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Ribonuclease T_1 Peptides. IV. Synthesis of a Protected Heptapeptide Corresponding to Sequence 17—23

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A protected heptapeptide corresponding to sequence 17—23 of ribonuclease T_1, namely benzyloxycarbonyl-L-seryl-L-threonyl-L-alanyl-L-glutaminyl-L-alanyl-L-alanyl-glycine ethyl ester (XIV), was synthesized by two routes. One consisted of the coupling of benzyloxycarbonyl-seryl-threonine azide with a pentapeptide ester, alanyl-glutaminyl-alanyl-alanyl-glycine ethyl ester. The other consisted of the coupling of benzyloxycarbonyl-seryl-threonyl-alanine azide with a tetrapeptide ester, glutaminyl-alanyl-alanyl-glycine ethyl ester. These four peptide segments were built up by the method of stepwise synthesis. Optical rotations, melting points and chromatographic behavior of XIV prepared by these two routes were found to be identical. Stereochemical homogeneity of the protected heptapeptide ester XIV was established by the digestion of the deblocked heptapeptide ester with leucine aminopeptidase.

Syntheses of the protected *N*-terminal undecapeptide (1—11) of ribonuclease T_1 containing asparagine residue in the 3rd position,[1] the protected pentapeptide (12—16)[2] and the heptapeptide (24—30)[3] were previously reported. In the present paper, we will describe the synthesis of the protected heptapeptide corresponding to the sequence 17—23, that is Z–Ser–Thr–Ala–Gln–Ala–Ala–Gly–OEt (XIV).[4] This heptapeptide sequence contains several functional groups, two hydroxys of serine and threonine and an acid amide of glutamine, in a rather short peptide, so the preparation of this compound seems to be interesting as an object of organic synthesis of a peptide.

The sequence of reaction for the synthesis of the desired compound XIV is shown in Fig. 1. The syntheses of the two C-terminal peptide segments, H–Gln–Ala–Ala–Gly–OEt·HCl (VI) and H–Ala–Gln–Ala–Ala–Gly–OEt·HCl (VIII), were achieved follows. Z–Ala–Ala–Gly–OEt (III) was prepared by coupling of H–Ala–Gly–OEt[5] with benzyloxycarbonyl-alanine *p*-nitrophenyl ester or a mixed

1) M. Ohno, T. Kato, N. Mitsuyasu, M. Waki, S. Makisumi and N. Izumiya, This Bulletin, **40**, 204 (1967).

2) M. Waki, N. Mitsuyasu, T. Kato, S. Makisumi and N. Izumiya, *ibid.*, **41**, 669 (1968).

3) T. Kato, N. Mitsuyasu, M. Waki, S. Makisumi and N. Izumiya, *ibid.*, **41**, 2480 (1968).

4) The following abbreviations are used; Z-, benzyloxycarbonyl; –OEt, ethyl ester; –ONp, *p*-nitrophenyl ester; –OBzl, benzyl ester; $-N_2H_3$, hydrazide; $-N_3$, azide; MA, mixed anhydride method; DMF, dimethylformamide; DMSO, dimethyl sulfoxide. Amino acid symbols donote the L-configuration.

5) W. Grassmann and E. Wünsch, *Chem. Ber.*, **91**, 449 (1958).

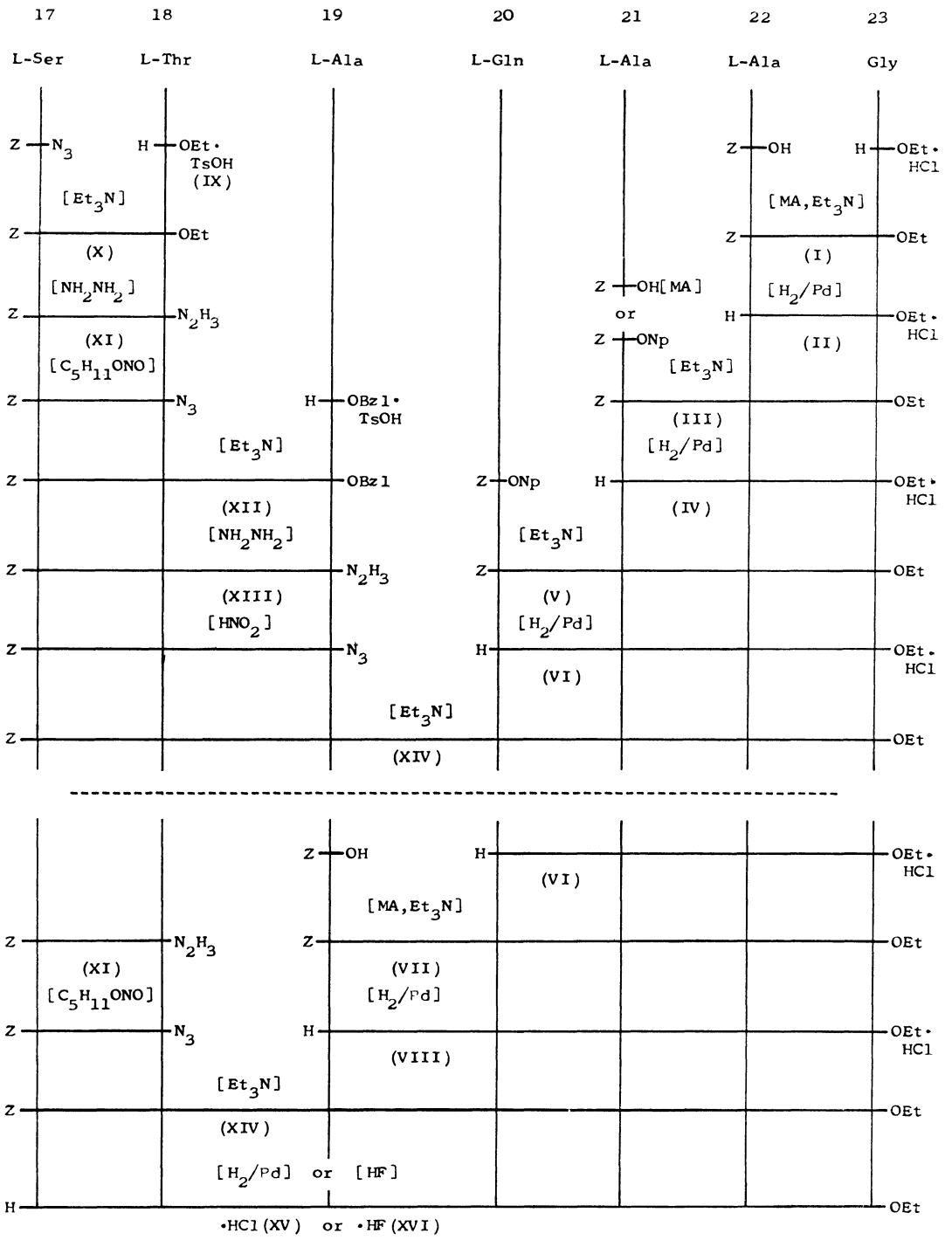

Fig. 1. Schematic diagram of synthesis of the heptapeptide ester.

anhydride[6] of benzyloxycarbonyl-alanine. The compound III was catalytically hydrogenated to yield the tripeptide ester hydrochloride (IV), which was then coupled with benzyloxycarbonyl-glutamine *p*-nitrophenyl ester. The protected tetrapeptide (V) obtained was suspended in methanol containing an equivalent hydrogen chloride and then hydrogenated to afford the tetrapeptide ester hydrochloride (VI) in a good yield. Z-Ala-Gln-

6) J. R. Vaughan, Jr. and R. L. Osato, *J. Amer. Chem. Soc.*, **73**, 5553 (1951).

Ala–Ala–Gly–OEt (VII) was prepared by coupling of benzyloxycarbonyl-alanine with VI by the mixed anhydride method in DMF because of poor solubility of VII. Compound VII thus obtained was hydrogenated as described for the preparation of VI to give the pentapeptide ester hydrochloride (VIII).

Synthesis of Z–Ser–Thr–N_2H_3 (XI) was reported previously.[7] Another *N*-terminal peptide segment, Z–Ser–Thr–Ala–N_2H_3 (XIII) was prepared as follows. The azide derived from the corresponding hydrazide XI was coupled with alanine benzyl ester to afford the acyl tripeptide ester, Z–Ser–Thr–Ala–OBzl (XII). The product obtained was converted to the hydrazide (XIII) by treatment with 2 equivalent of hydrazine.

For the synthesis of the desired protected heptapeptide ester, Z–Ser–Thr–Ala–Gln–Ala–Ala–Gly–OEt (XIV), two routes were developed (Fig. 1). The acyldipeptide azide derived from XI using isoamyl nitrite[8] was allowed to react with the pentapeptide ester (VIII) in DMF to give the desired protected heptapeptide ester (XIV) as a crystalline product. Compound XIV was obtained by condensation of the acyltripeptide azide derived from XIII with the tetrapeptide ester (VI) in a solvent of DMF. The optical rotation, melting point and chromatographic behavior of XIV prepared by these two routes were found to be identical.

For decarbobenzoxylation of the protected heptapeptide ester XIV, an attempt was made to perform catalytic hydrogenation of XIV in the presence of an equivalent of hydrogen chloride in methanol or DMF, but pure heptapeptide ester could not be obtained because of the insoluble character of XIV in an ordinary solvent. When XIV was subjected to hydrogenolysis in the presence of an equivalent of ethanolic hydrogen chloride in hexamethyl phosphoramide,[9] pure crystalline heptapeptide ester hydrochloride (XV) could be obtained. Thus, it was found that hexamethyl phosphoramide was a useful solvent for the hydrogenation of a sparingly soluble compound. The decarbobenzoxylation of XIV was performed by treatment of anhydrous hydrogen fluoride,[10] and pure crystalline heptapeptide ester hydrofluoride (XVI) was isolated. Homogeneity of the peptides, XV and XVI, was ascertained by thin-layer chromatography and by amino acid analysis on the acid hydrolysates of XV and XVI. Stereospecific homogeneity of the peptide XV was ascertained by digestion with leucine aminopeptidase.

Experimental

Melting points were uncorrected. Most of the optical rotations were measured on a Yanagimoto Photometric Polarimeter, OR-20 type, and some on a JASCO Model DIP–SL. Prior to analysis, samples were dried over phosphorus pentoxide at 60°C and 2 mmHg to a constant weight. Paper chromatography was carried out on Toyo Roshi No. 52 chromatography paper with the following solvent systems; R_f^{I}, *n*-butanol–acetic acid–pyridine–water, 4: 1: 1: 2 v/v. Thin-layer chromatography was carried out on Merck silica gel G with the following solvent systems; R_f^{1}, *n*-butanol–acetic acid–pyridine–water, 4: 1: 1: 2 v/v; R_f^{2}, *s*-butanol–formic acid–water, 75: 15: 10 v/v; R_f^{3}, chloroform–methanol, 5: 1 v/v; R_f^{4}, *n*-butanol–acetic acid–water, 4: 1: 2 v/v. Spots of materials possessing a free amino group on chromatograms were detected by spraying ninhydrin, and those of the amino group-blocked materials, by spraying 47% hydrobromic acid and then ninhydrin.

Z-Ala-Ala-Gly-OEt (III). (*a*) *Mixed Acid Anhydride Method.*[6] To a chilled solution of benzyloxycarbonyl-alanine (4.68 g, 21 mmol) and triethylamine (2.94 m*l*, 21 mmol) in tetrahydrofuran (40 m*l*), isobutyl chloroformate (2.75 m*l*, 21 mmol) was added. After 15 min, a chilled mixture of H–Ala–Gly–OEt·HCl[5] (II) (4.42 g, 21 mmol), triethylamine (2.94 m*l*, 21 mmol) and chloroform (40 m*l*) was added to the solution. The reaction mixture was stirred for one hour at 0°C, allowed to stand overnight at room temperature, and then evaporated to dryness *in vacuo*. The residual oil was extracted with ethyl acetate, and the solution was washed successively with a 3% sodium bicarbonate solution, 0.5 M citric acid and water, and dried over sodium sulfate. The filtered solution was concentrated *in vacuo* and the residual oil was crystallized after addition of ether and petroleum ether (5.0 g). It was recrystallized from ethanol–ether. Yield, 4.42 g (55%); mp 174—177°C; R_f^{1} 0.85; $[\alpha]_D^{15}$ −5.6° (*c* 2, DMF).

Found: C, 57.08; H, 6.43; N, 11.17%. Calcd for $C_{18}H_{25}O_6N_3$: C, 56.98; H, 6.64; N, 11.08%.

(*b*) *Active Ester Method.*[11] To a solution of II (0.84 g, 4 mmol) in a mixture of triethylamine (0.62 m*l*, 4 mmol) and DMF (5 m*l*), a solution of benzyloxycarbonyl-alanine *p*-nitrophenyl ester (1.38 g, 4 mmol) in DMF (5 m*l*) was added. The reaction mixture was allowed to stand overnight at room temperature, evaporated *in vacuo* and then extracted with ethyl acetate. The solution was treated by the same procedure as described above. Yield, 1.00 g (66%); mp 176—177°C; R_f^{1} 0.87; $[\alpha]_D^{15}$ −5.2° (*c* 2, DMF).

H-Ala-Ala-Gly-OEt·HCl (IV). A solution of III (2.66 g, 7 mmol) in a mixture of 0.52 N ethanolic hydrogen chloride (14.8 m*l*, 7.7 mmol) and ethanol (80 m*l*) was hydrogenated in the presence of palladium black. After 5 hr the filtrate from the catalyst was evaporated *in vacuo* and the residual oil was crystallized after addition

7) N. Mitsuyasu, M. Waki, S. Makisumi and N. Izumiya, *Memoirs of the Faculty of Science, Kyushu University, Ser. C. Chemistry*, **6**, 145 (1969).

8) J. Honzl and J. Rudinger, *Collect. Czech. Chem. Commun.*, **26**, 2333 (1961); E. Wünsch and A. Zwick, *Chem. Ber.*, **99**, 101 (1966).

9) U.S. 2487859 (1949), Eastman Kodak Co.; a product of Japan Oil Seal Industries Co. was used.

10) S. Sakakibara, Y. Shimonishi, M. Okada and Y. Kishida, "Peptides," Proc. 8th European Peptide Symp., ed. by H. C. Beyermann *et al.*, North Holland Publishing Co., Amsterdam (1967), p. 44; S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada and H. Sugihara, This Bulletin, **40**, 2164 (1967).

11) M. Bodanszky, *Nature*, **175**, 685 (1955).

of ethyl acetate and ether. Yield, 1.88 g (95%); mp 187—188°C; R_f^1 0.57; R_f^I 0.70; $[\alpha]_D^{20}$ −2.8° (*c* 1, DMF).

Found: C, 42.58; H, 7.13; N, 14.60%. Calcd for $C_{10}H_{19}O_4N_3 \cdot HCl$: C, 42.63; H, 7.16; N, 14.91%.

Z-Gln-Ala-Ala-Gly-OEt (V). To a solution of IV (3.10 g, 11 mmol) in a mixture of triethylamine (1.68 m*l*, 12 mmol) and DMF (15 m*l*), was added a solution of benzyloxycarbonyl-glutamine *p*-nitrophenyl ester (4.42 g, 11 mmol) in DMF (5 m*l*). After it had been stood overnight at room temperature, the reaction mixture was diluted with 200 m*l* of water. The crystalline product deposited was collected by filtration and washed successively with a 3% sodium bicarbonate solution, 0.5 N citric acid and water (4.12 g). It was recrystallized from DMF-ethanol-ether. Yield, 4.04 g (73%); mp 223—225°C; R_f^1 0.75; $[\alpha]_D^{15}$ −18.0° (*c* 1, DMSO).

Found: C, 54.66; H, 6.64; N, 13.71%. Calcd for $C_{23}H_{33}O_8N_5$: C, 54.43; H, 6.55; N, 13.80%.

H-Gln-Ala-Ala-Gly-OEt·HCl (VI). A suspension of V (2.54 g, 5 mmol) in a mixture of methanol (75 m*l*) and 0.48 N methanolic hydrogen chloride (11.7 m*l*, 5.5 mmol) was hydrogenated in the presence of palladium black. After 7 hr the filtrate from the catalyst was concentrated *in vacuo* and the residual oil was crystallized after addition of ether. Yield, 1.82 g (89%); mp 176—179°C; R_f^1 0.56; R_f^I 0.58; $[\alpha]_D^{15}$ −6.3° (*c* 0.24, DMF).

Found: C, 43.44; H, 6.92; N, 16.62%. Calcd for $C_{15}H_{27}O_6N_5 \cdot HCl \cdot 1/2H_2O$: C, 43.01; H, 6.98; N, 16.72%.

Z-Ala-Gln-Ala-Ala-Gly-OEt (VII). To a chilled solution of benzyloxycarbonyl-alanine (0.89 g, 4 mmol) and triethylamine (0.56 m*l*) in tetrahydrofuran (5 m*l*), isobutyl chloroformate (0.52 m*l*, 4 mmol) was added. After 15 min, a precooled solution of VI (1.64 g, 4 mmol) in a mixture of triethylamine (0.56 m*l*) and DMF (25 m*l*) was added to the above solution. The reaction mixture was stirred for one hour at 0°C, allowed to stand overnight at room temperature and then concentrated *in vacuo*. Water (100 m*l*) was added to the residual oil and the crystalline product deposited was collected by filtration and washed as described for V (1.58 g). It was recrystallized from DMF-methanol-ether. Yield, 1.47 g (62%); mp 254—255°C; R_f^1 0.78; R_f^2 0.68; $[\alpha]_D^{15}$ −20.0° (*c* 1, DMSO).

Found: C, 53.38: H, 6.85; N, 14.50%. Calcd for $C_{26}H_{38}O_9N_6 \cdot 1/2H_2O$: C, 53.14; H, 6.69; N, 14.30%.

H-Ala-Gln-Ala-Ala-Gly-OEt·HCl (VIII). A suspension of VII (0.29 g, 0.5 mmol) in a mixture of DMF (10 m*l*) and 0.48 N methanolic hydrogen chloride (1.14 m*l*, 0.55 mmol) was hydrogenated in the presence of palladium black. After 6 hr the filtrate from the catalyst was evaporated *in vacuo* and the residual oil was crystallized after the addition of ethanol and ether. Yield, 0.23 g (97%); mp 206—208°C; R_f^1 0.52; $[\alpha]_D^{15}$ −17.0° (*c* 1, DMSO).

Found: C, 43.46; H, 7.16; N, 16.80%. Calcd for $C_{18}H_{32}O_7N_6 \cdot HCl \cdot H_2O$: C, 43.33; H, 7.07; N, 16.84%.

Z-Ser-Thr-Ala-OBzl (XII). To a chilled (−5°C) solution of Z-Ser-Thr-N_2H_3[7] (XI) (6.74 g, 19 mmol) in a mixture of 2 N hydrochloric acid (35 m*l*) and DMF (15 m*l*), N sodium nitrite (20 m*l*) was added. After it had been stood for 10 min, the precipitated azide was extracted with ethyl acetate (200 m*l*), washed with a 3% sodium bicarbonate solution and water, and then dried over sodium sulfate at 0°C. To the filtrate from the desiccant was added a precooled mixture of alanine benzyl ester *p*-toluenesulfonate[12] (8.08 g, 23 mmol) and triethylamine (3.22 m*l*, 23 mmol) in ethyl acetate (60 m*l*). After the reaction mixture had been stirred for 2 days at 0°C, it was washed successively with a 3% sodium bicarbonate solution, 0.5 M citric acid and water, dried over sodium sulfate, and then evaporated *in vacuo*. The residual oil was crystallized by the addition of ether and petroleum ether and the crystals were collected by filtration (5.11 g). It was recrystallized from ethanol-ether-petroleum ether. Yield, 4.82 g (51%); mp 149—150°C; R_f^1 0.87; R_f^3 0.49; R_f^4 0.95, $[\alpha]_D^{15}$ −7.0° (*c* 1, DMF).

Found: C, 59.47; H, 6.31; N, 8.28%. Calcd for $C_{25}H_{31}O_8N_3$: C, 59.84; H, 6.23; N, 8.38%.

Z-Ser-Thr-Ala-N_2H_3 (XIII). A solution of XII (2.50 g, 5 mmol) and hydrazine hydrate (0.485 m*l*, 10 mmol) in DMF (20 m*l*) was allowed to stand overnight at room temperature, and the reaction mixture was evaporated *in vacuo*. The residual oil was solidified by the addition of ether, and the crystals were collected by filtration (2.12 g). It was recrystallized from hot water. Yield, 1.17 g (60%); mp 212—214°C; R_f^1 0.77; R_f^3 0.36; R_f^4 0.77; $[\alpha]_D^{15}$ −9.5° (*c* 1, DMSO).

Found: C, 49.88; H, 6.48; N, 16.29%. Calcd for $C_{18}H_{27}O_7N_5 \cdot 1/2H_2O$: C, 49.76; H, 6.50; N, 16.12%.

Z-Ser-Thr-Ala-Gln-Ala-Ala-Gly-OEt (XIV). (*a*) *Condensation of Z-Ser-Thr-N_3 and H-Ala-Gln-Ala-Ala-Gly-OEt·HCl. (VIII).* To a chilled (−5°C) solution of XI (142 mg, 0.4 mmol) in a mixture of DMF (4 m*l*) and 0.45 N hydrogen chloride-tetrahydrofuran (1.79 m*l*) was added isoamyl nitrite[8] (0.054 m*l*, 0.4 mmol). After 10 min, triethylamine (0.112 m*l*, 0.8 mmol) was added. To the solution was added a precooled mixture of VIII (192 mg, 0.4 mmol), triethylamine (0.056 m*l*, 0.4 mmol) and DMF (10 m*l*). The reaction mixture was then stirred for 2 days at 0°C and then evaporated *in vacuo*. The residual oil was crystallized by the addition of water. The crystals were collected by filtration and washed as described for V (153 mg). It was recrystallized from DMSO-ethanol-ether. Yield, 138 mg (45%); mp 251—253°C; R_f^1 0.82; R_f^2 0.79; R_f^3 0.65; $[\alpha]_D^{20}$ −15.8° (*c* 1, DMSO).

Found: C, 50.46; H, 6.69; N, 14.15%. Calcd for $C_{33}H_{50}O_{13}N_8 \cdot H_2O$: C, 50.25; H, 6.55; N, 14.21%.

(*b*) *Condensation of Z-Ser-Thr-Ala-N_3 and H-Gln-Ala-Ala-Gly-OEt (VI).* To a chilled solution of XIII (213 mg, 0.5 mmol) in a mixture of acetic acid (0.5 m*l*), water (2 m*l*) and N hydrochloric acid (0.6 m*l*) was added N sodium nitrite (0.6 m*l*). After 10 min, the reaction mixture was diluted with water (20 m*l*). The precipitated azide was collected by filtration, washed with a 3% sodium bicarbonate solution and water, and then dried *in vacuo* at 0°C. To a chilled solution of VI (205 mg, 0.5 mmol) dissolved in a mixture of DMF (10 m*l*) and triethylamine (0.07 m*l*, 0.5 mmol) was added the azide prepared before. The solution was stirred for 2 days at 0°C and then diluted with water (30 m*l*). The precipitate was collected by filtration and washed as described for V (211 mg). It was recrystallized from DMSO-ethanol-ether. Yield, 196 mg (51%); mp 251—252°C; R_f^1 0.83; $[\alpha]_D^{20}$ −15.6° (*c* 1, DMSO).

Found: C, 50.34; H, 6.63; N, 14.11%. Calcd for $C_{33}H_{50}O_{13}N_8 \cdot H_2O$: C, 50.25; H, 6.65; N, 14.21%.

12) S. Makisumi and N. Izumiya, *Nippon Kagaku Zasshi*, **78**, 662 (1957).

H-Ser-Thr-Ala-Gln-Ala-Ala-Gly-OEt·HCl (XV). A solution of XIV (39 mg, 50 μmol) dissolved in hexamethyl phosphoramide[9] (6 m*l*) containing 3.4 N ethanolic hydrogen chloride (0.018 m*l*, 60 μmol) was hydrogenated in the presence of palladium black. After 24 hr, the solution was diluted with petroleum ether (50 m*l*) and an oil was precipitated. In order to remove hexamethyl phosphoramide completely, the resulting oil was washed twice with petroleum ether by decantation and then triturated with ethanol and ether to afford powder (32 mg). It was recrystallized from DMSO-ethanol-ether. Yield, 28 mg (83%); mp 220—225°C (decomp.); R_f^1 0.55; amino acid ratios in acid hydrolysate,[13] Thr 0.90, Ser 0.86, Glu 1.00, Gly 1.00, Ala, 3.33.

In order to establish the stereochemical homogeneity of XV, a small amount of XV was digested by leucine aminopeptidase following essentially the same procedure as that of Hofmann and Yajima;[14] amino acid ratios[13] in the digest, Thr+Gln 1.78, Ser 1.11, Gly 0.80, Ala 3.31.

H-Ser-Thr-Ala-Gln-Ala-Ala-Gly-OEt·HF (XVI). Compound XIV (39 mg, 50 μmol) was placed in a vessel together with a drop of anisole.[10] HF (1 m*l*) was added into the vessel using an HF-reaction apparatus,[10] and the mixture was allowed to react at 0°C for 1 hr with stirring. Excess HF was removed under reduced pressure at 0°C, and the residue was kept at reduced pressure (3 mmHg) for 5 hr at room temperature over calcium chloride and sodium hydroxide. The oily residue was washed with ethanol by decantation and then dissolved in DMSO. After the insoluble material in small amount was filtered off, the filtrate was evaporated *in vacuo*. The residual oil was crystallized with the addition of ethanol and the crystals were collected by filtration. Yield, 29 mg (92%); mp 235—240°C (decomp.); R_f^1 0.50; amino acid ratios in acid hydrolysate,[13] Thr 0.93, Ser 0.93, Glu 0.99, Gly 1.00, Ala 3.21.

13) We are indebted to Mr. K. Noda in this laboratory for the amino acid analysis.

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