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# Ribonuclease $T_1$ Peptides. III. Synthesis of a Protected Heptapeptide Corresponding to Sequence 24—30

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A protected heptapeptide corresponding to sequence 24—30 of ribonuclease $T_1$, namely benzyloxycarbonyl-*O*-benzyl-L-tyrosyl-L-glutaminyl-L-leucyl-L-histidyl-$\gamma$-*t*-butyl-L-glutamyl-$\beta$-*t*-butyl-L-aspartyl-glycine ethyl ester (XI), was synthesized by coupling of benzyloxycarbonyl-*O*-benzyl-L-tyrosyl-L-glutaminyl-L-leucine azide with a tetrapeptide ester, L-histidyl-$\gamma$-*t*-butyl-L-glutamyl-$\beta$-*t*-butyl-L-aspartyl-glycine ethyl ester, which is derived from $N^{\alpha}$,$N^{im}$-dibenzyloxycarbonyl tetrapeptide ester by the catalytic hydrogenolysis. These tri- and tetrapeptide components were prepared with stepwise syntheses free from racemization.

The syntheses of the protected *N*-terminal undecapeptide (1—11) of ribonuclease $T_1$ containing asparagine residue in the 3rd position and the protected pentapeptide (12—16) were previously reported.[1,2] In the present paper, we will describe the synthesis of the protected heptapeptide corresponding to sequence 24—30, that is benzyloxycarbonyl-*O*-benzyl-L-tyrosyl-L-glutaminyl-L-leucyl-L-histidyl-$\gamma$-*t*-butyl-L-glutamyl-$\beta$-*t*-butyl-L-aspartyl-glycine ethyl ester (XI).

It has been known that one or more histidine residue in ribonuclease $T_1$ molecule play an important role to the enzymatic activity.[3] Hence the organic synthesis of peptide fragment of the enzyme containing histidine was supposed to be valuable to study the relationship of the structure and function of the enzyme molecule. Moreover this heptapeptide sequence contains acidic, basic and phenolic functional groups in a rather short peptide, so the preparation of this compound seemed to be interesting as an object of organic synthesis of a peptide.

The scheme of reactions used for the synthesis of XI was shown in Fig. 1. Benzyloxycarbonyl-$\beta$-*t*-butyl-L-aspartyl-glycine ethyl ester (I) was prepared by coupling of benzyloxycarbonyl-$\beta$-*t*-butyl-L-aspartic acid dicyclohexylammonium salt[4] with glycine ethyl ester hydrochloride using dicyclohexylcarbodiimide[5] as a coupling reagent. Furnished semisolid of I was catalytically hydrogenated to yield the dipeptide ester hydrochloride (II), which was then coupled with benzyloxycarbonyl-$\gamma$-*t*-butyl-L-glutamic acid[6] in the same manner as used for I. The protected tripeptide derivative (III) obtained was subjected to hydrogenolysis and then coupled with $N^{\alpha}$,$N^{im}$-dibenzyloxycarbonyl-L-histidine.[7] Crystals of the tetrapeptide derivative (V) thus obtained were again hydrogenated and then used to obtain the required heptapeptide derivative. The preparation of the *N*-terminal tripeptide (IX) was carried out as follows: L-leucine ethyl ester was coupled with benzyloxycarbonyl-L-glutamine *p*-nitrophenyl ester[8] to furnish the protected dipeptide ester (VII), and after the hydrogenolysis of VII, the dipeptide ester (VIII) was coupled with benzyloxycarbonyl-*O*-benzyl-L-tyrosine *p*-nitrophenyl ester.[8] The tripeptide ester derivative (IX) obtained was converted to the corresponding hydrazide (X) by the treatment with hydrazine.

The tripeptide hydrazide (X) was converted to the corresponding azide, and the azide was coupled with the tetrapeptide ester (VI). The protected heptapeptide ester derivative (XI) obtained was catalytically hydrogenated to remove the benzyloxycarbonyl and *O*-benzyl protecting groups,

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) S. Yamagata, K. Takahashi and F. Egami, *J. Biochem.*, **52**, 261 (1962); F. Egami, *Nippon Kagaku Zasshi (J. Chem. Soc. Japan, Pure Chem. Sect.)*, **87**, 909 (1966).
4) R. Schwyzer and H. Dietrich, *Helv. Chim. Acta*, **44**, 2003 (1961); E. Wünsch and A. Zwick, *Z. Physiol. Chem.*, **328**, 235 (1962); E. Schröder and E. Klieger, *Ann.*, **673**, 208 (1964).
5) J. C. Sheehan and G. P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955).
6) R. Schwyzer and H. Kappeler, *Helv. Chim. Acta*, **44**, 1991 (1961).
7) F. Sakiyama, K. Okawa, J. Yamakawa and S. Akabori, This Bulletin, **31**, 926 (1958); A. Patchornik, A. Berger and E. Katchalsky, *J. Am. Chem. Soc.*, **79**, 6416 (1957).
8) M. Bodanszky and V. du Vigneaud, *ibid.*, **81**, 5688 (1959).

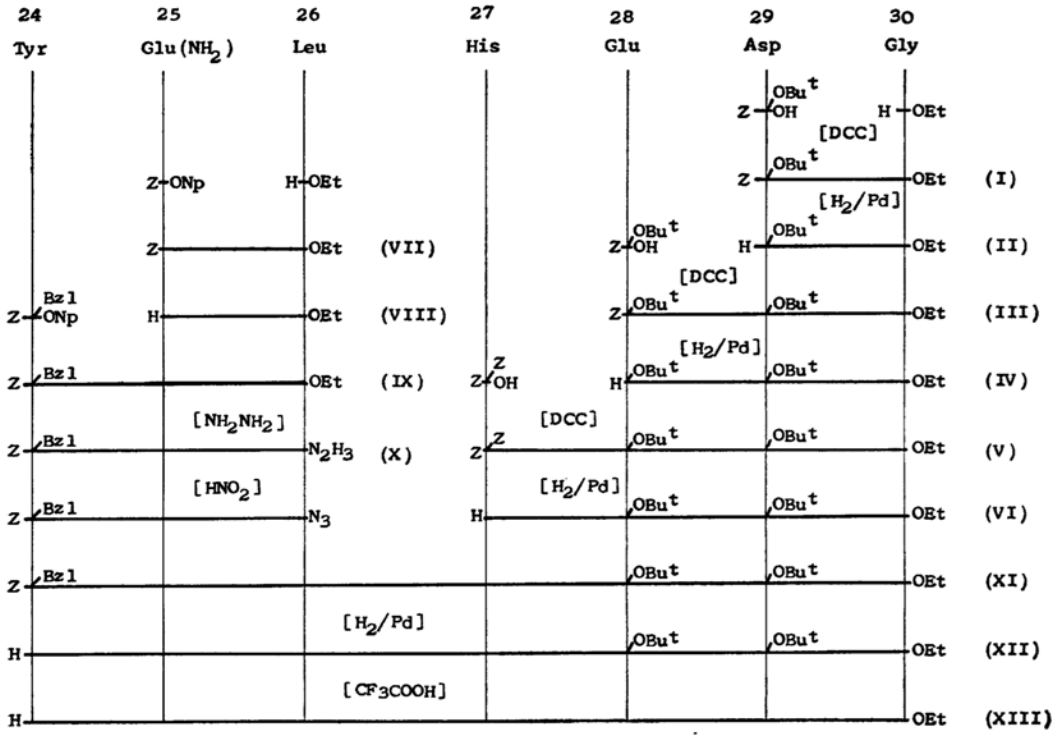


Fig. 1. Schematic diagram of synthesis of the heptapeptide ester. Z-, benzyloxycarbonyl; Bzl-, benzyl; -OBu$^t$, $t$-butyl ester; -ONp, $p$-nitrophenoxy ester; DCC, dicyclohexylcarbodiimide.

and the heptapeptide ester acetate (XII) obtained was then treated with trifluoroacetic acid to secure the heptapeptide ethyl ester trifluoroacetate (XIII). The peptide XIII was homogeneous as judged by the paper and thin-layer chromatography and its acid hydrolysate gave the theoretically expected amino acid ratios. Furthermore, the stereospecific purity of the peptide (XIII) was ascertained by the digestion with leucine aminopeptidase.

The syntheses of several peptides related to the ribonuclease $T_1$ enzyme are being continued in this laboratory.

## Experimental

The melting points were not corrected. The paper chromatography and thin-layer chromatography were carried out on Toyo Roshi No. 52 paper and 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. The developing solvent system most commonly used was $n$-butanol : acetic acid : pyridine : water (4 : 1 : 1 : 2). The optical rotations were measured on a Yanagimoto Photometric Polarimeter, OR-20 type. Prior to analysis, the samples were dried over anhydrous phosphorus pentoxide at 70°C and 2 mmHg to a constant weight.

**Benzyloxycarbonyl-$\beta$-$t$-butyl-L-aspartyl-glycine Ethyl Ester (I).** A suspension of benzyloxycarbonyl-$\beta$-$t$-butyl-L-aspartic acid dicyclohexylammonium salt[4] (5.047 g, 10 mmol) and glycine ethyl ester hydrochloride (1.396 g, 10 mmol) in chloroform (30 m$l$) was stirred for 30 min and the mixture was chilled to −5°C. Dicyclohexylcarbodiimide[5] (2.06 g, 10 mmol) was added to the mixture, the mixture was stirred for 2 hr at 0°C, and then it was allowed to stand overnight at 0°C. The mixture was then evaporated *in vacuo*, ethyl acetate (30 m$l$) was added to the residue, and the mixture was stored for several hours in a refrigerator. The dicyclohexylurea and dicyclohexylammonium hydrochloride deposited were filtered off (4.346 g, 99%), and the filtrate was washed successively with water, 10% citric acid and 4% sodium bicarbonate, and dried over sodium sulfate. The filtrate from the salt was evaporated and the residual semisolid was collected with the aid of a mixture of ether and petroleum ether (3.71 g, 91%). A portion of this was reprecipitated from ethyl acetate - ether - petroleum ether, and the semisolid obtained was analyzed; $R_f$ 0.90 on thin-layer.

Found: C, 58.43; H, 6.99; N, 7.26%. Calcd for $C_{20}H_{28}N_2O_7$: C, 58.81; H, 6.91; N, 6.86%.

**$\beta$-$t$-Butyl-L-aspartyl-glycine Ethyl Ester Hydrochloride (II·HCl).** A solution of I (3.26 g, 8 mmol) in ethanol (30 m$l$) was hydrogenated in the presence of palladium black at 0°C. After 15 min, 0.52 N hydrogen chloride in ethanol (13.1 m$l$) was added into the solution, and hydrogenolysis was continued for 4 hr at 0°C. The filtrate from the catalyst was evaporated *in vacuo* at low temperature; yield of the hygroscopic crystals, 2.49 g (100%); $R_f$ 0.95 on paper, 0.71 on thin-layer.

**Benzyloxycarbonyl-γ-*t*-butyl-L-glutamyl-β-*t*-butyl-L-aspartyl-glycine Ethyl Ester (III).** A mixture of benzyloxycarbonyl-γ-*t*-butyl-L-glutamic acid dicyclohexylammonium salt[6] (3.371 g, 6.5 mmol), II·HCl (2.03 g, 6.5 mmol), dicyclohexylcarbodiimide (1.339 g, 6.5 mmol) and chloroform (30 ml) was stirred for 2 hr at −5°C and then left to stand overnight at 0°C. The mixture was evaporated *in vacuo*, and ethyl acetate was added. After filtration, the ethyl acetate solution (*ca.* 100 ml) was washed with water, dilute acid and alkali as described for I, and dried. The filtrate from the salt was evaporated and the resulting crystals were collected by filtration with the aid of petroleum ether; yield, 3.438 g (90%); $R_f$ 0.92 on thin-layer. A portion (134 mg) of the crystals was recrystallized from ether-petroleum ether for analysis; 119 mg; mp 58—60°C; $[\alpha]_D^{30}$ −20.0° (*c* 1, dimethylformamide).

Found: C, 58.44; H, 7.39; N, 6.92%. Calcd for $C_{29}H_{43}N_3O_{10}$: C, 58.67; H, 7.30; N, 7.08%.

**γ-*t*-Butyl-L-glutamyl-β-*t*-butyl-L-aspartyl-glycine Ethy Ester Hydrochloride (IV·HCl).** A solution of III (3.002 g, 5.06 mmol) in ethanol (25 ml) was hydrogenated in the presence of palladium black and 0.52 N hydrogen chloride in ethanol (10.2 ml) as described for the preparation of II. The filtrate from the catalyst was evaporated *in vacuo*; yield of the oily product (IV·HCl), 2.717 g (109%); $R_f$ 0.97 on paper, 0.80 on thin-layer.

**$N^{\alpha}$,$N^{im}$-Dibenzyloxycarbonyl-L-histidyl-γ-*t*-butyl-L-glutamyl-β-*t*-butyl-L-aspartyl-glycine Ethyl Ester (V).** A mixture of $N^{\alpha}$,$N^{im}$-dibenzyloxycarbonyl-L-histidine[7] (2.27 g, 5 mmol), IV·HCl (the oily product from 5 mmol of III), triethylamine (0.73 ml), dicyclohexylcarbodiimide (1.03 g, 5 mmol) and chloroform (20 ml) was stirred for 2 hr at −5°C, allowed to stand overnight at 0°C, and evaporated *in vacuo*. Ethyl acetate (50 ml) was added to the residue, and the dicyclohexylurea deposited was filtered off (993 mg). The filtrate was washed with water, dilute citric acid and sodium bicarbonate solution as described previously. The solution dried was evaporated *in vacuo* and the residual oil was crystallized after the addition of ether and petroleum ether. Recrystallization from ethyl acetate - ether - petroleum ether gave 3.14 g (73%) of pure substance; $R_f$ 0.93 on thin-layer; mp 85—87°C; $[\alpha]_D^{17}$ −8.0° (*c* 1, dimethylformamide).

Found: C, 59.40; H, 6.65; N, 9.41%. Calcd for $C_{43}H_{56}N_6O_{13}$: C, 59.71; H, 6.53; N, 9.72%.

**L-Histidyl-γ-*t*-butyl-L-glutamyl-β-*t*-butyl-L-aspartyl-glycine Ethyl Ester Dihydrochloride (VI·2HCl).** A solution of V (2.112 g, 2.5 mmol) in ethanol (30 ml) was hydrogenated at 0°C in the presence of palladium black and 0.52 N hydrogen chloride in ethanol (8 ml) as described for the preparation of II·HCl. The filtrate from the catalyst was evaporated *in vacuo*; yield of the oily product (VI·HCl), 1.696 g (101%); $R_f$ 0.98 on paper, 0.70 on thin-layer.

**Benzyloxycarbonyl-L-glutaminyl-L-leucine Ethyl Ester (VII).** To a solution of L-leucine ethyl ester hydrochloride[9] (1.469 g, 7.5 mmol) dissolved in a mixture of triethylamine (1.16 ml, 8.5 mmol) and dimethylformamide (15 ml), a solution of benzyloxycarbonyl-L-glutamine *p*-nitrophenyl ester[8] (3.011 g, 7.5 mmol) in dimethylformamide (8 ml) was added. The solution was then allowed to stand overnight at room temperature and then poured into 225 ml of water. The crystalline product deposited was collected by filtration and washed successively with 3% sodium bicarbonate, M citric acid and water, and dried; 2.39 g. Recrystallization from ethanol - ether - petroleum ether gave 2.13 g (67%) of pure substance; $R_f$ 0.97 on thin-layer; mp 128—130°C; $[\alpha]_D^{20}$ −9.4° (*c* 1.0, dimethylformamide).

Found: C, 59.74; H, 7.33; N, 9.90%. Calcd for $C_{21}H_{31}N_3O_6$: C, 59.84; H, 7.41; N, 9.97%.

**L-Glutaminyl-L-leucine Ethyl Ester Hydrochloride (VIII·HCl).** To a solution of VII (2.11 g, 5 mmol) dissolved in methanol (30 ml) was added 0.72 N methanolic hydrogen chloride (7.63 ml, 5.5 mmol). The solution was hydrogenated in the presence of palladium black. After 6 hr the filtrate from the catalyst was evaporated to dryness *in vacuo*; yield of the oily product (VIII·HCl), 1.667 g (103%). The homogeneity of the oil was ascertained by paper and thin-layer chromatography; $R_f$ 0.80 on paper and 0.75 on thin-layer.

**Benzyloxycarbonyl-*O*-benzyl-L-tyrosyl-L-glutaminyl-L-leucine Ethyl Ester (IX).** To a solution of VIII·HCl (1.667 g, 5 mmol) dissolved in a mixture of triethylamine (0.78 ml, 5.5 mmol) and dimethylformamide (25 ml), a solution of benzyloxycarbonyl-*O*-benzyl-L-tyrosine *p*-nitrophenyl ester[8] (2.663 g, 5 mmol) in dimethylformamide (12 ml) was added. The solution was then allowed to stand overnight at room temperature and then poured into 200 ml of water. The crystalline product deposited was collected by filtration, washed and dried as described for VII. Recrystallization from methanol - ether - petroluem ether gave 2.725 g (86%) of IX; $R_f$ 0.98 on thin-layer; mp 210—211°C; $[\alpha]_D^{15}$ −16.2° (*c* 1, dimethylformamide).

Found: C, 65.83; H, 6.90; N, 8.25%. Calcd for $C_{37}H_{46}N_4O_8$: C, 65.86; H, 6.87; N, 8.30%.

**Benzyloxycarbonyl-*O*-benzyl-L-tyrosyl-L-glutaminyl-L-leucine Hydrazide (X).** A solution of IX (2.022 g, 3 mmol) and hydrazine hydrate (3 ml, 60 mmol) in dimethylformamide (12 ml) was allowed to stand for 2 days at room temperature. The solution was then evaporated *in vacuo* in order to remove excess hydrazine, and then 120 ml of water was added to the residual solution. The resulting crystals (1.932 g) were collected by filtration and recrystallized from dimethylformamide-ethanol to give 1.74 g of X (90%); $R_f$ 0.84 on thin-layer; mp 237°C; $[\alpha]_D^{15}$ −13.6° (*c* 1, dimethylformamide).

Found: C, 62.03; H, 6.76; N, 12.39%. Calcd for $C_{35}H_{44}N_6O_7 \cdot H_2O$: C, 61.93; H, 6.83; N, 12.38%.

**Benzyloxycarbonyl-*O*-benzyl-L-tyrosyl-L-glutaminyl-L-leucyl-L-histidyl-γ-*t*-butyl-L-glutamyl-β-*t*-butyl-L-aspartyl-glycine Ethyl Ester (XI).** Into a solution of X (1.652 g, 2.5 mmol) in a mixture of acetic acid (70 ml) and dimethylformamide (14 ml), there were added successively N hydrochloric acid (2.75 ml), sodium nitrite (190 mg) and N hydrochloric acid (2.75 ml) under stirring below −5°C. The faintly turbid solution changed to transparent in a few minutes. Then cold water (160 ml) was added to the solution, and the stirring was continued at 0°C for 30 min. The precipitate was then collected, washed successively with water, 4% sodium bicarbonate and water, and dried *in vacuo* at 0°C. The azide was then

9) F. Rohman, *Ber.*, **30**, 1978 (1897).

added to a chilled solution of VI·HCl (1.696 g, 2.5 mmol) in a mixture of dimethylformamide (40 m*l*) and triethylamine (0.73 m*l*). The solution was stirred for 3 days at 0°C, and diluted with 800 m*l* of cold water. The precipitate was collected, washed with 4% sodium bicarbonate, 10% citric acid and water, and dried; yield, 2.342 g. Recrystallization from methanol-dioxane-ether gave 2.11 g (69%) of XI; $R_f$ 0.85 on thin-layer; mp 213—214°C; $[\alpha]_D^{29}$ −21.1° (*c* 1, dimethylformamide).

Found: C, 59.71; H, 6.78; N, 10.94%. Calcd for $C_{62}H_{84}N_{10}O_{16}\cdot H_2O$: C, 59.88; H, 6.97; H, 11.26%.

**L-Tyrosyl-L-glutaminyl-L-leucyl-L-histidyl-L-glutamyl-L-aspartyl-glycine Ethyl Ester Ditrifluoroacetate (XIII·2CF$_3$COOH).** A solution of XI (50 mg, 40 $\mu$mol) in 80% acetic acid (1 m*l*) was subjected to hydrogenolysis in the presence of palladium black. After 1 hr, the filtrate from the catalyst was evaporated to dryness; yield of hygroscopic crystals (XII·2AcOH), 51 mg; $R_f$ 0.75 on thin-layer. XII·2AcOH (48 mg) was dissolved in trifluoroacetic acid (0.4 m*l*), and the solution was left to stand for 30 min and evaporated to dryness. The resulting crystals were collected by filtration with the aid of ether (46 mg); mp 180—185°C; $[\alpha]_D^{26}$ −10.0° (*c* 0.3, acetic acid); $R_f$ 0.80 on paper, 0.71 on thin-layer; amino acid ratios in acid hydrolysate, $Tyr_{1.0}$, $Leu_{1.2}$, $His_{0.9}$, $Glu_{2.2}$, $Asp_{0.9}$, $Gly_{0.9}$.

Found: C, 44.91; H, 5.67; N, 12.30%. Calcd for $C_{39}H_{56}N_{10}O_{14}\cdot 2C_2HO_2F_3\cdot 2H_2O$: C, 44.79; H, 5.42; N, 12.15%.

The digestion of XIII·HCl by leucine aminopeptidase was carried out by the procedure of Hofmann and Yajima.[10] The paper chromatography of the digest with a solvent system of *n*-butanol : acetic acid : water (4 : 1 : 2) revealed the presence of seven ninhydrin-positive components with $R_f$ values identical with those of authentic samples of aspartic acid ($R_f$, 0.25), histidine (0.28), glutamine (0.31), glycine (0.33), glutamic acid (0.36), tyrosine (0.54) and leucine (0.76).

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