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Studies on Histidyl Peptides. I

Imidazole Protected Histidine in the Synthesis of C-Terminal and N-Terminal Histidyl Peptides

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Carbobenzoxy-*im*.benzyl-L-histidine and *im*.benzyl-L-histidine benzyl ester have been used as starting materials in the synthesis of C-terminal and N-terminal histidyl peptides.

It has been suggested during the last few years that histidine probably constitutes a portion of the active center of various hydrolytic enzymes. This suggestion is strongly supported by the evidence that photo-oxidation of the residues of histidine in ribonuclease, trypsin, lysozyme, etc., is accompanied by the loss of enzymic activity. The esteratic properties of various imidazole compounds have been extensively studied [1], but whether the histidine residue is the primary site of reaction of the various organophosphorus enzyme inhibitors, remains a subject of intense controversy [2–4]. The tempting hypothesis that a phosphoryl group is transferred from the imidazole group of histidine to a hydroxyl group of an adjacent residue of serine, needs to be established on the basis of experimental evidence.

We therefore consider it worth while to undertake the synthesis of various peptides containing histidine and serine for two reasons. Primarily to study their behavior towards organophosphorus enzyme inhibitors, and secondly, to study the sensitivity of various proteolytic enzymes towards these peptides when phosphorylated at different functional groups. This is a part of the study of the biological importance of phosphorylated peptides [5,6] which is carried out in this laboratory.

A report of the synthesis of such peptides, involving the sequences histidylserine and serylhistidine, as well as several other C-terminal and N-terminal histidyl peptides will be presented in this paper. During this synthetic work, the recently reported procedure [7] *, employing imidazole-benzyl-L-histidine,

* *Footnote added in proof.* A preliminary note appeared in *J. Org. Chem.* **21** (1956) 1550 and it is noteworthy that Schwyzer has successfully employed imidazole protected histidine in the recent synthesis of a pentapeptide, [*Nature* **182** (1958) 1669].

was exclusively used. The potentialities of this procedure have also been investigated in detail in the course of this work.

DISCUSSION AND RESULTS

The use of carbobenzoxy-*im*.benzyl-L-histidine in the synthesis of N-terminal histidyl peptides.

The basic nature of the imidazole group of histidine has presented formidable problems during the incorporation of an histidine residue into synthetic polypeptides.

Recently, Katchalski *et al.*[8] reported the synthesis of 1,N-dicarbobenzoxy-L-histidine, but it has since been noted, that this compound decarbobenzoxylates readily to produce N-carbobenzoxy-L-histidine.

Akabori *et al.*[9], employing 1,N-dicarbobenzoxy-L-histidine as starting material, have synthesized a number of histidyl dipeptide ester derivatives. It is, however, evident from the work of Katchalski, that any attempt to extend the peptide chain of these dicarbobenzoxy histidyl peptide esters on either the carboxyl or the amino end, will be complicated due to the elimination of the 1-carbobenzoxy group.

It is noteworthy that although a number of synthetic procedures are currently available for the synthesis of histidyl peptides [9–11], no synthesis of dipeptide L-histidyl-L-histidine in optically pure form has yet been reported.

Fischer and Suzuki [12] in 1905 prepared histidyl-histidine by alkaline hydrolysis of L-histidine anhydride, but this hydrolysis was accompanied by racemization. Almost thirty years later, Abderhalden and Leinert [13] hydrolysed L-histidine anhydride with acid, thinking that they could avoid racemization, but no product was isolated.

In this connection, we have succeeded in producing an unequivocal synthesis of an L-histidyl-L-histidine derivative by employing *im*.benzyl-L-histidine [14] as starting material.

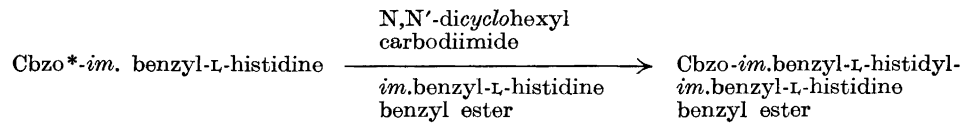
Coupling *im*.benzyl-L-histidine with one mole of benzyl chloroformate [15] in aqueous solution at 0°, at pH alkaline by addition of lithium hydroxide *, followed by acidification, resulted in the synthesis of carbobenzoxy-*im*. benzyl-L-histidine [7]. This compound is sparingly soluble in various organic solvents, such as chloroform, dioxane, ethylacetate, tetrahydrofuran, *etc.*, a fact which appeared to be a stumbling block in its usefulness in peptide synthesis. An attempt to convert carbobenzoxy-*im*.benzyl-L-histidine into its chloride hydrochloride on treatment with thionyl chloride, resulted in a crystalline product. Further work is in progress to identify the composition of this product.

Although the condensation of carbobenzoxy-*im*.benzyl-L-histidine with amino acid and peptide esters, employing the tetraethyl pyrophosphite procedure [16], holds promise due to its well established capacity to deal with

* Lithium hydroxide was chosen instead of sodium or potassium hydroxide since the latter forms less soluble salts with carbobenzoxy-*im*. benzyl-L-histidine which precipitated during the coupling, thus forming a mass.

amino acid and peptide derivatives of low solubility [17], we did not persist in pursuing this procedure since the carbodiimide method [18] operated with excellent results in our case.

A slight modification was, however, enforced due to the afore mentioned solubility properties of carbobenzoxy-*im*.benzyl-L-histidine in organic solvents. Carbobenzoxy-*im*.benzyl-L-histidine was therefore dissolved in dimethylformamide upon heating, and when the solution standing at room temperature had cooled to 40—50°, it was mixed with amino acid esters and carbodiimide. Condensation of carbobenzoxy-*im*.benzyl-L-histidine, with *im*.benzyl-L-histidine benzyl ester thus resulted in the production of carbobenzoxy-*im*.benzyl-L-histidyl-*im*.benzyl-L-histidine benzyl ester.



The conversion of the latter in to L-histidyl-L-histidine will be reported in a later communication.

In the investigated cases, reported below, the peptide ester derivatives thus formed were easily isolated in crystalline form from the dimethylformamide solution by addition of water.

Carbobenzoxy-*im*.benzyl-L-histidyl peptide esters can be extended on either the carboxyl or the amino end by hydrolysis of the ester group, or selective splitting of the N-carbobenzoxy group by hydrogen bromide [16,19] or catalytic hydrogenation [15].

The use of *im*.benzyl-L-histidine benzyl ester in the synthesis of histidine peptides

All the amino acid benzyl esters used in the course of this work were prepared in accordance with the directions of Cipera and Nicholls [20]. Following their method, we also conveniently esterified *im*.benzyl-L-histidine to give the *im*.benzyl-L-histidine benzyl ester dibenzenesulfonate. The latter ester was obtained in crystalline form in about 80 % yield. It can readily recrystallize and remain unchanged for several days in the open. In contrast, *im*.benzyl-L-histidine methyl ester dihydrochloride[7], obtained by the Fischer procedure, appears to be extremely hygroscopic.

Condensation of carbobenzoxy-L-phenylalanine with L-histidine methyl ester employing the carboxylic-carbonic anhydride procedure has been recently reported [21]. This procedure, however, does not always produce satisfactory results, probably due to the undesired side-reaction with the imidazole group of histidine.

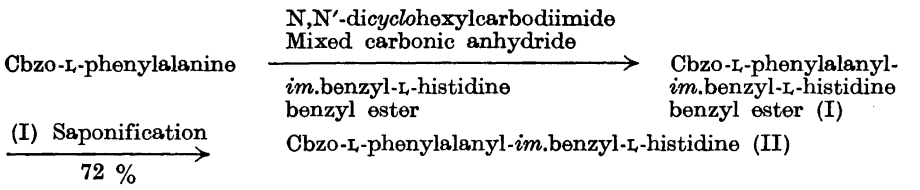
When carbobenzoxy amino acids were condensed with *im*.benzyl-L-histidine benzyl ester *via* the mixed carboxylic-carbonic anhydride [22,23] or the

* Cbzo stands for Carbobenzoxy.

carbodiimide procedure for peptide bond formation, the desired derivatives were obtained in good yield and in crystalline form. Carbobenzoxy-L-serine [24] was thus coupled with *im*.benzyl-L-histidine benzyl ester, employing the carbodiimide prodedure to give the product carbobenzoxy-L-seryl-*im*.benzyl-L-histidine benzyl ester * in crystalline form.

Merrifield and Woolley [25], during their synthetic studies with peptides possessing strepogeny activity, attempted the synthesis of carbobenzoxy-L-seryl-L-histidine methyl ester, to this end employing the azide and carbodiimide methods to condense carbobenzoxy-L-serine with histidine methyl ester. As the authors state, the oily product obtained was not homogeneous, and crystallized only after it had been purified by countercurrent distribution.

When carbobenzoxy-L-phenylalanine was condensed with *im*.benzyl-L-histidine benzyl ester by either of the aforementioned condensing methods, the product carbobenzoxy-L-phenylalanyl-*im*.benzyl-L-histidine benzyl ester (I) was obtained in good yield. Compound (I) was saponified, with no difficulty, with the equivalent amount of sodium hydroxide, and upon acidification with acetic acid, carbobenzoxy-L-phenylalanyl-*im*.benzyl-L-histidine (II) was obtained, after recrystallization, at a 72 % yield.



Hydrolysis of the ester (I) provides the carboxyl group of the *im*.benzyl-L-histidine residue free to link with other amino acids and peptides. Compound (II) was consequently condensed with L-leucine benzyl ester and L-aspartic acid dibenzyl ester by the carbodiimide procedure to produce carbobenzoxy-L-phenylalanyl-*im*.benzyl-L-histidyl-L-leucine benzyl ester (III) as well as the corresponding aspartate derivative.

Unreacted compound (II) was separated from (III), not by the usual procedure consisting in extraction with sodium or potassium bicarbonate solution [26]. Compound (II) and the corresponding derivatives reported here, form sparingly soluble salts in water with alkalies, triethylamine, dimethylamine, and ammonia.

Taking advantage of the widely different solubilities of (II) and (III) in ethylacetate, we were able to separate them by dissolving the residue in the above-mentioned solvent. Compound (II) is sparingly soluble in ethylacetate, precipitates and can be removed by filtration. Complete removal of (II) is accomplished by washing the ethylacetate layer thoroughly with 5 % sodium carbonate solution.

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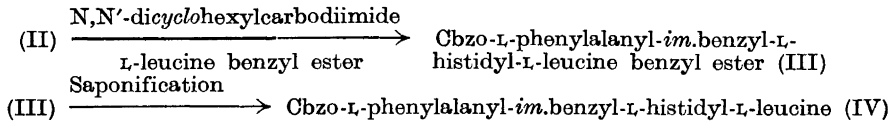
* The sequence serylhistidine has been identified in the B chain of insulin [Ryle A., Sanger F., Smith L. and Kitai R. *Biochem. J.* **60** (1955) 541] and it is rather interesting to note that the reverse sequence histidylserine occurs in glucagon [Bromer W., Sinn L. and Behrens O. *J. Am. Chem. Soc.* **79** (1957) 2807].

Compound (III) upon saponification, followed by acidification with acetic acid, produced carbobenzoxy-L-phenylalanyl-*im*.benzyl-L-histidyl-L-leucine (IV) in good yield.



Condensation of carbobenzoxy-L-phenylalanyl-*im*.benzyl-L-histidine azide with leucine benzyl ester does not always give satisfactory results due to the tendency of the azide to rearrange. A similar tendency has been observed in the case of carbobenzoxy-L-seryl-L-histidine azide by Merrifield and Wolley [25].

Compound (IV), dissolved in absolute ethanol, was selectively decarbobenzoxylated by catalytic hydrogenation in the presence of palladium black. The so formed L-phenylalanyl-*im*.benzyl-L-histidyl-L-leucine(V), being insoluble in absolute ethanol, precipitates during the hydrogenation. Selective splitting of the N-carbobenzoxy group frees the amino end for extension of the peptide chain, using conventional methods.

Compound (IV), dissolved in 50 % ethanol and hydrogenated for 24 h in the presence of palladium black, afforded the peptide L-phenylalanyl-L-histidyl-L-leucine with an $[\alpha]_D^{24}$ value of +30.5° as a 1.08 % solution in glacial acetic acid.

Since it is a well-known fact that *im*.benzyl-L-histidine easily splits by reduction with sodium in liquid ammonia [14] to produce free histidine, compound (IV) was reduced by this procedure. The tripeptide L-phenylalanyl-L-histidyl-L-leucine * thus obtained showed an optical rotation of $[\alpha]_D^{24}$ +29.5° as a 1 % solution in glacial acetic acid.

EXPERIMENTAL

Carbobenzoxy-im.*benzyl-L-histidine*. A solution of 1.22 g (0.005 mole) of *im*.benzyl-L-histidine in 5 ml of N LiOH was cooled in an ice-bath, and 1 ml of carbobenzoxy chloride, diluted in 3 ml of dioxane, was added in four portions while stirring. During the addition of the carbobenzoxy chloride the solution was kept alkaline by addition of N LiOH. When the last portion of the carbobenzoxy chloride was added, the solution was permitted to remain at room temperature for about 15 min. Addition of 20—30 ml of water, and acidification with acetic acid, caused the desired product to precipitate. It was then filtered off, washed well with water and finally with ether. It was recrystallized by dissolving in dimethylformamide and precipitating with ether. Yield 1.3 g (70 %), m.p. 210—213° (reported [7] 210—213°), $[\alpha]_D^{21}$ + 5.2° (*c* 5 in glacial acetic acid).

im.*Benzyl-L-histidine benzyl ester dibenzenesulfonate*. Into a 150 ml round-bottomed flask were placed 2.45 g (0.01 mole) of *im*.benzyl-L-histidine, 3.87 g (10 % excess) of benzenesulfonic acid monohydrate, 10 ml of benzyl alcohol and 20 ml of carbon tetrachloride. The mixture was then heated under reflux, the liberated water being removed azeotropically. A clear solution was obtained after reflux began. When water no longer

* The C-terminal sequence in valine-hypertensin has been shown to be phenylalanylhistidyl-leucine [Elliot, D. and Peart, W. *Nature* **177** (1956) 527].

distilled off (about the seventh addition of carbon tetrachloride), the reaction mixture was permitted to cool down to room temperature. Through addition of dry ether, the ester precipitated. It was then filtered and washed several times with ether, after which it was dried in a desiccator and then recrystallized by dissolving in *iso*propyl alcohol or dioxane, and precipitating by ether, yield 5.1 g (80 %), m.p. 174—177°, $[\alpha]_D^{18}$ + 8.0° (*c* 1.5 in dimethylformamide). (Found: N 6.50. Calc.for $C_{32}H_{33}O_8N_3S_2$: N 6.45).

Carbobenzoxy-im.*benzyl*-L-*histidyl*-L-*serine benzyl ester.* A suspension of 1.89 g (0.005 mole) of carbobenzoxy-*im*.benzyl-L-histidine in 30 ml of dimethylformamide was dissolved upon heating and then left to cool to 40—45°. This solution was mixed with a previously prepared solution of 1.76 g (0.005 mole) of L-serine benzyl ester benzenesulfonate, and 0.5 g (0.005 mole) of triethylamine in 10 ml of dimethylformamide. Di*cyclo*hexyl-carbodiimide (0.98 g, 0.005 mole) was immediately added and the solution was stirred overnight at room temperature. Di*cyclo*hexylurea was removed by filtration, and the solution was diluted by with 400—500 ml of water *. The ensuing oil crystallized upon scratching with a glass rod. It was cooled, filtered and washed several times with water, then recrystallized by dissolving in ethanol and precipitating with ether. Yield 1.7 g (60 %), m.p. 149—151°, $[\alpha]_D^{21}$ + 2.9° (*c* 5.8 in glacial acetic acid). When recrystallized again from ethylacetate, the m.p. remained the same. (Found: C 66.70; H 5.98; N 10.05. Calc. for $C_{31}H_{32}O_6N_4$: C 66.89; H 5.79; N 10.07).

Carbobenzoxy-im.*benzyl*-L-*histidyl*-im.*benzyl*-L-*histidine benzyl ester.* To 10 ml of dimethylformamide were added 3.25 g (0.005 mole of *im*.benzyl-L-histidine benzyl ester dibenzenesulfonate and 1.01 g (0.01 mole) of triethylamine. This solution was mixed with 1.86 g (0.005 mole) of cbzo-*im*.benzyl-L-histidine, dissolved in 30 ml of dimethylformamide (heated at 40—45°) and 1 g (about 0.005 mole) of di*cyclo*hexylcarbodiimide. The solution was stirred overninght at room temperature. The di*cyclo*hexylurea was then filtered off, washed twice with tetrahydrofuran, and the combined filtrates were diluted with 400 ml of water. Upon cooling and scratching with a glass rod, the desired product crystallized. It was filtered off, washed with water several times, and recrystallized from ethanol-ether. Yield 1.9 g (54 %) m.p. 137°, $[\alpha]_D^{21}$ —22.3° (*c* 5.3 in glacial acetic acid). A further 200 mg of the compound was obtained from the mother-liquor on addition of petroleum ether, m.p. 135°. (Found: C 70.50; H 5.77; N 11.95. Calc for $C_{51}H_{50}O_5N_6$: C 70.68; H 5.78; N 12.06).

Carbobenzoxy-im.*benzyl*-L-*histidylglycine benzyl ester.* A solution of 1.68 g (0.005 mole) of glycine benzyl ester *p*-toluenesulfonate in 10 ml of dimethylformamide was neutralized by addition of 0.5 g (0.005 mole) of triethylamine. It was then mixed with a hot solution (40—45°) of 1.86 g (0.005 mole) of cbzo-*im*.benzyl-L-histidine in 30 ml of dimethylformamide, and 0.98 g (0.005 mole) of di*cyclo*hexylcarbodiimide. The mixture was stirred overnight at room temperature. Di*cyclo*hexylurea was removed by filtration, and the solution was diluted by addition of 500 ml of water. On standing in the refrigerator, the oily product crystallized. It was recrystallized from ethanol-ether, yield 1.1 g (40 %), m.p. 111—112°. (Found: N 10.71. Calc. for $C_{30}H_{30}O_5N_4$: N 10.64).

Carbobenzoxy-im.*benzyl*-L-*histidylglycine.* To a solution of 1 g of the above ester in 15 ml of acetone, 2 ml of N NaOH was added. 5 min later, the product that far saponified, precipitated. Water (5 ml) was then added, and the precipitate was completely dissolved. The solution thus obtained was then allowed to remain for another 15 min at room temperature, and was then diluted with 150 ml of water. Acidification with acetic acid made the desired product precipitate, yield 0.75 g (90 %), m.p. 235—240°, $[\alpha]_D^{20}$ —5.5° (*c* 2.1 in glacial acetic acid). (Found: C 62.80; H 5.85; N 12.75. Calc. for $C_{23}H_{24}O_5N_4$: C 63.20; H 5.54; N 12.85).

Carbobenzoxy-im.*benzyl*-L-*histidyl*-L-*glutamic acid dibenzyl ester.* A solution of 2.49 g (0.005 mole) of L-glutamic acid dibenzyl ester *p*-toluenesulfonate and 0.5 g (0.005 mole) of triethylamine in 10 ml of dimethylformamide was mixed with a solution of 1.89 g

* Alternatively the dimethylformamide was distilled off *in vacuo* (1 mm pressure) at 50° and the remaining residue taken up in ethylacetate or chloroform. The solution was then washed successively with 5 % lithium carbonate and water, and dried over sodium sulphate. The solvent was evaporated *in vacuo*, and the remaining residue was treated as above.

(0.005 mole) of cbzo-*im*.benzyl-L-histidine pro-heated at 40—45°. Di*cyclo*hexylcarbodiimide (0.98 g, 0.005 mole) was immediately added, and the solution was stirred overnight at room temperature. Then di*cyclo*hexylurea was filtered off, and the solution was diluted with 400—500 ml of water. On standing overnight in the refrigerator, the desired product crystallized. Yield 2.8 g. It was recrystallized from ethylacetate-ether, yield 1.8 g (50 %), m.p. 89—91°. (Found: N 8.24. Calc. for $C_{40}H_{40}O_7N_4$: N 8.13).

Carbobenzoxy-im.*benzyl*-L-*histidyl*-L-*glutamic acid.* A solution of 1.72 g (2.5 mmole) of the above ester, and 5 ml of N NaOH in 10 ml of acetone, was stirred for 30 min at room temperature. At the end of that time, 10 ml of water was added, and the solution was filtered and diluted with further 200 ml of water. Upon acidification with acetic acid and cooling in the refrigerator, the product precipitated. Yield 1.15 g (90 %), m.p. 95—97° (recrystallized from ethanol-water). (Found: N 11.10. Calc for $C_{26}H_{28}O_7N_4$: N 11.19).

Carbobenzoxy-L-*phenylalanyl*-im.*benzyl*-L-*histidine. A. By the mixed anhydride procedure.* A solution containing 1.48 g (0.005 mole) of carbobenzoxy-L-phenylalanine, 10 ml of tetrahydrofuran and 0.5 g (0.005 mole) of triethylamine, was cooled to 0°, and 0.5 g (0.005 mole) of ethylchlorocarbonate was then added. After 5 min, 3.25 g (0.005 mole) of *im*.benzyl-L-histidine benzyl ester dibenzenesulfonate and 1.01 g. (0.01 mole) of triethylamine, dissolved in 20 ml of tetrahydrofuran, were added. Coupling was ensured by evolution of carbon dioxide. After 30 min the reaction mixture was diluted with 250—300 ml of water and the desired ester precipitated. The ester was filtered off, washed several times with bicarbonate solution and finally with water. It was then recrystallized by dissolving in ethanol and precipitating with ether, yield 1.9 g (61 %), m.p. 106—107°. (Found: N 8.90. Calc. for $C_{37}H_{36}O_5N_4$: N 9.08).

B. By the carbodiimide method. To a solution of 10 ml of tetrahydrofuran were added 3.25 g (0.005 mole) of *im*.benzyl-L-histidine benzyl ester dibenzenesulfonate, 1.01 g (0.01 mole) of triethylamine, 1.48 g (0.005 mole) of carbobenzoxy-L-phenylalanine and 0.98 g (0.005 mole) of di*cyclo*hexylcarbodiimide. After 15 min di*cyclo*hexylurea began to precipitate. The mixture was stirred at room temperature for 5—6 h and then acidified with a few drops of acetic acid. After 30 min the di*cyclo*hexylurea was removed by filtration, and the solution was diluted with water as described above. Alternatively, the tetrahydrofuran was evaporated *in vacuo* at 40° and the residue was taken up in chloroform. The chloroform layer was washed successively with bicarbonate solution and water. It was dried over sodium sulphate, and the solvent evaporated *in vacuo*. The solid residue was recrystallized from ethanol-ether in a manner similar to that described above, yield 2.1 g (70 %), m.p. 106—107°.

Saponification of the ester. The above ester (6.16 g, 0.01 mole) was dissolved in 20 ml of acetone, 11 ml (10 % excess) of N NaOH was added and the mixture was stirred at room temperature for 30 min. The thus formed sodium salt of the dipeptide derivative which precipitated in the meantime, was dissolved upon gentle heating, and the solution was quickly filtered and diluted with 300 ml of water. This made the slightly soluble sodium salt of the dipeptide precipitate and form a white mass. Upon acidification with acetic acid, the desired compound crystallized. After cooling, the product was filtered, washed several times with water and recrystallized by dissolving in 80 % ethanol, and precipitating with ether. Yield 3.7 g (72 %), m.p. 139—140° (reported [7] 140—141°), $[\alpha]_D^{12}$ + 28° (*c* 1 in glacial acetic acid).

Carbobenzoxy-L-*phenylalanyl*-im.*benzyl*-L-*histidyl*-L-*aspartic acid.* A solution of 1.21 g (2.5 mmole) of L-aspartic acid dibenzyl ester *p*-toluenesulfonate in 10 ml of dimethylformamide was neutralized by addition of 0.25 g (250 mmole) of triethylamine. This solution was mixed with 1.31 g (250 mmole) of cbzo-L-phenylalanyl-*im*.benzyl-L-histidine, and when the latter was completely dissolved, 0.49 g (2.5 mmole) of di*cyclo*hexylcarbodiimide was added. Di*cyclo*hexylurea was filtered off 6 h later and the solvent was evaporated to dryness at 1 mm pressure at 50°. Ethylacetate was added to the residue and concentrated *in vacuo*. This process was repeated four times. The residue was then taken up in ethylacetate (50 ml), cooled for one hour, and filtered. Afterwards the filtrate was washed twice with 5 % sodium carbonate solution, and dried over sodium sulphate. The solvent was evaporated *in vacuo* and the remaining residue solidified upon treatment with ether. Yield 1.5 g. The ester thus obtained was further saponified as follows:

The ester (2.04 g, 2.5 mmole) was suspended in 20 ml of acetone-ethanol (1:1) and 5 ml of N NaOH were added. Stirring for 30 min at room temperature, with gentle

heating at the end of this period, brought about the solution of the hydrolyzed ester. The solution was immediately filtered to remove traces of insoluble compound and then diluted with 200 ml of water, when the tripeptide sodium salt precipitated. The precipitate was acidified with acetic acid, filtered, washed several times with water and recrystallized from 60 % ethanol. Yield 1.2 g (80 %), m.p. 193—196° (reported [7] 193—196°), $[\alpha]_D^{12}$ +20° (*c* 1 in glacial acetic acid).

*Carbobenzoxy-*L*-phenylalanyl-*im.*benzyl-*L*-histidyl-*L*-leucine.* This compound was prepared in a manner similar to that used in the synthesis of the carbobenzoxy-L-phenylalanyl-*im.*benzyl-L-histidyl-L-aspartic acid. When carbobenzoxy-L-phenylalanyl-*im.*benzyl-L-histidine was coupled with leucine methyl or benzyl esters, an oily product was obtained. Saponification produced the desired product in an overall yield of 50 %, m.p. 96°. A sample of the product was recrystallized by dissolution in *iso*propyl alcohol and by precipitation with *iso*propyl ether, m.p. 160—165° (not sharp, reported [7] 163—165°).

L-*Phenylalanyl-*im.*benzyl-*L*-histidyl-*L*-leucine.* A solution of 1.55 g (2.5 mmole) of carbobenzoxy-L-phenylalanyl-*im.*benzyl-L-leucine (m.p. 96°) in absolute ethanol was hydrogenated in the presence of 300 mg of palladium black. Hydrogenation was carride out for half-an-hour during which time the desired product precipitated. The precipitate was dissolved by addition of water and heating. The catalyst was filtered off and the solution evaporated to dryness *in vacuo*. The residue was filtered by addition of ether, yield 1.09 g (90 %). Paper chromatography revealed besides the main product another faint ninhydrin-positive spot at the region of the free peptide phenylalanylhistidylleucine. Recrystallization from 50 % alcohol gave a homogeneous product, as was indicated by paper chromatography and paper electrophoresis, m.p. 162—164° (decomp.), $[\alpha]_D^{21}$ +23.3°; (*c* 1.03 in glacial acetic acid). (Found: C 66.10; H 6.80. Calc. for $C_{28}H_{35}O_4N_5$: C 66.51° H 6.97).

L-*Phenylalanyl-*L*-histidyl-*L*-leucine.* Carbobenzoxy-L-phenylalanyl-*im.*benzyl-L-histidyl-L-leucine (1.59 g, 2.5 mmole) was suspended in 50 ml of liquid ammonia and 0.34 g (100 % excess) of sodium was added in pieces. The blue color remained for about 20 min and then the excess of sodium was neutralized by addition of ammonium acetate. Ammonia was allowed to evaporate and the residue was taken up in 15 ml of water. The water layer was washed twice with ether and evaporated *in vacuo* at 40°. To the residue 5 ml of water was added and the solution reconcentrated. This process was repeated several times. At the end, the residue was treated with 3 ml of water and cooled in the refrigerator. The precipitated peptide was filtered off and washed with 1 ml of cold water. Yield 0.5 g (50 %), $[\alpha]_D^{24}$ +29.5° (*c* 1 in glacial acetic acid), [reported [7] $[\alpha]_D^{24}$ +30.5° (*c* 1.08 in glacial acetic acid)].

*Carbobenzoxy-*L*-seryl-*im.*benzyl-*L*-histidine benzyl ester.* A suspension of 3.25 g (0.005 mole) of *im.*benzyl-L-histidine benzyl ester dibenzenesulfonate in 30 ml of tetrahydrofuran was neutralized by addition of 1.01 g (0.01 mole) of triethylamine. 5 min later 1.19 g (0.005 mole) of carbobenzoxy-L-serine and 0.98 g (0.005 mole) of di*cyclo*hexylcarbodiimide were added while stirring. The mixture was permitted to remain overnight at room temperature and di*cyclo*hexylurea was then removed by filtration. The solution was diluted with 300 ml of water, causing the desired product to precipitate. The product was filtered off, washed several times with water, and purified by dissolution in hot ethylacetate and precipitated by addition of petroleum ether. Yield 2.2 g. Further recrystallization from ethanol-ether gave a product with a sharp m.p. 138.5—139.5°, yield 1.6 g (57 %), $[\alpha]_D^{21}$ −26.3° (*c* 8.4 in glacial acetic acid). (Found: C 66.70; H 5.92; N 10.01. Calc. for $C_{31}H_{32}O_6N_4$: C 66.89; H 5.79; N 10.07).

*N-Tritylglycyl-*im.*benzyl-*L*-histidine.* Coupling of tritylglycine [27] with *im.*benzyl-L-histidine benzyl ester was accomplished either by the mixed carboxylic-carbonic anhydride procedure, or the carbodiimide method, in a manner similar to that described for the preparation of carbobenzoxy-L-phenylalanyl-*im.*benzyl-L-histidine. At the end of the reaction the tetrahydrofuran was evaporated *in vacuo* and the residue was taken up in ethylacetate. The ethylacetate layer was then washed thoroughly with 5 % sodium carbonate solution and water. It was dried over sodium sulphate and the solvent evaporated *in vacuo*. No attempt was made to crystallize the remaining residue which was further hydrolyzed in the same way as described for the carbobenzoxy-L-phenylalanyl-*im.*benzyl-

L-histidine derivative. The product was recrystallized from ethanol-water, yield 60 %, $[\alpha]_D^{21}$ +24.2° (*c* 2.7 in tetrahydrofuran), m.p. 198—201° (reported [7] 198—201°).

*Carbobenzoxy-L-isoleucyl-*im.*benzyl-L-histidine benzyl ester.* This compound was prepared in a manner similar to that used in the synthesis of carbobenzoxy-L-phenylalanyl-*im.* benzyl-L-histidine. Thus 2.65 g (0.01 mole) of carbobenzoxy-L-*iso*leucine [21] was coupled with *im.*benzyl-L-histidine benzyl ester, employing either the mixed carboxylic-carbonic anhydride procedure or the carbodiimide method. After recrystallization from 70 % ethanol, 3.5 g (60 %), and 4 g (70 %), respectively, were obtained, m.p. 132—134°, $[\alpha]_D^{21}$ −21.8° (*c* 10.1 in glacial acetic acid). (Found: N 9.76. Calc. for $C_{34}H_{38}O_5N_4$: N 9.61).

im.*Benzyl-L-histidine methyl ester dihydrochloride.* A fast stream of hydrogen chloride was bubbled for 15 min into a suspension of 2.45 g (0.01 mole) of *im.*benzyl-L-histidine in 20 ml of absolute methanol. The obtained solution was then evaporated *in vacuo* and the residue dissolved by addition of a new portion of methanol. Hydrogen chloride was bubbled in again. After removal of the solvent *in vacuo*, the remaining oily residue was dried *in vacuo* for 3—4 h at 40°, making thus the oily product solidify. The product (3.1 g), being extremely hygroscopic, was further dried before use in a desiccator (P_2O_5) for 24 h. The ester obtained is pure enough for peptide synthesis. A sample of it was dissolved in the minimal amount of methanol, concentrated with hydrogen chloride, and upon addition of dry ether precipitated. The solvent was decanted and the precipitate was washed with ether and dried in a desiccator (P_2O_5). The thus recrystallized ester melted at 111—115°, (reported [7] 111—115°), when heated quickly.

*Carbobenzoxy-L-phenylalanyl-*im.*benzyl-L-histidine hydrazide.* A solution containing 2.99 g (0.01 mole) of carbobenzoxy-L-phenylalanine and 1.01 g (0.01 mole) of triethylamine in 20 ml of chloroform was cooled to 5° and treated with 1.08 g (0.01 mole) of ethylchlorocarbonate. After 5 min it was mixed with a solution of 3.30 g (0.01 mole) of *im.*benzyl-L-histidine methyl ester dihydrochloride, and 2.02 g (0.02 mole) of triethylamine in 30 ml of chloroform. Coupling was ensured by evolution of carbon dioxide. After 30 min the solution was washed with dilute bicarbonate solution and water. It was then dried over sodium sulphate and the solvent evaporated *in vacuo* at 40°. To the residue ethanol was added and concentrated again. The remaining oily residue (3.8 g, 70 %) was dissolved in 20 ml of hot ethanol and mixed with 1 g (200 % excess) of hydrazine monohydrate. The mixture was kept overnight at room temperature, and by addition of water the hydrazide precipitated completely. It was filtered off and recrystallized from 80 % ethanol, yield 2.8 g (75 %), m.p. 183—186° (reported [7] 183—186°).

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