Synthesis of Phenistidine and Derivatives[1]

M. K. STANFIELD AND D. W. WOOLLEY

Rockefeller Institute, New York, New York

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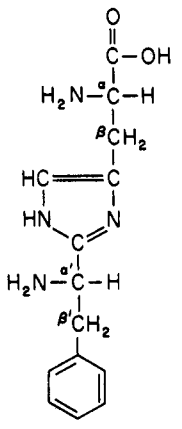
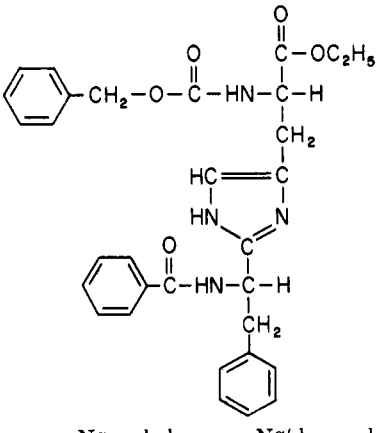
The new amino acid phenistidine [2-(α'-amino-β'-phenylethyl)histidine] has been synthesized for use in the investigation of the mechanism of enzyme action. The chloromethyl ketone derived from carbobenzoxy-α-ethyl aspartate (*viz.* ethyl 2-carbobenzoxyamino-4-keto-5-chloro-L-valerate) was caused to react with benzoylphenylalanineamidine to yield the N^{α}-carbobenzoxy-N^{α}-benzoylphenistidine ethyl ester. In similar fashion the corresponding $N^{\alpha'}$-acetyl derivative was prepared. Purification of these compounds proved very difficult, but was accomplished by chromatography on silicic acid columns. Attempts to benzylate the imidazole nitrogen of phenistidine by procedures successful with histidine failed. The retention of optical purity in the two reactants during the ring-forming condensation was investigated.

In order to test the hypothesis of Woolley, *et al.*,[2] on the reasons for the specificity of enzyme action, it is necessary to synthesize a complicated and previously unknown amino acid called phenistidine. The structure of this new amino acid is shown in Figure 1, along with its N^{α}-carbobenzoxy-$N^{\alpha'}$-benzoyl ethyl ester. These derivatives were needed to facilitate the incorporation of phenistidine into peptide chains for the testing of the postulates about the mechanism of enzyme action. The purpose of this paper is to describe the synthesis of phenistidine (previously called phenylalanohistidine[3]) and some of its derivatives.



phenistidine



N^{α}-carbobenzoxy-$N^{\alpha'}$-benzoylphenistidine ethyl ester

Figure 1.

The compounds proved to be very difficult to synthesize. Imidazoles with complicated acylaminoalkyl side chains in the 2-position, and with the side chain of histidine in the 4-position are virtually unknown. The only related example previously studied was 2-benzylhistidine[2] which lacked the acylamino group in the alkyl side chain. In the case of phenistidine the difficulties of synthesis were increased by the fact that the molecule contained two asymmetric carbon atoms so that four optical isomers are possible. The isomer desired for the enzymatic work would need to be the one in which both asymmetric centers belonged to the L series.

The synthesis was carried out by one of the two routes explored previously[2] for the preparation of 2-benzylhistidine. This was to condense a suitably substituted chloromethyl ketone (*viz.*, ethyl 2-carbobenzoxyamino-4-keto-5-chloro-L-valerate[2]) with a suitable amidine (*viz.*, benzoyl-L-phenylalanineamidine hydrochloride or the corresponding acetyl derivative). This reaction was found to occur best in ethanol with an excess of the amidine which was liberated from its hydrochloride by 1 equiv. of sodium ethoxide. During the reaction, however, the optical center of the amidine was racemized, as might be anticipated from the earlier work of Elliott[4] with acylamino acid esters. The optical center of the chloromethyl ketone seemed not to be altered as judged from examination of unreacted ketone. In order to avoid the partial racemization of the one center, many attempts were made to bring about the condensation without the use of alkali. Simple imidazoles have, in fact, been made by related condensations in nonalkaline solvents.[5,6] In the present work some of the desired product was obtained by long heating of the reactants either in acetic acid or in ethanol, or without solvent at 110°, but the yields by these procedures were too low to make it feasible to use them for preparative purposes (less than 5%). With the use of sodium ethoxide yields as high as 60% were obtained.

The purification of the products proved to be unusually difficult. By means of chromatography on columns of silicate acid this was best accomplished.

For incorporation of the protected amino acid into peptides it was desirable to protect the imidazole ring by benzylation. This is the maneuver which is frequently used for histidine itself. However, the benzylation of the imidazole ring in derivatives of phenistidine proved to be impractical. Treatment of the free amino acid in liquid ammonia with sodium and benzyl chloride yielded no trace of the desired product, even though this reaction succeeded with 2-benzylhistidine.[2] Some benzylation occurred when the fully protected amino ester (*i.e.*, N^{α}-carbobenzoxy-$N^{\alpha'}$-benzoylphenistidine ethyl ester) was treated with sodium hydride and benzyl chloride, but complete conversion did not occur and the benzylated, fully protected derivative could not be separated from starting material. In all of these trials, small amounts of the desired product which might have been formed were readily detectable. Hydrolysis of the total reaction mixture and paper electrophoresis of the hydrolysate revealed a characteristic spot due to im-benzylphenistidine (im = imidazolyl) if it were present.

(1) Supported in part by Grant A1260 from the U. S. Public Health Service.

(2) D. W. Woolley, J. W. B. Hershey, and I. H. Koehelik, *Proc. Natl. Acad. Sci. U. S.*, **48**, 709 (1962).

(3) D. W. Woolley, J. W. B. Hershey, and H. A. Jodlowski, *J. Org. Chem.*, **28**, 2012 (1963).

(4) D. F. Elliott, *J. Chem. Soc.*, 62 (1950).

(5) H. Bredereck, R. Gompper, and H. Wild, *Chem. Ber.*, **88**, 1351 (1955).

(6) J. W. Cornforth and H. T. Huang, *J. Chem. Soc.*, 1960 (1948).

Experimental

N-Benzoyl-L-phenylalaninenitrile.—This nitrile was formed by dehydration of N-benzoyl-L-phenylalanineamide with $POCl_3$ in pyridine solution exactly as described for the corresponding acetyl compound[3] except that chloroform instead of benzene was used for extraction, and the nitrile was recrystallized from benzene. It melted at 144–145°.

Anal. Calcd. for $C_{16}H_{14}N_2O$: C, 76.8; H, 5.6; N, 11.2. Found: C, 76.6; H, 5.8; N, 10.9.

N-Benzoyl-L-phenylalanineamidine Hydrochloride.—This compound had the great advantage over the corresponding acetyl compound[3] in that it crystallized readily, and thus could be purified without the need for countercurrent distribution. Its acyl group was also much more stable to acid so that much better yields were possible.

A solution of benzoyl-L-phenylalaninenitrile (5.0 g., 20 mmoles) in 35 ml. of dry chloroform was treated with 4 ml. of 10 *M* hydrogen chloride in absolute ethanol. The mixture was held at 25° for 2 hr., then at 4° overnight, and finally at 25° for 2 hr. The solvents were removed under reduced pressure at 25°; the residue was dissolved in 10 ml. of absolute ethanol at 0° and immediately mixed with 25 ml. of alcoholic ammonia (saturated solution). After the mixture had stood tightly stoppered for 4 days at 25°, it was concentrated under reduced pressure to dryness and the residue was fractionally crystallized from absolute ethanol by gradual addition of anhydrous ether and recrystallized. Ammonium chloride was removed in the first crop. Yield of pure amidine salt was 4.1 g., m.p. 221–223°.

Anal. Calcd. for $C_{16}H_{18}ClN_3O$: C, 63.3; H, 5.9; N, 13.5. Found: C, 63.2; H, 5.7; N, 13.6.

Countercurrent distribution in the system chloroform–methanol–water, 2:2:1, for 95 transfers gave a symmetrical peak with maximum in tube 65.

N^{α}-Carbobenzoxy-$N^{\alpha'}$-benzoylphenistidine Ethyl Ester.—A solution of N-benzoyl-L-phenylalanineamidine hydrochloride (1.52 g., 5.02 mmoles) and ethyl 2-carbobenzoxyamino-4-keto-5-chloro-L-valerate[2] (0.66 g., 2.0 mmoles) in 20 ml. of absolute alcohol was stirred and refluxed while a solution of sodium (0.115 g., 5.02 mmoles) in 18 ml. of absolute alcohol was dripped in during 5 hr. Refluxing and stirring were continued for an additional 5 hr. An anhydrous ethanolic solution of hydrogen chloride (9.9 *M*, 0.5 ml.) was added to the cooled solution which was concentrated to dryness under reduced pressure below 40°. The residue was freed from ethanol by repeated suspension in chloroform and evaporation, and was then triturated with 20 ml. of chloroform. In most runs part of the desired product remained insoluble in chloroform although some always dissolved.[7] To recover the chloroform-soluble portion, it was necessary to chromatograph on silicic acid.

The chloroform-insoluble gum, dissolved in the least possible volume of ethanol, was treated with 40 ml. of chloroform, 40 ml. of water, and enough sodium bicarbonate to give pH 7–8. The aqueous phase was washed with chloroform (three 35-ml. portions) and the combined chloroform extracts were dried with sodium sulfate. The aqueous phase was reserved for isolation of unreacted amidine. Filtration followed by evaporation gave a glass from which the desired product (0.291 g.) was obtained by solution in glacial acetic acid (1.5 ml.), addition of anhydrous benzene (20 ml.), followed by lyophilization. Occasionally crystals could be obtained from benzene with great difficulty. Thin layer chromatography on silicic acid in 3% methanol in chloroform gave a spot with R_f 0.50, detected by iodine vapor. When the substance was hydrolyzed in refluxing 6 *N* HCl and the hydrolysate was submitted to paper electrophoresis, pH 5.0, 0.1 *M* pyridine acetate, and the paper was sprayed with ninhydrin, a characteristic gray spot, R_{his} 0.72, was obtained along with a purple spot near the origin. The compound was unstable when stored for several months at 4° in the dark as shown by the appearance of several new spots detectable on thin layer chromatography. Decomposition also was indicated by the development of a brown color. This was in sharp contrast to the behavior of the corresponding histidine derivative (carbobenzoxyhistidine ethyl ester).

Anal. Calcd. for $C_{31}H_{32}N_4O_5$: C, 68.9; H, 6.0; N, 10.4. Found: C, 69.0; H, 6.1; N, 10.3 (Kjedahl).

To recover the remainder of the product which was present in the chloroform-soluble fraction described above, the solution was chromatographed on a silicic acid column (2 × 35 cm., 70 g. of Mallinckrodt silicic acid, 100 mesh, predried at 110°, poured in chloroform, and washed until translucent). After introduction of the sample, the column was eluted with chloroform (1 l.). Evaporation of the eluate yielded unchanged chloro ketone (*viz.*, ethyl 2-carbobenzoxyamino-4-keto-5-chloro-L-valerate) in chromatographically and optically pure condition (0.655 g.).

The eluent was then changed to ethanol–chloroform, 2:98, and the appearance of the desired product was monitored by thin layer chromatography and by hydrolysis and paper electrophoresis (see above). The product began to appear at about 200 ml. after the solvent change and the majority of the material was eluted between 300 and 600 ml. The material was recovered by evaporation of the appropriate fractions to give 0.389 g. The total yield was therefore 0.680 g. (1.25 mmoles).

Unchanged benzoylphenylalanineamidine hydrochloride (252 mg.) was recovered from the aqueous phase mentioned above by acidification with hydrochloric acid to pH 6, evaporation, and recrystallization from alcohol and ether. The recovered amidine was racemic as shown by hydrolysis with hydrochloric acid isolation of the phenylalanine, and measurement of its rotation.

N^{α}-Carbobenzoxy-$N^{\alpha'}$-acetylphenistidine Ethyl Ester.—This compound was prepared in the way described for the corresponding $N^{\alpha'}$-benzoyl compound except that N-acetyl-L-phenylalanineamidine hydrochloride[3] (2.5 g., 10.3 mmoles) was used along with the chloro ketone (1.33 g., 4.5 mmoles) instead of the benzoylamidine. The chloroform-soluble part of the reaction mixture was separated by column chromatography on silicic acid. The acetyl derivative did not move on the column with 2 or 3% ethanol in chloroform as did the benzoyl derivative. Therefore, after 700 ml. of the eluent with 3% ethanol had been collected, the solvent was changed to 20% ethanol in chloroform, and the desired substance was collected soon after the change. It showed R_f 0.60 in silicic acid thin layer chromatography with methanol–chloroform, 1:9, and gave the characteristic gray spot with ninhydrin when it was hydrolyzed and submitted to electrophoresis on paper at pH 5.0. The final product was crystallized from benzene, m.p. 111°. The yield was 630 mg.

Anal. Calcd. for $C_{26}H_{30}N_4O_5$: C, 65.3; H, 6.3; N, 11.7. Found: C, 64.3; H, 5.9; N, 11.9.

The material eluted from the silicic acid column with 3% ethanol was studied at some length. Although it could not be obtained crystalline it was much purified by further chromatography and seemed to consist of the oxazole corresponding to the phenistidine derivative; *i.e.*, the material seemed to be ethyl α-carbobenzoxamido-β-[2-(α'-acetamido-β'-phenylethyl)-4-oxazoyl]propionate. When hydrolyzed with acid it yielded phenylalanine, aspartic acid, and a spot which gave a characteristic pink color with ninhydrin on the paper strips after electrophoresis in pyridine acetate at pH 5.0. This "pink spot" moved in electrophoresis with a mobility of 1.08 relative to histidine. Both in paper electrophoresis and in paper chromatography with phenol–aqueous HCl it behaved as did 2,5-diamino-4-ketovaleric acid which is formed when the imidazole ring of histidine esters or amides is cleaved by acid chlorides and the product is hydrolyzed.[8] These are the products one would expect the oxazole corresponding to phenistidine to yield on acid hydrolysis, but there was no unequivocal proof of the structure.

This material which yielded the pink ninhydrin spot on acid hydrolysis was formed in considerable amounts in the reaction between the acetylamidine and the chloro ketone, but only in very small amounts when the benzoylamidine was used.

Formation of Phenistidine Derivatives in Nonalkaline Reaction Conditions.—Many experiments were conducted in an effort to form phenistidine without the accompanying racemization of the phenylalanine portion which took place when sodium ethoxide was used. The analytical method used to determine the extent of the condensation reaction was the following. The crude reaction mixtures were hydrolyzed with 6 *N* hydrochloric acid, and after evaporation the hydrolysates were examined by paper electrophoresis as indicated in the preceding sections, and the amount of the phenistidine which had been formed was measured by the ninhydrin reaction on the material which moved

(7) In some runs this precipitate did not form and the entire chloroform-soluble reaction mixture was separated on columns of silicic acid.

(8) A. Windaus, W. Dörries, and H. Jensen, *Ber.*, **54B**, 2745 (1921).

at pH 5.0 with a mobility relative to histidine of 0.72. Any phenistidine which might have been formed was thus readily detected. Small amounts were formed when the acetylamidine hydrochloride and the chloro ketone were fused without solvent at 110°. Maximal yield was obtained after 24 hr., but it never exceeded 2%, and much decomposition occurred during such long periods of reaction. Small amounts were also formed when the two reactants (the acetylamidine hydrochloride and the chloro ketone) were dissolved in refluxing acetic acid for 20 hr. or in formamide at 100° for 1 hr., but the yield did not exceed 1%.

Phenistidine.—The free amino acid was obtained either by hydrolysis of the pure derivatives described above, or by hydrolysis of crude reaction mixtures of the acylamidine and the chloromethyl ketone. The pure free amino acid was isolated from a fusion reaction (110°, 18 hr.) of acetyl-L-phenylalanineamidine hydrochloride (0.735 g., 3 mmoles) and ethyl 2-carbobenzoxyamino-4-keto-5-chloro-L-valerate (0.990 g., 3 mmoles). The crude reaction mixture was hydrolyzed in refluxing 6 *N* hydrochloric acid for 3 hr., and, after removal of excess hydrogen chloride by repeated evaporation under reduced pressure, the hydrolysate was neutralized with ammonia and distributed countercurrently for 294 transfers in the system 1-butanol–ethanol–water–concentrated aqueous ammonia, 45:5:49:1. Monitoring by paper electrophoresis showed that the phenistidine was maximal in tube 50. The contents of tubes 40–60 were freed of solvents and distributed countercurrently in the system 1-butanol–water–acetic acid, 10:9:1, for the 146 transfers. Phenistidine was maximal in tube 8. It was obtained pure as the monoacetate salt (23 mg.) by evaporation of the contents of tubes 1–14 and precipitation with alcohol.

Anal. Calcd. for $C_{16}H_{22}N_4O_4$: N, 16.8. Found: N, 16.9.

In paper electrophoresis it gave a single spot with the characteristic gray color when heated with ninhydrin and moved relative to histidine 0.72 at pH 5.0 in 0.1 *M* pyridine acetate. In ascending paper chromatography it gave a single spot with R_f 0.39 in *sec*-butyl alcohol–water–concentrated aqueous ammonia, 25:9:1; R_f 0.86 in phenol–0.1 *N* hydrochloric acid, 9:2; and R_f 0.55 in 1-propanol–water, 2:1.

When hydrolyzed for 20 hr. in refluxing 6 *N* hydrochloric acid it was not entirely stable. Paper electrophoresis of the hydrolysate at pH 5.0 showed, in addition to the characteristic gray spot, small amounts of a neutral spot which was identified as phenylalanine by paper chromatography and of aspartic acid.

2-Alkylidene-2H-indole Intermediates. The Lithium Aluminum Hydride Hydrogenolysis of 2-Indolecarbinol Derivatives[1]

LLOYD J. DOLBY AND DAVID L. BOOTH[2]

Department of Chemistry, University of Oregon, Eugene, Oregon 97403

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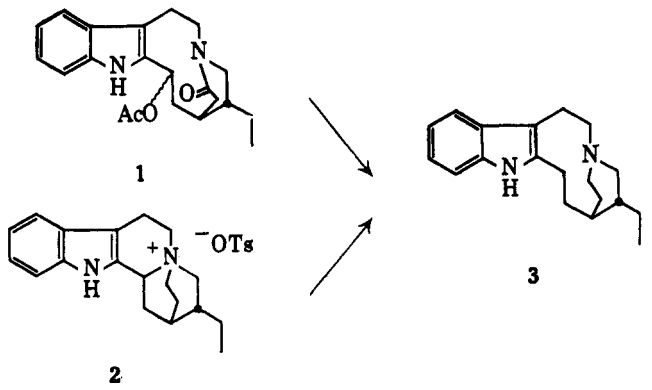
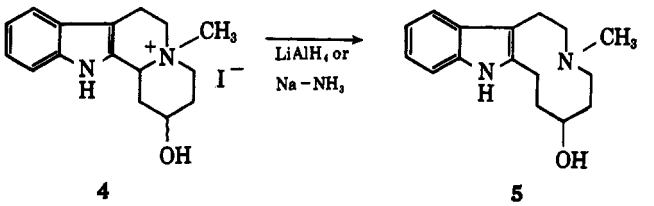
The action of lithium aluminum hydride results in hydrogenolysis of the oxygen function of several 2-indolecarbinol derivatives. We propose that these reactions proceed by an elimination–addition sequence involving 2-alkylidene-2H-indole intermediates. The observation that methylation of the indole nitrogen inhibits the hydrogenolysis reaction is taken as firm evidence for this hypothesis.

We recently reported that lithium aluminum hydride reduction of the acetoxylactam **1** and the quaternary ammonium salt **2** gives rise to the same tetracyclic base **3**.[3] It was suggested that these lithium aluminum hydride reductions involve 2-alkylidene-2H-indole intermediates. We wish to present further results which indidate the intervention of these intermediates.



The generality of this type of cleavage reaction is shown by the lithium aluminum hydride reduction of the tetracyclic quaternary ammonium salt **4**, prepared by reduction and methylation of 1,2,3,4,6,7,12,12b-octahydro-2-ketoindolo[2,3-*a*]pyridocoline.[4] The action of lithium aluminum hydride in refluxing N-methylmorpholine converts the quaternary ammonium salt **4** to the tricyclic alcohol **5**, obtained in 45% yield The same tricyclic alcohol is obtained in 76% yields from the Birch reduction of **4** as anticipated from previous studies.[5] The structure of the tricyclic alcohol **5** is supported by its proton magnetic resonance spectrum which shows an N-methyl group at τ 7.77 but no signal which could be ascribed to a C-methyl group.



We propose that these reactions take place by an elimination–addition sequence involving an intermediate 2-alkylidene-2H-indoles. Similar mechanisms have been advanced to account for the hydrogenolysis of 3-indolecarbinol derivatives[6] and 2- and 3-pyrrolecarbinols.[7] A consequence of this hypothesis is that N-methylation prevents the hydrogenolysis reaction by blocking the elimination which undoubtedly proceeds *via* the indole anion. The effect has been observed with 3-indolecarbinol derivatives[6] and also in the pyrrole series.[7a]

(1) Supported by a grant from the National Institutes of Health (Grant NB 03232-03).

(2) U. S. Public Health Service Predoctoral Fellow.

(3) L. J. Dolby and S. Sakai, *J. Am. Chem. Soc.*, **86**, 1890 (1964).

(4) G. B. Kline, *ibid.*, **81**, 2251 (1959).

(5) E. Leete, *Chem. Ind.* (London), 692 (1960).

(6) E. Leete, *J. Am. Chem. Soc.*, **81**, 6023 (1959).

(7) R. L. Hinman and S. Theodropulos, *J. Org. Chem.*, **28**, 3052 (1963); (b) W. Herz and C. F. Courtney, *J. Am. Chem. Soc.*, **76**, 576 (1954); (c) A. Treibs and H. Derra-Scherer, *Ann.*, **589**, 188 (1954); (d) A. Treibs and H. Scherer, *ibid.*, **577**, 139 (1952).