[From the William G. Kerckhoff Laboratories of the Biological Sciences, California Institute of Technology, and the Chemical Laboratory, University of California]

THE SYNTHESIS OF *d,l*-CITRULLINE FROM NON-BIOLOGICAL PRECURSORS[1]

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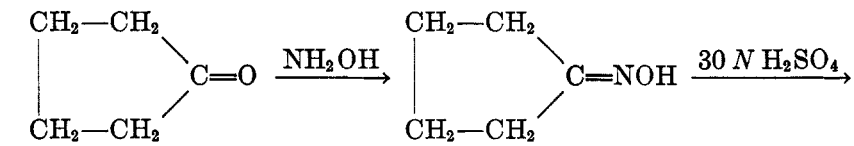
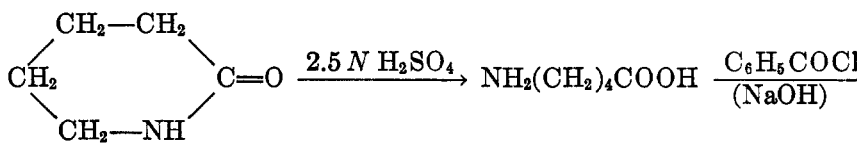
Citrulline has been prepared by the tryptic digestion of casein (1); the action of putrefied pancreas (2), bacteria (3), or alkali (4) on arginine; the hydrolysis of α-monobenzoyl-δ-carbamylornithine (5, 6); and the reaction of ornithine monosulfate with urea in the presence of cupric oxide (7, 8). The synthesis of dibenzoylcitrulline benzoylamide, α-monobenzoylcitrulline methyl ester, and α-monobenzoylcitrullineamide has also been described (9). The arginine which was utilized as starting material in these syntheses of citrulline was isolated from protein sources.

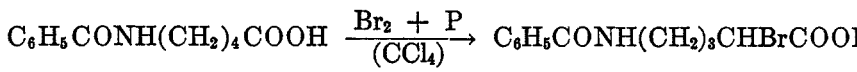
As has been pointed out by Marvel and Stoddard (10) and Rose (11) it is important that synthetic amino acids be used in determining the nutritional requirements of microorganisms, and for other metabolic purposes, in order that small amounts of naturally-occurring substances which might seriously affect the results of such studies may be excluded. For this reason a synthesis of *d,l*-citrulline has been devised by which this amino acid may be prepared in large amounts from substances of non-biological origin. The steps in this synthesis are shown below.



I. Cyclopentanone — II. Cyclopentanone oxime



III. α-Piperidone — IV. δ-Amino-*n*-valeric acid



V. δ-Benzoylamino-*n*-valeric acid — VI. *d,l*-α-Bromo-δ-benzoylamino-*n*-valeric acid

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$$\xrightarrow{NH_3} C_6H_5CONH(CH_2)_3CH(NH_2)COOH \xrightarrow[(HCl)]{H_2O}$$

VII. *d,l*-α-Amino-δ-benzoylamino-*n*-valeric acid (*d,l*-δ-benzoylornithine)

$$NH_2(CH_2)_3CH(NH_2 \cdot HCl)COOH \xrightarrow{CuO + NH_2CONH_2}$$

VIII. *d,l*-α,δ-Diamino-*n*-valeric acid monohydrochloride (*d,l*-ornithine monohydrochloride)

$$[NH_2CONH(CH_2)_3CH(NH_2)COO]_2Cu \xrightarrow{H_2S}$$

IX. Copper salt of *d,l*-δ-carbamylornithine

$$NH_2CONH(CH_2)_3CH(NH_2)COOH$$

X. *d,l*-Citrulline

Experimental procedures thought to be of special interest in connection with the authors' synthesis are (a) the use of hydroxylamine sulfate to convert cyclopentanone (I) to the corresponding oxime (II) in place of sodium nitrite and sodium bisulfite employed by Eck and Marvel (12) for the synthesis of cyclohexanone oxime and (b) the preparation of δ-benzoylamino-*n*-valeric acid (V) from cyclopentanone oxime (II) in about 71% over-all yield. According to Schniepp and Marvel (13) the over-all yield is only 29% of the theoretical amount when the intermediate substances (III) and (IV) are isolated.

d,l-Ornithine monohydrochloride may be prepared by the described method somewhat more conveniently than from γ-phthalimidopropylmalonic ester (14), β-vinylacrylic acid (15), γ-phthalimidopropylthalimidomalonic ester (16), piperidine (17), acrolein (18), or α-aminoadipic acid and hydrazoic acid (19). According to the authors' preliminary experiments, as well as the investigations of Maeda and Nozoe (20), it seems probable that the synthesis of *d,l*-proline from *d,l*-α-bromo-δ-benzoylamino-*n*-valeric acid, prepared by the described series of reactions, is as convenient as any others (21) which have been reported.

EXPERIMENTAL

Cyclopentanone oxime (II). Three hundred thirty-six grams (4.0 moles) of cyclopentanone[2] (b.p. 126–128°/750 mm., uncorr.), 1000 ml. of water, and 720 g. (4.0 moles)

[2] Cyclopentanone may be obtained from commercial sources but that used for the present purpose was prepared from adipic acid essentially according to the method of Thorpe and Kon (22). The adipic acid was obtained from the E. I. du Pont de Nemours and Co., Wilmington, Delaware. Yields of cyclopentanone (b.p., 126–128°, uncorr.) averaging 81.5% were obtained from 1600 to 1800 g. quantities of adipic acid.

of technical (about 90%) hydroxylamine sulfate[3] are placed in a 5-l. round-bottomed flask. The mixture is brought to *p*H 6 by the addition of a saturated solution of technical sodium hydroxide (about 240 ml.) while it is being mechanically stirred. During the addition of the alkali, ice is added as necessary to keep the reaction-mixture at about 25°. The mixture is allowed to stand about thirty minutes. The precipitated cyclopentanone oxime and sodium sulfate, suspended in about 2.5 liters of liquid, are collected on a Büchner funnel and dried at room temperature.

The crude mixture is suspended in benzene (1 ml. per gram of solute), the suspension is heated to 40° and filtered, and the undissolved solid is washed twice with benzene. The filtrate, containing the cyclopentanone oxime, is distilled at atmospheric pressure in an oil-bath at about 125° to remove the benzene. The distillation is continued under reduced pressure using a water-pump. The yield of product, b.p. 93–97°/24 mm. (uncorr.) and m.p. 53.5–54.5° (uncorr.), from four runs averaged 368 g. (93% of the theoretical amount).

*δ-Benzoylamino-*n*-valeric acid*[4] (V). One hundred twenty grams (1.2 moles) of cyclopentanone oxime is dissolved cautiously in 240 ml. of cold approximately 30 *N* sulfuric acid solution. This solution is divided into twelve equal parts, each of which (about 30 ml.) is placed in a 500-ml. conical flask. Rearrangement of the oxime to α-piperidone is effected according to Wallach's (23) method by heating each flask with a free flame until a vigorous reaction occurs. The black solution is rinsed into a 5-liter round-bottomed flask using 250 ml. of water per flask. The combined solutions are refluxed with 10 g. of Norit "A" for two hours, after which the mixture is cooled and filtered.

The colorless filtrate is brought to *p*H 7 by the addition of a saturated solution of technical sodium hydroxide (about 400 ml.). This solution is made strongly alkaline by the addition of 150 ml. of a saturated solution of technical sodium hydroxide and is cooled and stirred vigorously with a mechanical stirrer while 120 ml. (1.0 mole) of technical benzoyl chloride is added over a period of thirty minutes. During this process a saturated solution of sodium hydroxide is added in 10-ml. portions as required to maintain the experimental solution basic to phenolphthalein. When the benzoyl chloride has been added, the solution is stirred for an additional thirty minutes. The suspended sodium sulfate is removed by filtration and washed twice with water.

The combined filtrate and washings are made strongly acid to Congo red by the slow addition of about 200 ml. of concentrated technical hydrochloric acid. The precipitated δ-benzoylamino-*n*-valeric acid is collected on a 6-inch Büchner funnel, washed twice with water to remove sodium sulfate, washed twice with 250-ml. portions of isopropyl ether to remove benzoic acid and other side-reaction products, and dried in air. The yield of product, m.p.[5] 90° ± 1° (uncorr.), from six runs averaged 190 g. (71%).

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[3] The hydroxylamine sulfate was obtained from the Commercial Solvents Co., Terre Haute, Indiana.

[4] This synthesis is a modification of the procedures reported by Schniepp and Marvel (13), Fischer and Zemplén (17), and Maeda and Nozoe (20).

[5] According to Schotten (24) and Gabriel (25) the melting point of δ-benzoylamino-*n*-valeric acid is 94°. Salkowski (26) and Wallach (23) found, however, that some samples liquefied at 94°, re-solidified, and then melted at 106–107°. A product melting at 105–106° was prepared by Schniepp and Marvel (13). The authors' product, precipitated from methanol by the addition of isopropyl ether, invariably melted

d,l-*δ-Benzoylornithine*[4] (VII). Four hundred grams (1.8 moles) of δ-benzoylamino-*n*-valeric acid, 20 g. of dry red phosphorus, and 1.5 liters of dry carbon tetrachloride are placed in a 3-l. three-necked flask equipped with a dropping-funnel, a mercury-sealed mechanical stirrer, and a one-meter water-cooled condenser connected to an efficient water-trap. Two hundred milliliters of dry bromine is placed in the dropping-funnel, the stirrer is started, and the bromine is added at a rate such that the hydrogen bromide is evolved rapidly and the heat of reaction causes the mixture to reflux vigorously. When all of the bromine has been added the flask is immersed in a water-bath, which is maintained at 70–85°, until the bromine has disappeared from the condenser (45–60 minutes). The mixture in the flask is cooled, the carbon tetrachloride is decanted, and a liter of water is added to the residual material in the flask. The mixture is stirred and powdered sodium bicarbonate (about 500 g.) is added slowly until the solid dissolves and further additions of bicarbonate cause no effervescence. The temperature of the reaction-mixture should be maintained at 25–30° with ice during the addition of the bicarbonate.

The mixture is filtered to remove excess bicarbonate and the alkali-insoluble side-reaction product, N-benzoyl-β,β-dibromo-α-piperidone.[6] The filtrate, containing the sodium salt of *d,l*-α-bromo-δ-benzoylamino-*n*-valeric acid in a volume of about 2.5 liters, is made strongly acid to Congo red by the addition of concentrated technical hydrochloric acid. The aqueous layer above the oily bromo acid is decanted and the residual oil is washed three times with water by decantation. The oily bromo acid is separated and four liters of 15 *N* ammonium hydroxide is added to the oil.

After amination has proceeded for three days the reaction-mixture is distilled under reduced pressure on a water-bath using a water-pump. The product is washed three times with ice-water to remove bromides and is dried at 40°. It melts with decomposition in 9–11 seconds when plunged into a bath at 274° (uncorr.).[7] The average yield of *d,l*-δ-benzoylornithine from six runs was 81 g. (19%).

d,l-*Ornithine monohydrochloride*[8] (VIII). One hundred seventy grams (0.72 mole) of *d,l*-δ-benzoylornithine and 3 liters of concentrated C. P. hydrochloric acid are placed in a 5-liter round-bottomed flask equipped with a water-cooled condenser which is connected to a water-trap. The solution is refluxed for twenty hours and then cooled for three hours in an ice-bath. The suspended benzoic acid is collected on a Büchner funnel and washed with ice-water to remove the mother liquor. The

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over the range, 94–105° (uncorr.). Recrystallization of this material from water gave a product which melted at 104–105° (uncorr.). The product obtained by precipitation of the latter material from methanol by isopropyl ether melted at 94–105° (uncorr.). These observations would appear to support the view of Salkowski (26) that δ-benzoylamino-*n*-valeric acid may exist in two crystalline forms.

[6] The yield of purified N-benzoyl-β,β-dibromo-α-piperidone from six runs averaged 96 g. (18%).

[7] The melting point bath, m.p. about 225°, consisted of an equal molar mixture of sodium nitrate and potassium nitrate. Soft glass melting point tubes of uniform bore, 0.6 mm. inside diameter and 0.8 mm. outside diameter, were used for the melting point determinations. Melting (or decomposition) temperatures of amino acids determined by this procedure, which is a modification of that described by Dunn and Brophy (27), are believed to be more significant than those obtained by other methods.

[8] A similar synthesis has been reported by Maeda and Nozoe (20).

combined filtrate and washings are distilled to dryness under reduced pressure on a water-bath using a water-pump. About 500 ml. of water is added and the distillation procedure is repeated to remove as much hydrochloric acid as possible.

The thick, yellowish residues from two runs, each starting with 170 g. of *d,l*-δ-benzoylornithine, are combined and dissolved in 800 ml. of hot 96% ethanol. The warm alcoholic solution is brought to *p*H 6-7, measured with wet nitrazine paper, by the slow addition of about 105 ml. of 15 *N* ammonium hydroxide solution. If the alkali is added slowly enough the *d,l*-ornithine monohydrochloride will separate as a finely divided solid. An oil tends to form when neutralization of the acid is too rapid. After all of the alkali has been added the mixture is allowed to stand for one hour. The suspended *d,l*-ornithine monohydrochloride is collected on a Büchner funnel and washed twice with 200-ml. portions of 96% ethanol. The yield of dry, crude product is about 250 g.

In order to remove the chief contaminant, ammonium chloride, the crude product is suspended for about ten minutes in 800 ml. of boiling 96% ethanol. The suspended *d,l*-ornithine monohydrochloride is collected immediately on a Büchner funnel, washed twice with 200-ml. portions of 96% ethanol and dried at 40°. The yield of product,[9] which melts with decomposition in 9 seconds when plunged into a bath at 233°, is 237 g. (97% of the theoretical amount).

Copper d,l-*citrullinate*[10] (IX). A mixture of 35 g. (0.21 mole) of *d,l*-ornithine monohydrochloride and 20 g. of cupric oxide in 200 ml. of water is boiled for thirty minutes. The mixture is filtered to remove excess cupric oxide. Fifty grams of urea is added to the filtrate and the mixture is evaporated on a steam-bath to a volume of about 175 ml. This solution is transferred to a 200-ml. round-bottomed flask, the stopper is wired in the flask, and the flask is immersed for three hours in a boiling water-bath. Within thirty minutes the copper salt begins to precipitate and after two hours it forms almost a solid mass. The flask is cooled overnight in a refrigerator. The copper salt is collected, washed three times with 100-ml. portions of water, and dried at 50°. It decomposes with effervescence in 25 seconds to give a reddish product when plunged into a bath at 260°. The average yield of copper *d,l*-citrullinate from five runs was 31 g.

d,l-*Citrulline*[10] (X). Two hundred eleven grams of copper *d,l*-citrullinate is suspended in 3.5 l. of water, the suspension is heated nearly to boiling while being stirred with a mechanical stirrer, and hydrogen sulfide is passed into the suspension for two hours. If the colloidal cupric sulfide does not coagulate sufficiently to filter well, stirring of the hot solution is continued for thirty to sixty minutes and, if necessary, an equal volume of 96% ethanol is added. The cupric sulfide is collected on a Büchner funnel and washed with water. If the filtrate is blue the treatment with hydrogen sulfide should be repeated. The filtrate is treated with 10 g. of Norit "A" to remove small amounts of colloidal cupric sulfide.

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[9] A solution containing 68.6 g. of the crude product dissolved in 50 ml. of hot water was decolorized with 1 g. of Norit "A." Three hundred milliliters of 96% ethanol was added to the filtrate and the mixture was allowed to stand overnight in the refrigerator. The precipitate was collected, washed with 100 ml. of 96% ethanol, and dried at 40°. The yield of product, which melted with effervescence in 9 seconds when plunged into a bath at 233°, was 58.6 g. (85% recovery). *Anal.* Less than 0.004% Fe, P_2O_5, and heavy metals; Cl^-, 100.0 and 100.0% of the theoretical amount.

[10] This synthesis is essentially that described by Kurtz (7) who prepared 1.51 g. of *d,l*-citrulline.

The volume of the colorless filtrate is reduced to about 400 ml. by distillation under reduced pressure on a water-bath using a water-pump. An equal volume of 96% ethanol is added to the hot residual solution and the mixture is allowed to stand three hours in an ice-bath. The crystalline *d,l*-citrulline is collected, washed with two 500-ml. portions of 96% ethanol, and dried at 40°. The yield of product, which melted with effervescence in nine seconds when plunged into a bath at 246°, was 136 g. A second crop of *d,l*-citrulline (16.7 g.) was isolated from the mother liquor which was reduced in volume and treated with ethanol as described. The total yield was 152.7 g. (62%, based on *d,l*-ornithine monohydrochloride).

The 136 g. of first crop *d,l*-citrulline was dissolved in 400 ml. of water and the solution was treated with 1 g. of Norit "A." The carbon suspension was filtered and washed. Twelve hundred milliliters of 96% ethanol was added to the hot solution and the mixture was cooled three hours in an ice-bath. The crystals were collected, washed with 350 ml. of 96% ethanol, and dried at 40°.

The yield of product, which melted with effervescence in 8 seconds when plunged into a bath at 246°, was 126.7 g. (93% recovery).

Anal. Less than 0.004% Cl^-, NH_3, Fe, P_2O_5, and heavy metals; formol titration with the glass electrode: m. eq. found, 2.264 (2.260 theor.) and 2.287 (2.281 theor.), average per cent of the theoretical, 100.2; moisture, 0.06%.

By recrystallization from water of 70.7 g. of *d,l*-citrulline, for which analyses are given above, 57.4 g. of first crop and 7.9 g. of second crop were obtained. The product (first crop) melted with effervescence in 9 seconds when plunged into a bath at 248°.

Anal. Moisture, 0.05%; and formol titration with the glass electrode: 100.3 average per cent of the theoretical amount.

SUMMARY

A synthesis of *d,l*-citrulline from substances of non-biological origin in seven main steps by which more than 100 grams of the analytically pure amino acid were prepared at one time is described.

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