*Synthesis of Histidyl Peptides. II. Synthesis and Properties of Dicarbobenzyloxyhistidylamino Acid Esters**

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In the previous paper[1], a report was made of the snythesis of some *N*-α-*N*-*Im*-dicarbobenzyloxy-L-histidyl-L-amino acid esters including neutral, acidic, hydroxyl and sulfur-containing amino acids from dicarbobenzyloxyhistidine and respective amino acid esters by the dicyclohexylcarbodiimide method and it was observed that this reagent was useful for the preparation of these peptide derivatives. However, other possible methods for the synthesis of the dicarbobenzyloxyhistidyl peptide ester have not yet been investigated. In the present communication, some recent studies on the application of the other peptide bond forming methods for the preparation of dicarbobenzyloxy peptide esters are described, and additional syntheses of some of dicarbobenzyloxyhistidyl-amino acid esters by the dicylohexylcarbodiimide method are also included.

In order to find the most suitable method to prepare dicarbobenzyloxyhistidylamino acid ester from its constituent, three different methods (the mixed anhydride, the *p*-nitrophenyl ester and the dicyclohexylcarbodiimide methods) were compared in points of easiness of purification, purity and yield of the product. Because of the relative ease in the preparation of crystalline material, dicarbobenzyloxy-L-histidyl-L-phenylalanine methyl ester was chosen as a model peptide in the present investigation.

In the first place, the mixed anhydride method using alkylchloroformate[2] was employed as a synthetic method.

When dicarbobenzyloxy derivative of L-histidine is recrystallized from methyl alcohol, this material is known to contain one molecule of methyl alcohol which is the essential factor in getting stable crystals[1]. Since methyl alcohol was known to react with alkylchloroformate, twice molar amounts of the reagent were brought to reaction with dicarbobenzyl-

* Part I; The previous report (Ref. 1) was Part I of this series. Preliminary study was presented at the 14th Annual Meeting of the Chemical Society of Japan, Tokyo, April, 1961.

1) S. Akabori, K. Okawa and F. Sakiyama, *Nature*, **181**, 772 (1958); F. Sakiyama, K. Okawa, T. Yamakawa and S. Akabori, This Bulletin, **31**, 926 (1958).

2) J. R. Vaughan, *J. Am. Chem. Soc.*, **73**, 3547 (1951); Th. Wieland, H. Bernhard, *Ann.*, **572**, 190 (1951); R. A. Boissonas, *Helv. Chim. Acta*, **34**, 884 (1951).

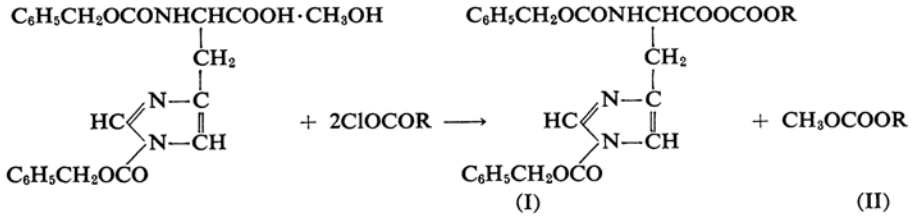

Fig. 1. Reaction of *N*-α-*N*-*Im*-dicarbobenzyloxyhistidine methanolate with alkyl chloroformate.

oxy-L-histidine methanolate[3] in dry tetrahydrofuran to prepare the mixed anhydride (I). Both the mixed anhydride (I) and alkyl-methyl carbonate (II) were formed in the reaction mixture (Fig. 1). By the coupling of the ester component with the mixed anhyride (I), the crystalline model peptide derivative was obtained in a 46% yield when isobutyl chloroformate was used. The same product was also obtained in a 35% yield by using ethyl chloroformate in place of the isobutyl derivative. When the reaction mixture was immediately warmed up to the refluxing point, after completion of the addition of L-phenylalanine methyl ester to the mixed anhydride (I), the yield increased significantly higher (50%).

After the first crop of the model peptide derivative was collected, no ninhydrin positive substances was detected in the mother liquor by paper chromatography.

It seems that no side reaction of alkyl methyl carbonate (II) with the methyl ester of L-phenylalanine occurs at this temperature. Therefore the absence of a free amino group might be due to the acylation of the amino group of phenylalanine ester with an excess of alkyl chloroformate. Next, the model peptide was prepared using a reduced amount of the reagent, one and a half equivalents of alkyl-chloroformate to a unit amount of disubstituted histidine, in stead of two, by a similar refluxed procedure. The yield of the model peptide derivative in this experiment was much higher than the previous. This fact suggested that the methyl alcohol was partly liberated from dicarbobenzyloxyhistidine methanolate[4] and the amount of the reagent was sufficient even under the above conditions, or that the reagent was preferably reacted with phenylalanine ester than alcohol.

In the second place, *p*-nitrophenyl ester of dicarbobenzyloxy-L-histidine was prepared in an 85% yield in chloroform by the Bodanszky's procedure[5] and also obtained by the procedure via the mixed anhydride[6]. This *p*-nitrophenyl ester could not be obtained in a crystalline state, in contrast to those of the other amino acid derivatives. Coupling of this ester with L-phenylalanine methyl ester afforded the model peptide derivative in a yield of 58%. This relatively low yield of the product might be attributed to the presence of another unsubstituted tertiary nitrogen in the imidazole nucleus and partly to the steric hindrance by the bulky substituent adjacent to the carboxyl group in the histidine residue.

Thirdly the model peptide derivative was prepared by the condensation using dicyclohexylcarbodiimide[7]. No difficulties were encountered in the procedure of the peptide synthesis by this reagent as mentioned in the previous paper. The most satisfactory result was given in the present case.

Some pysico-chemical properties of this model peptides obtained by the above described different methods were compared as is shown in Table I and Fig. 2.

It was concluded from the above results that the dicyclohexylcarbodiimide method was the most suitable for the formation of the peptide bond in which dicarbobenzyloxyhistidine was concerned. By this method additional dicarbobenzyloxy-L-histidyl-L-amino acid esters containing alanine, valine, aspartic acid, tyrosine, *S*-benzylcysteine, methionine and serine were synthesized. The author also describes the preparation of the benzyl ester of dicarbobenzyloxy-L-histidyl-L-threonine. The benzyl ester of the threonine was prepared by an esterification procedure similar to that of L-histidine benzyl ester[8]. The DL-isomer of threonine benzyl ester tosylate was obtained in fine crystals. Efforts to crystallize the L-isomer failed, but L-threonine benzyl ester could be obtained in fine needles[9]. *N*-α-Carbobenzyloxy-L-histidyl-L-threonine hydrazide from this benzl ester was identical with same compound obtained from the methyl ester.

3) Dicarbobenzyloxy-L-histidine methanolate represents crystalline dicarbobenzyloxy-L-histidine with one molecule of methyl alcohol.

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

5) D. F. Elliot, D. W. Russel, *Biochem. J.*, **66**, 49P (1957); M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959).

6) M. Rothe and F. W. Kunitz, *Ann.*, **609**, 97 (1957); Details by this procedure will be presented elsewhere.

7) J. C. Sheehan and G. P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955).

8) S. Akabori. S. Sakakibara and S. Shiina, This Bulletin, **31**, 784 (1958).

9) F. Sakiyama, unpublished data.

TABLE 1. DICARBOBENZYLOXY-L-HISTIDYL-L-PHENYLALANINE METHYL ESTER OBTAINED BY MEANS OF VARIOUS METHODS

Method	Reaction solvent	Yield	M. p. °C	$[\alpha]_D^{20}$ *8	Anal.*9 C	H	N
DCCD*1	Chloroform	93	123~124, 132	+54.2	65.74	5.58	9.56
Mixed anhydride with *iso*-BuOCOCl*2	THF*4	46*5, 50*6, 71*7	123~124, 132~133	+53.5	65.86	5.49	9.52
Mixed anhydride with EtOCOCl*3	THF	35	123~124, 132~133	—	65.53	5.56	9.55
p-Nitrophenol	Chloroform	58	123~124, 132~133	+54.4	65.90	5.48	9.58

*1 DCCD: dicyclohexylcarbodiimide
*2 *iso*-BuOCOCl: isobutyl chloroformate
*3 EtOCOCl: ethyl chloroformate
*4 THF: tetrahydrofuran
*5 The molar ratio between dicarbobenzyloxy-L-histidine and alkyl chloroformate is 1 to 2 and the reaction mixture of the mixed anhydride and phenylalanine methyl ester was not warmed.
*6 The molar ratio is similar to *5, but the reaction mixture was warmed to reflux.
*7 The molar ratio is 1 to 1.5 and the reaction mixture was transiently warmed to reflux.
*8 *c* 2.5 in chloroform
*9 Calcd. for $C_{32}H_{32}O_7N_4$: C, 65.74; H, 5.52; N, 9.59%

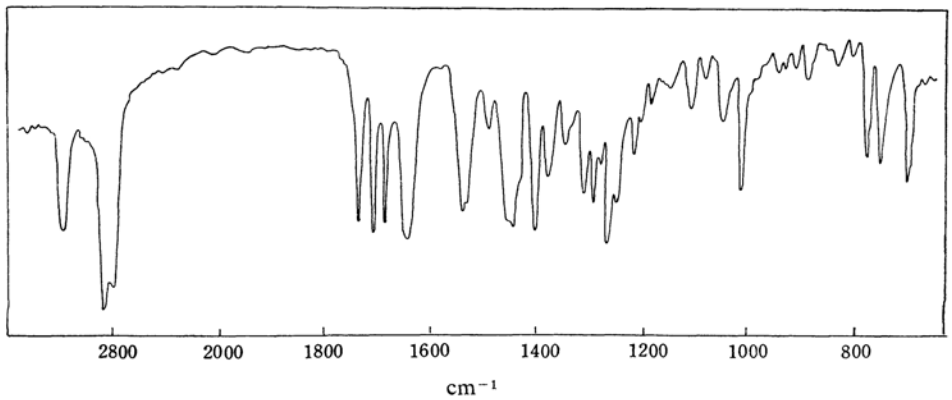

Fig. 2. IR-Spectrum of *N*-α-*N*-*Im*-dicarbobenzyloxy-L-histidyl-L-phenylalanine methyl ester obtained by the DCCD method.

It is interesting to note that the imidazole linked carbobenzyloxy group of dicarbobenzyloxyhistidine is relatively less stable than that of dicarbobenzyloxyhistidylamino acid ester, when the crystalline material is allowed to stand in the atmosphere for a long time.

When the dicarbobenzyloxyhistidine was exposed to alkaline media, the labile imidazole-linked carbobenzyloxy group was eliminated and *N*-α-carbobenzyloxyhistidine was produced. Therefore it was expected that some difficulties would be encountered during the alkaline hydrolysis of the disubstituted histidyl peptide ester owing to the presence of the labile carbobenzyloxy group. In the present paper, the problems concerning the alkaline hydrolysis of the dicarbobenzyloxy-L-histidylamino acid ester are also investigated.

As has been reported[10], *N*-α-carbobenzyloxyhistidylamino acid esters, which were prepared by means of the azide method, were thoroughly hydrolyzed by an equimolar amount of alkali in aqueous alcoholic solution. The alkaline hydrolysis of dicarbobenzyloxyhistidylamino acid esters was thus explored under similar conditions. But only low yield of the hydrolysis product was obtained. Since no detectable cleavage of the peptide bond and no destruction of the imidazole nucleus were found, it appears probable that the low yield was based on the incomplete hydrolysis.

It seems probable that the incompleteness of the hydrolysis reaction was due to the presence of the alkaline labile carbobenzyloxy group. For example, a hydrolysis product of dicarbobenzyloxy-L-histidyl-L-phenylalanine

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

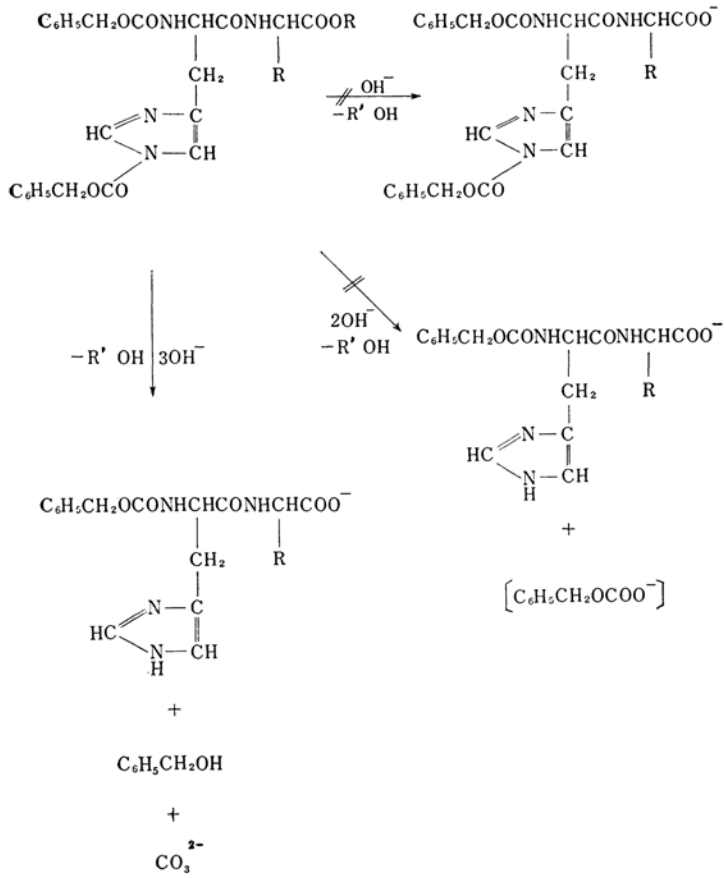

Fig. 3. Alkaline hydrolysis of the dicarbobenzyloxyhistidyl amino acid ester.

ethyl ester was identified to be *N*-α-carbobenzyloxy-L-histidyl-L-phenylalanine and not the dicarbobenzyloxy dipeptide. The carbobenzyloxy group linked to the nitrogen of the imidazole nucleus in the histidyl peptide derivative was quite stable comparing with that of dicarbobenzyloxy histidine in the crystalline state. However, in the alkaline or basic medium, this carbobenzyloxy group was also unstable. In alkaline media, hydroxide ions might simultaneously attack both the labile carbobenzyloxy group and the carboxyl ester group. Generally, the active acyl group linked to imidazole was readily convertible to the acyl anion and imidazole by hydroxdie ions. Thus the formation of the acyl anion might result in the consumption of hydroxide ions. If the acyl anion produced was stable in alkaline media, twice molar amounts of alkali would be sufficient for the hydrolysis reaction as is shown in Fig. 3. But the use of twice molar amounts of alkali also brought the incomplete hydrolysis of the ester group. For example, *N*-α-carbobenzyloxy-L-histidyl-L-leucine, which used to be readily obtained as crystals, was not crystallized merely by acidification of the reaction mixture but the isolation yield was 60%.

The complete hydrolysis was finally obtained by the use of three-fold molar equivalents of alkali in alcohol, dioxane or acetone. Thus the following mechanism for the hydrolysis of dicarbobenzyloxyhistidylamino acid ester with aqueous alkali would be considered: hydroxide ions attacked not only the –CO–N= bond in the imidazole-linked carbobenzyloxy group but also the –CO–O– bond in the carboxyl ester group, and the intermediate monobenzyl carbonate ions produced from the former might be converted into benzyl alcohol and carbonate ions by the action of hydroxide ions as is shown in Fig. 3. This carbonate forming reaction would prevent the complete hydrolysis of the ester group when equi- or twice molar amounts of alkali are used.

The infrared spectra of these *N*-α-carbobenzyloxy-L-histidylamino acids showed the absence of the absorption band at near 1750 cm^{-1}.

N-α-*N*-*Im*-Dicarbobenzyloxy-L-histidyl-L-phenylalanine prepared by the direct carbobenzyloxylation of the *N*-α-carbobenzyloxy dipeptide revealed the above characteristic absorption[11] owing to the presence of the imidazole-linked carbobenzyloxy group as is shown in Fig. 4.

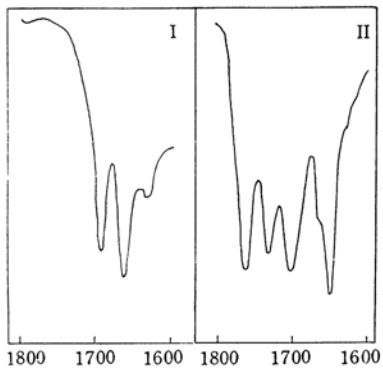

Fig. 4. Infrared spectra of *N*-α-monocarbobenzyloxy- and *N*-α-*N*-*Im*-dicarbobenzyloxy-L-histidyl-L-phenylalanine.

I *N*-α-Carbobenzyloxy-L-histidyl-L-phenylalanine.
II *N*-α-*N*-*Im*-Dicarbobenzyloxy-L-histidyl-L-phenylalanine.
(Nujol mull. Rock-salt optics. Hitachi infrared spectrophotometer)

A few of *N*-α-carbobenzyloxyl-L-histidylamino acids were obtained by this hydrolysis procedure. The threonine derivative was obtained in a poor yield in contrast to the *O*-benzylserine derivative. The fact seems to be due to the presence of a free hydroxyl group in threonine residue. It was observed that the carbobenzyloxyhistidylamino acids are generally hardly soluble in ordinary oranic solvents, as is expected from their dipolar properties.

Experiental

All melting points were uncorrected. The R_f values described were those by *n*-butanol-acetic acid-water (4 : 1 : 1, by volume) and the spot of *N*-α-carbobenzyloxyhistidylamino acid was detected by the use of Pauly's reagent.

***N*-α-*N*-*Im*-Dicarbobenzyloxy-L-histidyl-L-phenylalanine Methyl Ester.**—*A) By the Mixed Anhydride Method.* — i) The mixed anhydride method with isobutyl chloroformate:

Dicarbobenzyloxy-L-histidine methanolate (4.5 g., 0.01 M) was dissolved in anhydrous tetrahydrofuran (10 ml.) and triethylamine (3 ml.). Then isobutyl chloroformate (2.7 g., 0.02 M) was added dropwise to the above solution with vigorous stirring at −16 ~ −10°C and the temperature was maintained at −10°C for 15 min. L-Phenylalanine methyl ester was added to the above mixed anhydride solution at −15 ~ −20°C with vigorous stirring. After being kept overnight at room temperature, white precipitates were filtered off and the filtrate was evaporated to syrup, which was first dissolved in methyl alcohol and then gradually crystallized out. The fine needles were collected. Yield 2.5 g. The second crop (0.2 g.) was obtained from the mother liquor. Total yield was 2.7 g. (46%). Recrystallization was performed from methyl alcohol.

ii) The mixed anhydride method with isobutyl chloroformate under heating:

After the addition of the ester component to the anhydride solution was complete, the mixture was warmed to the refluxing point and then immediately cooled to room temperature. The filtrate, after triethylamine hydrochloride had been removed, was concentrated and treated with methyl alcohol. The yields were 50% (when dicarbobenzyloxy-L-histidine methanolate/isobutyl chloroformate=1/2), and 71% (when the above ratio=2/3), respectively.

iii) The mixed anhydride method with ethyl chloroformate:

The procedure of synthesis was similar to i).

B) By the p-Nitrophenyl Ester Method. — i) Dicarbobenzyloxy-L-histidine *p*-nitrophenyl ester: A solution of dicyclo hexylcarbodiimide (1.1 g.) in chloroform (6 ml.) was added into a chloroform solution (20 ml.) containing dicarbobenzyloxy-L-histidine methanolate (2.3 g.) and *p*-nitrophenol (0.83 g.). After being kept at room temperature for 5 hr., two drops of glacial acetic acid was added to the above mixture. After one hour, dicyclohexylurea was separated and filtered off. The filtrate was evaporated to syrup in vacuo. By the addition of ether, a gelatinous product was precipitated, filtered off and thoroughly washed with ether. The removal of ether gave a powder-like *p*-nitrophenyl ester with slight yellowish color. Yield 2.4 g. (85%). Sample for analysis was reprecipitated from ethyl alcohol but could not be obtained in fine crystals, m. p. 86 ~ 90°C.

Found: C, 61.53; H, 4.55; N, 10.35. Calcd. for $C_{28}H_{24}O_8N_4$: C, 61.76; H, 4.44; N, 10.29%.

ii) Dicarbobenzyloxy-L-histidyl-L-phenylalanine methyl ester: Dicarbobenzyloxy-L-histidine *p*-nitrophenyl ester (2.2 g.) was dissolved in warm chloroform (7 ml.) and the chloroform solution was mixed with L-phenylalanine methyl ester which was prepared from its hydrochloride (1.1 g.) and triethylamine in chloroform. After being kept at room temperature overnight, the solvent was removed and resulting syrup was treated with methyl alcohol, and then chilled as was described in A i). Yield 1.35 g. (58%).

C) By the Dicyclohexylcarbodiimide Method.—Dicarbobenzyloxy-L-histidine methanolate (2.3 g.) was dissolved in chloroform (7 ml.). A solution of L-phenylalanine methyl ester, which was prepared from its hydrochloride (1.4 g.) and triethylamine, was added into the above solution. After the addition of dicyclohexylcarbodiimide (1.1 g.) in chloroform was complete, the reaction mixture was

11) In general, dicarbobenzyloxyhistidine and dicarbobenzyloxyhistidyl amino acid esters show strong absorption at 1750~1760 cm⁻¹.

TABLE II. DICARBOBENZYLOXY-L-HISTIDYLAMINO ACID ESTERS OBTAINED BY THE DCCD METHOD

Dicarbobenzyloxy derivative of L-histidyl-	Yield %	M. p. °C	$[\alpha]_D^{20}$*	Solvent for recrystallization	Anal. Found C	Found H	Found N	Calcd. C	Calcd. H	Calcd. N
L-Alanine ethyl ester	77	131~132	+42.0	Ethyl acetate-petroleum ether	62.30	5.90	10.97	62.05	5.79	10.72
L-Valine methyl ester	76	120~121	+37.6	Ethyl acetate	62.87	6.07	10.82	62.65	6.01	10.44
L-Aspartic acid dimethyl ester	72	100~102	+40.9	Ethyl acetate-petroleum ether	59.46	5.39	9.93	59.34	5.34	9.89
S-Benzyl-L-cysteine methyl ester	66	137~137.5	+27.6	Methyl alcohol-ether	62.99	5.39	9.55	62.83	5.42	9.09
L-Threonine ethyl ester	65	106~108	+21.3	Ethyl acetate	60.43	5.90	10.18	60.83	5.84	10.14
L-Phenylalanine ethyl ester	94	159~161	+50.0	Ethyl alcohol	65.91	5.70	9.77	66.21	5.89	9.39
L-Tyrosine methyl ester	—	129~131	+41.6	Ethyl alcohol-ether	64.18	5.55	9.15	63.98	5.37	9.33
L-Serine methyl ester	50	152~152.5	+25.6	Ethyl alcohol	59.31	5.44	11.11	59.53	5.38	10.68
Glycine benzyl ester	82	112.5~113.5	+21.7	Ethyl acetate	65.50	5.33	9.94	65.25	5.30	9.82
L-Methionine benzyl ester	72	125	+32.9	Ethyl alcohol	63.56	5.70	8.72	63.34	5.63	8.69
L-Glutamic acid dibenzyl ester	79	117~118	+24.5	Ethyl alcohol-petroleum ether	67.26	5.60	7.45	67.20	5.50	7.63

* *c* 2, in chloroform

allowed to stand overnight at room temperature. The cyclohexylurea precipitated was filtered off and the filtrate was evaporated. The syrupy material was dissolved in a small amount of methyl alcohol and chilled. The fine needles precipitated were collected and weighed. Yield, 2.7 g. (93%). By recrystallization from methyl alcohol 2.3 g. of pure materials were obtained (80%).

This peptide derivative has double melting points. The crystal melted at 123~124°C after softening at 117~119°C and meanwhile (within from thirty seconds to a few minutes) the molten materials solidified and melted again at 131~132°C.

Dicarbobenzyloxy-L-histidylamino acid esters described in Table II were synthesized by the carbodiimide method[1] from dicarbobenzyloxy-L-histidine methanolate and respective amino acid esters.

DL-Threonine Benzyl Ester *p*-Toluenesulfonate. —DL-Threonine (5 g.), *p*-toluenesulfonic acid monohydrate (10 g.) and benzyl alcohol (50 ml.) were dissolved in chloroform (100 ml.). The mixture was refluxed for thirty hours by using Wieland's esterification apparatus, in which silica gel was used as a desiccant. The solvent and excess benzyl alcohol were removed under reduced pressure and the oily product obtained was gradually crystallized by a minute trituration in ether (200 ml.) under cooling. After being kept in a refrigerator overnight, the crystals were filtered and washed thoroughly with ether until no smell of benzaldehyde was detected. Recrystallization of the crude product (12.5 g., 89%) from methyl alcohol-ether gave fine crystals. m. p. 113°C. R_f 0.72, yellow spot with ninhydrin.

Found: C, 57.46; H, 5.82; N, 3.59. Calcd. for $C_{18}H_{23}O_6NS$: C, 56.68; H, 6.08; N, 3.67%.

Since an attempt to crystallize the L-isomer by the same procedure failed, and a slightly brownish paperchromatographically pure L-threonine benzyl ester *p*-toluenesulfonate was used as a starting material for the peptide synthesis without further purification.

Dicarbobenzyloxy-L-histidyl-L-threonine Benzyl Ester. — Dicarbobenzyloxy-L-histidyl-L-threonine benzyl ester was prepared in a 71% yield. Recrystallization from ethyl alcohol or ethyl alcohol-petroleum ether gave fine crystals. M. p. 130~131°C. $[\alpha]_D^{20}$ +22.5° (*c* 2, in chloroform).

Found: C, 64.46; H, 5.66; N, 9.40. Calcd. for $C_{33}H_{34}O_8N_4$: C, 64.47; H, 5.57; N, 9.11%.

***N*-α-Carbobenzyloxy-L-histidyl-L-threonine Hydrazide.**—Warm methanolic solution of dicarbobenzyloxy-L-histidyl-L-threonine benzyl ester (600 mg.) was cooled to room temperature and 80% hydrazine hydrate (0.3 ml.) was added. After 42 hr. at room temperature, white crystals (350 mg., 82%) precipitated, and were collected, washed with water and recrystallized from ethyl alcohol. M. p. 185.5~186°C (decomp.).

Found: C, 51.20; H, 6.29; N, 19.72. Calcd. for $C_{18}H_{24}O_5N_6 \cdot H_2O$: C, 51.18; H, 6.20; N, 19.90%.

The same product was also obtained from the methyl ester of dicarbobenzyloxy-L-histidyl-L-threonine by a similar procedure.

***N*-α-Carbobenzyloxy-L-histidylglycine.**—Into dicarbobenzyloxy-L-histidylglycine methyl ester (478 mg.) suspended in methyl alcohol (4 ml.), N sodium

hydroxide (3 ml.) was portionwise added with shaking. By the addition of the first 2 ml. of alkali the suspension became clear. After being kept for half an hour at room temperature, the clear solution was diluted with water (5 ml.) and acidified with N hydrochloric acid to pH 4.8~5.0. Yield, 300 mg. (83%). Recrystallization from hot dimethylformamide-ether gave pure *N*-α-carbobenzyloxy-L-histidyl-glycine, m. p. 230°C (decomp.); lit.[12], 230~231°C (decomp.). $[\alpha]_D^{31.2}$ −20.5° (*c* 1.05, in 5% aqueous hydrogen carbonate), R_f, 0.78.

Found: C, 55.00; H, 5.34; N, 16.39. Calcd. for $C_{16}H_{18}O_6N_4$: C, 55.48; H, 5.24; N, 16.17%.

This procedure is referred to below as method-i-a.

***N*-α-Carbobenzyloxy-L-histidyl-L-alanine.**—Into a suspension of dicarbobenzyloxy-L-histidyl-L-alanine ethyl ester (600 mg.) in ethyl alcohol (3 ml.), N sodium hydroxide (3 ml.) was added. The resulting solution was allowed to stand for half an hour at room temperature and then acidified with N hydrochloric acid to pH 4.8~5.0. Fine white needles (285 mg.) were obtained by scratching and chilling, m. p. 214~216.5°C (decomp.). Additional crystals (30 mg.) were obtained from the mother liquor. Total yield, 87%. Pure materials recrystallized from water melted at 218~219°C with decomposition after sintering at 215°C. $[\alpha]_D^{31.2}$ −25.5° (*c* 1.00, in 5% sodium hydrogen carbonate).

Found: C, 56.36; H, 5.60; N, 15.81. Calcd. for $C_{17}H_{21}O_5N_4Cl$: C, 56.65; H, 5.59; N, 15.55%.

This procedure is referred to below as method-i-b.

***N*-α-Carbobenzyloxy-L-histidyl-L-valine.**—A solution of dicarbobenzyloxy-L-histidyl-L-valine methyl ester (536 mg.) in methyl alcohol (4 ml.) was treated with N sodium hydroxide (3 ml.) at room temperature for half an hour and then the resulting solution was acidified. In this case, contrary to the other dipeptide derivative no precipitates were produced. The solution then evaporated to dryness under reduced pressure. The residues were dissolved in ethyl alcohol and the insoluble material (probably sodium chloride) was filtered off. The filtrate was diluted with ether. Fine crystals were collected (385 mg., 95%), m. p. 196~197.5°C (decomp.). Recrystallization from ethyl alcohol-ether gave fine crystals after being kept in a refrigerator about three weeks, m. p. 187~187.5°C (decomp.) sintered at 175°C. $[\alpha]_D^{31.2}$ −20.0° (*c* 1.08, in water).

Found: C, 56.52; H, 6.23; N, 13.95. Calcd. for $C_{19}H_{25}O_5N_4 \cdot H_2O$: C, 56.17; H, 6.45; N, 13.79%.

This procedure is referred to below as method-ii.

***N*-α-Carbobenzyloxy-L-histidyl-L-leucine.**—By method-i-b *N*-α-carbobenzyloxy-L-histidyl-L-leucine was obtained from dicarbobenzyloxy dipeptide ester in a 90% yield. Recrystallization from ethyl alcohol-water gave pure materials, m. p. 188~189°C (decomp.) $[\alpha]_D^{31.2}$ −9.9° (*c* 1.00 in 5% aqueous sodium hydrogen carbonate).

Found: C, 57.09; H, 6.87; N, 13.42. Calcd. for $C_{20}H_{26}O_5N_4 \cdot H_2O$: C, 57.13; H, 6.87; N, 13.33%.

***N*-α-Carbobenzyloxyl-L-histidyl-L-phenylalanine.**—Dicarbobenzyloxy-L-histidyl-L-phenylalanine ethyl ester (600 mg.) was hydrolyzed by method-i-a and *N*-α-carbobenzyloxy-L-histidyl-L-phenylalanine (420 mg.) was obtained in an almost theoretical yield. Recrystallization from a large amount of 25% aqueous ethyl alcohol gave fine crystals, m. p. 231~232°C (decomp.) (lit., 227~228[13]).

Found: C, 63.28; H, 5.58; N, 13.15. Calcd. for $C_{24}H_{26}O_6N_4$: C, 63.29; H, 5.54; N, 12.84%.

The alkaline hydrolysis in aqueous dioxane solution also proceeded smoothly in a homogeneous solution but only colloidal precipitates resulted from acidification. The hydrolysis in the acetone solution was, at first, in a heterogeneous state and after a few minutes the solution became clear but the sodium carbonate was precipitated at the end of the reaction.

By method-i-a the dipeptide derivatives mentioned below were also prepared.

***N*-α-Carbobenzyloxy-L-histidyl-*O*-benzyl-L-serine.**—Yield, 80%. M. p. 210~211°C (decomp.). Recrystallization from dimethylformamide-ether.

Found: C, 61.51; H, 5.72; N, 12.04. Calcd. for $C_{24}H_{26}O_6N_4$: C, 61.79; H, 5.61; N, 12.01%.

***N*-α-Carbobenzyloxy-L-histidyl-L-tyrosine.**—Yield, 88%. M. p. 240°C (decomp.). Recrystallization from ethyl alcohol-water.

Found: C, 61.05; H, 5.35; N, 12.38. Calcd. for $C_{24}H_{26}O_7N_4$: C, 60.89; H, 5.52; N, 12.30%.

***N*-α-Carbobenzyloxy-L-histidyl-*S*-benzyl-L-cysteine.**—Yield, 94%. M. p. 197~199°C. (lit., 191~192°C[14]).

Found: C, 59.87; H, 5.35; N, 11.63. Calcd. for $S_{24}H_{26}O_5N_4S$: C, 59.58; H, 5.43; N, 12.09%.

***N*-α-Carbobenzyloxy-L-histidyl-L-aspartic Acid.**—By method-i-b, dicarbobenzyloxy-L-histidyl-L-aspartic acid dimethyl ester (2.3 g.) in methyl alcohol (8 ml.) was treated with four molar equivalents of alkali because of the presence of two carboxyl groups in the aspartic acid residue. Yield, 1.3 g. (75%). M. p. 222~222.5°C (decomp.). Recrystallization from dimethylformamide did not raise the melting point. $[\alpha]_D^{31.2}$ −16.9° (*c* 1.05, 5% aqueous sodium hydrogen carbonate) (lit., m. p. 222°C[14]).

Found: C, 53.15; H, 5.19; N, 13.83. Calcd. for $C_{18}H_{20}O_7N_4$: C, 53.47; H, 4.98; N, 13.86%.

***N*-α-Carbobenzyloxy-L-histidyl-L-glutamic Acid.**—By the procedure mentioned above, *N*-α-carbobenzyloxy-L-histidyl-L-glutamic acid was prepared in an 83% yield and recrystallized from water, m. p. 169~172°C. $[\alpha]_D^{31.2}$ −21.1 (*c* 1.00, in a 5% aqueous sodium hydrogen carbonate) (lit., 169~171°C[14]).

Found: C, 53.19; H, 5.48; N, 12.98. Calcd. for $C_{19}H_{22}O_7N_4 \cdot (1/2)H_2O$: C, 53.40; H, 5.42; N, 13.11%.

***N*-α-Carbobenzyloxy-L-histidyl-L-threonine.**—The *N*-α-carbobenzyloxy derivative of this dipeptide was isolated in a 50% yield by method-ii and recrystallized from ethyl alcohol-ether, m. p. 189.5~190°C (decomp.). $[\alpha]_D^{16}$ −23.6° (*c*, 1.00, in 5% aqueous sodium hydrogen carbonate).

Found: C, 55.38; H, 5.68; N, 14.35. Calcd. for $C_{18}H_{22}O_6N_4$: C, 55.24; H, 5.66; N, 14.32%.

12) G. Weitzel, F. Schneider and A-M. Fretzdorff, *Z. physiol. Chem.*, **307**, 23 (1957).

13) K. Hofmann, H. Kappeler, A. E. Furlenmeier, M. E. Wooner, E. T. Schwartz and T. A. Thompson, *J. Am. Chem. Soc.*, **79**, 1641 (1957).

14) F. Schneider, *Z. physiol. Chem.*, 320, 82 (1960).

Dicarbobenzyloxy-L-histidyl-L-phenylalanine.—*N*-α-Carbobenzyloxy-L-histidyl-Lphenylalanine (420 mg.) was dissolved in a mixture of sodium hydroxide (1 ml.), water (7 ml.) and solid sodium carbonate (100 mg.). Into the above solution, carbobenzyloxychloride (171 mg.) was portionwise added under cooling and stirring. An oily product was separated, then gradually solidified, and finally gelled. After half an hour, the solution was acidified to Congo red and the gel was filtered, washed with alcohol and dried. White powders (300 mg.) were obtained and reprecipitated from the mixture of ethyl alcohol and ethyl acetate-ether. M. p. 123°C.

Found: C, 63.84; H, 5.40; N, 9.31. Calcd. for $C_{31}H_{30}O_7N_4 \cdot H_2O$: C, 63.25; H, 5.48; N, 9.52%.

Summary

Three different methods for the synthesis of dicarbobenzyloxy-L-histidylamino acid esters were studied. The general procedure of the hydrolysis of dicarbobenzyloxyhistidylamino acid esters were reported and the mechanism of this hydrolysis reaction was also discussed.

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