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Ribonuclease T_1 Peptides. II. Synthesis of a Protected Pentapeptide Corresponding to Sequence 12—16

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A protected pentapeptide corresponding to sequence 12—16 of ribonuclease T_1, namely benzyloxycarbonyl-L-seryl-L-seryl-L-seryl-β-*t*-butyl-L-aspartyl-L-valine ethyl ester (XI), was synthesized by coupling of benzyloxycarbonyl-seryl-serine azide with a tripeptide ester, seryl-β-*t*-butyl-aspartyl-valine ethyl ester, which is derived from benzyloxycarbonyl-seryl-β-*t*-butyl-aspartyl-valine ethyl ester by the hydrogenolysis. The hydrogenolysis and the subsequent treatment with trifluoroacetic acid of the protected pentapeptide ester (XI) yielded a neutral pentapeptide ester, seryl-seryl-seryl-aspartyl-valine ethyl ester, as a crystalline product.

The synthesis of the protected *N*-terminal undecapeptide (1—11) of ribonuclease T_1 containing asparagine residue in the 3rd position was previously reported.[1] In the present paper, we will describe the synthesis of the protected pentapeptide corresponding to sequence 12—16, that is benzyloxycarbonyl-L-seryl-L-seryl-L-seryl-β-*t*-butyl-L-aspartyl-L-valine ethyl ester (XI).

This pentapeptide sequence possesses several functional groups, three hydroxyl ones of serine and an acidic one of aspartic acid, in a rather short peptide, so the preparation of this peptide derivatives seemed to be interesting as an object of organic synthesis.

In the synthesis of the protected pentapeptide (XI), we first selected a route involving the coupling of benzyloxycarbonyl-seryl-seryl-serine azide derived from the corresponding hydrazide (VI) with β-*t*-butyl-aspartyl-valine ethyl ester (VIII). However, the coupling reaction did not afford the desired product (XI); it was found that the product isolated was benzyloxycarbonyl-seryl-seryl-serine amide. In this connection, it would be noteworthy that several workers have reported the formation of acylamino acid or peptide amide from the corresponding azide during the peptide synthesis.[2]

The successful synthesis of the desired product (XI) was performed through the sequence of reactions as shown in Fig. 1. Benzyloxycarbonyl-seryl-serine ethyl ester (II) was prepared by coupling benzyloxycarbonyl-serine azide obtained from the corresponding hydrazide[3] using isoamyl nitrite[4] with serine ethyl ester (I). The dipeptide ester (II) obtained was converted to the hydrazide (III) by the treatment with hydrazine. Benzyloxycarbonyl-β-*t*-butyl-aspartyl-valine ethyl ester (VII) was prepared by coupling benzyloxycarbonyl-β-*t*-butyl-aspartic acid[5] with valine ethyl ester[6] using dicyclohexylcarbodiimide[7] as a coupling reagent or by the mixed anhydride method.[8] The oily product (VII) was hydrogenated to yield the dipeptide ester hydrochloride (VIII·HCl), which was then coupled with benzyloxycarbonyl-serine azide derived from the corresponding hydrazide[3] to give the protected tripeptide ester (IX). This protected peptide ester (IX) was subjected to hydrogenolysis to yield the tripeptide ester (X), which was coupled with benzyloxycarbonyl-seryl-serine azide derived from III as the same manner used for the preparation of II. This coupling gave the desired protected pentapeptide ester (XI) as a crystalline product.

Thus we could prepare the protected pentapeptide ester (XI) by coupling of benzyloxycarbonyl-dipeptide azide derived from III with the tripeptide ester (X). In this connection, it should

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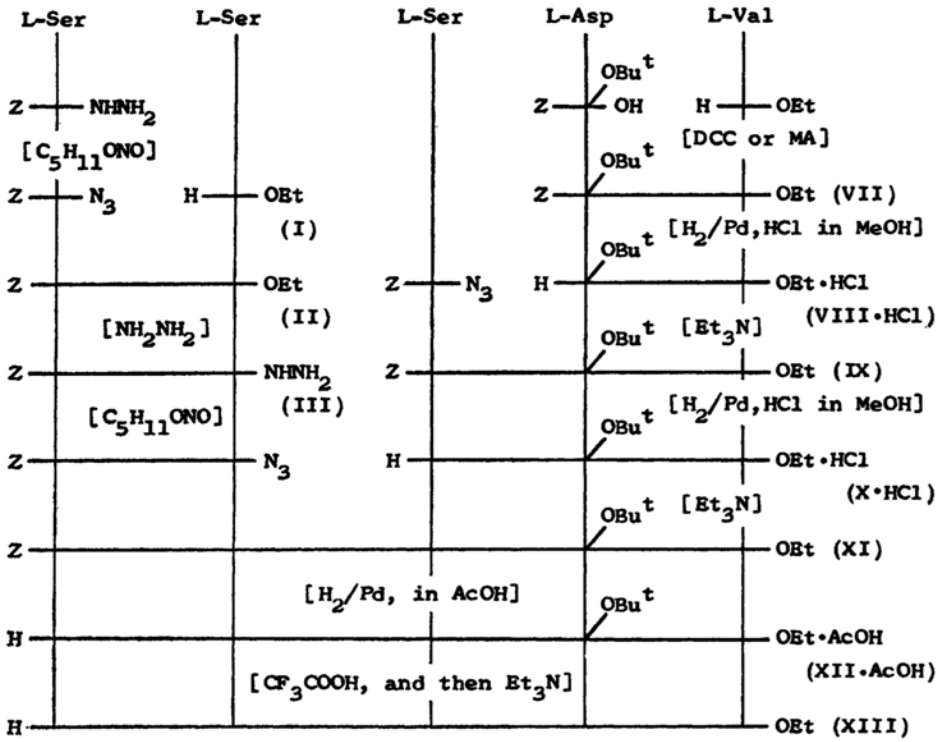

Fig. 1. Schematic diagram of synthesis of the pentapeptide ester. Z-, Benzyloxycarbonyl; -OBu^t, t-butyl ester; DCC, dicyclohexylcarbodiimide; MA, mixed anhydride method.

be pointed out that Hofmann *et al.*[9] reported the successful synthesis of the protected hexapeptide t-butyl ester (sequence 15—20 of ribonuclease A) by coupling of benzyloxycarbonyl-seryl-serine azide with threonyl-seryl-alanyl-alanine t-butyl ester and Scoffone *et al.*[10] similarly prepared the protected pentapeptide ester (sequence 16—20 of ribonuclease A) from benzyloxycarbonyl-seryl-threonine azide and seryl-alanyl-alanine t-butyl ester.

The protected pentapeptide ester (XI) was hydrogenated in a solvent of acetic acid to remove the benzyloxycarbonyl group, and the pentapeptide ester acetate (XII·AcOH) obtained was then treated with trifluoroacetic acid to remove the β-t-butyl group. The resulting pentapeptide ethyl ester (XIII) was obtained as a crystalline product. The homogeneity of the peptide (XIII) was ascertained by paper and thin-layer chromatography and the expected molar ratio of the constituent amino acids was observed in the acid hydrolysate of XIII by the amino acid analysis. Furthermore, the stereospecific purity of the peptide (XIII) was ascertained by the digestion with leucine aminopeptidase.

9) K. Hofmann, W. Hass, M. J. Smithers and G. Zanetti, *ibid.*, **87**, 631 (1965).
10) E. Scoffone, F. Marchiori, R. Rocchi, G. Vidali, A. M. Tamburro, A. Scatturin and A. Marzotto, *Tetrahedron Letters*, **9**, 943 (1966).

Experimental

All the melting points are uncorrected. The paper and thin-layer chromatographies were carried out on Toyo Roshi No. 52 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.

L-Serine Ethyl Ester p-Toluenesulfonate (I·TsOH). This compound was prepared according to the general procedure of Kato *et al.*[6] A suspension of L-serine (10.51 g) and p-toluenesulfonic acid monohydrate (19.02 g) in a mixture of ethanol (60 ml) and carbon tetrachloride (300 ml) was refluxed for 48 hr, and the water liberated was removed as an azeotropic mixture. The reaction mixture was then evaporated to dryness *in vacuo* to give an oily product; yield, 30.66 g (100%); R_f 0.67.[11]

Benzyloxycarbonyl-L-seryl-L-serine Ethyl Ester (II). To a chilled (−5°C) solution of benzyloxycarbonyl-serine hydrazide[3] (5.06 g, 20 mmol) in dimethylformamide (60 ml) containing 12.12 ml of 3.3 N hydrogen chloride in ethyl acetate, there were stirred isoamyl nitrite[4] (2.34 g, 20 mmol). After 10 min, triethylamine (5.6 ml, 40 mmol) was added. To the solution was added a mixture of I·TsOH (6.11 g, 20 mmol) and

11) The R_f value of the thin-layer chromatography with Merck silica gel refers to a solvent system of n-butanol - acetic acid - pyridine - water (4 : 1 : 1 : 2, v/v).

triethylamine (2.8 ml, 20 mmol) in dimethylformamide (40 ml). The reaction mixture was then stirred for 2 days at 0°C and evaporated *in vacuo*. The residue was dissolved in ethyl acetate (120 ml), and the solution was washed successively with a 4% sodium bicarbonate solution, 3% hydrochloric acid and water, dried over sodium sulfate, and then evaporated *in vacuo*. The crystalline residue was collected with the aid of ether-petroleum ether. It was recrystallized from ethyl acetate-petroleum ether; yield, 5.0 g (71%); mp 127—129°C; $[\alpha]_D^{22}$ −8.3° (*c* 1, methanol); R_f 0.82.[11]

Found: C, 54.10; H, 6.26; N, 7.95%. Calcd for $C_{16}H_{22}O_7N_2$: C, 54.23; H, 6.26; N, 7.91%.

Benzyloxycarbonyl-L-seryl-L-serine Hydrazide (III). A solution of II (3.18 g, 9 mmol) and hydrazine hydrate (1.09 ml, 22.5 mmol) in ethanol (20 ml) was allowed to stand overnight at room temperature. The excess hydrazine was evaporated *in vacuo*, and the resulting crystals were collected by filtration with the aid of cold ethanol. It was recrystallized from hot water; yield, 1.96 g (64%); mp 221—222°C (decomp.); $[\alpha]_D^{25}$ −30.0° (*c* 1, N hydrochloric acid); R_f 0.75.[11] Hofmann *et al.* had prepared this compound (III) previously from benzyloxycarbonyl-seryl-serine methyl ester with hydrazine; mp 221—222°C (decomp.); $[\alpha]_D^{20}$ −30.5° (*c* 1, N hydrochloric acid).[9]

Benzyloxycarbonyl-L-seryl-L-seryl-L-serine Ethyl Ester (IV). III (1.26 g, 3.7 mmol) in dimethylformamide (8 ml) containing 2.24 ml of 3.3 N hydrogen chloride in ethyl acetate was treated with isoamyl nitrite (432 mg, 3.7 mmol) for 10 min, and then triethylamine (1.03 ml, 7.4 mmol) as described above. To this solution was added a mixture of I·TsOH (1.13 g, 3.7 mmol) and triethylamine (0.51 ml, 3.7 mmol) in dimethylformamide (20 ml). The mixture was stirred for 3 days at 0°C and then evaporated *in vacuo* to a small volume. The precipitate which formed upon the addition of water was collected (the mother liquor was set aside for further isolation of IV), washed with a small amount of 4% sodium bicarbonate solution, 3% hydrochloric acid and water, and dried; yield, 0.43 g.

The mother liquor was extracted with chloroform, and the extract was washed with water, and dried. The filtrate was evaporated, and the residual syrup was solidified by the addition of ether and petroleum ether; yield, 0.56 g. The combined product (0.99 g) was recrystallized from methanol - ether - petroleum ether; yield, 0.90 g (56%); mp 184—185°C; R_f 0.77.[11]

Found: C, 50.95; H, 6.24; N, 9.30%. Calcd for $C_{19}H_{27}O_9N_3 \cdot \frac{1}{2}H_2O$: C, 50.66; H, 6.27; N, 9.33%.

Benzyloxycarbonyl - L - seryl - L - seryl - L - serine Methyl Ester (V). To a chilled solution of III (0.96 g, 2.8 mmol) in a mixture of glacial acetic acid (10 ml) and water (15 ml), there were stirred 6 N hydrochloric acid (1.13 ml) and a 0.4 M sodium nitrite solution (7.05 ml). After 5 min, the azide was extracted with ethyl acetate (100ml), and the organic layer was washed with a 4% sodium bicarbonate solution and water, and dried. The filtrate was added to a mixture of serine methyl ester hydrochloride[12] (0.44 g, 2.8 mmol) and triethylamine (0.4 ml) in dimethylformamide (20 ml). The mixture was stirred for 2 days at 0°C and evaporated *in vacuo*. The precipitate which formed upon the addition of 2% hydrochloric acid was collected, and washed with water and dried; yield, 0.37 g (30%); mp 195—196°C; R_f 0.73.[11]

Found: C, 50.76; H, 6.06; N, 9.79%. Calcd for $C_{18}H_{25}O_9N_3$: C, 50.58; H, 5.90; N, 9.83%.

Benzyloxycarbonyl - L - seryl - L - seryl - L - serine Hydrazide (VI). To a solution of IV (358 mg, 0.81 mmol) in methanol (6 ml), hydrazine hydrate (0.16 ml, 3.24 mmol) was added and the solution was allowed to stand for 2 days at 30°C. The reaction mixture was then evaporated *in vacuo*. The resulting crystals were collected with the aid of cold ethanol; yield, 327 mg (92%); mp 232—234°C (decomp.); R_f 0.68.[11]

Found: C, 47.48; H, 5.99; N, 16.06%. Calcd for $C_{17}H_{25}O_8N_5$: C, 47.77; H, 5.90; N, 16.39%.

VI was also prepared from V (261 mg, 0.61 mmol) and hydrazine hydrate (0.59 ml) by the same procedure as described above; yield, 157 mg (60%); mp 233—234°C (decomp.); R_f 0.68.[11]

Benzyloxycarbonyl-β-*t*-butyl-L-aspartyl-L-valine Ethyl Ester (VII). *a) Mixed Anhydride Method.* To a chilled solution of benzyloxycarbonyl-aspartic acid β-*t*-butyl ester[5] (404 mg, 1.25 mmol) and triethylamine (0.175 ml) in tetrahydrofuran (6 ml), isobutyl-chloroformate (0.164 ml, 1.25 mmol) was added. After 15 min, a mixture of valine ethyl ester *p*-toluenesulfonate[6] (397 mg, 1.25 mmol), triethylamine (0.175 ml) and chloroform (3 ml) was added to the solution. The reaction mixture was allowed to stand overnight and then evaporated to dryness *in vacuo*. The residual oil was dissolved in ethyl acetate (15 ml), and the solution was washed successively with 0.5 M citric acid, a 4% sodium bicarbonate solution and water, dried over sodium sulfate, and then evaporated to dryness *in vacuo*. The product was obtained as an oil; yield, 483 mg (86%); R_f 0.94.[11]

b) Dicyclohexylcarbodiimide Method. To a solution of the dicyclohexylammonium salt of benzyloxycarbonyl-aspartic acid β-*t*-butyl ester[5] (4.04 g, 8 mmol), valine ethyl ester *p*-toluenesulfonate[6] (2.54 g, 8 mmol) in chloroform (80 ml) was added dicyclohexylcarbodiimide[7] (1.65 g, 8 mmol) at 0°C. The reaction mixture was stirred for 1 hr at 0°C and then kept overnight in a refrigerator. The mixture was evaporated *in vacuo*, and ethyl acetate (100 ml) was added to the residue. After the insoluble dicyclohexylurea was filtered off, the filtrate was treated following the same procedure as described above; yield of the oily VII, 3.17 g (88%); R_f 0.94.[11]

β-*t*-Butyl-L-aspartyl-L-valine Ethyl Ester Hydrochloride (VIII·HCl). A solution of VII (3.38 g, 7.5 mmol) in methanol (20 ml) was hydrogenated in the presence of palladium black at 0°C. After 5 min, 0.408 N methanolic hydrogen chloride (18.38 ml) was added and hydrogenolysis was continued for 2 hr at 0°C. The filtrate from the catalyst was evaporated to dryness *in vacuo* at room temperature, and the yield of the oily product was 2.44 g (92 %). The oily product was crystallized upon the standing in a refrigerator for several days. A portion (352 mg) of this crystal was recrystallized from methanol-ether; yield, 226 mg; mp 147—148°C; R_f 0.77.[11] This sample was dried over phosphorus pentoxide for 2 hr at 45°C and 2 mmHg for the analysis.

Found: C, 50 40; H, 8 24; N, 7.95%. Calcd for

12) S. Guttmann and R. A. Boissonnas, *Helv. Chim. Acta*, **41**, 1867 (1958).

$C_{15}H_{28}O_5N_2 \cdot HCl \cdot \frac{1}{4}H_2O$: C, 50.41; H, 8.04; N, 7.83%.

Benzyloxycarbonyl-L-seryl-β-t-butyl-L-aspartyl-L-valine Ethyl Ester (IX). This compound was obtained by the same procedure as described for the preparation of II. Benzyloxycarbonyl-serine hydrazide (1.77 g, 7 mmol) and VIII·HCl (2.47 g, 7 mmol) afforded 2.28 g of the crude product. It was recrystallized from methanol - ether - petroleum ether; yield, 2.07 g (55%); mp 134—137°C; $[\alpha]_D^{25}$ −29.0° (*c* 1, methanol); R_f 0.96.[11]

Found: C, 57.96; H, 7.37; N, 8.01%. Calcd for $C_{26}H_{39}O_9N_3$: C, 58.08; H, 7.31; N, 7.82%.

L-Seryl-β-t-butyl-L-aspartyl-L-valine Ethyl Ester Hydrochloride (X·HCl). IX (1.85 g, 3.4 mmol) was subjected to hydrogenolysis in the presence of palladium black using 0.408 N methanolic hydrogen chloride (8.48 m*l*) as has been described in the case of VIII·HCl. The product was obtained as an oil; yield, 1.41 g (94%); R_f 0.76,[11] 0.94.[13]

Benzyloxycarbonyl-L-seryl-L-seryl-L-seryl-β-t-butyl-L-aspartyl-L-valine Ethyl Ester (XI). This compound was prepared by the same procedure as described for the preparation of II. III (1.17 g, 3.4 mmol) and X·HCl (1.50 g, 3.4 mmol) gave 2.14 g of the crystalline product. It was recrystallized from methanol-ether; yield, 1.62 g (67%); mp 174—175°C; $[\alpha]_D^{25}$ −26.4° (*c* 0.3, methanol); R_f 0.91.[11]

Found: C, 52.74; H, 6.96; N, 9.63%. Calcd for $C_{32}H_{49}O_{13}N_5 \cdot H_2O$: C, 52.66; H, 7.04; N, 9.60%.

L-Seryl-L-seryl-L-seryl-β-t-butyl-L-aspartyl-L-valine Ethyl Ester Acetate (XII·AcOH). XI (107 mg, 0.15 mmol) in acetic acid (5 m*l*) was subjected to hydrogenolysis in the presence of palladium black at 0°C. The filtrate from the catalyst was evaporated to dryness *in vacuo* at room temperature and the oily residue was triturated with ether. The hygroscopic powder was collected by filtration; yield, 91 mg (95%). The homogeneity was ascertained by the chromatographies; R_f 0.74,[11] 0.83,[13] 0.24.[14]

L-Seryl-L-seryl-L-seryl-L-aspartyl-L-valine Ethyl Ester (XIII). XII·AcOH (73 mg) was dissolved in trifluoroacetic acid (2.5 m*l*) and the solution was left to stand for 30 min at room temperature. The solution was evaporated to dryness *in vacuo* to yield a hygroscopic solid. The residue was dissolved in water and the solution was neutralized with the addition of triethylamine (*ca.* 0.025 m*l*). Then, the solution was evaporated to dryness and the remaining crystals were collected by filtration with the aid of ethanol; yield, 40 mg (67%); mp 182—183°C; $[\alpha]_D^{25}$ −22.7° (*c* 0.15, acetic acid); R_f 0.51,[13] 0.02;[14] amino acid ratios in acid hydrolysate, $Ser_{2.9}Asp_{1.0}Val_{1.1}$.[15]

Found: C, 44.57; H, 6.80; N, 12.90%. Calcd for $C_{20}H_{35}O_{11}N_5 \cdot H_2O$: C, 44.52; H, 6.91; N, 12.98%.

The digestion of XIII by leucine aminopeptidase was carried out by the procedure of Hofmann and Yajima.[16] The paper chromatography[13] of the digest revealed the presence of only three ninhydrine-positive components with R_f values identical with those of authentic samples of aspartic acid (R_f 0.20), serine (0.28) and valine (0.58).

13) The R_f value of the paper chromatography refers to the same solvent system described.[11]

14) The R_f value of the thin-layer chromatography with Merck silica gel refers to a solvent system of chloroform-methanol (5 : 1, v/v).

15) The authors are indebted to Mr. Kosaku Noda for the amino acid analysis.

16) K. Hofmann and H. Yajima, *J. Am. Chem. Soc.*, **83**, 2289 (1961).