Synthesis of Carnosine, Anserine, and Isoanserine

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A new method for the synthesis of carnosine, anserine, and isoanserine is described. N-Phthalyl-β-alanine is condensed by the mixed anhydride method with histidine, 1-methylhistidine, and 3-methylhistidine, respectively, in aqueous solution. Hydrazinolysis of the phthalyl group yields the above dipeptides. An independent proof of structure for anserine is presented also.

Carnosine and anserine are dipeptides occurring in vertebrate skeletal muscle. Despite numerous publications relating to their physiological role, no valid biochemical function has as yet been assigned to them. Recent evidence[1] suggests that the two histidine peptides may provide a major portion of the buffering action necessary to maintain a physiological pH range in skeletal muscles during and immediately after strenuous exercise.

The constitution of carnosine has been established as β-alanyl-L-histidine by degradation and synthesis,[2-5] and that of anserine as β-alanyl-1-methyl-L-histidine.[6] Synthetic anserine was first obtained by Behrens and du Vigneaud[7] by condensation of 1-methyl-L-histidine methyl ester (prepared by hydrolysis of natural L-anserine and esterification of the 1-methyl-L-histidine thus obtained) with N-carbobenzoxy-β-alanyl azide followed by hydrolysis and hydrogenation of the reaction product.

In this paper we report a new more convenient method for the synthesis of carnosine, anserine, and "*iso*-anserine," together with independent proof of structure for anserine. The mixed anhydride obtained from N-phthalyl-β-alanine and ethyl chlorocarbonate was condensed with the lithium salt of L-histidine in aqueous solution to give a 60% yield of N-phthalyl-β-alanyl-L-histidine. Hydrazinolysis of the phthalyl group furnished L-carnosine in 96% yield. Similarly a 54% yield of N-phthalyl-β-alanyl-3-methyl-L-histidine was obtained from N-phthalyl-β-alanine and 3-methyl-L-histidine. After cleavage of the phthalyl group β-alanyl-3-methyl-L-histidine (isoanserine) was isolated as the nitrate in 54% yield. This peptide has not been encountered in nature and is described here for the first time.[8] The same procedure furnished N-phthalyl-β-alanyl-1-methyl-L-histidine in 65% yield and L-anserine nitrate in 98% yield. The free bases were obtained by removal of the nitrate ion with Dowex-3 resin. The optical rotation and melting point of L-anserine agreed with the values reported for the naturally occurring compound, but differed from those found for L-isoanserine.

1-Methyl-L-histidine, L-α-amino-β-(1-methyl-5-imidazolyl)propionic acid, and 3-methyl-L-histidine, L-α-amino-β-(1-methyl-4-imidazolyl)propionic acid, were prepared by treatment of L-histidine with sodium and methyl iodide in liquid ammonia[9] and separation of the isomers on a Dowex-50 column (α-picolinium form) essentially according to McManus.[10] 1-Methyl-L-histidine was racemized by heating with acetic anhydride in acetic acid according to Bergmann and Zervas.[11] The infrared spectrum of the racemic product was found to be identical with that of 1-methyl-DL-histidine of unequivocal constitution, synthesized according to Jones and McLaughlin,[12] thus proving the 1-position of the methyl group of 1-methyl-L-histidine obtained by methylation of L-histidine. These findings together with the synthesis described above provide independent confirmation of the structure of L-anserine as β-alanyl-1-methyl-L-histidine. The infrared spectrum of 3-methyl-DL-histidine obtained by racemization of 3-methyl-L-histidine with barium hydroxide was recorded for comparison.

It is interesting to note that racemization of 3-methyl-L-histidine with acetic anhydride in acetic acid[11] led to a mixture of three ninhydrin-positive substances from which 3-methyl-DL-histidine could be isolated only in poor yield and with great difficulty. Racemization with barium hydroxide[13] yielded 3-methyl-DL-histidine and a second more basic component, which were separated on an Amberlite CG-50 column. The nature of the second component has not yet been ascertained. 1-Methyl-L-histidine when racemized by barium hydroxide likewise gave two ninhydrin-positive materials, whereas L-histidine under the same conditions furnished DL-histidine only.

There are few references in the literature concerning the use of free amino acids in the synthesis of peptides. The simplicity of procedure and fair yields of peptides obtained in the present approach are therefore noteworthy.

Experimental[14]

N-Phthalyl-β-alanyl-L-histidine.—A solution of 5.26 g. of phthalyl-β-alanine[5] and 2.4 g. of freshly distilled triethylamine in 100 ml. of ethylene glycol dimethyl ether (dried over calcium hydride) was cooled to −5° in an ice–methanol bath. The solution was stirred mechanically and 2.6 g. of ethyl chlorocarbonate was added dropwise. The reaction mixture was stirred for an additional 10 min., filtered to remove triethylammonium chloride, and added all at once with vigorous stirring to an ice-cooled solution of 3.1 g. of L-histidine in 60 ml. of water contain-

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(14) Melting points are uncorrected. Microanalyses were by A. Elek Microanalytical Laboratories, Los Angeles, Calif.

ing 1 mole equiv. of lithium hydroxide at pH 9.6. Vigorous evolution of carbon dioxide ensued, and the initially clear solution became turbid with formation of a gelatinous precipitate. After standing at 5° for 2 hr., the reaction mixture (pH 7.5) was adjusted to pH 5.0 with 1 *N* hydrochloric acid (approximately 20 ml.) and filtered. The filtrate was evaporated to dryness at low temperature and pressure and the resulting frothy glass-like residue was triturated with 200 ml. of methanol to give a white granular solid. Water (10 ml.) was added and the mixture was heated to boiling. The resulting solution was allowed to cool, whereupon it deposited crystalline N-phthalyl-β-alanyl-L-histidine in 3.5-g. yield. Evaporation of the mother liquors to dryness and recrystallization of the residue from a hot mixture of 50 ml. of methanol and 8 ml. of water furnished 0.8 g. of a second crop. The total yield was 4.3 g. (60%), m.p. 230–235° dec., $[\alpha]^{22}D$ +21.0°; lit.[5] m.p. 221–224° dec., $[\alpha]^{22}D$ +21.5°. In a large-scale experiment 1500 g. of phthalyl-β-alanine tri-*n*-butylammonium salt was condensed with 685 g. of L-histidine as outlined above. Filtration of the mixed anhydride solution was omitted since tri-*n*-butylammonium chloride is soluble in ethylene glycol dimethyl ether. The yield of recrystallized product was 57%.

L-Carnosine was obtained by hydrazinolysis of N-phthalyl-β-alanyl-L-histidine according to Turner,[5] m.p. 255–260° dec., $[\alpha]^{23}D$ +20.1° (*c* 2, water); lit.[5,15] m.p. 250–253° dec. and 260° dec., $[\alpha]^{22}D$ +21.7° and $[\alpha]^{25}D$ +20.5°.

N-Phthalyl-β-alanyl-1-methyl-L-histidine.—A solution of 8.8 g. of phthalyl-β-alanine and 8.1 g. of freshly distilled tri-*n*-butylamine in 180 ml. of ethylene glycol dimethyl ether (dried over calcium hydride) was stirred mechanically, cooled to −5°, and treated with 4.5 g. of ethyl chlorocarbonate as described above. After stirring for an additional 10 min., the mixed anhydride solution was added to 75 ml. of a cold aqueous solution of the lithium salt of 7.5 g. of 1-methyl-L-histidine (Calbiochem, Los Angeles, Calif.) with vigorous stirring. Stirring was continued for an additional 30 min. at 10°. The reaction mixture then was adjusted to pH 5.5 with 1 *N* hydrochloric acid and evaporated to dryness. The glossy white residue was distilled successively with 200 ml. of methanol and then with a mixture of 100 ml. of methanol and 100 ml. of 2-propanol. It then was boiled gently with 200 ml. of methanol while approximately 15 ml. of water was added cautiously until one phase was obtained. The solution was filtered and cooled to give 9.65 g. (65%) of crystalline product, m.p. 222–223°. After recrystallization from a mixture of 340 ml. of methanol and 34 ml. of water the product was dried *in vacuo* over phosphorus pentoxide, yielding 7.65 g., m.p. 228–230°.

Anal. Calcd. for $C_{18}H_{18}N_4O_5$: N, 15.13. Found: N, 15.13.

L-Anserine Nitrate.—N-Phthalyl-L-anserine (38.2 g.) was dissolved in 120 ml. of water and treated with a solution of 5.9 g. of hydrazine (95%) in 33 ml. of methanol. After standing at room temperature for 3 days, the reaction mixture was diluted with water and acidified with acetic acid. The precipitate of phthalyl hydrazide was removed by filtration and the filtrates were evaporated to dryness at low temperature and pressure. Traces of acetic acid were removed by two successive distillations with 25 ml. of water. The solid residue then was dissolved in 75 ml. of water containing 4.8 ml. (1 mole equiv.) of concentrated nitric acid. The solution was evaporated to dryness at low temperature and pressure, and the residue was redissolved in 25 ml. of methanol. 2-Propanol (150–200 ml.) then was added to the solution and the mixture was stored at 5° overnight. Anserine nitrate was obtained as shiny needles, 18 g., m.p. 220°. Concentration of the mother liquors and addition of 2-propanol yielded two further crops of material (8.4 and 4 g.) of similar quality. The total yield was 98%. The crude product was recrystallized by heating with 300 ml. of methanol and adding water (50–60 ml.) until one phase was obtained. The solution was refrigerated overnight and filtered. The precipitate of anserine nitrate was collected and dried at 60° over phosphorus pentoxide *in vacuo*, 20.5 g., m.p. 226–228°, lit.[7] m.p. 225°.

Anal. Calcd. for $C_{10}H_{17}N_5O_6$: C, 39.60; H, 5.65; N, 23.09. Found: C, 39.67; H, 5.72; N, 23.22.

L-Anserine.—A solution of 1 g. of anserine nitrate in 50 ml. of water was shaken with 5 g. of Dowex 3-X4 (free base) resin for 50 min. and filtered. The resin was washed twice with water and the pooled filtrates were evaporated to dryness. Traces of water were removed by three distillations with 10 ml. of 2-propanol. The residue was crystallized by heating with 10 ml. of methanol and adding water dropwise until one phase was obtained. Upon cooling, 0.45 g. of L-arserine was obtained, m.p. 240–242° dec. after drying at 60° over phosphorus pentoxide *in vacuo*, $[\alpha]^{23}D$ +11.4° (*c* 5, water); lit.[7,16] m.p. 238–239° dec., $[\alpha]^{30}D$ +12.25° and $[\alpha]^{15}D$ +11.26°.

Anal. Calcd. for $C_{10}H_{16}N_4O_3$: C, 49.99; H, 6.72; N, 23.33. Found: C, 50.10; H, 6.47; N, 23.11.

N-Phthalyl-L-isoanserine.—The mixed anhydride prepared from 8.8 g. of N-phthalyl-β-alanine and 4.5 g. of ethyl chlorocarbonate in ethylene glycol dimethyl ether as described above, was condensed with 7.5 g. of 3-methyl-L-histidine (Calbiochem, Los Angeles, Calif.). The reaction mixture was adjusted to pH 5.5 and evaporated to dryness at low temperature and pressure. The sirupy residue was distilled successively with 200 ml. of methanol, a mixture of 100 ml. of methanol and 100 ml. of 2-propanol, and finally with 100 ml. of methanol to give a white, dry powder. The material was dissolved in 100 ml. of methanol, and 100 ml. of 2-propanol was added to the solution. The precipitate which formed on cooling was collected, washed with 2-propanol and ether, and dried over phosphorus pentoxide, yielding 10.1 g. A second crop (5.7 g.) was obtained from the mother liquors after further refrigeration. The material was recrystallized from a mixture of 150 ml. of methanol and 7 ml. of water in 8.5-g. (54%) yield. The product was a hydrate and melted at 152–155°. A sample was recrystallized and dried at 60° over phosphorus pentoxide *in vacuo*, m.p. 233–235° dec.

Anal. Calcd. for $C_{18}H_{18}N_4O_5$: C, 58.37; H, 5.16; N, 15.13. Found: C, 58.40; H, 5.00; N, 15.11.

L-Isoanserine Nitrate.—A solution of 1.8 g. of N-phthalyl-L-isoanserine in 6 ml. of water was treated with 0.3 g. of hydrazine (95%) in 2 ml. of methanol. After 3 days at room temperature, the reaction mixture was diluted with 10 ml. of water, acidified with acetic acid, and filtered. The filtrate was evaporated to dryness and the residue was distilled twice with 100 ml. of water to remove traces of acetic acid. The material then was dissolved in 50 ml. of water containing 0.4 ml. of concentrated nitric acid (20% excess) and the solution was evaporated to dryness. The residue was dissolved in a minimum volume of hot methanol. L-Isoanserine crystallized upon cooling, yielding 0.75 g. (54%), m.p. 178–180°. After a second crystallization from 15 ml. of methanol and 0.75 ml. of water and drying at 60° over phosphorus pentoxide *in vacuo*, the product melted at 183–185°.

Anal. Calcd. for $C_{10}H_{17}N_5O_6$: C, 39.60; H, 5.65; N, 23.10. Found: C, 39.36; H, 5.73; N, 23.03.

L-Isoanserine.—The free base was prepared by treatment of the nitrate with Dowex 3X-4 (free base) resin as described for L-anserine. The crude product was recrystallized from methanol containing 7% water and dried at 60° over phosphorus pentoxide *in vacuo*, m.p. 239–243° dec., $[\alpha]^{21}D$ +33.5° (*c* 1, water).

Anal. Calcd. for $C_{10}H_{16}N_4O_3$: C, 49.99; H, 6.71; N, 23.33. Found: C, 49.79; H, 6.91; N, 23.05.

3-Methyl-DL-histidine.—A solution of 480 mg. of 3-methyl-L-histidine in 12 ml. of a saturated solution of barium hydroxide was heated to 150–160° in a sealed tube for 20 hr. After cooling to room temperature, carbon dioxide gas was passed through the reaction mixture and the precipitate of barium carbonate was removed by filtration. The filtrate was concentrated to a small volume, filtered once more, and evaporated to dryness. The residue was dissolved in 3 ml. of water. Paper chromatograms run on Whatman No. 1 paper (11.5 × 12.5 in.) in the solvent system 2-propanol–water (75:25) revealed the presence of two ninhydrin-positive components: 3-methyl-DL-histidine with R_f 0.22 and an unknown with R_f 0.38. The solution was then applied to a 12 × 0.9 cm. column of Amberlite CG-50 H-form resin. Elution with water (approximately 2500 ml.) yielded chromatographically pure 3-methyl-DL-histidine. The unknown remaining on the resin was eluted with 0.025 *M* acetic acid. The first eluate containing 3-methyl-DL-histidine was evaporated to dryness. The residue was dissolved in 7 ml. of methanol. The solution was filtered and evaporated to dryness. The residual solid was dissolved in 0.5 ml. of methanol, and 5 ml. of 2-propanol was added with stirring. Crystallization of 3-methyl-DL-histidine was induced by scratching with a glass rod. After cooling, filtering, and drying over phosphorus pentoxide

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in vacuo, 370 mg. of product was obtained. It was further purified by recrystallization from a mixture of 3 ml. of methanol, 3 ml. of 2-propanol, and 5 drops of water, yielding 290 mg., m.p. 224–226° dec., $[\alpha]^{22}D$ 0.00 (*c* 3, water). The compound was chromatographically homogeneous in three different solvent systems: (1) 2-propanol–water (75:25), (2) pyridine–acetone–ammonia (6 *N*) (45:30:25), and (3) 2-propanol–formic acid–water (8:1:1).

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Synthesis and Reactions of Anhydrous Lithium Cyanide[1]

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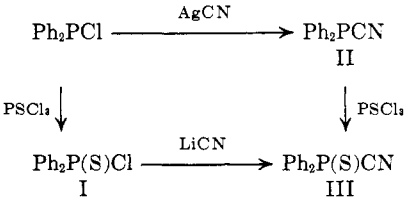
A new synthesis of anhydrous lithium cyanide is reported. Its reactivity is compared with that of silver cyanide. With organic halogen compounds it yields the normal cyano derivative. Its reactions with phenacyl bromide, diphenylchlorophosphine oxide and sulfide, and with diphenyldicyanosilane are described.

Anhydrous lithium cyanide has been found to be an effective reagent for replacing the chloro group by the cyano group in some compounds which do not react with silver cyanide. The latter reagent is usually employed for such replacements on halo phosphines.[2,3] However, it fails to react with diphenylchlorophosphine sulfide, diphenylchlorophosphine oxide, or diphenyldichlorosilane. Anhydrous lithium cyanide reacts readily with these compounds to give good yields of the cyano derivatives. This reactivity is not surprising in view of the good solubility of lithium cyanide in solvents of high dielectric constant, its low melting point, and the high charge density of the unhydrated lithium ion.

Anhydrous lithium cyanide is easily prepared in high purity and yield by the reaction of liquid hydrogen cyanide with *n*-butyllithium. Anhydrous lithium cyanide has been synthesized previously by the treatment of lithium foil with 50% hydrogen cyanide in benzene[4] and by the reaction of anhydrous hydrogen cyanide with an ether suspension of hydrated lithium hydroxide.[5]

The synthesis of diphenylcyanophosphine sulfide by the use of anhydrous lithium cyanide can serve to illustrate the greater reactivity of lithium cyanide than silver cyanide. The sulfide III was synthesized by the following two routes: (1) by the reaction of phosphorus thiochloride with diphenylcyanophosphine (II), and (2) by the reaction of anhydrous lithium cyanide with diphenylchlorophosphine sulfide (I).



The reaction of I with silver cyanide yielded only starting material. However, the reaction of anhydrous lithium cyanide with I at room temperature yielded III (70%). The recovered residue (26%) proved to be undistillable material of various degrees of polymerization. The synthesis of III can also be carried out, however, in high yield by the reaction of phosphorus thiochloride with II.

Neither potassium cyanide nor silver cyanide reacted with diphenylchlorophosphine oxide, only starting material being recovered. However, with lithium cyanide an undistillable solid product was obtained in 59% yield. This product gave the correct analysis for diphenylcyanophosphine oxide but is undoubtedly polymeric.

The greater reactivity of anhydrous lithium cyanide is illustrated again in its reaction with diphenyldichlorosilane. There was no reaction between silver cyanide and diphenyldichlorosilane in refluxing benzene overnight or at 160–185° for 6 hr. without solvent. Lithium cyanide and diphenyldichlorosilane in refluxing benzene gave diphenyldicyanosilane in 68% yield. Silver cyanide does react with diphenyldibromosilane to give diphenyldiisocyanosilane in 80% yield, as described by McBride.[6]

Lithium cyanide reacted readily with phenacyl bromide in dimethylformamide solution to form benzoylacetonitrile, indicating that it yields the cyano and not the isocyano derivative.

Experimental

Lithium Cyanide.—*n*-Butyllithium (64 g., 1.0 mole) in hexane (Foote Mineral Co.) was transferred by filter stick under nitrogen pressure into a 1-l. three-necked, round-bottomed flask fitted with a pressure-equalizing dropping funnel, a nitrogen inlet tube, and a cold finger trap cooled with trichloroethylene–Dry Ice to −14°. A solution of anhydrous hydrogen cyanide (26 g., 0.96 mole) in 32 ml. of dry benzene was added dropwise and with rapid stirring to the *n*-butyllithium at 0–5°. There was immediate precipitation of a white solid. After the addition was complete, the reaction mixture was stirred for an additional 15 min. The contents of the flask were rapidly transferred under nitrogen to a 500 ml. filter apparatus equipped with a large coarse fritted disk and a 40–50 ground glass joint to facilitate pouring from the reaction flask under dry nitrogen. The white solid was washed four times with dry benzene and four times with petroleum ether (b.p. 30–60°) and dried under vacuum at 100–110° to yield 33.1 g. (quantitative yield), m.p. 161–162° (lit.[4] m.p. 160°).

Benzylacetonitrile.—Lithium cyanide and phenacyl bromide in acetonitrile or in dimethylformamide yielded benzoylacetonitrile, m.p. 80.5–80.7°. By vapor phase chromatography the product

(1) Presented at the XIXth International Congress of Pure and Applied Chemistry, London, July 10–17, 1963.

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