for $C_{61}H_{82}O_{17}N_{10}S \cdot H_2O$: C, 58.17; H, 6.56; N, 11.12. Found: C, 58.25; H, 6.82; N, 11.08.

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