BULLETIN OF THE CHEMICAL SOCIETY OF JAPAN VOL. 40 204—208 (1967)

Ribonuclease T_1 Peptides. I. Synthesis of a Protected *N*-Terminal Undecapeptide Containing Asparagine Residue in Third Position

Motonori Ohno, Tetsuo Kato, Nobuo Mitsuyasu, Michinori Waki,
Satoru Makisumi and Nobuo Izumiya

Laboratory of Biochemistry, Faculty of Science, Kyushu University, Hakozaki, Fukuoka

(Received June 21, 1966)

A protected undecapeptide containing the possible *N*-terminal sequence of ribonuclease T_1, namely benzyloxycarbonyl-L-alanyl-*S*-benzyl-L-cysteinyl-L-asparaginyl-L-tyrosyl-L-threonyl-*S*-benzyl-L-cysteinyl-glycyl-L-seryl-L-asparaginyl-*S*-benzyl-L-cysteinyl-L-tyrosine ethyl ester (XXI), was synthesized by coupling of benzyloxycarbonyl-alanyl-*S*-benzylcysteinyl-asparaginyl-tyrosine azide with heptapeptide ester, threonyl-*S*-benzylcysteinyl-glycyl-seryl-asparaginyl-*S*-benzylcysteinyl-tyrosine ester, which is derived from *p*-methoxybenzyloxycarbonyl-heptapeptide ester by the action of trifluoroacetic acid. The optical homogeneity of the protected undecapeptide (XXI) was established by deblocking of XXI and subsequently digesting the resulting free undecapeptide with leucine aminopeptidase.

Ribonuclease T_1, one of ribonucleases from *Aspergillus oryzae*,¹⁾ is a simple protein composed of one hundred and four amino acid residues and has two intramolecular disulfide bridges. In 1965, Takahashi has described the complete amino acid sequence of this protein, in which the 3rd amino acid residue from the *N*-terminus has been assigned to L-aspartic acid.²⁾ In this structure the sequence alanyl-cysteinyl-aspartyl-tyrosyl-threonyl-cysteinyl-glycyl-seryl-asparaginyl-cysteinyl-tyrosyl- is present. This peptide segment is located in the *N*-terminal position and involves the intrachain disulfide bridges with another cysteinyl residue in 103rd position of the ribonuclease T_1 molecule. In the same year, Kasai *et al.* have reported that the 3rd amino acid residue is L-asparagine.³⁾

A program has been undertaken in this Laboratory, which has been directed toward the synthesis and enzymic evaluation of certain structural features of this protein. To this end certain peptides containing the above mentioned peptide segment with the cysteine residues protected are being synthesized. The present paper will describe the synthesis of the protected undecapeptide containing L-asparagine residue in the 3rd position from *N*-terminus, benzyloxycarbonyl-L-alanyl-*S*-benzyl-L-cysteinyl-L-asparaginyl-L-tyrosyl-L-threonyl-*S*-benzyl-L-cysteinyl-glycyl-L-seryl-L-asparaginyl-*S*-benzyl-L-cysteinyl-L-tyrosine ethyl ester (XXI).

In the synthesis of the protected undecapeptide (XXI), which is outlined in Fig. 1, we selected a route involving the coupling of the benzyloxycarbonyl-tetrapeptide azide derived from the corresponding hydrazide (XX) with the heptapeptide ethyl ester (XIV) which is derived from the *p*-methoxybenzyloxycarbonyl-heptapeptide ethyl ester (XIII) by the action of trifluoroacetic acid. The protected heptapeptide (XIII) was prepared by coupling the *p*-methoxybenzyloxycarbonyl tripeptide azide derived from XII with the tetrapeptide ester (VII). The derivative (XII or VII) of either the tripeptide or tetrapeptide was built up by stepwise elongation of the peptide chain from the carboxyl toward the amino end. In order to minimize the possibility of racemization, the azide procedure was used wherever two peptides were to be connected.⁴⁾ Coupling of a benzyloxycarbonyl-amino acid with amine component was mainly accomplished by carboxy activation through the use of the *p*-nitrophenyl ester.⁴⁾

The benzyl group was used for the protection of the sulfhydryl function of the cysteine residues.⁵⁾ The benzyloxycarbonyl group was used to protect the amino function in a number of steps. It has been recognized that the benzyloxycarbonyl group in a benzyloxycarbonyl-peptide ethyl ester is readily removed by the action of hydrogen bromide in acetic acid;⁶⁾ many intermediate peptide ethyl ester

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1) K. Sato and F. Egami, *J. Biochem. (Tokyo)*, **44**, 753 (1957).

2) K. Takahashi, *J. Biol. Chem.*, **240**, 4117 (1965); *The Protein, Nucleic Acid and Enzyme (Tampakushitsu-Kakusan-Koso)*, **10**, 1277 (1965).

3) H. Kasai, K. Takahashi and T. Ando, Proceedings of the Symposium on Enzyme Chem., Tokushima, p. 77 (1965).

4) K. Hofmann and P. G. Katsoyannis, "The Proteins," Vol. I, Academic Press, New York (1963), p. 54.

5) J. L. Wood and V. du Vigneaud, *J. Biol. Chem.*, **130**, 109 (1939).

6) D. Ben-Ishai and A. Berger, *J. Org. Chem.*, **17**, 1564 (1952).

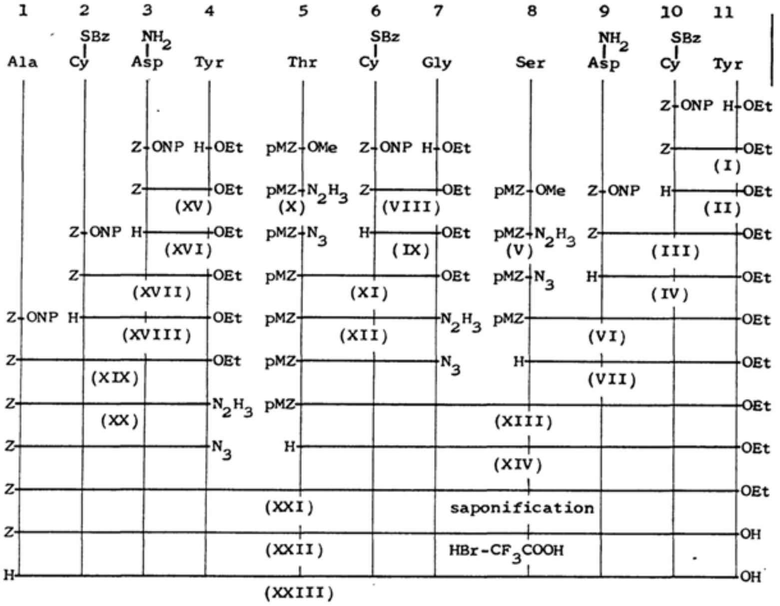

Fig. 1. Schematic diagram of synthesis of undecapeptides. Z, benzyloxycarbonyl; pMZ, *p*-methoxybenzyloxycarbonyl; SBz, *S*-benzyl; ONP, *p*-nitrophenoxy, N_2H_3; hydrazide.

hydrobromides (II and others) containing *S*-benzylcysteine residues were obtained in this manner. The *p*-methoxybenzyloxycarbonyl group was used for the protection of the amino function of the hydroxy-amino acid residues.[7] This group in the protected peptides (VI and XIII) was easily removed by the action of trifluoroacetic acid without the side reaction such as an acylation of the hydroxy group of hydroxy-amino acids, serine and threonine.[8]

An attempt was made to ascertain by enzymic method the optical homogeneity of the protected undecapeptide (XXI). The benzyloxycarboxyl-undecapeptide (XXII), which was obtained by the saponification of XXI, was debenzyloxycarboxylated by the exposure to bubbled hydrogen bromide in trifluoroacetic acid, and the undecapeptide (XXIII) obtained was incubated with leucine aminopeptidase. The paper chromatographic analysis of the leucine aminopeptidase digest revealed the presence of ninhydrin positive compounds with R_f values corresponding to the expected amino acids only. The absence of ninhydrin positive components corresponding to any of the intermediates suggests that digestion was complete. This implies that no racemization of the constituent amino acids had occurred during the synthetic processes.

Experimental

The melting points were not corrected. The optical rotations were measured on a Yanagimoto Photometric Polarimeter, OR-20 type. The paper chromatography and thin-layer chromatography were carried out on Toyo Roshi No. 52 chromatography paper and on Merck silica gel G, respectively. Spots of materials possessing a free amino group on a thin-layer plate were detected by spraying ninhydrin, and those of the amino group-blocked materials, by spraying 47% hydrobromic acid and then ninhydrin. Prior to analysis, the samples were dried over phosphorus pentoxide at 90°C and 2 mmHg to constant weight.

Benzyloxycarbonyl-*S*-benzyl-L-cysteinyl-L-tyrosine Ethyl Ester (I). To a solution of 4.90 g (20 mmol) of L-tyrosine ethyl ester hydrochloride dissolved in a mixture of 2.8 m*l* (20 mmol) of triethylamine and 20 m*l* of dimethylformamide, was added a solution of 9.32 g (20 mmol) of benzyloxycarbonyl-*S*-benzyl-L-cysteine *p*-nitrophenyl ester[9] in 20 m*l* of dimethylformamide. The solution was then allowed to stand overnight at room temperature and then diluted with 500 m*l* of water. The oil separated was extracted twice with 60 m*l* portions of ethyl acetate, and the combined ethyl acetate solution was washed successively with a 3% sodium bicarbonate solution, N hydrochloric acid and water, dried over sodium sulfate, and then evaporated *in vacuo*. When the oily residue was treated with

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[7] F. Weygand and K. Hunger, *Chem. Ber.*, **95**, 1 (1962).

[8] The use of hydrogen bromide in acetic acid for the removal of the protecting group from the protected peptides containing hydroxy-amino acid causes acetylation of the hydroxy function. This type of acetylation had been observed in the syntheses of serine peptides (E. D. Nicolaides and H. A. Dewald, *J. Org. Chem.*, **28**, 1926 (1963)) and of threonine peptides (J. M. Stewart and D. W. Woolley, *Biochemistry*, **3**, 700 (1964)) relating to bradykinin.

[9] M. Bodansky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959).

petroleum ether, it was obtained as a semi-solid mass. It weighed 9.72 g.

***S*-Benzyl-L-cysteinyl-L-tyrosine Ethyl Ester Hydrobromide (II).** To a suspension of 9.72 g (18 mmol) of I in a mixture of 1.0 m*l* of anisole and 25 m*l* of acetic acid, was added 50 m*l* of 3 N hydrogen bromide in acetic acid. After it had stood for one hour at room temperature, the solution was evaporated to dryness *in vacuo*. The oily residue was washed several times with ether by decantation and dried in a desiccator with calcium chloride and sodium hydroxide. It weighed 8.18 g; R_f 0.85,[11] 0.82.[12]

Benzyloxycarbonyl-L-asparaginyl-*S*-benzyl-L-cysteinyl-L-tyrosine Ethyl Ester (III). To a solution of 8.72 g (*ca.* 18 mmol) of II in 20 m*l* of dimethylformamide, was added 3 m*l* of triethylamine followed by a solution of 6.54 g (16.9 mmol) of benzyloxycarbonyl-L-asparagine *p*-nitrophenyl ester[9] in 15 m*l* of dimethylformamide. After it had stood overnight at room temperature, the solution was diluted with 700 m*l* of water. The crystalline product deposited was collected by filtration and washed successively with a 3% sodium bicarbonate solution, N hydrochloric acid and water (8.0 g). It was recrystallized from dimethylformamide-water. Yield, 7.26 g (66%); mp 148—152°C; $[\alpha]_D^{20}$ $-37.2°$ (*c* 1.07, dimethylformamide).

Found: C, 61.32; H, 6.06; N, 8.47%. Calcd for $C_{33}H_{38}O_8N_4S$: C, 60.91; H, 5.89; N, 8.61%.

L-Asparaginyl-*S*-benzyl-L-cysteinyl-L-tyrosine Ethyl Ester Hydrobromide (IV). To a suspension of 3.91 g (6 mmol) of III in a mixture of 0.5 m*l* of anisole and 10 m*l* of acetic acid, was added 20 m*l* of 3 N hydrogen bromide in acetic acid. After it had stood for one hour at room temperature, the solution was evaporated to dryness in vacuo. The oily residue was obtained as described in the preparation of II. It weighed 3.44 g; R_f 0.85,[11] 0.80.[12]

***p*-Methoxybenzyloxycarbonyl-L-serine Hydrazide (V).** To a suspension of 6.24 g (40 mmol) of L-serine methyl ester hydrochloride[13] in 160 m*l* of ethyl acetate, was added 6.20 m*l* (44 mmol) of triethylamine, and the heterogeneous solution was vigorously stirred for one hour. Triethylamine hydrochloride was filtered off, and the filtrate was concentrated to a volume of about 80 m*l* in vacuo. To this was added 9.12 g (44 mmol) of *p*-methoxybenzyloxycarbonyl azide[7] and the solution was then allowed to stir for 2 days at 35°C and washed with N citric acid and water, dried over sodium sulfate, and then evaporated *in vacuo*. The oily residue was dissolved in 8 m*l* of dimethylformamide, and 16 m*l* (320 mmol) of hydrazine hydrate was added thereto. The solution was allowed to stand for 2 days at room temperature. After the solution in which crystals partially deposited was kept *in vacuo* at 40°C in order to remove excess hydrazine, the mixture was diluted with 200 m*l* of water. After it had been kept in a refrigerator for several hours, the crystals deposited were collected by filtration and washed with cold water (4.74 g). It was recrystallized from methanol-ether. Yield, 3.71 g (33%); mp 177—178°C; $[\alpha]_D^{20}$ $+7.0°$ (*c* 1.17, dimethylformamide).

Found: C, 50.97; H, 6.22; N, 14.86%. Calcd for $C_{12}H_{17}O_5N_3$: C, 50.88; H, 6.05; N, 14.38%.

***p*-Methoxybenzyloxycarbonyl-L-seryl-L-asparaginyl-*S*-benzyl-L-cysteinyl-L-tyrosine Ethyl Ester (VI).** To a chilled (−5°C) solution of 1.70 g (6 mmol) of V dissolved in a mixture of 20 m*l* of water, 1.5 m*l* of acetic acid and 12 m*l* of N hydrochloric acid, was added 3.3 m*l* of 2 N sodium nitrite. After it had stood for 5 min at −5°C, the solution was diluted with 170 m*l* of cold water. The azide separated as a viscous mass was extracted twice with 30 m*l* portions of ethyl acetate, and the combined ethyl acetate solution was washed with a 3% sodium bicarbonate solution and water, and then dried over sodium sulfate at 0°C. The ethyl acetate solution of the azide was added to a chilled solution of 3.88 g (*ca.* 6.5 mmol) of IV dissolved in a mixture of 1.5 m*l* of triethylamine and 10 m*l* of dimethylformamide. The solution was allowed to stir for 2 days at 0°C and then concentrated *in vacuo* in order to remove ethyl acetate. The concentrated solution was diluted with 400 m*l* of water, and the precipitate was collected by filtration and washed successively with a 3% sodium bicarbonate solution, M citric acid and water (2.82 g). It was recrystallized from ethanol-ether-petroleum ether. Yield, 2.58 g (56%); mp 174—177°C; $[\alpha]_D^{20}$ $-25.5°$ (*c* 1.02, dimethylformamide).

Found: C, 58.28; H, 6.04; N, 8.89%. Calcd for $C_{37}H_{45}O_{11}N_5S$: C, 57.87; H, 5.91; N, 9.12%.

L-Seryl-L-asparaginyl-*S*-benzyl-L-cysteinyl-L-tyrosine Ethyl Ester Trifluoroacetate (VII). A solution of 3.14 g (4 mmol) of VI dissolved in a mixture of 1.6 m*l* of anisole and 16 m*l* of trifluoroacetic acid was allowed to stand for 5 min at 0°C and then evaporated to dryness *in vacuo*. The oily residue solidified quickly by adding ether. Yield, 2.80 g; R_f 0.79.[11]

Benzyloxycarbonyl-*S*-benzyl-L-cysteinylglycine Ethyl Ester (VIII). This compound was prepared by the same procedure as described for the preparation of III. Six and fifty-two hundredths grams (14 mmol) of benzyloxycarbonyl-*S*-benzyl-L-cysteine *p*-nitrophenyl ester and 1.88 g (14 mmol) of glycine ethyl ester hydrochloride afforded 5.48 g of the crude product. It was recrystallized from ethyl acetate-petroleum ether. Yield, 5.30 g (88%); mp 98—99°C, lit.[14] 98—99°C; $[\alpha]_D^{20}$ $-42.2°$ (*c* 1.0, dimethylformamide).

Found: C, 61.20; H, 6.20; N, 6.40%. Calcd for $C_{22}H_{26}O_5N_2S$: C, 61.38; H, 6.09; N, 6.51%.

***S*-Benzyl-L-cysteinylglycine Ethyl Ester Hydrobromide (IX).** This compound was prepared by the same procedure as described for the preparation of II. Treatment of 2.58 g (6 mmol) of VIII with hydrogen bromide in acetic acid gave an oily product. It weighed 2.07 g; R_f 0.88,[11] 0.80,[12] 0.63.[15]

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[10] B. Iselin, M. Feurer and R. Schwyzer (*Helv. Chim. Acta*, **38**, 1508 (1955)) obtained this compound as an analytically pure solid.

[11] The R_f of the thin-layer chromatography with Merck silica gel G refers to the *n*-butanol-acetic acid-pyridine-water (4 : 1 : 1 : 2 v/v) system.

[12] The R_f of the thin-layer chromatography with Merck silica gel G refers to the *t*-butanol-formic acid-water (75 : 15 : 10 v/v) system.

[13] E. Fischer and W. A. Jacobs, *Ber.*, **39**, 2949 (1906).

[14] This compound had been synthesized by the isocyanate method and by the phosphazo method (S. Goldschmidt and J. Christian, *Chem. Ber.*, **86**, 1116 (1953)).

[15] The R_f of the paper chromatography refers to the *n*-butanol-acetic acid-pyridine-water (4 : 1 : 1 : 2 v/v) system.

***p*-Methoxybenzyloxycarbonyl-L-threonine Hydrazide (X).** This compound was prepared by the same procedure as described for the preparation of V. An oily *p*-methoxybenzyloxycarbonyl-L-threonine methyl ester, which was prepared from 6.21 g (30 mmol) of *p*-methoxybenzyloxycarbonyl azide and 3.99 g (30 mmol) of L-threonine methyl ester,[16] was treated with 15 m*l* (300 mmol) of hydrazine hydrate to afford crystalline product (3.41 g). It was recrystallized from methanol-ether-petroleum ether. Yield, 3.06 g (33%); mp 194—195°C; $[\alpha]_D^{20}$ $+10.2°$ (*c* 1.12, dimethylformamide).

Found: C, 52.35; H, 6.49; N, 14.09%. Calcd for $C_{13}H_{19}O_5N_3$: C, 52.51; H, 6.44; N, 14.13%.

***p*-Methoxybenzyloxycarbonyl-L-threonyl-*S*-benzyl-L-cysteinylglycine Ethyl Ester (XI).** This compound was obtained by the same procedure as described for the preparation of VI. Two and eighty-five hundredths grams (9.6 mmol) of X and 3.94 g (*ca.* 10.5 mmol) of IX afforded 3.56 g of the crude product. It was recrystallized from ethyl acetate-petroleum ether. Yield, 3.34 g (62%); mp 134—136°C; $[\alpha]_D^{20}$ $-16.8°$ (*c* 1.01, dimethylformamide).

Found: C, 57.50; H, 6.27; N, 7.63%. Calcd for $C_{27}H_{35}O_8N_3S$: C, 57.74; H, 6.28; N, 7.48%.

***p*-Methoxybenzyloxycarbonyl-L-threonyl-*S*-benzyl-L-cysteinylglycine Hydrazide (XII).** A solution of 3.34 g (5.94 mmol) of XI and 6 m*l* (120 mmol) of hydrazine hydrate in 12 m*l* of dimethylformamide was allowed to stand for 2 days at room temperature. The solution was then evaporated in vacuo in order to remove excess hydrazine, after which 200 m*l* of water was added to the residual solution; the resulting crystals were collected by filtration (2.92 g). It was recrystallized from dimethylformamide-ether. Yield, 2.76 g (90%); mp 214—215°C; $[\alpha]_D^{20}$ $-20.8°$ (*c* 1.08, dimethylformamide).

Found: C, 54.54; H, 6.16; N, 12.88%. Calcd for $C_{25}H_{33}O_7N_5S$: C, 54.84; H, 6.08; N, 12.79%.

***p*-Methoxybenzyloxycarbonyl-L-threonyl-*S*-benzyl-L-cysteinylglycyl-L-seryl-L-asparaginyl-*S*-benzyl-L-cysteinyl-L-tyrosine Ethyl Ester (XIII).** To a chilled (−5°C) solution of 1.92 g (3.5 mmol) of XII in 20 m*l* of dimethylformamide, was added 9 m*l* of N hydrochloric acid, followed by 1.9 m*l* of 2 N sodium nitrite. After it had stood for 5 min at −5°C, the solution was diluted with 200 m*l* of water. The precipitated azide was collected by filtration and washed with a 3% sodium bicarbonate solution and water, and then dried *in vacuo* at 0°C to a constant weight. The azide was added to a chilled (−5°C) solution of 2.50 g (*ca.* 3.5 mmol) of VII in a mixture of 20 m*l* of dimethylformamide and 0.55 m*l* of triethylamine. The solution was allowed to stir for 2 days at 0°C and then diluted with 250 m*l* of water. The precipitate was collected by filtration and washed successively with a 3% sodium bicarbonate solution, 0.5 M citric acid and water (3.02 g). It was recrystallized from dimethylformamide-ether. Yield, 2.82 g (72%); mp 210—212°C (decomp.); $[\alpha]_D^{20}$ $-21.8°$ (*c* 0.55, dimethylformamide).

Found: C, 55.99; H, 5.93; N, 10.01%. Calcd for $C_{53}H_{66}O_{15}N_8S_2 \cdot H_2O$: C, 55.97; H, 6.03; N, 9.86%.

L-Threonyl-*S*-benzyl-L-cysteinylglycyl-L-seryl-L-asparaginyl-*S*-benzyl-L-cysteinyl-L-tyrosine Ethyl Ester Trifluoroacetate (XIV). This compound was obtained by the same procedure as described for the preparation of VII. Treatment of 2.24 g (2 mmol) of XIII with trifluoroacetic acid in the presence of anisole afforded the crystalline product. Yield, 2.04 g (96%); mp 174—176°C, $[\alpha]_D^{20}$ $-20.2°$ (*c* 0.55, dimethylformamide), R_f 0.80.[11]

Found: N, 10.62%. Calcd for $C_{46}H_{59}O_{14}N_8S_2F_3$: N, 10.49%.

Benzyloxycarbonyl-L-asparaginyl-L-tyrosine Ethyl Ester (XV). This compound was prepared by the same procedure as described for the preparation of III. Eleven and sixty hundredths grams (30 mmol) of benzyloxycarbonyl-L-asparagine *p*-nitrophenyl ester and 6.90 g (30 mmol) of L-tyrosine ethyl ester hydrochloride afforded 7.48 g of the crude product. It was recrystallized from dimethylformamide-water. Yield, 6.20 g (47%); mp 174—175°C; $[\alpha]_D^{20}$ $+1.9°$ (*c* 1.05, dimethylformamide).

Found: C, 60.38; H, 6.03; N, 9.17%. Calcd. for $C_{23}H_{27}O_7N_3$: C, 60.38; H, 5.95; N, 9.19%.

L-Asparaginyl-L-tyrosine Ethyl Ester Hydrobromide (XVI). This compound was prepared by the same procedure as described for the preparation of II. Treatment of 4.43 g (10 mmol) of XV with hydrogen bromide in acetic acid afforded an oily product. It weighed 3.62 g; R_f 0.78,[11] 0.71,[12] 0.61.[15]

Benzyloxycarbonyl-*S*-benzyl-L-cysteinyl-L-asparaginyl-L-tyrosine Ethyl Ester (XVII). This compound was prepared by the same procedure as described for the preparation of III. Three and seventy-two hundredths grams (8 mmol) of benzyloxycarbonyl-*S*-benzyl-L-cysteine *p*-nitrophenyl ester and 3.44 g (*ca.* 8.8 mmol) of XVI gave 4.95 g of the crystalline product. It was recrystallized from dimethylformamide-water. Yield, 4.59 g (88%); mp 154—155°C; $[\alpha]_D^{20}$ $-22.6°$ (*c* 0.98, dimethylformamide).

Found: C, 60.41, H, 5.91; N, 8.33%. Calcd for $C_{33}H_{38}O_8N_4S$: C, 60.91; H, 5.89; N, 8.61%.

***S*-Benzyl-L-cysteinyl-L-asparaginyl-L-tyrosine Ethyl Ester Hydrobromide (XVIII).** This compound was prepared by the same procedure as described for the preparation of II. Treatment of 4.02 g (6.5 mmol) of XVII with hydrogen bromide in acetic acid gave an oil. It weighed 3.68 g; R_f 0.80,[11] 0.75,[12] 0.82.[15]

Benzyloxycarbonyl-L-alanyl-*S*-benzyl-L-cysteinyl-L-asparaginyl-L-tyrosine Ethyl Ester (XIX). This compound was prepared by the same procedure as described for the preparation of III. Two and seventeen hundredths grams (6.5 mmol) of benzyloxycarbonyl-L-alanine *p*-nitrophenyl ester[17] and 3.86 g (*ca.* 7 mmol) of XVIII afforded 3.25 g of the crude product. It was recrystallized from dimethylformamide-water. Yield, 3.10 g (68%); mp 184—187°C; $[\alpha]_D^{20}$ $-14.6°$ (*c* 0.91, dimethylformamide).

Found: C, 59.66; H, 6.02; N, 9.45%. Calcd for $C_{36}H_{43}O_9N_5S$: C, 59.90; H, 6.00; N, 9.70%.

Benzyloxycarbonyl-L-alanyl-*S*-benzyl-L-cysteinyl-L-asparaginyl-L-tyrosine Hydrazide (XX). This compound was obtained by the same procedure as described for the preparation of XII. Two and eighty-two hundredths grams (4 mmol) of XIX and 4.0 m*l* (80 mmol) of hydrazine hydrate gave 2.58 g of the crude product. It was recrystallized from

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16) J. Degraw, L. Goodman, B. Weinstein and B. R. Baker, *J. Org. Chem.*, **27**, 576 (1962).

dimethylformamide-ether. Yield, 2.46 g (87%) ; mp 255—257°C (decomp.); $[\alpha]_D^{20}$ −29.8° (*c* 1.13, dimethylformamide).

Found: C, 57.20; H, 5.88; N, 13.62%. Calcd for $C_{34}H_{41}O_8N_7S$: C, 57.69; H, 5.84; N, 13.85%.

Benzyloxycarbonyl-L-alanyl-*S*-benzyl-L-cysteinyl-L-asparaginyl-L-tyrosyl-L-threonyl-*S*-benzyl-L-cysteinylglycyl-L-seryl-L-asparaginyl-L-benzyl-L-cysteinyl-L-tyrosine Ethyl Ester (XXI). The azide derived at −5°C from 1.06 g (1.5 mmol) of XX by the action of 1.6 m*l* of N sodium nitrite in a mixture of 4 m*l* of N hydrochloric acid and 15 m*l* of dimethylformamide was precipitated by dilution with 200 m*l* of water, collected by filtration, washed with a 3% sodium bicarbonate solution and water, and then dried *in vacuo* at 0°C. The azide was then added to a chilled (−5°C) solution of 1.60 g (1.5 mmol) of XIV dissolved in a mixture of 0.24 m*l* of triethylamine and 12 m*l* of dimethylformamide, and the solution was allowed to stir for 2 days at 0°C. The solution was evaporated *in vacuo* and the resulting gelatinous residue was triturated with water. The crystalline product was collected by filtration and washed with a 3% sodium bicarbonate solution, 0.5 M citric acid and water (1.73 g). It was recrystallized from dimethylformamide-ether. Yield, 1.62 g (66%); mp 246—247°C (decomp.); $[\alpha]_D^{20}$ −27.3° (*c* 0.55, dimethylformamide).

Found: C, 56.26; H, 5.77; N, 10.98%. Calcd for $C_{78}H_{95}O_{20}N_{13}S_3 \cdot 2H_2O$: C, 56.20; H, 5.99; N, 10.92%.

Benzyloxycarbonyl-L-alanyl-*S*-benzyl-L-cysteinyl-L-asparaginyl-L-tyrosyl-L-threonyl-*S*-benzyl-L-cysteinylglycyl-L-seryl-L-asparaginyl-*S*-benzyl-L-cysteinyl-L-tyrosine (XXII). To a solution of 0.163 g (0.1 mmol) of XXI in 2 m*l* of dimethylformamide, was added 0.2 m*l* of 2 N sodium hydroxide. After 4.5 hr, the solution was acidified with 0.3 m*l* of 2 N hydrochloric acid and then diluted with 15 m*l* of water. The gelatinous product was collected by centrifugation and washed with water (0.11 g). It was recrystallized from dimethylformamide-ether. Yield, 0.085 g (53%); mp 150—155°C; $[\alpha]_D^{20}$ −23.6° (*c* 0.5, dimethylformamide), R_f 0.77.11)

Found: C, 54.95; H, 6.05; N, 11.28%. Calcd for $C_{76}H_{91}O_{20}N_{13}S_3 \cdot 2H_2O$: C, 55.69; H, 5.84; N, 11.11%.

L-Alanyl-*S*-benzyl-L-cysteinyl-L-asparaginyl-*S*-tyrosyl-L-threonyl-*S*-benzyl-L-cysteinylglycyl-L-seryl-L-asparaginyl-*S*-benzyl-L-cysteinyl-L-tyrosine (XXIII). Into the solution of 0.038 g (0.024 mmol) of XXII dissolved in a mixture of 0.2 m*l* of anisole and 2 m*l* of trifluoroacetic acid, a slow stream of hydrogen bromide was bubbled for 2 hr at 0°C and then for 0.5 hr at room temperature with exclusion of moisture. The solution was evaporated *in vacuo* and the oily residue was triturated with ether to afford the crystalline product. It was collected by filtration and washed with ether (0.030 g). The purity was ascertained by paper chromatography, R_f 0.65.15) This product was used as such for the leucine aminopeptidase digestion.

The Digestion of XXIII by Leucine Aminopeptidase. The digestion by leucine aminopeptidase was carried out by the procedure of Hofmann and Yajima.18) The two dimensional paper chromatography was applied using the solvent systems of *n*-butanol-acetic acid-pyridine-water (4:1:1:2 v/v) and 80% pyridine. The paper chromatography of the digest revealed the presence of only seven ninhydrin-positive components with R_f values identical with those of authentic samples of *S*-benzylcysteine, tyrosine, alanine, threonine, serine, glycine and asparagine, respectively.

We wish to express our thanks to Dr. K. Takahashi, Professor T. Ando and Professor F. Egami of the University of Tokyo for their continuing interest during this work. We wish also to thank Kyowa Hakko Co., Ltd. and Ajinomoto Co., Inc. for supplying some amino acids.

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