*Synthesis of Histidyl Peptides**

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Peptides containing L-histidine have frequently been obtained from biologically active substances such as ribonuclease[1], insulin[2], bacitracin[3] and strepogenin[4], and it has recently been suggested that the histidyl residue constitutes an essential part of the active sites of α-chymotrypsin, cholinesterase, lysozyme[5], and so on. Despite these findings indicating the importance of histidyl peptides in biochemistry, no satisfactory methods have so far been worked out for their convenient synthesis. This appears to be due mainly to the fact that there have been no successful procedures available to protect the imidazole nucleus of histidine.

Even when the α-amino group of histidine is masked with an acyl group, the molecule still behaves as a zwitter-ion owing to the presence of the imidazole ring, and this character affords unavoidable difficulties to the subsequent reactions. First of all, the presence of unprotected imidazole residue in the molecule often leads to various undesirable side reactions during the peptide synthesis. Furthermore, restricted solubility in organic solvents of the acylated derivative, e. g., *N*-α-carbobenzyloxyhistidine, is not favorable for their coupling with amino acid esters. The use of *N*-α-carbobenzyloxyhistidine is also impractical in view of the poor yield obtainable in its synthesis from histidine by the direct carbobenzyloxylation method[6].

Theodropoulos[7] has synthesized histidyl peptides using *N-Im*-benzyl-L-histidine prepared by the procedure of du Vigneaud[8]. Although this method is free from most of the defects mentioned above, it is not suitable for practical purpose because of the fact that the benzyl derivative is not easily accessible. Of late, Amiard et al. reported the preparation of the dityrityl histidine through which some of the histidyl peptides were synthesized[9]. The trityl method was valuable for the limited instance as it was rather tedious.

Holley and Sondheimer[10] have prepared a number of histidyl peptides employing the azide procedure in which the imino group of the imiazole ring was not protected. This method, though widely used as the only practical means for the synthesis of histidyl peptides[11], could not be regarded as a very excellent one because of the difficulty associated with the preparation of the intermediate *N*-α-carbobenzyloxyhistidine azide and its instability.

In the present paper the authors wish to report the preparation of dicarbobenzyloxy-L-histidine and its availability for the synthesis of histidyl peptides.

Preparation and Properties of Dicarbobenzyloxyhistidine. — Using sodium carbonate in place of sodium hydroxide, the authors succeeded in the percarbobenzyloxylation of L-histidine and obtained the α, *Im*-dicarbobenzyloxy derivative in 81% yield. Sodium hydroxide was also effective under limited conditions, but the yield in this case was relatively low.

The dicarbobenzyloxy compound thus obtained was found to be rather unstable

* A part of this investigation has been reported in "*Nature*", **181**, 772 (1958).

1) C. H. W. Hirs, *Fed. Proc.*, **16**, 196 (1957); R. R. Redfield and C. B. Anfinsen, *J. Biol. Chem.*, **221**, 385 (1956).

2) H. Brown, F. Sanger and R. Kitai, *Biochem. J.*, **60**, 556 (1956).

3) J. Porath, *Nature*, **172**, 871 (1953); I. M. Lockhart and E. P. Abraham, *Biochem. J.*, **58**, 633 (1954); L. C. Craig et al., *J. Am. Chem. Soc.*, **77**, 731 (1955).

4) R. B. Merrifield and D. W. Woolley, ibid., **78**, 358 (1956).

5) The late Dr. C. Fromageot suggested in the discussion that the histidyl residue might be the active site of lysozyme. (International Symposium on Enzyme Chemistry. Tokyo, Oct., 1957).

6) M. Bergmann and L. Zervas, *Ber.*, **65**, 1199 (1932).

7) D. Theodropoulos, *J. Org. Chem.*, **21**, 1550 (1956).

8) V. du Vigneaud and O. K. Behlens, *J. Biol. Chem.*, **117**, 27 (1937).

9) G. Amiard, R. Heymes and L. Velluz, *Bull. soc. chim. France*, 191 (1956).

10) R. Holley and E. Sondheimer, *J. Am. Chem. Soc.*, **76**, 1326 (1954).

11) R. F. Fischer et al., ibid., **76**, 5076 (1954); R. A. Boissonas et al., *Experientia*, **12**, 446 (1956); D. W. Woolley, et al., *J. Am. Chem. Soc.*, **78**, 4646 (1956); K. Hofmann et al., ibid., **76**, 1641 (1957); R. Schwyzer, et al., *Helv. chim. Acta*, **40**, 614 (1957).

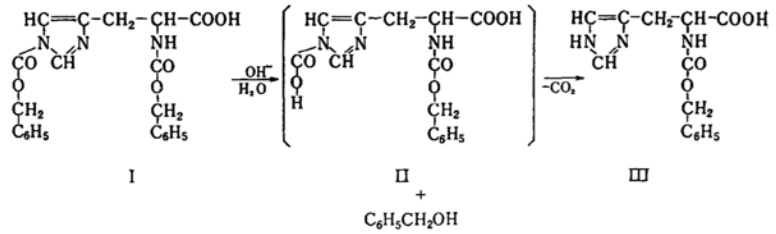

Fig. 1. Decomposition of dicarbobenzyloxy histidine in alkaline media.

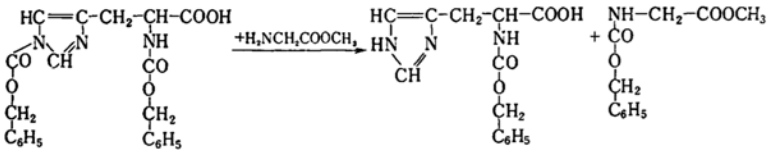

Fig. 2. The transfer of the carbobenzyloxy group.

in alkaline media. The compound was con vertible into *N*-α-carbobenzyloxyhistidine with elimination of the imidazole-linked carbobenzyloxy group (Fig. 1). It was thought that, in alkaline solution, dicarbobenzyloxyhistidine (I) was partly split into an intermediate II and benzyl alcohol by the action of hydroxyl ions. Then, II might spontaneously changed to form the *N*-α-carbobenzyloxy derivative (III) and carbon dioxide. The low yield of the dicarbobenzyloxy compound encountered in the presence of sodium hydroxide may have been due to such instability of the product in alkaline reaction.

In non-aqueous basic media the carbobenzyloxy residue linked to imidazole appeared to act as an active acyl group. When the dicarbobenzyloxy derivative and glycine methyl ester were incubated in dioxane at 50~60°C for one hour, a gelatinous precipitate of silky lustre was formed. Carbobenzyloxy glycine methyl ester and *N*-α-carbobenzyloxy histidine were isolated and identified from the precipitate. These compounds must have been formed by the transcarbobenzyloxylation reaction as illustrated in Fig. 2. This mechanism received some support from the fact that no transfer reaction but the decomposition took place in basic media if an acceptor compound was absent in the reaction mixture. Bergmann[12] and Wieland[13] have already observed that acetyl or amino acyl groups linked to the imidazole nucleus can be transfered to suitable acceptors under mild conditions.

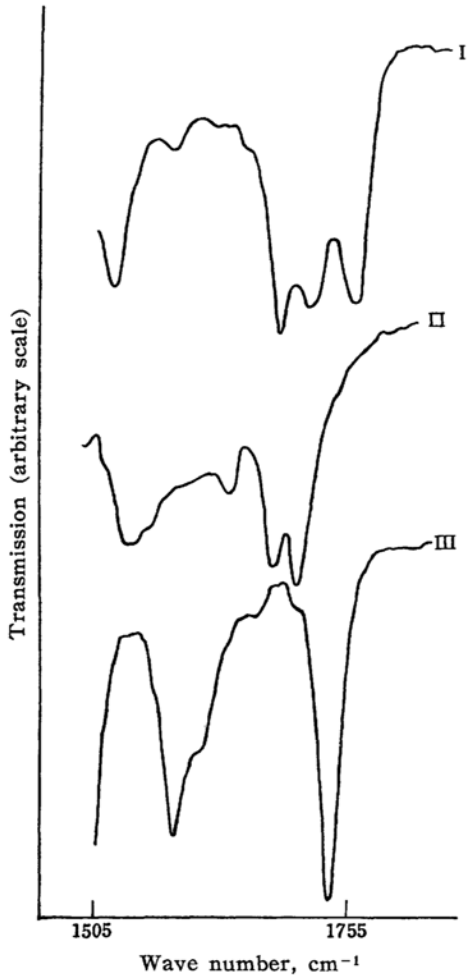

Fig. 3. Infrared absorption spectra of carbobenzyloxy derivatives of L-histidine. I, DCH; II, α-MCH; III, *Im*-MCH. (Rock salt, Hilger H800)

12) M. Bergmann and L. Zervas, *Z. physiol. Chem.*, **175**, 154 (1928).
13) Th. Wieland and G. Schneider, *Ann.*, **580**, 159 (1953).

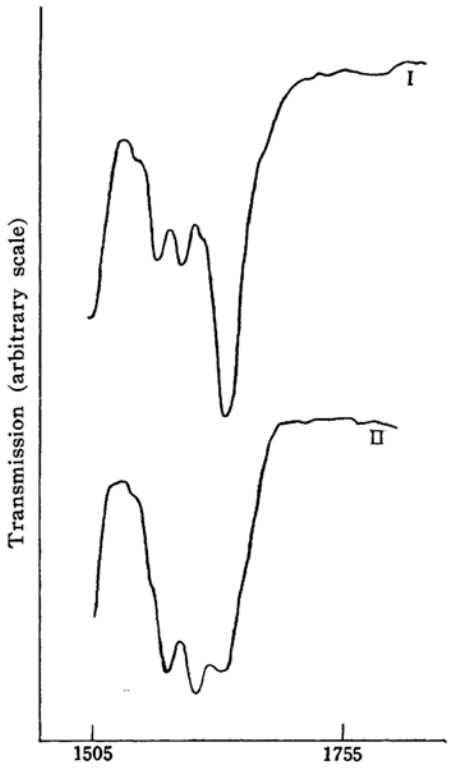

Fig. 4. Infrared absorption spectra of L-histidine (I) and its hydrochloride hydrate (II). (Rock salt, Hilger H800)

As a by-product, a small amount of monocarbobenzyloxy-L-histidine was isolated from the crude dicarbobenzyloxy derivative which was obtained by the reaction between L-histidine and the equivalent mole of carbobenzyloxychloride. It melted at 194~195°C with decomposition and gave a positive ninhydrin and a negative Pauly's reaction. These facts, together with the result of the infrared absorption analysis, were utilized to identify the compound as *N-Im*-carbobenzyloxyhistidine.

Infrared absorption spectra of dicarbobenzyloxy-L-histidine (DCH), α-carbobenzyloxy-L-histidine (α-MCH) and *Im*-carbobenzyloxy-L-histidine (*Im*-MCH) are shown in Fig. 3. Those of L-histidine and its hydrochloride hydrate are also shown in Fig. 4 for comparison. The band at 1759 cm^{-1} and that at 1733 cm^{-1} were assumed to correspond to the —CO—N= grouping which was formed by the combination of the imidazole nucleus with the carbobenzyloxy group.

These findings might be in good agreement with Otting's observation[14] that those characteristic absorption maxima of several acyl imidazoles appeared in the region of 1744~1748 cm^{-1}.

Synthesis of the Histidyl Peptide.— Several peptide derivatives of L-histidine, methyl ester of dicarbobenzyloxy-L-histidylglycine, -L-leucine, -L-threonine, -L-methionine, -*O*-benzyl-L-serine and -L-glutamic acid** were synthesized from dicarbobenzyloxy-L-histidine monohydrate and the respective amino acid ester with the aid of *N, N'*-dicyclohexylcarbodiimide[15]. Dicarbobenzyloxy-L-histidine was superior to the monocarbobenzyloxy compound as a starting material in respect to the higher solubility, the easier preparations and less possibility of undergoing some side reactions.

Dicarbobenzyloxy-L-histidyl glycine methyl ester was converted into the α-monocarbobenzyloxy dipeptide by alkaline hydrolysis in dimethylformamide[16]. L-Histidyl-L-leucine was prepared from the corresponding dipeptide ester by treatment with concentrated hydrochloric acid at 37°C[17]. The resulting product possessed properties identical with those reported by Holley and Sondheimer[10].

The synthesis of the L-histidyl-L-threonyl-L-valyl-L-glutamic acid derivative, the sequence of which was found in a heme-linked peptide obtained from cytochrome c[18], was explored by the following route. This involved the condensation of dicarbobenzyloxy-L-histidine and the tripeptide ester which was prepared from its carbobenzyloxy derivative by the action of hydrogen bromide in dioxane[19]. The coupling by the Sheehan's reagent proceeded smoothly as in the case of the dipeptide derivative, giving an yield of 50%.

N-Carbobenzyloxy-L-threonine was prepared using sodium carbonate in place of sodium bicarbonate[20] or sodium hydroxide, and was isolated as fine crystals, the yield of which was quite satisfactory. The tripeptide derivative, carbobenzyloxy-L-threonyl-L-valyl-L-glutamic acid dimethyl

14) W. Otting, *Ber.*, **89**, 1940 (1956).
15) J. C. Sheehan and G. P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955).
** The diethyl ester was exceptionally used for the peptide synthesis.
16) G. Weitzel, F. Schneider and A-M. Fretzdorff, *Z. physiol. Chem.*, **307**, 23 (1957).
17) R.B. Merrifield and D. W. Woolley, *J. Am. Chem. Soc.*, **78**, 4646 (1956).
18) H. Tuppy and S. Paleus, *Acta chim. Scand.*, **9**, 353 (1955).
19) K. Okawa, This Bulletin, **30**, 977 (1957).
20) G. Riley, J. H. Turnbull and W. Wilson, *J. Chem. Soc.*, **1957**, 1373.

ester, was derived from the above compound and L-valyl-L-glutamic acid ester in 78% yield by the carbodiimide method.

In a preliminary experiment, it was observed that both the two carbobenzyloxy groups in dicarbobenzyloxy-L-histidyl-L-threonine methyl ester were quantitatively removed by the action of hydrogen bromide in dioxane as in the case of dicarbobenzyloxy-L-histidine. Then the dipeptide ester produced was coupled with carbobenzyloxy-*S*-benzyl-L-cysteine by carbodiimide and the carbobenzyloxy tripeptide ester was produced (m. p. 114~115°C). Elementary analysis of the product was in good agreement with carbobenzyloxy-*S*-benzyl-L-cysteinyl-L-histidyl-L-threonine methyl ester ($C_{29}H_{35}O_7N_5S$). Furthermore, dicarbobenzyloxy-L-histidyl-L-threonine methyl ester was subjected to the reaction with 80% hydrazine hydrate and the hydrazide of the monocarbobenzyloxy dipeptide, containing one molecule of water, was obtained in a moderate yield (m. p. 186°C). This substance may also be utilized for the synthesis of the L-histidyl-L-threonyl peptide through the azide.

It could be concluded from the results of the present investigation that dicarbobenzyloxy histidine can be utilized for the synthesis of the histidyl peptides by the Sheehan's method though no general procedure for the hydrolysis of dicarbobenzyloxy peptide derivatives has been established.

At the end of this investigation, we had a chance to read the paper by Dr. E. Katchalski[21] on the carbobenzyloxy derivative of histidine, in which he reported the synthesis by a somewhat different method of dicarbobenzyloxy histidine and its chemical properties. It was found that the dicarbobenzyloxy histidine obtained by us showed coincidence with his product in all respects.

Experimental

N*-α, *N*-*Im*-Dicarbobenzyloxy-L-histidine.**— L-Histidine monohydrochloride monohydrate (10 g.) was dissolved in 48 ml. of 2 N sodium hydroxide solution and after addition of 2 N sodium carbonate solution (75 ml.), carbobenzyloxychloride (17.2 g.) was portion-wise dropped into the above solution under vigorous stirring and cooling for about half an hour. It was about five minutes until the white precipitates began to appear. Stirring was continued for 20 to 30 min. at room temperature and the solution was acidified to Congo red with 6 N hydrochloric acid under cooling. The gelatinous mass produced was separated from the supernatant by decantation. The product was first dissolved and then gradually crystallized out when it was triturated with ethanol. The white crystals were collected and washed with a small amount of ethanol. The yield was about 17g. (81%), m.p. 95~98°C (decomp.). Recrystallization from acetone-cold water gave white needles of dicarbobenzyloxy-L-histidine monohydrate (14g. 66%). m.p. 103~105°C (decomp.). $[\alpha]_D^{14}$ +34.01 (c. 28.99 mg. in 1 ml. of ethyl acetate).

Anal. Found: C, 59.86; H, 5.11; N, 9.46. Calcd. for $C_{22}H_{21}O_6N_3 \cdot H_2O$: C, 59.64; H, 5.25; N, 9.52%. Crystallization water: Found: 4.19; Calcd. 4.08%

When the dicarbobenzyloxy derivative was recrystallized from methanol, it was found that the compound, which melted at 105~107°C with decomposition, had one molecule of alcohol in place of water.

Anal. Found: C, 60.70; H, 5.50; N, 9.18. Calcd. for $C_{22}H_{21}O_6N_3 \cdot CH_3OH$: C, 60.65; H, 5.53; N, 9.23%.

The product is readily soluble in acetone, dioxane, tetrahydrofuran, chloroform and dimethylformamide, moderately soluble in warm methanol and ethanol, slightly soluble in benzene****, ethyl acetate and ether, and insoluble in petroleum ether and water. The negative Pauly's reaction was given at 0°C, but the solution turned to deep red color at room temperature as it elapsed. It was unstable to moisture and heat, and it decomposed to a material with some oily product, both when it was exposed to the atmosphere and when it was kept in the Abderhalden's apparatus (acetone in jacket) to dry the sample for analysis. This gelatinous substance washed with acetone and ethyl acetate gave hygroscopic powder, from which *N*-α-carbobenzyloxyhistidine was isolated in 70~80% yield by recrystallization from ethanol. Although little decomposition was encountered when it was kept over phosphorous pentoxide, it seemed preferable for the peptide synthesis to use freshly prepared crystals than the stored one. Both the carbobenzyloxy groups were quantitatively removed by the Ben-Ishai's procedure and the paper chromatography in the system of *n*-butanol-acetic acid-water (4 : 1 : 1 by volume) showed no spots except one corresponding to histidine.

The reaction of dicarbobenzyloxy-L-histidine with glycine methyl ester. — Dicarbobenzyloxy-L-histidine monohydrate (2.2 g.) and glycine methyl ester (prepared from its hydrochloride (1.3 g.) and ammonia-chloroform solution) were dissolved in 20 ml. of absolute dioxane and the mixture was kept at 50~60°C for 3 hours. The white precipitate produced was separated

21) A. Patchornik, A. Berger and E. Katchalski, *J. Am. Chem. Soc.* **79**, 6416 (1957).

*** Vigorous shaking of the vessel containing the mixture of histidine, alkali and acylchloride also gave the dicarbobenzyloxy derivative in the same yield.

**** Though, in a preliminary paper published in "Nature", it was reported that the dicarbobenzyloxy derivative of histidine was soluble in benzene, ethyl acetate and ether, further invesitigation on the solubility showed that the derivative in pure state had the least solubility in those solvents.

from the supernatant by decantation and was washed with ethyl acetate. The resulting white crystals were recrystallized from ethanol and identified to be *N*-α-carbobenzyloxy-L-histidine, which gave the positive Pauly's reaction. m. p. 165~166°C $[\alpha]_D^{25}$ −24.56 (c. 11.7 mg. in 1 ml. of 6 N hydrochloric acid).

Anal. Found: N, 14.50: Calcd. for $C_{14}H_{15}O_4N_3$: N, 14.50%.

On the paper chromatogram it showed an Rf. 0.64 in the system of *n*-butanol-acetic acid-water (4 : 1 : 5 by volume) and an Rf. 0.79 in that of the same solvents (4 : 1 : 1 by volume). Carbobenzyloxy glycine was obtained from the supernatant and washing by concentration and hydrolysis. When dicarbobenzyloxyhistidine was subjected to the reaction with twice equivalent mole of triethylamine under the same condition except the absence of any primary amine, it partly decomposed to give *N*-α-carbobenzyloxy-L-histidine.

***N-Im*-Carbobenzyloxy-L-histidine.**—From L-histidine monohydrochloride monohydrate (5.1 g. 0.025 M), 2 N sodium carbonate and an equivalent mole of carbobenzyloxychloride (5.1 g. 0.03 M), the crude material was obtained by the same procedure as the preparation of the dicarbobenzyloxy derivative. The crystals of this compound happened to separate from the solution when the crude carbobenzyloxy derivative, most of which was composed of the dicarbobenzyloxy derivatives, was recrystallized from the mixture of acetone-water (3 : 1 by volume). The yield was very poor. It was slightly soluble in water and in organic solvents. m. p. 193~194°C (decomp.).

Anal. Found: N, 14.44. Calcd. for $C_{14}H_{15}O_4N_3$: N, 14.53%.

Dicarbobenzyloxy-L-histidyl amino acid esters.—The same procedure as in the synthesis of dicarbobenzyloxy-L-histidyl-L-threonine methyl ester was applied to other histidyl peptide derivatives.

Dicarbobenzyloxy-L-histidyl-L-threonine methyl ester.—Freshly prepared dicarbobenzyloxy-L-histidine monohydrate (4.4 g.) was dissolved in 33 ml. of absolute chloroform and the solution was added to the chloroform solution containing L-threonine methyl ester (prepared from its hydrochloride (2.5 g.) and 1.5 N ammonia-chloroform solution (14 ml.) and *N*, *N'*-dicyclohexylcarbodiimide (2.1 g.). Dicyclohexylurea was precipitated after a few minutes with evolution of heat. The solution was cooled with water for several minutes and allowed to stand overnight at room temperature. About 1 ml. of glacial acetic acid was added and the precipitate produced was filtered off after an hour. The filtrate was washed with 0.2 N hydrochloric acid, 1% sodium bicarbonate and water successively, dried over anhydrous sodium sulfate and concentrated in vacuo to a paste-like mass. The residue was triturated with ether and the resulting crystals were collected. White needles of dicarbobenzyloxy-L-histidyl-L-threonine methyl ester (4.25 g.) were obtained in 78.8% yield. m. p. 140~144°C. After recrystallization from ethyl acetate-petroleum ether, the melting point was raised to 149~150°C. $[\alpha]_D^{14}$ +12.74 (c. 30.5 mg. in 1 ml. of glacial acetic acid).

Anal. Found: C, 59.61; H, 5.77; N, 10.21. Calcd. for $C_{27}H_{30}O_8N_4$: C, 60.21; H, 5.61; N. 10.40%.

Dicarbobenzyloxy-L-histidyl glycine methyl ester.—By the same procedure, fine crystals (3.4 g.) were obtained in 68% yield from dicarbobenzyloxy-L-histidine (4.4 g.) and glycine methyl ester. The dicarbobenzyloxy dipeptide ester was recrystallized from ethyl acetate. m. p. 68~70°C. $[\alpha]_D^{10}$ +10.47 (c. 19.4 mg. in 1 ml. of methanol).

Anal. Found: C, 59.75; H. 5.68; N, 10.23. Calcd. for $C_{25}H_{26}O_4N_7 \cdot CH_3OH$: C, 59.31; H, 5.74; N, 10.64%.

Dicarbobenzyloxy-L-histidyl-L-leucine methyl ester.—L-Leucine methyl ester, prepared from its hydrochloride (2.4 g.), was coupled with dicarbobenzyloxy-L-histidine monohydrate (2.8 g.) by carbodiimide. Dicarbobenzyloxy-L-histidyl-L-leucine methyl ester (2.4 g.) was obtained as a white precipitate in 68% yield. Recrystallization from ethyl acetate-petroleum ether gave fine crystals. m. p. 101~103°C. $[\alpha]_D^{14}$ +21.44° (c. 29.0 mg. in 1 ml. of ethyl acetate).

Anal. Found: C, 63.14; H, 6.31; N, 10.44. Calcd. for $C_{29}H_{34}O_7N_4$: C, 63.26; H, 6.22; N, 10.18%.

Dicarbobenzyloxy-L-histidyl-L-glutamic acid diethyl ester.—From dicarbobenzyloxy-L-histidine monohydrate (1.4 g.) and L-glutamic acid diethyl ester was prepared dicarbobenzyloxy-L-histidyl-L-glutamic acid diethyl ester (1.25 g. 70%), melting at 78~80°C. $[\alpha]_D^{14}$ +0.21 (c. 28.5 mg. in 1 ml. of ethyl acetate).

Anal. Found: C, 60.42; H, 5.92; N, 9.06; Calcd. for $C_{31}H_{36}O_9N_4$: C, 61.17; H, 5.96; N, 9.19%.

Dicarbobenzyloxy-L-histidyl-*O*-benzyl-L-serine methyl ester. — The coupling reaction between dicarbobenzyloxy-L-histidine monohydrate (1.4 g.) and *O*-benzyl-L-serine methyl ester by the carbodiimide procedure gave 1.5 g. of dicarbobenzyloxy-L-histidyl-*O*-benzyl-L-serine methyl ester: yield 81%. m. p. 113~115°C. $[\alpha]_D^{14}$ +19.80 (c. 29.6 mg. in 1 ml. of ethyl acetate).

Anal. Found: C, 64.75; H, 5.57; N, 9.54. Calcd. for $C_{33}H_{34}O_8N_4$: C, 64.48; H, 5.58; N, 9.12%.

Dicarbobenzyloxy-L-histidyl-L-methionine methyl ester.—White crystals (1.5 g.) of dicarbobenzyloxy-L-histidyl-L-methionine methyl ester were prepared from dicarbobenzyloxy-L-histidine monohydrate (1.3 g.) and L-methionine methyl ester. The dipeptide derivative (m. p. 110~113°C) was recrystallized from chloroform-petroleum ether. $[\alpha]_D^{22}$ +9.07 (c. 28.0 mg. in 1 ml. of glacial acetic acid).

Anal. Found: C, 59.10; H, 5.55: N, 9.72. Calcd. for $C_{28}H_{32}O_7N_4S$: C, 59.14; H, 5.67; N, 9.85%.

Carbobenzyloxy-L-histidyl glycine. — The dimethylformamide solution (2 ml.) containing the dicarbobenzyloxy dipeptide ester (2 g.) was adjusted to pH 9.0~9.2 with 2 N sodium hydroxide solution under cooling. The solution was kept for 30 min. at room temperature and then acidified to pH 5.0 with 1 N hydrochloric acid. By concentration and addition of acetone a swollen

material (1 g.) was obtained. m. p. 140~145°C Recrystallization from methyl alcohol gave the carbobenzyloxy dipeptide (0.5 g.) melting at 237~238°C.

Anal. Found: C, 54.40; H, 5.47; N, 15.79. Calcd. for $C_{16}H_{18}O_6N_4$: C, 55.48; H, 5.24; N, 16.17%.

Carbobenzyloxy-L-threonyl-L-valyl-L-glutamic acid dimethyl ester.—Carbobenzyloxy-L-threonine (2.5 g.) was dissolved in 5 ml. of chloroform and the solution was added to the chloroform solution containing *N, N'*-dicyclohexylcarbodiimide (2.1 g.) and L-valyl-L-glutamic acid dimethyl ester (prepared from its hydrobromide (4.3 g.) and triethylamine). As the reaction proceeded with evolution of heat, the resulting dicyclohexylurea was precipitated. After 3 hours the precipitate was filtered off, a few drops of glacial acetic acid was added to the filtrate and the solution was kept overnight at 5°C. The filtrate liberated from dicyclohexylurea (0.3 g.) was washed with 0.2 N hydrochloric acid, 0.25 N sodium carbonate and water successively, and dried. By concentration and dilution with petroleum ether, white crystals (3.9 g.) were obtained. From the filtrate an additional 0.1 g. of the product was obtained. The two crops were combined and recrystallized from ethyl acetate-petroleum ether. The yield was 3.6 g. (72%). m. p. 172~174°C. (sintered at 145~150°C). It gave the positive biuret reaction. $[\alpha]_D^{10}$ −56.5 (c. 23 mg. in 1 ml. of methanol).

Anal. Found: C, 56.70; H, 7.33; N, 8.47. Calcd. for $C_{24}H_{35}O_9N_3$: C, 56.57; H, 6.92; N, 8.25%.

Dicarbobenzyloxy-L-histidyl-L-threonyl-L-valyl-L-glutamic acid dimethyl ester. — Into the dimethylformamide solution containing L-threonyl-L-valyl-L-glutamic acid dimethyl ester (1.4 g.) and *N, N'*-dicyclohexylcarbodiimide (0.7 g.) was added the dimethylformamide solution (3 ml.) of dicarbobenzyloxy-L-histidine monohydrate (1.4 g.) under stirring. The resulting dicyclohexylurea appeared within 5 minutes and was filtered off after 30 minutes. The filtrate was allowed to stand overnight at 5°C. A small amount of dicyclohexylurea precipitated was filtered and the solvent of the filtrate was replaced by chloroform (20 ml.). Then it was washed with 0.2 N hydrochloric acid and water, successively, and dried over anhydrous sodium sulfate. The fluffy precipitate appeared when twice the volume of petroleum ether was added to the above solution. Additional material was obtained from the mother liquor by dilution with petroleum ether for the total of 1.2 g. (50%) of white powders. m. p. 95~98°C. $[\alpha]_D^{10}$ +20.1 (c. 16.4 mg. in 1 ml. of chloroform).

Anal. Found: C, 57.53; H, 6.71; N, 10.65. Calcd. for $C_{38}H_{48}N_6O_{12}\cdot H_2O$: C, 57.13; H, 6.31; N, 10.72%.

The dicarbobenzyloxy tetrapeptide ester was positive to the biuret reaction and its acid hydrolysate gave all the constitutional amino acids on the paper chromatogram.

Carbobenzyloxy-L-valyl-L-glutamic acid dimethyl ester.—From the syrupy carbobenzyloxy-L-valine (15 g.), glutamic acid dimethyl ester (prepared from its hydrochloride (17 g.)) and *N, N'*-dicyclohexylcarbodiimide (12 g.) was obtained the carbobenzyloxy dipeptide ester in 80% yield using dioxane as a reaction solvent. m. p. 117~120°C (sintered at 102~103°C). A sample for the analysis was twice recrystallized from ethyl acetate-petroleum ether. m. p. 122~123°C (sintered at 115~116°C). $[\alpha]_D^{10}$ −130.7 (c. 16 mg. in 1 ml. of methanol).

Anal. Found: C, 59.24; H, 7.08; N, 6.76. Calcd. for $C_{20}H_{28}O_7N_2$: C, 59.10; H, 6.94; N, 6.89%.

L-Threonyl-L-valyl-L-glutamic acid dimethyl ester and L-valyl-L-glutamic acid dimethyl ester were quantitatively obtained by decarbobenzyloxylation with hydrogen bromide in dioxane from the respective carbobenzyloxy derivative.

***N*-Carbobenzyloxy-L-threonine.**—To the cold solution of 2 N sodium hydroxide (25 ml.) containing L-threonine (6 g.) was added 2 N sodium carbonate (38 ml.) and carbobenzyloxychloride (8.5 g.). The mixture was shaken vigorously for 15 minutes at room temperature. Excess of carbobenzyloxychloride precipitated was removed into ether and the aqueous layer was acidified to Congo red with 6 N hydrochloric acid. Then the oily product precipitated was extracted into ethyl acetate (30 ml. three times) and the united extracts were dried over anhydrous sodium sulfate. Concentration, followed by addition of petroleum ether, afforded silky crystals (12 g. 95%). m. p. 95~98°C. Recrystallization from ethyl acetate-petroleum ether gave white needles. m. p. 103~105°C. $[\alpha]_D^{25}$ −2.4 (c. 11.7 mg. in 1 ml. of ethanol).

Anal. Found: C, 57.50; H, 6.25; N, 5.67. Calcd. for $C_{12}H_{15}O_5N$: C, 56.91; H, 5.97; N, 5.53%.

L-Histidyl-L-leucine. — Dicarbobenzyloxy-L-histidyl-L-leucine methyl ester (2.4 g.) was suspended in 20 ml. of concentrated hydrochloric acid and was kept at 37°C for 50 minutes. The opaque solution was spread on a glass plate and evaporated to dryness over concentrated sulfuric acid under reduced pressure. The residue was dissolved in a small amount of 99% ethanol and triethyl amine was added after the removal of insoluble substance. A fluffy material precipitated was collected. Yield 0.5 g. (42%). The dipeptide was recrystallized from 33% ethanol. m. p. 212~214°C (decomp.) $[\alpha]_D^{14}$ −43.5 (c. 10 mg. in 1 ml. of 0.1 N sodium hydroxide) (lit.[9] $[\alpha]_D^{22}$ −43.5 (c. 1.0, 0.1 N sodium hydroxide)).

Anal. Found: N, 20.54. Calcd. for $C_{12}H_{20}O_3N_4$: N, 20.88%.

Summary

N-α,N-Im-Dicarbobenzyloxyhistidine was prepared in good yield using sodium carbonate, and some histidyl peptide derivatives were synthesized by Sheehan's method using the compound as a starting material.

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