β-3,4-Dihydroxyphenylserine (XII). A. From High-melting β-3,4-Dibenzyloxyphenylserine.—A suspension of 3.94 g. (0.01 mole) of β-3,4-dibenzyloxyphenylserine (m.p. 180°) in 50 ml. of 50% methyl alcohol was hydrogenated at atmospheric pressure using 0.2 g. of 5% palladium-on-charcoal catalyst. In the course of the reduction, it was necessary to add 0.2 g. of fresh catalyst. After eight hours, 465 ml. of hydrogen had been absorbed (theory 480 ml.) and the reduction was stopped. Product had crystallized but it redissolved on warming and the catalyst was removed by filtration. The alcohol was removed by vacuum concentration and the aqueous solution was treated with acid-washed Darco. The almost colorless filtrate was concentrated to 5 ml. and 15 ml. of ethyl alcohol was added slowly. After standing at −20° for 18 hours, the crystalline product was collected and washed with 50% alcohol. It weighed 1.83 g. (85.9%) and melted with decomposition at 199–200°. Analyses showed that the compound was hydrated.

Anal. Calcd. for $C_9H_{11}O_5N \cdot H_2O$: C, 46.75; H, 5.67; N, 6.06. Found: C, 46.84; H, 5.66; N, 6.06.

B. From Low-melting β-3,4-Dibenzyloxyphenylserine.—A solution of 12.22 g. (0.03 mole) of β-3,4-dibenzyloxyphenylserine monohydrate (m.p. 146°) in a mixture of 30 ml. of water, 30 ml. of ethyl alcohol and 15 ml. of 2 *N* lithium hydroxide was hydrogenated at atmospheric pressure using 2.0 g. of 5% palladium-on-charcoal catalyst. After 1490 ml. of hydrogen had been absorbed (theory 1470 ml.), the reduction ceased and 6 ml. of concentrated hydrochloric acid was added to the reaction mixture. After filtration through acid-washed Darco, the light yellow filtrate was neutralized with 2 *N* lithium hydroxide (22 ml.). Crystallization was rapid and, after cooling the mixture at 0° for 24 hours, 5.94 g. (93.0%) of product was obtained. Due to the insolubility of the compound, it was purified by dissolving it in alkali and then reprecipitating by the addition of acid. The product had a gray tinge and melted with decomposition at 220–225°.

Anal. Calcd. for $C_9H_{11}O_5N$: C, 50.70; H, 5.20; N, 6.57. Found: C, 50.15; H, 5.36; N, 6.51.

WEST POINT, PA.

[CONTRIBUTION FROM THE NEW YORK STATE AGRICULTURAL EXPERIMENT STATION, CORNELL UNIVERSITY]

The Synthesis of L-Histidyl Peptides[1]

BY ROBERT W. HOLLEY AND ERNEST SONDHEIMER

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L-Histidyl peptides have been synthesized for the first time, using a modified carbobenzoxy azide peptide synthesis procedure.

In spite of the widespread occurrence of L-histidine in proteins, the synthesis of L-histidyl peptides has not previously been described.[2] Presumably this is due to the presence of the imidazole ring in the histidine side chain, which would complicate many peptide synthesis procedures. The imidazole ring is weakly basic, with pK_a approximately 6, and under certain conditions undergoes ring opening or substitution reactions. The reaction conditions which can be used in peptide synthesis are, therefore, limited. For example, it is unlikely that the histidine carboxyl could be converted to an acid chloride without having the imidazole ring protected in some way.

Two DL-histidyl peptides have been described, but the procedures used in their preparation are not applicable to the synthesis of L-histidyl peptides. Bergmann and Zervas[3] prepared DL-histidylglycine by way of an oxazolone, obtained from acetyl-DL-histidine and acetic anhydride, which reacted with glycine ethyl ester to give acetyl-DL-histidylglycine ethyl ester. Hydrolysis of the latter with dilute hydrochloric acid gave a low yield of DL-histidylglycine. Presumably this procedure could be used for the preparation of other DL-histidyl peptides, but it is not applicable to the synthesis of L-histidyl peptides since the intermediate oxazolone would be racemized. Fischer and Suzuki[4] prepared histidylhistidine by mild alkaline hydrolysis of L-histidine anhydride (3,6-di-(4-imidazolemethyl)-2,5-piperazinedione). As would be expected, the hydrolysis was accompanied by racemization.[5]

Of the various peptide synthesis procedures which might be applicable to the synthesis of L-histidyl peptides, it seemed to us that the carbobenzoxy azide procedure was the most promising, although difficulties were anticipated in the isolation of the basic carbobenzoxy-L-histidyl azide and there was the possibility of reaction of the azide with the imidazole ring present in the azide molecule.

Carbobenzoxy-L-histidine methyl ester was prepared by acylation of L-histidine methyl ester with carbobenzoxy chloride in chloroform containing triethylamine. The ester, an oil, was converted to the crystalline hydrazide by treatment with hydrazine hydrate in absolute ethanol. Potentiometric titration indicated the presence of two basic groups, pK_a 6.1 and 2.5, corresponding to the imidazole ring and the hydrazide, respectively.

In the standard carbobenzoxy azide procedure, the hydrazide is converted to the azide by treatment with nitrous acid in acid solution, and the azide is extracted into an inert organic solvent. Carbobenzoxy-L-histidyl azide, however, is basic, and is not extractable from acid solution. This difficulty was overcome by preparation of the azide in aqueous acid, followed by basification of the solution. The azide, obtained in high yield as an oil, was extracted into an inert organic solvent, and was allowed to react with an amino acid ester. The carbobenzoxy-L-histidylamino acid esters were obtained in good yield. There was no indication of reaction of the azide with the imidazole ring present within the azide molecule.

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(2) J. S. Fruton, *Advances in Protein Chemistry*, **5**, 1 (1949).

(3) M. Bergmann and L. Zervas, *Z. physiol. Chem.*, **175**, 154 (1928).

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TABLE I

Compound	Yield, %	Solvent for recryst.,[a] ml./100 mg.	M.p., °C.	$[\alpha]^{22}D$	Nitrogen, % Calcd.	Found[b]
Carbobenzoxy-L-histidine hydrazide	56	1.7 water	171–173	.....	23.1	23.2[c]
Carbobenzoxy-L-histidyl-L-alanine ethyl ester	82	0.25 ethanol	149.5–152	−25° (*c* 1, ethanol)	14.4	14.4[d]
Carbobenzoxy-L-histidyl-L-leucine methyl ester	73	1.3 acetone + 4 water[e]	125–128	−25.5 (*c* 1, ethanol)	13.5	13.5[f]
Carbobenzoxy-L-histidyl-L-serine methyl ester	66	0.5 ethanol	140–142	.....	14.4	14.5[g]
Carbobenzoxy-L-histidyl-L-alanine	98	1 water	211–214[h]	−6 (*c* 1, 50% ethanol)	15.6	15.8
Carbobenzoxy-L-histidyl-L-leucine	89	0.2 ethanol + 0.5 water	185–188	−7 (*c* 1, ethanol)	13.9	13.8
L-Histidyl-L-alanine	95	0.4 water + 2 ethanol	198–200[h]	−27 (*c* 1, 0.10 *N* NaOH)	24.8	24.6
L-Histidyl-L-leucine	94	4, 30% ethanol	214–217[h]	−43.5 (*c* 1, 0.10 *N* NaOH)	20.9	20.8
Carbobenzoxy-L-histidylglycine hydrazide	71[i]	1.1, 95% ethanol	179.5–181	.....	23.3	23.7
Carbobenzoxy-L-histidyl-L-alanine hydrazide	82	3, 95% ethanol	202.5–204	.....	22.5	22.1[k]
Carbobenzoxy-L-histidyl-L-leucine hydrazide	66	0.3, 95% ethanol	175–177	.....	20.2	20.1

[a] Recovery from recrystallizations averaged 80%. [b] Analyses by Dr. G. Weiler and Dr. F. B. Strauss, Oxford, England. Samples were dried before analysis. [c] Calcd.: C, 55.43; H, 5.65. Found: C, 55.67; H, 5.82. [d] Calcd.: C, 58.75; H, 6.23. Found: C, 58.59; H, 6.03. [e] This compound always crystallized very slowly. [f] Calcd.: C, 60.56; H, 6.78. Found: C, 60.73; H, 6.90. [g] Calcd.: C, 55.38; H, 5.68. Found: C, 54.99; H, 5.47. [h] Decomp. [i] Over-all yield, starting with carbobenzoxy-L-histidine hydrazide. [k] Calcd.: C, 54.53; H, 5.92. Found: C, 54.71; H, 5.90.

Subsequent transformations were carried out by standard procedures. The carbobenzoxy-L-histidylamino acid esters were saponified to the carbobenzoxy-L-histidylamino acids, which on hydrogenolysis, using palladium black, gave the dipeptides. Alternatively, for use in the preparation of higher L-histidyl peptides, the esters were converted to the corresponding hydrazides.

The new compounds which have been prepared and characterized are listed in Table I.

Experimental[6]

Carbobenzoxy-L-histidine Hydrazide.—A solution prepared from 1.50 g. (6.2 millimoles) of L-histidine methyl ester dihydrochloride, 12.4 ml. of purified chloroform and 1.73 ml. (12.4 millimoles) of redistilled triethylamine was cooled in ice and treated with 0.68 ml. (3.1 millimoles) of freshly prepared carbobenzoxy chloride solution.[7] The mixture was stirred and after two minutes 0.87 ml. (6.2 millimoles) of triethylamine was added. After the solid dissolved, 0.68 ml. (3.1 millimoles) more carbobenzoxy chloride solution was added. After a few minutes the mixture was removed from the ice-bath and left at room temperature 30 minutes. The chloroform solution was washed three times with 5 ml. of water, dried over sodium sulfate, and evaporated to dryness. Absolute ethanol was added and evaporated three times, and finally the oil was dissolved in 3 ml. of absolute ethanol and 0.62 ml. of 100% hydrazine hydrate was added. The solution was left at room temperature. The hydrazide started to crystallize in about 30 minutes. After 24 hours, the mixture was cooled and the hydrazide was collected by filtration. It was washed with ether and dried *in vacuo;* 0.93 to 1.17 g. (50–62%), m.p. 166–169°. It was recrystallized from 17 ml. of water to give 0.7 to 0.9 g., m.p. 171–173°. It gave a negative ninhydrin test. Potentiometric titration indicated the presence of two basic groups, pK_a 6.1 and 2.5, corresponding to the imidazole and hydrazide groups. Analyses are given in Table I.

Carbobenzoxy-L-histidyl-L-alanine Ethyl Ester.—A mixture of 360 mg. (2.34 millimoles) of L-alanine ethyl ester hydrochloride and 8 ml. of ether was cooled in an ice-bath, and 3 ml. of cold 50% (w./v.) potassium carbonate solution was added. The mixture was equilibrated, and the ethereal solution was separated and dried over sodium sulfate at 0° during the preparation of the azide described below.

A solution of 606 mg. (2.00 millimoles) of carbobenzoxy-L-histidine hydrazide in 6.0 ml. (6.0 meq.) of 1.00 *N* hydrochloric acid was mixed with 8 ml. of purified ethyl acetate and cooled in an ice-bath. A cold solution of 140 mg. (2.0 millimoles) of sodium nitrite in 0.5 ml. of water was added. After 2 minutes, 2.4 ml. of cold 50% potassium carbonate solution was added and the mixture was equilibrated. The ethyl acetate solution was separated and the aqueous solution was extracted with an additional 1 ml. of ethyl acetate. The combined ethyl acetate solutions were dried a few minutes over sodium sulfate at 0°.

The solution of L-alanine ethyl ester was filtered through a cotton plug, and the solution of the azide was poured through the same filter. The flasks and filter were rinsed with 1 ml. of ethyl acetate. The reaction solution was left at 0° for 24 hours. Crystals began to form in less than 1 hour. The product was collected by filtration, washed with a little cold ethyl acetate, and dried in air and finally *in vacuo;* 636 mg. (82%), m.p. 146–151°. It was recrystallized from 1.5 ml. of absolute ethanol; recovery 88%, m.p. 149.5–152°.

In the preparations of **carbobenzoxy-L-histidyl-L-leucine methyl ester and carbobenzoxy-L-histidyl-L-serine methyl ester** the reaction mixtures were left overnight at 0° and then 24 hours at room temperature. The serine derivative, which had crystallized, was collected by filtration. The leucine derivative, isolated by evaporation of the solution after it had been washed twice with water, was obtained as a glass which crystallized during four days at room temperature, under petroleum ether.

Carbobenzoxy-L-histidyl-L-alanine.—To a solution of 370 mg. (0.95 millimole) of carbobenzoxy-L-histidyl-L-alanine ethyl ester in 2.0 ml. of methanol was added 0.95 ml. (1.06 meq.) of 1.12 *N* sodium hydroxide. After 30 minutes at room temperature 1.06 ml. of 1.00 *N* hydrochloric acid was added and the methanol and water were removed *in vacuo*. The residue was recrystallized from 4 ml. of water; 336 mg. (98%), m.p. 210–214° dec. Recrystallization of 100 mg. from 1 ml. of water gave 86 mg., m.p. 211–214° dec. A sample was recrystallized again for analysis. It gave a negative ninhydrin test. **Carbobenzoxy-L-histidyl-L-leucine** was prepared in the same way.

L-Histidyl-L-alanine.—To a solution of 260 mg. (0.75 millimole) of carbobenzoxy-L-histidyl-L-alanine in 6 ml. of methanol, 3 ml. of water and 0.15 ml. of glacial acetic acid was added 60 mg. of palladium black, and hydrogen was bubbled through at room temperature with shaking for 2 hours. The catalyst was removed by filtration, and the solution was evaporated to dryness *in vacuo*. Absolute ethanol was added and evaporated twice; 165 mg. (97%), m.p. 186–190° dec. The product was recrystallized twice using 0.4 ml. of water and 2 ml. of ethanol per 100 mg.; recovery 105 mg., m.p. 198–200° dec. It gave a positive ninhydrin test. For analysis, the compound was dried 5 minutes *in vacuo* at 100° over phosphorus pentoxide. Prolonged drying at 100° gave material with a nitrogen analysis corresponding to the calculated value for the diketopiperazine. **L-Histidyl-L-leucine** was prepared in the same way.

Carbobenzoxy-L-histidyl-L-alanine Hydrazide.—To a solution of 94 mg. (0.24 millimole) of carbobenzoxy-L-histidyl-L-alanine ethyl ester in 0.25 ml. of absolute ethanol was added 0.025 ml. of hydrazine hydrate, and the solution was refluxed 15 minutes. The solution was cooled to 0° and the product collected by filtration. It was washed with 0.2 ml. of absolute ethanol and 1 ml. of ether, and dried *in vacuo;* 77 mg. (82%), m.p. 188–193°. For analysis it was recrys-

(6) All melting points were determined on a microscope hot-stage and are corrected. Amino acid ester hydrochlorides were prepared at room temperature by the method of R. L. M. Synge, *Biochem. J.*, **42**, 99 (1948).

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tallized twice from 95% ethanol (3 ml. per 100 mg.); 43 mg., m.p. 202.5–204°.

Carbobenzoxy-L-histidylglycine hydrazide and carbobenzoxy-L-histidyl-L-leucine hydrazide were prepared in the same way except that the reactions were carried out for 24 hours at room temperature.

GENEVA, NEW YORK

[CONTRIBUTION FROM THE NATIONAL CANCER INSTITUTE, NATIONAL INSTITUTES OF HEALTH]

Preparation and Properties of 2,4-Dinitrophenyl-L-amino Acids

BY KRISHNARAU R. RAO[1] AND HERBERT A. SOBER

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The crystalline 2,4-dinitrophenyl derivatives of the following L-amino acids have been prepared for the first time: valine, norvaline, isovaline, leucine, isoleucine, alloisoleucine, α-aminononylic acid, serine, γ-hydroxy-α-aminobutyric acid, ε-hydroxy-α-aminocaproic acid, threonine, allothreonine, S-benzylcysteine, hydroxyproline, aspartic acid, glutamine, α,γ-diaminobutyric acid, histidine, as well as those of DL-ethionine, DL-methionine, DL-glutamic acid, DL-pipecolic acid and γ-aminobutyric acid. The derivatives of L-glutamic and allohydroxy-L-proline were obtained as hygroscopic yellow solids. The purification of many of these compounds required anhydrous conditions. The molar rotations of one of the optical enantiomorphs of the above as well as those of alanine, α-aminobutyric acid, asparagine, arginine, ornithine, lysine, cystine, phenylalanine, tryptophan, tyrosine and proline, have been determined in *N* NaOH or 4% $NaHCO_3$ and in glacial acetic acid and vary from 2 to 40 times that of the parent amino acid. Ultraviolet absorption data and molar extinction values were obtained and the chromatographic behavior on paper in several solvent systems was examined.

The dinitrophenyl (DNP) derivatives of the amino acids glycine, DL-alanine, DL-histidine, DL-leucine and L-asparagine were first prepared by Abderhalden and Blumberg[2] using 1-chloro-2,4-dinitrobenzene which reacts with the free amino group. Sanger[3–5] found that 1-fluoro-2,4-dinitrobenzene (FDNB) was a more suitable reagent capable of reacting near neutrality at room temperature with the free amino groups of proteins and peptides. He evolved thereby an elegant method of identifying the N-terminal residues of proteins and peptides based on the stability of the DNP-amino acid to acidic or enzymatic hydrolysis.

Preliminary experiments indicated that the melting points of the DNP derivatives of optically active amino acids differed in many instances from those of the racemic form and that the molecular rotation of the optically active derivative was much larger than that of the parent amino acid. Except for asparagine,[2] arginine,[5] lysine,[6,7] ornithine,[4] cystine,[5,6] phenylalanine,[3] tryptophan,[4,5] proline,[5,6] tyrosine,[6] alanine[8] and α-aminobutyric acid,[8] all the amino acids whose crystalline DNP derivatives have hitherto been prepared were racemic.

Optical configuration has been correlated with biological activity and is also believed to be implicated in the effect of several natural antimetabolites. It seemed likely that the characterization of DNP derivatives of amino acid enantiomorphs would help in the determination of optical configuration as well as in the identification of amino acids present singly or in combination in natural compounds. Such an application was made recently by Alderton[8] while this work was in progress. He prepared the DNP derivatives of L-alanine and L-α-aminobutyric acid and determined their optical rotation in alkali in order to elucidate the optical configuration of the α-carbon in a sulfide amino acid in the antibiotic peptide, subtilin.

The availability of a large variety of pure amino acid enantiomorphs, prepared by the resolution procedure of Greenstein and collaborators (*cf.* Greenstein, Birnbaum and Otey[9] for details), made possible the preparation of a large number of DNP-L-amino acids. These derivatives were shown to be homogeneous by chromatography on paper in various solvents and were characterized with respect to optical rotation, ultraviolet absorption and melting point.

Experimental

L-Amino acids, obtained by resolution of the corresponding racemic amino acids and determined to be better than 99.9% optically pure,[9] were kindly donated by Dr. J. P. Greenstein and were employed for the preparation of the derivatives except for hydroxy-L-proline, L-cystine hydrochloride, DL-glutamic acid and DL-methionine,[10] L-asparagine[11] and DL-ethionine[12] which were commercial preparations recrystallized before use. DL-Pipecolic acid was kindly donated by Dr. Alton Meister and γ-aminobutyric acid by Dr. H. Steinman.

The coupling reaction was performed by shaking the amino acid with FDNB[13] in the presence of a slight excess of sodium bicarbonate for 2–5 hours in 50% ethanol at room temperature. Protection of DNP-glutamic acid and DNP-aspartic acid from light at all stages of their preparation as advocated by Mills[14] was helpful in increasing yields and inducing crystallization. After the reaction was completed, alcohol was removed at room temperature and the excess FDNB was extracted by shaking thrice with ether. Ether was removed and the aqueous solution was acidified with 6 *N* HCl till strongly acid and the solid or oil that separated was washed several times with small quantities of ice-cold water. Anhydrous conditions were found to be essential for crystallization in many cases although crystallization took place on standing in the cold in a few instances. Rep-

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