The Synthesis of Cyclo-pentaglycyl-L-lysyl and Its Hydrolysis by Trypsin

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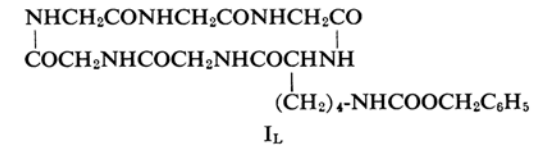
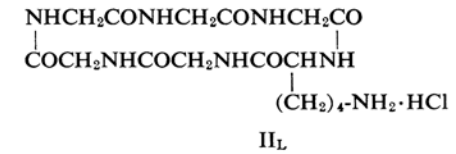
In a previous paper from this laboratory, it was shown that L-phenylalanyl- and L-valyl-L-lysine anhydrides are not hydrolyzed by trypsin at the lysine carbonyl linkage.[1] Although the reason why the dipeptide anhydrides are not susceptible to the enzyme is not yet clear, it is possible that a certain minimum ring size is necessary in order that a cyclic peptide can be hydrolyzed by the enzyme. From the literature, it appears that cyclic tri-, tetra- and pentapeptides are difficult to synthesize,[2] but cyclic hexapeptides have been prepared without difficulty.[2,3] Therefore, we attempted to synthesize a cyclic hexapeptide containing a lysine residue.

In the present investigations, the synthesis of cyclo-pentaglycyl-ε-benzyloxycarbonyl-L-lysyl (I_L) was studied in detail in an attempt to obtain fundamental information about the syntheses of cyclic peptides; then, cyclo-pentaglycyl-L-lysyl hydrochloride (II_L), which had been obtained by the hydrogenolysis of I_L, was tested by the trypsin.



I_L



II_L

1) N. Izumiya, T. Kato, Y. Fujita, M. Ohno and M. Kondo, This Bulletin, 37, 1809 (1964).
2) R. Schwyzer, *CIBA Foundation Symposium of Amino Acids and Peptides with Antimetabolic Activity*, 171 (1958).
3) T. Kato, M. Kondo, M. Ohno and N. Izumiya, This Bulletin, 38, 1202 (1965).

Various methods for the synthesis of cyclo-pentaglycyl-ε-benzyloxycarbonyllysyl were undertaken. Since the cyclization of a linear active ester gave a better yield than the other cyclization methods,[2] we first attempted to prepare the cyclic peptide by the active ester method. In the present series of syntheses of the cyclic peptide, a benzyloxycarbonyl group was used for the selective blocking of the ε-amino group of the lysine residue.

$$\text{TRI-Gly}_2\text{-OH} + \varepsilon\text{-Z-L-Lys-OEt}\cdot\text{TsOH} \xrightarrow[\text{M.A. 77\%}]{\text{NEt}_3}$$

$$\underset{\text{III}}{\text{TRI-Gly}_2\text{-}\varepsilon\text{-Z-L-Lys-OEt}} \xrightarrow{\text{HCl-MeOH}}$$

$$\underset{\text{IV}}{\text{H-Gly}_2\text{-}\varepsilon\text{-Z-L-Lys-OEt}\cdot\text{HCl}} \xrightarrow[\text{TRI-Gly}_3\text{-OH, M.A. 68\%}]{\text{NEt}_3}$$

$$\underset{\text{V}}{\text{TRI-Gly}_5\text{-}\varepsilon\text{-Z-L-Lys-OEt}} \xrightarrow[91\%]{\text{OH}^-\text{ and }\text{H}^+}$$

$$\underset{\text{VI}}{\text{TRI-Gly}_5\text{-}\varepsilon\text{-Z-L-Lys-OH}} \xrightarrow{\text{NPS, pyridine}}$$

$$\underset{\text{VII}_{PR}}{\text{TRI-Gly}_5\text{-}\varepsilon\text{-Z-Lys-ONP}} \xrightarrow{\text{CF}_3\text{COOH, H}_2\text{O}}$$

$$\underset{\text{VIII}_{PR}}{\text{H-Gly}_5\text{-}\varepsilon\text{-Z-Lys-ONP}\cdot\text{CF}_3\text{COOH}} \xrightarrow[33\%\text{ from VI}]{\text{pyridine}}$$

$$\varepsilon\text{-Z-}\underbrace{\text{Lys Gly}_5}_{\text{I}_{PR}} \xrightarrow[\text{HCl-MeOH}]{\text{H}_2/\text{Pd}} \underbrace{\text{Lys Gly}_5}_{\text{II}_{PR}}\cdot\text{HCl}$$

Fig. 1. Synthesis of cyclic hexapeptide via VII_{PR}: TRI, trityl; Z, benzyloxycarbonyl; TsOH, *p*-toluenesulfonic acid; NEt_3, triethylamine; M.A., mixed anhydride method; NPS, di-*p*-nitrophenyl sulfite; ONP, *p*-nitrophenoxy.

Figure 1 indicates the sequence of reactions via the linear hexapeptide *p*-nitrophenyl ester, in which ε-benzyloxycarbonyllysine was the *C*-terminal amino acid. The product obtained in a fairly good yield by the cyclization reaction of VIII_{PR} was found to be a monomer with a molecular weight in agreement with that of cyclic hexapeptide (I_{PR}). However, it was found that the lysine residue in the compound I_{PR} is partially racemized, since the specific rotation of I_{PR} was +0.5, whereas that of optically-pure cyclo-pentaglycyl-ε-benzyloxycarbonyl-L-lysyl (I_L) was +0.9. When calculations are based on the specific rotation, I_{PR} is found to contain about 78 per cent of the L-compound (I_L) and 22 per cent of the D-compound. Furthermore, II_{PR} derived from I_{PR} was not completely hydrolyzed by trypsin. The cyclo-pentaglycyllysyl which remained unchanged appeared to have a D-configuration (see Fig. 8). We assume that a partial racemization had taken place at the step of the *p*-nitrophenyl esterification of VI by di-*p*-nitrophenyl sulfite and pyridine, since Iselin and Schwyzer had reported that a partially-racemized benzyloxycarbonyl dipeptide *p*-nitrophenyl ester was obtained when benzyloxycarbonyl dipeptide was treated with di-*p*-nitrophenyl sulfite and pyridine.[4] Furthermore, since it has been reported that no racemization occurs when the benzyloxycarbonyl dipeptide *p*-nitrophenyl ester which was obtained from benzyloxycarbonyl amino acid and amino acid *p*-nitrophenyl ester is coupled with the amino acid ethyl ester,[5] no racemization may be expected at the step of the formation of I_{PR} from VIII_{PR} by intramolecular peptide bond formation. Therefore, a glycine or proline residue should be selected as the *C*-terminal amino acid of the *N*-blocked peptide in the case of its *p*-nitrophenyl esterification with di-*p*-nitrophenyl sulfite and a base.

$$\text{VI} \xrightarrow[71\%]{\text{AcOH, H}_2\text{O}} \underset{\text{IX}}{\text{H-Gly}_5\text{-}\varepsilon\text{-Z-L-Lys-OH}}$$

$$\xrightarrow[\text{less than 4.7\%}]{\text{DCC}} \text{I}_{PR} \text{ or } \text{I}_L$$

Fig. 2. Synthesis of cyclic hexapeptide with IX and DCC: DCC, dicyclohexylcarbodiimide.

Figure 2 indicates an attempt to cyclize pentaglycyl-ε-benzyloxycarbonyl-L-lysine with two equivalents of dicyclohexylcarbodiimide. The yields were, however, found to be very poor. Contrary to this study, syntheses of cyclic hexa-,[6] hepta-[7] and octapeptide[8] from the pertinent linear peptides with carbodiimide have been reported.

Figures 3 and 4 indicate attempts to cyclize the linear hexapeptide azides. Although it has been reported that cyclo-hexaglycyl is obtained in a good yield by dimerization reaction from triglycine azide,[9] both triglycyl-ε-benzyloxycarbonyl-L-lysylglycylglycine azide and pentaglycyl-ε-benzyloxycarbonyl-L-lysine azide gave only a very small amount of I_L. Sheehan and McGregor reported that the cyclization of tripeptide azide did not give the desired

4) B. Iselin and R. Schwyzer, *Helv. Chim. Acta*, **43**, 1760 (1960).
5) M. Goodman and K. C. Stueben, *J. Am. Chem. Soc.*, **81**, 3980 (1959).
6) K. D. Kopple and K. E. Nitecki, ibid., **83**, 4103 (1961); **84**, 4457 (1962); T. Wieland and K. W. Ohle, *Ann.*, **605**, 179 (1957).
7) R. O. Studer, W. Lergier and K. Vogler, *Helv. Chim. Acta*, **46**, 612 (1963); K. Vogler, R. O. Studer, P. Lanz, W. Lergier, E. Böhni and B. Fust, ibid., **46**, 2823 (1963).
8) K. Vogler, R. O. Studer, W. Lergier and P. Lanz, ibid., **43**, 1751 (1960); R. O. Studer, K. Vogler and W. Lergier, ibid., **44**, 131 (1961).
9) J. C. Sheehan and W. L. Richardson, *J. Am. Chem. Soc.*, **76**, 6329 (1954); **77**, 6391 (1955).

$$\text{BOC-Gly-OH} + \text{IV} \xrightarrow[88\%]{\text{DCC}}$$

$$\text{BOC-Gly}_3\text{-}\varepsilon\text{-Z-L-Lys-OEt} \xrightarrow[93\%]{\text{OH}^- \text{ and } \text{H}^+} \underset{\text{X}}{}$$

$$\text{BOC-Gly}_3\text{-}\varepsilon\text{-Z-L-Lys-OH} \xrightarrow[77\%]{\text{H-Gly}_2\text{-OBz, DCC}} \underset{\text{XI}}{}$$

$$\text{BOC-Gly}_3\text{-}\varepsilon\text{-Z-L-Lys-Gly}_2\text{-OBz} \xrightarrow[93\%]{\text{NH}_2\text{NH}_2} \underset{\text{XII}}{}$$

$$\text{BOC-Gly}_3\text{-}\varepsilon\text{-Z-L-Lys-Gly}_2\text{-NHNH}_2 \xrightarrow{\text{HCl-MeOH}} \underset{\text{XIII}}{}$$

$$\text{H-Gly}_3\text{-}\varepsilon\text{-Z-L-Lys-Gly}_2\text{-NHNH}_2\cdot 2\text{HCl} \xrightarrow{\text{HNO}_2}$$

$$\text{H-Gly}_3\text{-}\varepsilon\text{-Z-L-Lys-Gly}_2\text{-N}_3\cdot\text{HCl} \xrightarrow{\text{NaHCO}_3} \text{Trace of } \text{I}_\text{L}$$

Fig. 3. Attempt to synthesize cyclic hexapeptide via XIII: BOC, *t*-butyloxycarbonyl.

$$\text{V} \xrightarrow[71\%]{\text{NH}_2\text{NH}_2} \text{TRI-Gly}_5\text{-}\varepsilon\text{-Z-L-Lys-NHNH}_2 \xrightarrow[77\%]{\text{HCl-MeOH}} \underset{\text{XIV}}{}$$

$$\text{H-Gly}_5\text{-}\varepsilon\text{-Z-L-Lys-NHNH}_2\cdot 2\text{HCl} \xrightarrow{\text{HNO}_2} \underset{\text{XV}}{}$$

$$\text{H-Gly}_5\text{-}\varepsilon\text{-Z-L-Lys-N}_3\cdot\text{HCl} \xrightarrow{\text{NaHCO}_3} \text{Trace of } \text{I}_\text{L}$$

Fig. 4. Attempt to synthesize cyclic hexapeptide via XIV.

cyclic hexapeptide.[10]

Figure 5 illustrates the sequence of reactions

$$\text{pMZ-Gly-OH} + \text{IV} \xrightarrow[87\%]{\text{NEt}_3,\ \text{M.A.}} \underset{\text{XVI}}{}$$

$$\text{pMZ-Gly}_3\text{-}\varepsilon\text{-Z-L-Lys-OEt} \xrightarrow[79\%]{\text{NH}_2\text{NH}_2} \underset{\text{XVII}}{}$$

$$\text{pMZ-Gly}_3\text{-}\varepsilon\text{-Z-L-Lys-NHNH}_2 \xrightarrow{\text{HNO}_2} \underset{\text{XVIII}}{}$$

$$\text{pMZ-Gly}_3\text{-}\varepsilon\text{-Z-L-Lys-N}_3 \xrightarrow[71\%\text{ from XVIII}]{\text{H-Gly}_2\text{-OBz}} \underset{\text{XIX}}{}$$

$$\text{pMZ-Gly}_3\text{-}\varepsilon\text{-Z-L-Lys-Gly}_2\text{-OBz} \xrightarrow[75\%]{\text{OH}^- \text{ and } \text{H}^+} \underset{\text{XX}}{}$$

$$\text{pMZ-Gly}_3\text{-}\varepsilon\text{-Z-L-Lys-Gly}_2\text{-OH} \xrightarrow{\text{NPS}} \underset{\text{XXI}}{}$$

$$\text{pMZ-Gly}_3\text{-}\varepsilon\text{-Z-L-Lys-Gly}_2\text{-ONP} \xrightarrow{\text{CF}_3\text{COOH, anisole}}$$

$$\text{H-Gly}_3\text{-}\varepsilon\text{-Z-L-Lys-Gly}_2\text{-ONP}\cdot\text{CF}_3\text{COOH} \xrightarrow[42\%\text{ from XX}]{\text{pyridine}}$$

$$\varepsilon\text{-Z-L-}\underbrace{\text{Lys Gly}_5}_{\text{I}_\text{L}} \xrightarrow[66\%]{\text{H}_2/\text{Pd, HCl}} \text{L-}\underbrace{\text{Lys Gly}_5}_{\text{II}_\text{L}}\cdot\text{HCl}$$

Fig. 5. Synthesis of cyclic hexapeptide via XXI: pMZ, *p*-methoxybenzyloxycarbonyl.

via the peptide active ester in which glycine is the *C*-terminal amino acid. The optical purity of the L-lysine residue should be maintained in this reaction sequence, since the coupling of *N*-blocked peptide hydrazide (XVIII) and the dipeptide ester is performed

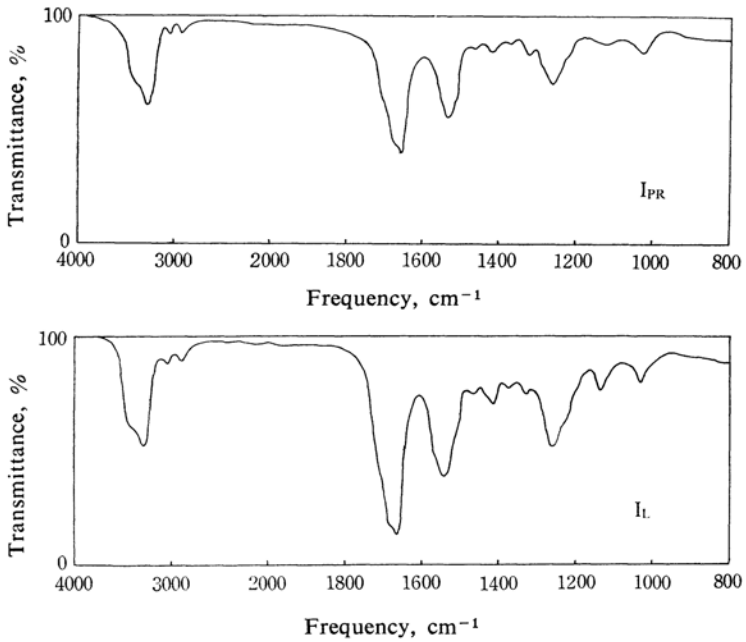


Fig. 6. Infrared spectra of cyclo-pentaglycyl-ε-benzyloxycarbonyllysyl (I_{PR}) and cyclo-pentaglycyl-ε-benzyloxycarbonyl-L-lysyl (I_L).

10) J. C. Sheehan and D. N. McGregor, ibid., **84**, 3000 (1962).

by the azide method, in which the *C*-terminal amino acid residue is known to be remarkably resistant to racemization.[11] Furthermore, the *p*-nitrophenyl esterification of XX does not cause any racemization since *C*-terminal amino acid is glycine. During this study, we found that the *p*-methoxybenzyloxycarbonyl group was superior to trityl or *t*-butyloxycarbonyl as a group protecting the *N*-terminal amino group of a linear peptide *p*-nitrophenyl ester. The *p*-methoxybenzyloxycarbonyl group is easily, in a few minutes, released at 0°C with trifluoroacetic acid in the absence of water, while the trityl group requires successive treatments with trifluoroacetic acid and water. The experiment, as shown in Fig. 1, indicates that the sensitive *p*-nitrophenyl ester group is degraded partially in the presence of water. Furthermore, we found that the *t*-butyloxycarbonyl peptide could not afford the desired *t*-butyloxycarbonyl peptide *p*-nitrophenyl ester with di-*p*-nitrophenyl sulfite and a base.[12] The yield of I_L from XXI was fairly good, as is shown in Fig. 5. The infrared absorption spectra of I_L was identical with that of I_{PR} (Fig. 6), as were the R_f values of II_{PR} and II_L in the paper chromatography. The hydrogenolysis of I_L yielded the desired cyclic peptide, II_L. The homogeneity of II_L wa asscertained by chromatography and by elemental analyses.

It was observed that cyclo-pentaglycyl-L-lysyl is completely hydrolyzed by trypsin to pentaglycyl-L-lysine. This agrees well with the fact that a linear peptide containing L-lysine is susceptible to the hydrolytic action of trypsin at the linkage of lysine carbonyl.[13] An authentic pentaglycyl-L-lysine hydrochloride (XXII) was synthesized, as Fig. 7 shows. This finding is of great interest, because this is the first example of how trypsin can hydrolyze a cyclic peptide. However, glycyl-L-lysine anhydride, a cyclic dipeptide, was found not to be hydrolyzed by trypsin, but it did not inhibit the hydrolysis of α-benzoyl-L-arginine amide by trypsin.[14] These enzymatic experiments show that even a cyclic dipeptide containing a L-lysine residue has no affinity to trypsin, while a cyclic hexapeptide, cyclo-pentaglycyl-L-lysyl, can form an enzyme-substrate complex which is finally decomposed to the enzyme and the product. Further experiments on the preparation of cyclic peptides, cyclo-(glycyl$_n$-L-lysyl)$_2$ (n=2 and 4), and on their susceptibility to trypsin are in progress in this laboratory.

$$\text{IX} \xrightarrow[80\%]{H_2/Pd,\ HCl\text{-}MeOH} \underset{\text{XXII}}{\text{H-Gly}_5\text{-L-Lys-OH}\cdot\text{HCl}}$$

Fig. 7. Synthesis of pentaglycyl-L-lysine hydrochloride.

Experimental

The melting points were not corrected. The optical rotations were measured on a Yanagimoto Photometric Polarimeter, OR-20 type. The paper chromatography was carried out on Toyo Roshi No. 52 chromatography paper. The thin-layer chromatography was carried out on Merck silica gel G. The developing solvents were *n*-butanol - acetic acid - pyridine - water (4 : 1 : 1 : 2) and *t*-butanol - formic acid - water (75 : 15 : 10). 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 per cent hydrobromic acid and then ninhydrin.

Trityldiglycyl - ε - benzyloxycarbonyl - L - lysine Ethyl Ester (III).—The mixed anhydride prepared at −5°C from 18.7 g. (50 mmol.) of tritylglycylglycine,[15] 6.7 ml. (50 mmol.) of isobutyl chloroformate and 7.0 ml. (50 mmol.) of triethylamine in 150 ml. of tetrahydrofuran was coupled with 24.0 g. (50 mmol.) of ε-benzyloxycarbonyl-L-lysine ethyl ester *p*-toluenesulfonate[16] dissolved in a chilled mixture of 7.0 ml. (50 mmol.) of triethylamine and 150 ml. of chloroform. The reaction mixture was allowed to stand overnight and then evaporated to dryness in vacuo. The oily residue was dissolved in 250 ml. of ethyl acetate, and the solution was washed successively with 100-ml. portions of 0.5 N acetic acid, a 3% sodium bicarbonate solution and water, dried over sodium sulfate, and then evaporated to dryness in vacuo. The oily residue was then solidified by adding petroleum ether. Yield: 25.7 g. (77%). For analysis, the product was recrystallized from ethyl acetate - ether - petroleum ether; m. p. 107—108°C, $[\alpha]_D^{20}$ −7.1° (*c* 2, methanol).

Found: C, 70.42; H, 6.57; N, 8.45. Calcd. for $C_{39}H_{44}O_6N_4$: C, 70.46; H, 6.67; N, 8.43%.

Diglycyl-ε-benzyloxycarbonyl-L-lysine Ethyl Ester Hydrochloride (IV).—To a solution of 6.64 g. (10 mmol.) of III in 50 ml. of methanol, 15 ml. of N hydrogen chloride in methanol was added; then the solution was boiled for 2 min. The solution was concentrated in vacuo and ether was added to the residue. The resulting white crystals were collected by filtration and washed with ether (3.72 g.). The product was recrystallized from methanol - ether. Yield: 3.44 g. (75%); m. p. 144—146°C, $[\alpha]_D^{20}$ −13.2° (*c* 2, methanol).

Found: C, 52.17; H, 6.71; N, 11.84. Calcd. for $C_{20}H_{30}O_6N_4\cdot HCl$: C, 52.34; H, 6.59; N, 12.21%.

11) N. F. Albertson, "Organic Reactions." Vol. 12 (1962), p. 168.
12) M. Ohno and N. Izumiya, unpublished.
13) H. Neurath and G. W. Schwert, *Chem. Revs.*, **46**, 69 (1950); N. Izumiya, T. Yamashita, H. Uchio and K. Kitagawa, *Arch. Biochem. Biophys.*, **90**, 170 (1960).
14) T. Yamamoto and N. Izumiya, unpublished.
15) R. Schwyzer, B. Iselin, W. Rittel and P. Sieber, *Helv. Chim. Acta*, **39**, 872 (1956).
16) T. Kato, S. Makisumi, M. Ohno and N. Izumiya, *J. Chem. Soc. Japan, Pure Chem. Sec.* (*Nippon Kagaku Zasshi*), **83**, 1151 (1962).

Tritylpentaglycyl-ε-benzyloxycarbonyl-L-lysine Ethyl Ester (V).—The mixed anhydride prepared at −5°C from 4.32 g. (10 mmol.) of trityldiglycylglycine,[15] 1.3 ml. (10 mmol.) of isobutyl chloroformate and 1.4 ml. (10 mmol.) of triethylamine in a mixture of 30 ml. of tetrahydrofuran and 20 ml. of chloroform was coupled with 4.59 g. (10 mmol.) of IV dissolved in a chilled mixture of triethylamine and 30 ml. of chloroform. The reaction mixture was allowed to stand overnight and then evaporated to dryness in vacuo. The oily residue was dissolved in 200 ml. of chloroform, and the solution was washed successively with 0.5 M citric acid, a 3% sodium bicarbonate solution and water, dried over sodium sulfate, and then evaporated to dryness in vacuo. The oily residue was easily solidified by adding ether and petroleum ether (6.19 g.). The product was recrystallized from acetone-ether. Yield: 5.68 g. (68%); m. p. 94—96°C, $[\alpha]_D^{20}$ −4.7° (*c* 2, dimethylformamide).

Found: C, 64.35; H, 6.56; N, 11.33. Calcd. for $C_{45}H_{53}O_9N_7$: C, 64.65; H, 6.39; N, 11.73%.

Tritylpentaglycyl-ε-benzyloxycarbonyl-L-lysine (VI).—To a solution of 1.80 g. (2.15 mmol.) of V in a mixture of 10 ml. of dioxane and 5 ml. of methanol, 2.5 ml. of N sodium hydroxide was added. After 2 hr., the solution was concentrated in vacuo to remove methanol, and the residual solution was diluted with water and acidified with 3.0 ml. of M citric acid under cooling. The precipitate was collected by filtration under cooling and washed with cold water. The product was recrystallized from ethanol-ether. Yield: 1.58 g. (91%). For analysis, this was further recrystallized from ethanol-ether; m. p. 109—112°C, $[\alpha]_D^{20}$ −6.5° (*c* 2, dimethylformamide).

Found: C, 63.25; H, 6.20; N, 12.22. Calcd. for $C_{43}H_{49}O_9N_7$: C, 63.92; H, 6.11; N, 12.14%.

Tritylpentaglycyl-ε-benzyloxycarbonyllysine *p*-Nitrophenyl Ester (VII_{PR}).—A solution of 1.62 g. (2 mmol.) of VI and 1.30 g. (4 mmol.) of di-*p*-nitrophenyl sulfite[4] dissolved in 10 ml. of pyridine was allowed to stand at room temperature for 15 hr. The solvent was then removed in vacuo, and the oily residue was solidified by adding a mixture of ether and petroleum ether (1 : 1) and scratching. The product was collected by filtration and washed with a mixture of ether and petroleum ether (1 : 1) until the yellow color could not be discerned upon the addition of N sodium hydroxide to the filtrate. A faintly yellow product was obtained. Yield: 1.80 g.; $[\alpha]_D^{20}$ −1.6° (*c* 2, dimethylformamide).

Found: N, 11.43. Calcd. for $C_{49}H_{52}O_{11}N_8$: N, 11.93%.

The solution of 3.04 mg. of this product in 50 ml. of methanol was diluted with N sodium hydroxide to 100 ml. ($c=3.27\times10^{-5}$ M). The absorption of this solution was measured at 404 mμ and compared with a standard curve which had been determined with the benzyloxycarbonyl-L-leucine *p*-nitrophenyl ester in the same solvent.[17] The value of the absorbance of this sample corresponded to $c=3.46\times10^{-5}$ M. Therefore, the *p*-nitrophenyl ester content of this product was estimated to be 106%.

Pentaglycyl-ε-benzyloxycarbonyllysine *p*-Nitrophenyl Ester Trifluoroacetate ($VIII_{PR}$).—A solution of 1.0 g. of VII_{PR} dissolved in 18 ml. (25 g.) of trifluoroacetic acid was cooled to −5°C, and then 15 ml. of cold water was added slowly to this solution. After 15 min., the precipitate of triphenylcarbinol was filtered off. The filtrate was lyophilized, and the oily residue was solidified by adding ether and scratching. The powdery solid was collected by filtration and washed thoroughly with a mixture of ether and petroleum ether (1 : 1) until the yellow color could not be discerned upon the addition of N sodium hydroxide to the filtrate. A faintly yellow powder was obtained; 0.660 g. (apparent yield, 86%).

The *p*-nitrophenyl ester content of this product was estimated by the same method as has been described previously except that a mixture of dimethylformamide and N sodium hydroxide (1 : 1) was used as a solvent and the measurement was performed at 411 mμ. The *p*-nitrophenyl ester content was estimated to be 81%. Therefore, the true yield of the compound $VIII_{PR}$ was 70%.

Cyclo-pentaglycyl-ε-benzyloxycarbonyllysyl (I_{PR}).—A solution of 0.610 g. (0.762 mmol.) of $VIII_{PR}$ in a mixture of 15 ml. of dimethylformamide and 0.15 ml. of acetic acid was stirred drop by drop into 140 ml. of pyridine kept at 55—60°C over a 9-hr. period. The solution was stirred at this temperature for another 2 hr. and then evaporated to dryness in vacuo. The dried residue was dissolved in 160 ml. of a hot mixture of water and methanol (1 : 1), and insoluble materials were filtered off. The filtrate was passed successively through columns (1×14 cm.) of Dowex-50 (H^+ form) and Dowex-1 (OH^- form) which had been washed with a mixture of water and methanol (1 : 1). The columns were then washed with a mixture of water and methanol (1 : 1). The filtrate and washings (total 400 ml.) were combined and evaporated to dryness in vacuo. The weight of the dried residue was 0.229 g. (39% from VII_{PR}). The crude cyclic product was recrystallized from methanol-water. Yield: 0.193 g. (33% from VII_{PR}); m. p. 261—262°C, $[\alpha]_D^{20}$ +0.5° (*c* 1.33, dimethylformamide).

Found: C, 51.90; H, 6.33; N, 17.19. Calcd. for $C_{24}H_{34}O_8N_7\cdot1/2H_2O$: C, 51.62; H, 6.33; N, 17.59%.

The air-dried compound lost 1.62% of its weight after being dried for 2 hr. at 130°C, 0.1 mmHg. Calcd. for $1/2H_2O$: 1.62%. The molecular weight was determined by the micro Rast method with a solvent of hexahydro-*p*-aminobenzoic acid lactam.[18] Calcd.: 549. Found: 557, 510.

Cyclo-pentaglycyllysyl Hydrochloride (II_{PR}).—A solution of 5.56 mg. (0.01 mmol.) of I_{PR} dissolved in a mixture of 0.4 ml. of 0.025 N hydrochloric acid and 0.2 ml. of water was hydrogenolyzed in the presence of palladium black. After the filtrate had been evaporated to dryness, the residue was filtered with the aid of acetone; yield, 3.0 mg. The homogeneity of II_{PR} was established by

17) R. Schwyzer and P. Sieber, *Helv. Chim. Acta*, **40**, 624 (1957).

18) G. Wendt, *Chem. Ber.*, **75**, 425 (1942).

chromatography (R_f[19] 0.27, R_f[20] 0.42 and R_f[21] 0.13). These values of R_f are identical as those of analytically-pure cyclo-pentaglycyl-L-lysyl hydrochloride (II_L).

Pentaglycyl-ε-benzyloxycarbonyl-L-lysine (IX).—When 1.0 g. (1.24 mmol.) of VI suspended in 20 ml. of 50 per cent aqueous acetic acid was boiled for 2 min., it dissolved and triphenylcarbinol was deposited.[22] Ten milliliters of water was then added, and the solution was kept in a refrigerator for several hours, after which the carbinol was removed by filtration (0.298 g.). The filtrate was evaporated to dryness in vacuo. The product was filtered with the aid of ethanol (0.630 g.). This was recrystallized from water-ethanol. Yield: 0.500 g. (71%); m. p. 213—214°C (decomp.), $[\alpha]_D^{20}$ −2.4° (*c* 2, 0.2 N hydrochloric acid).

Found: C, 49.44; H, 6.28; N, 17.13. Calcd. for $C_{24}H_{35}O_9N_7 \cdot 1/2H_2O$: C, 49.39; H, 6.39; N, 16.80%.

An Attempt to Cyclize IX by Dicyclohexylcarbodiimide.—To a suspension of 0.234 g. (0.414 mmol.) of IX in 10 ml. of dimethylformamide, 0.170 g. (0.828 mmol.) of dicyclohexylcarbodiimide was added. The reaction mixture was kept at 0°C with stirring for 24 hr. and then evaporated to dryness in vacuo. The residue was extracted with 50 ml. of a hot mixture of water and methanol (1 : 1). After the extract had been cooled, the dicyclohexylurea deposited was removed by filtration and the filtrate was passed successively through columns of Dowex-50 (H^+ form) and Dowex-1 (OH^- form). The filtrate and washings were then combined and evaporated to dryness in vacuo (0.011 g.). This product contained cyclo-pentaglycyl-ε-benzyloxycarbonyllysyl since the hydrogenolyzed material gave a single spot of R_f 0.27[19] which was identical with II_{PR}.

***t*-Butyloxycarbonyltriglycyl-ε-benzyloxycarbonyl-L-lysine Ethyl Ester (X).**—To a stirred solution of 3.50 g. (20 mmol.) of *t*-butyloxycarbonylglycine[23] and 9.18 g. (20 mmol.) of IV in 200 ml. of chloroform, 2.8 ml. (20 mmol.) of triethylamine was added. The solution was then cooled to 0°C, and 4.12 g. (20 mmol.) of dicyclohexylcarbodiimide was stirred into it. The reaction mixture was stirred at 0°C for one hour and then kept in a refrigerator overnight. Dicyclohexylurea was removed by filtration. The filtrate was washed successively with 0.5 M citric acid, a 3% sodium bicarbonate solution and water, dried over sodium sulfate, and then evaporated to dryness in vacuo (9.88 g.). The product was twice recrystallized from ethyl acetate-petroleum ether. Yield: 8.88 g. (79%); m. p. 115—117°C, $[\alpha]_D^{20}$ −8.4° (*c* 2, methanol).

Found: C, 56.11; H, 7.35; N, 11.71. Calcd. for $C_{27}H_{41}O_9N_5$: C, 55.94; H, 7.13; N, 12.08%.

***t*-Butyloxycarbonyltriglycyl-ε-benzyloxycarbonyl-L-lysine (XI).**—To a solution of 8.42 g. (15 mmol.) of X in 45 ml. of methanol, 18 ml. of N sodium hydroxide was added. Saponification was carried out at room temperature for 2 hr. After the removal of the methanol in vacuo, the aqueous solution was acidified with 23 ml. of M citric acid under cooling and the oil deposited was extracted two times with 50-ml. portions of ethyl acetate. The combined ethyl acetate solution was then washed with water, dried over sodium sulfate, and evaporated to dryness in vacuo. The residue was solidified by adding petroleum ether. Yield: 7.44 g. (93%). For analysis, the product was twice recrystallized from ethyl acetate-ether; m. p. 66—67°C, $[\alpha]_D^{20}$ +2.6° (*c* 2, methanol).

Found: C, 54.11; H, 6.60; N, 12.22. Calcd. for $C_{25}H_{37}O_9N_5$: C, 54.43; H, 6.76; N, 12.70%.

***t*-Butyloxycarbonyltriglycyl-ε-benzyloxycarbonyl-L-lysylglycylglycine Benzyl Ester (XII).**—To a stirred, faintly cloudy solution of 5.52 g. (10 mmol.) of XI and 3.94 g. (10 mmol.) of glycylglycine benzyl ester *p*-toluenesulfonate in 200 ml. of chloroform, 1.4 ml. (10 mmol.) of triethylamine was added. The solution was then cooled to 0°C and 2.06 g. (10 mmol.) of dicyclohexylcarbodiimide was added to it. After the mixture had been stirred at 0°C for 4 hr., it was kept in a refrigerator for 2 days and then evaporated in vacuo. The residue was filtered with the aid of water and petroleum ether, and washed with 0.5 M citric acid, a 3% sodium bicarbonate solution and water. The dried mixture was dissolved in methanol and kept in a refrigerator for several hours, and the dicyclohexylurea was removed by filtration. These operations were repeated several times, thus removing almost all the dicyclohexylurea. Finally the filtrate was evaporated to dryness in vacuo. The oily residue was solidified by adding ether and petroleum ether. Yield: 5.80 g. (77%). For analysis, the product was twice recrystallized from methanol-ethyl acetate-ether; m. p. 132—156°C, $[\alpha]_D^{20}$ −0.5° (*c* 2, dimethylformamide).

Found: C, 56.83; H, 6.51; N, 12.38. Calcd. for $C_{36}H_{49}O_{11}N_7 \cdot 1/2H_2O$: C, 56.54; H, 6.35; N, 12.82%.

***t*-Butyloxycarbonyltriglycyl-ε-benzyloxycarbonyl-L-lysylglycylglycine Hydrazide (XIII).**—A solution of 1.51 g. (2 mmol.) of XII and 0.20 g. (4 mmol.) of hydrazine hydrate in 14 ml. of methanol was allowed to stand at room temperature for 24 hr. The solution was then evaporated to dryness in vacuo, after which the oily residue was immediately solidified by adding a mixture of ether and petroleum ether (1 : 1). This product was recrystallized from methanol-ether. Yield: 1.27 g. (93%); m. p. 159—172°C.

Found: C, 50.17; H, 6.66; N, 18.41. Calcd. for $C_{29}H_{45}O_{10}N_9 \cdot H_2O$: C, 49.92; H, 6.79; N, 18.07%.

An Attempt to Synthesize Cyclic Hexapeptide via XIII by the Azide Method.—A solution of 1.01 g. (1.49 mmol.) of XIII in a mixture of 8.5 ml.

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

20) The R_f of the paper chromatography refers to the *t*-butanol-formic acid-water (75 : 15 : 10) system.

21) 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) system.

22) L. Zervas and D. M. Theodoropoulos, *J. Am. Chem. Soc.*, **78**, 1359 (1956); G. C. Stelakatos, D. M. Theodoropoulos and L. Zervas, ibid., **81**, 2884 (1959).

23) This was prepared from glycine and *t*-butyloxycarbonyl azide by the general method developed by Schwyzer et al. (R. Schwyzer, H. Kappeler, B. Iselin, W. Rittel and H. Zuber, *Helv. Chim. Acta*, **42**, 2622 (1959)).

of methanol and 20 ml. of 2.84 N hydrogen chloride in methanol was allowed to stand at room temperature for 2 hr.[24] The solution was then evaporated to dryness in vacuo, and the residue was kept under high vacuum for 2 hr. The main portion of the residue was regarded as triglycyl-ε-benzyloxycarbonyl-L-lysylglycylglycine hydrazide dihydrochloride. The residue was dissolved in 8 ml. of 0.2 N hydrochloric acid. To the solution was then added a solution of 0.111 g. (1.6 mmol.) of sodium nitrate in 1 ml. of water under cooling. After it had stood for 15 min. at 0°C, the solution was poured into 1.5 l. of cold water containing 5.6 g. of sodium bicarbonate. The solution was kept at 4°C for 24 hr., and then the pH was brought to 5 with 2 N hydrochloric acid. The solution was passed successively through columns of Dowex-50 (H^+ form) and Dowex-1 (OH^- form). The filtrate and washings were combined and evaporated to dryness in vacuo. Only a few milligrams of I_L were obtained.

Tritylpentaglycyl-ε-benzyloxycarbonyl-L-lysine Hydrazide (XIV).—A solution of 0.836 g. (1 mmol.) of V and 0.150 g. (3 mmol.) of hydrazine hydrate in 5 ml. of methanol was allowed to stand at room temperature for 24 hr. The solution was then concentrated in vacuo to a small volume, ether was added, and the resulting crystals were collected by filtration (0.70 g.). The product was recrystallized from methanol-ether. Yield: 0.580 g. (71%); m. p. 162—167°C, $[\alpha]_D^{20}$ −3.0° (*c* 2, dimethylformamide).

Found: C, 62.70; H, 6.36; N, 14.75. Calcd. for $C_{43}H_{51}O_8N_9$: C, 62.83; H, 6.25; N, 15.34%.

Pentaglycyl-ε-benzyloxycarbonyl-L-lysine Hydrazide Dihydrochloride (XV).—A solution of 0.476 g. (0.58 mmol.) of XIV in a mixture of 8 ml. of methanol and 2.4 ml. of 0.5 N hydrogen chloride in methanol was boiled for 2 min. Then the solution was evaporated in vacuo, and the resulting crystals were filtered with the aid of a mixture of ethanol and ether. The product was recrystallized from methanol-ethanol-ether. Yield: 0.292 g. (77%); m. p. 196—198°C.

Found: N, 18.33. Calcd. for $C_{24}H_{39}O_8N_9Cl_2 \cdot 2H_2O$: N, 18.29%.

An Attempt at the Cyclization of XV by the Azide Method.—To a cold solution of 0.255 g. (0.39 mmol.) of XV dissolved in 4 ml. of 0.1 N hydrochloric acid, a cold solution of 0.028 g. (0.40 mmol.) of sodium nitrate in 0.3 ml. of water was added. After it had stood for 15 min. at 0°C, the golden yellow solution was poured into 300 ml. of cold water containing 1.8 g. of sodium bicarbonate. The solution was kept at 4°C for 45 hr., and the pH was adjusted to 5 with 2 N hydrochloric acid. The solution was passed successively through columns of Dowex-50 and Dowex-1. The filtrate and washings were combined and evaporated to dryness in vacuo. Only a trace of I_L was thus obtained.

***p*-Methoxybenzyloxycarbonylglycine (XVI).**—The synthetic procedure[25] was revised as follows. A mixture of 2.25 g. (30 mmol.) of glycine, 45 ml. of water, 45 ml. of dioxane, 6.72 g. (80 mmol.) of sodium bicarbonate and 8.07 g. (39 mmol.) of *p*-methoxybenzyloxycarbonyl azide was stirred at room temperature for 24 hr. The solution was then evaporated in vacuo in order to remove the dioxane. After it had been extracted with ethyl acetate, the solution was acidified with 80 ml. of M citric acid and then extracted with ethyl acetate. The ethyl acetate solution was dried and evaporated to dryness in vacuo. The crystals were washed with petroleum ether. Yield: 6.93 g. (97%); m. p. 95—96°C. Reported value, m. p. 94—96°C.[25]

***p*-Methoxybenzyloxycarbonyltriglycyl-ε-benzyloxycarbonyl-L-lysine Ethyl Ester (XVII).**—The mixed anhydride prepared from 3.59 g. (15 mmol.) of XVI, 1.95 ml. (15 mmol.) of isobutyl chloroformate and 2.10 ml. (15 mmol.) of triethylamine in 30 ml. of chloroform was coupled with 6.89 g. (15 mmol.) of IV dissolved in a chilled mixture of 2.10 ml. (15 mmol.) of triethylamine and 60 ml. of chloroform. After the organic solvents had been removed in vacuo, water was added to the residue and it was kept in a refrigerator. The product was collected by filtration and washed successively with M citric acid, a 3% sodium bicarbonate solution and water (9.0 g.). The product was recrystallized from methanol-ether-petroleum ether. Yield: 8.2 g. (87%); m. p. 108—112°C, $[\alpha]_D^{20}$ −6.1° (*c* 2, dimethylformamide).

Found: C, 57.19; H, 6.38; N, 10.93. Calcd. for $C_{31}H_{41}O_{10}N_5$: C, 57.84; H, 6.42; N, 10.88%.

***p*-Methoxybenzyloxycarbonyltriglycyl-ε-benzyloxycarbonyl-L-lysine Hydrazide (XVIII).**—A solution of 7.53 g. (12 mmol.) of XVII and 12 ml. (240 mmol.) of hydrazine hydrate in 24 ml. of dimethylfomamide was allowed to stand at room temperature for 2 days. The excess hydrazine was then evaporated in vacuo, and the residual solution was diluted with 400 ml. of water. The product was collected by filtration (6.30 g.). This was recrystallized from methanol-ether. Yield: 5.82 g. (79%); m. p. 182—184°C, $[\alpha]_D^{20}$ −3.9° (*c* 2, dimethylformamide).

Found: C, 55.17; H, 6.25; N, 15.79. Calcd. for $C_{29}H_{39}O_8N_7$: C, 55.31; H, 6.24; N, 15.57%.

***p*-Methoxybenzyloxycarbonyltriglycyl-ε-benzyloxycarbonyl-L-lysylglycylglycine Benzyl Ester (XIX).**—To a cooled solution of 4.30 g. (7 mmol.) of XVIII in 20 ml. of dimethylformamide and 7.7 ml. of N hydrochloric acid, a cold solution of 0.531 g. (7.7 mmol.) of sodium nitrate in 3 ml. of water was added. After it had stood for 5 min. at −5°C, the solution was diluted to a volume of 350 ml. with cold water and extracted two times with 80-ml. portions of ethyl acetate. The ethyl acetate solution was twice washed with a sodium bicarbonate solution and with water, dried over sodium sulfate, and then added to a cold solution of 2.56 g. (7 mmol.) of glycylglycine benzyl ester *p*-toluenesulfonate in 0.98 ml. (7 mmol.) of triethylamine and 4 ml. of dimethylformamide. The reaction mixture was kept at 3°C with stirring for 2 days. A gelatinous material deposited in the course of the reaction. The mixture was then evaporated in vacuo in order to remove the ethyl acetate, and the resulting solution was diluted with cold water. The product deposited was collected

24) E. Klieger and H. Gibian, *Ann.*, **649**, 183 (1961).
25) F. Weygand and K. Hunger, *Chem. Ber.*, **95**, 1 (1962).

by filtration and washed with a citric acid solution, a sodium bicarbonate solution and water (4.08 g.). The product was recrystallized from dimethylformamide - ether - petroleum ether. Yield: 4.0 g. (71%); m. p. 183—186°C, $[\alpha]_D^{20}$ −2.2° (*c* 2, dimethylformamide).

Found: C, 57.96; H, 6.27; N, 11.82. Calcd. for $C_{40}H_{49}O_{12}N_7$: C, 58.60; H, 6.02; N, 11.96%.

p-Methoxybenzyloxycarbonyltriglycyl-ε-benzyloxycarbonyl-L-lysylglycylglycine (XX).—Into a suspension of 2.41 g. (3 mmol.) of XIX in 35 ml. of dioxane, 33 ml. of 0.1 N sodium hydroxide was stirred at 0°C. By thin-layer chromatography,[21] the hydrolysis was found to be complete after 1.5 hr.; it gave only one detectable product. The solution was then evaporated in vacuo in order to remove the dioxane and then further treated with a mixture of ether and petroleum ether (1 : 1) in order to extract the residual dioxane. The acidification of the aqueous solution with 4 ml. of M citric acid yielded a gelatinous product. After the solution had stood in a refrigerator overnight, the product was collected by filtration and washed with cold water (1.83 g.). The product was recrystallized from dimethylformamide - water. Yield: 1.64 g. (75%); m. p. 190—195°C, $[\alpha]_D^{20}$ −3.8° (*c* 2, dimethylformamide).

Found: C, 54.49; H, 6.06; N, 12.87. Calcd. for $C_{33}H_{43}O_{12}N_7$: C, 54.31; H, 5.94; N, 13.44%.

p-Methoxybenzyloxycarbonyltriglycyl-ε-benzyloxycarbonyl-L-lysylglycylglycine p-Nitrophenyl Ester (XXI).—To a solution of 1.05 g. (1.43 mmol.) of XX in a mixture of 0.24 ml. (1.72 mmol.) of triethylamine and 6 ml. of dimethylformamide, 1.02 g. (3.14 mmol.) of di-*p*-nitrophenyl sulfite was added. The solution was then allowed to stand at room temperature overnight. The solvent was removed in vacuo, and the oily residue was solidified by adding a mixture of ether and petroleum ether (1 : 1) and scratching. The product was then filtered with the aid of petroleum ether (1.53 g.). The *p*-nitrophenyl ester content in this product was estimated to be 106% by means of the method described in the preparation of VII_{PR}. This product was used for the next reaction without further purification.

Cyclo-pentaglycyl-ε-benzyloxycarbonyl-L-lysyl- (I_L).—To a mixture of 1.53 g. of XXI and 1.3 ml. of anisole, 13 ml. of trifluoroacetic acid was added at −5°C.[25] After 10 min., the reaction mixture formed a reddish-brown solution. The removal of the trifluoroacetic acid in vacuo and the addition of ether yielded a faintly brown powder. The product was collected by filtration, washed with ether, and then dissolved in a mixture of 0.5 ml. of acetic acid and 25 ml. of dimethylformamide. A solution containing triglycyl-ε-benzyloxycarbonyl-L-lysylglycylglycine *p*-nitrophenyl ester trifluoroacetate was stirred drop by drop into 300 ml. of pyridine kept at 70—80°C over a 5.5-hr. period. The solution was then stirred at 70°C for another hour. After the solvents had been removed in vacuo, the residue was dissolved in 140 ml. of methanol and a small amount of insoluble material was filtered off. The methanolic solution was diluted with 140 ml. of water and passed successively through columns (2.5×10 cm.) of Dowex-50 and Dowex-1. The filtrate and washings were then combined and evaporated to dryness in vacuo. The residue was dissolved in 50 ml. of methanol, and insoluble materials were filtered off. The filtrate was concentrated to a small volume (0.4 ml.), and 4 ml. of ether and then 4 ml. of petroleum ether were added, to afford crystals; yield, 0.435 g. This gave only one spot in a paper chromatogram, R_f 0.70.[21] Careful recrystallization from methanol - ether - petroleum ether afforded 0.335 g. (42% from XX); m. p. 262°C, $[\alpha]_D^{20}$ +0.9° (*c* 1.33, dimethylformamide).

Found: C, 52.35; H, 5.99; N, 17.77. Calcd. for $C_{24}H_{34}N_8O_7$: C, 52.54; H, 6.25; N, 17.87%.

Cyclo-pentaglycyl-L-lysyl Hydrochloride (II_L).—A solution of 0.083 g. (0.15 mmol.) of I_L in a mixture of 6 ml. of methanol and 0.165 ml. of N hydrochloric acid was hydrogenolyzed in the presence of palladium black. The filtrate was then evaporated in vacuo and the concentrated solution was diluted to a volume of 0.5—0.6 ml. with water, whereupon a very small amount of an insoluble material was filtered off. After the filtrate had been concentrated to a small volume (0.3 ml.), addition of 0.3 ml. of ethanol and then 2 ml. of acetone yielded crystals. Yield: 0.045 g. (66%); m. p. 247°C (decomp.), $[\alpha]_D^{20}$ +15.6° (*c* 1.3, water); R_f 0.27,[19] 0.42[20] and 0.13[21].

Found: C, 39.87; H, 6.82; N, 20.46. Calcd. for $C_{16}H_{30}O_6N_7Cl \cdot 2H_2O$: C, 39.46; H, 6.83; N, 20.13%.

Pentaglycyl-L-lysine Hydrochloride (XXII).—A solution of 0.300 g. (0.53 mmol.) of IX dissolved in a mixture of 7 ml. of water and 3 ml. of 0.2 N hydrochloric acid was hydrogenolyzed in the presence of palladium black. The filtrate was evaporated to a small volume, and ethanol was added to afford crystals (0.230 g.). The product was recrystallized from water - ethanol. Yield: 0.202 g. (80%); m. p. 210—212°C (decomp.), $[\alpha]_D^{20}$ 0° (*c* 1.33, water).

Found: C, 40.03; H, 6.62; N, 20.03. Calcd. for $C_{16}H_{30}O_7N_7Cl \cdot 1/2H_2O$: C, 40.29; H, 6.76; N, 20.55%.

The Action of Trypsin on Glycyl-L-lysine Anhydride Hydrochloride, II_{PR} and II_L.—Hydrolysis experiments of substrates by trypsin[26] were carried out at pH 7.8, 30°C. The initial concentration of the substrates was 0.01 M, and the enzyme concentration was 0.05 mg. protein nitrogen per milliliter. The progress of the reactions was checked by thin-layer and paper chromatographies in the course of time. Table I shows the compositions of the reaction mixtures. Number 2 in Table I indicates the control experiment without the enzyme. No. 1 was performed in order to determine whether or not the trypsin autolysis took place. The control experiments (1 and 2) showed that neither the hydrolysis of cyclo-pentaglycyllysyl in the absence of the enzyme nor the autolysis of trypsin occurred. Experiment No. 3 indicated that glycyl-L-lysine anhydride hydrochloride[27] was not hydrolyzed at

26) This was a salt-free, crystalline sample from the Nutritional Biochemicals Corporation, U. S. A.

27) The synthesis of the compound (by M. Kondo and N. Izumiya) is unpublished; R_f 0.34.[19]

TABLE I. COMPOSITION OF THE MIXTURES OF THE ENZYME REACTION

Experimental No.	Substrate	Weighed amount of substrate	0.2 M Tris buffer (pH 7.8)	Trypsin solution (0.2 mg. protein nitrogen per ml.)	Water	Total volume
1			0.50 ml.	0.25 ml.	0.25 ml.	1.0 ml.
2	Lys Gly$_5$·HCl (II_{PR})	0.01 mmol.	0.50		0.50	1.0
3	L-Lys Gly·HCl	0.01	0.50	0.25	0.25	1.0
4	Lys Gly$_5$·HCl (II_{PR})	0.01	0.50	0.25	0.25	1.0
5	L-Lys Gly$_5$·HCl (II_L)	0.01	0.50	0.25	0.25	1.0

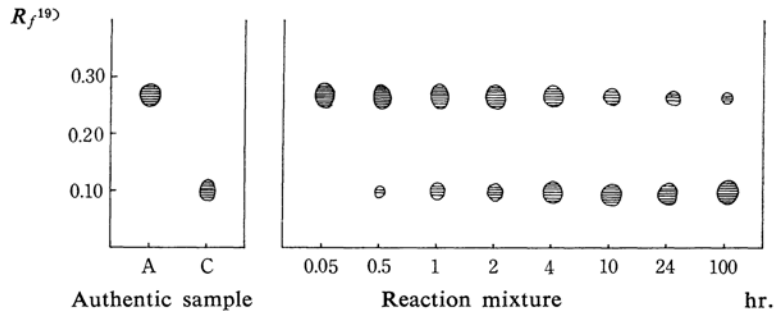


Fig. 8. Paper chromatograms of reaction mixtures of the experiment No. 4 in Table I.
Authentic sample: A, cyclo-pentaglycyllysyl hydrochloride (II_{PR}); C, pentaglycyl-L-lysine hydrochloride (XXII).

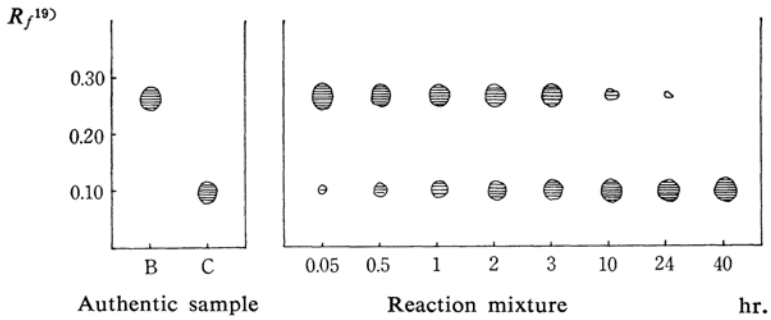


Fig. 9. Paper chromatograms of reaction mixtures of the experiment No. 5 in Table I.
Authentic sample: B, cyclo-pentaglycyl-L-lysyl (II_L); C, pentaglycyl-L-lysine hydrochloride (XXII).

all by trypsin, even after 48 hrs.' incubation. Cyclo-pentaglycyllysyl hydrochloride (II_{PR}) was partially hydrolyzed by the enzyme (Fig. 8). After 100 hrs.' incubation, a great part of the II_{PR} was hydrolyzed to the linear pentaglycyl-L-lysine, as may be seen in Fig. 8, while the rest of the material remained unchanged as the cyclic hexapeptide, presumably of the D-configuration. On the other hand, cyclo-pentaglycyl-L-lysyl hydrochloride (II_L) was completely hydrolyzed by trypsin after 40 hr. (Fig. 9). That the product of the two latter reactions was the corresponding linear hexapeptide, pentaglycyl-L-lysine, was proved by the chromatographic comparison of it with an authentic sample of pentaglycyl-L-lysine hydrochloride (XXII) synthesized as has been described above.

Summary

A cyclic hexapeptide, cyclo-pentaglycyl-L-lysyl hydrochloride (II_L), has been synthesized in an attempt to obtain fundamental information about the syntheses of cyclic peptides; it has also been subjected to the action of trypsin.

p-Methoxybenzyloxycarbonyltriglycyl-ε-benzyloxycarbonyl-L-lysylglycylglycine (XX) has

been prepared by the saponification of the di-*N*-blocked hexapeptide ester which had been obtained by the condensation of *p*-methoxybenzyloxycarbonyltriglycyl-ε-benzyloxycarbonyl-L-lysine azide with the glycylglycine benzyl ester. XX has been converted to the relevant *p*-nitrophenyl ester by the action of di-*p*-nitrophenyl sulfite, and, after the removal of the *p*-methoxybenzyloxycarbonyl group with trifluoroacetic acid, the hexapeptide *p*-nitrophenyl ester has been transformed, by means of a large amount of pyridine, to the cyclic benzyloxycarbonyl-substituted hexapeptide. It has then been hydrogenolyzed in the presence of palladium black to furnish the desired product, II_L.

Several different synthetic methods for obtaining II_L have been adopted. However, they are not satisfactory with regard to the optical purity of the L-lysine residue or the yield.

It has been observed that II_L is completely hydrolyzed to pentaglycyl-L-lysine by trypsin. This appears to be the first example of how trypsin can hydrolyze a cyclic peptide. A smaller cyclic peptide, glycyl-L-lysine anhydride, could not be hydrolyzed.

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