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α,ε-Diamino-β-hydroxypimelic Acid. I. Synthesis of Isomer A

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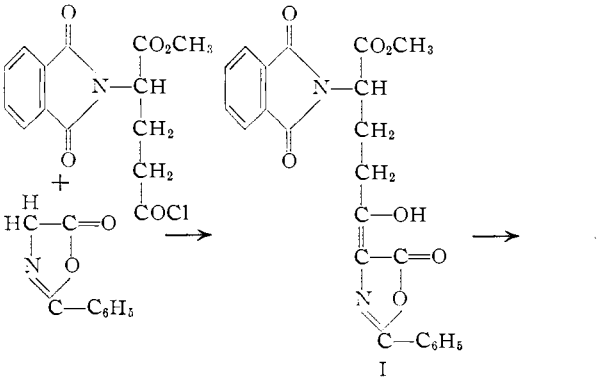
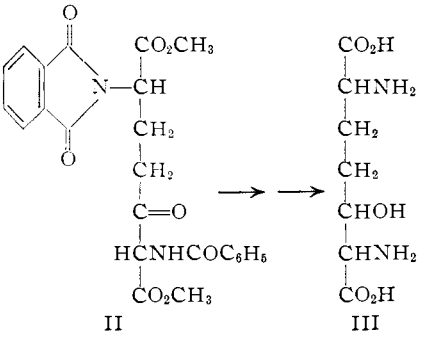
α,ε-Diamino-β-hydroxypimelic acid has been synthesized and one of the four possible racemic isomers has been isolated in a pure form. The acid chloride of phthaloyl-DL-glutamic acid α-methyl ester was condensed with 2-phenyl-5-oxazolone. The condensation product was converted by methanolysis to dimethyl α-benzamido-β-keto-ε-phthalimidopimelate. Reduction of the ketone and hydrolysis of protecting groups gave the desired amino acid.

Degradation of "tabtoxinine,"[1] the amino acid contained within the phytopathogenic toxin of *Pseudomonas tabaci*,[2] has shown it to be α,ε-diamino-β-hydroxypimelic acid (HDAP)(III). Since this was the first report of the occurrence of this amino acid in nature, the synthesis of HDAP was undertaken not only as confirmation of the structure of tabtoxinine and elucidation of its stereochemistry, but also with a view toward a further investigation of the biological properties of this compound.

The basic step in the synthesis was the condensation of the acid chloride[3] of phthaloyl-DL-glutamic acid α-monomethyl ester with 2-phenyl-5-oxazolone. A similar condensation of another acid chloride with this oxazolone has been described.[4] Although the desired product, 2-phenyl-4-(DL-γ-phthalimido-γ-carbomethyoxybutyryl)-5-oxazolone (I), was obtained in only a fair yield, it could be separated from the chief impurity, a highly colored neutral substance, by extraction into very dilute sodium bicarbonate solution.



I



II III

Methanolysis of the purified condensation product converted it quantitatively into dimethyl α-benzamido-β-keto-ε-phthalimidopimelate (II). It was to be expected that this compound, having two asymmetric carbon atoms, would be obtained as a mixture of the two diastereoisomers which would be readily interconvertible by enolization. The formation of an immediate and intense color when the keto ester was treated with ferric chloride indicated that this enolization was extensive. Repeated recrystallization of the keto ester failed to separate one isomer, and the analytically pure compound did not melt sharply.

Reduction of the keto ester with sodium borohydride in methanol solution gave a mixture of hydroxy ester isomers, one of which could be separated by crystallization. Subsequent evidence showed that it was one of the four possible racemic dimethyl α-benzamido-β-hydroxy-ε-phthalimidopimelates; it was called isomer A. The other three isomers could not be separated in pure form. The keto ester could also be satisfactorily reduced by high-pressure hydrogenation over Raney nickel, but in this case the hydroxy ester could not be crystallized. Hydrogenation under the conditions used saturated the nucleus of the phthalyl residue, but not that of the benzoyl, as shown by isolation of hexahydrophthalic and benzoic acids after hydrolysis.

α,ε-Diamino-β-hydroxypimelic acid, having three asymmetric carbon atoms and no molecular symmetry, should exist as eight optical isomers (four DL-pairs). While the exact stereochemistry of tabtoxinine is not known, preliminary work[1] had indicated that both amino groups were in the L-configuration. The HDAP isomer A described herein is not the same as tabtoxinine, although the synthetic and natural amino acids had the same R_f on paper chromatograms and mixtures of the two were not resolved. The difference between the natural and synthetic isomers was shown by chromatography on Dowex-50 cation exchange resin by the analytical procedure of Moore and Stein,[5] and by paper chromatography of their dinitrophenyl (DNP) derivatives. Chromatography of DNP derivatives proved extremely valuable, as it was the only available method for distinguishing the four synthetic isomers, one of which was identical with tabtoxinine. Work in progress on separation of the diastereoisomers and determination of their absolute configurations will be reported subsequently.

(1) D. W. Woolley, G. Schaffner and A. C. Braun, *J. Biol. Chem.*, **198**, 807 (1952).

(2) D. W. Woolley, G. Schaffner and A. C. Braun, *ibid.*, **215**, 485 (1955).

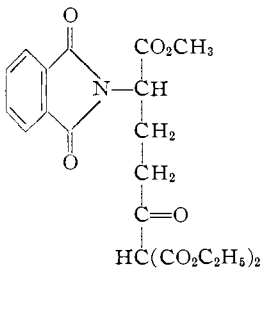
(3) J. C. Sheehan and W. A. Bolhofer, THIS JOURNAL, **72**, 2469 (1950).

(4) H. E. Carter, J. B. Harrison and D. Shapiro, *ibid.*, **75**, 4705 (1953).

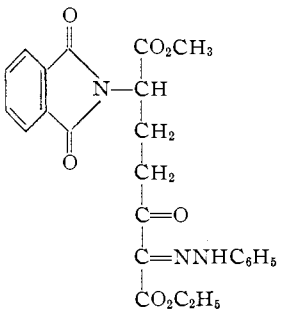
(5) S. Moore and W. H. Stein, *J. Biol. Chem.*, **192**, 663 (1951).

An attempt to carry through the synthesis of HDAP from L-glutamic acid with the retention of the optical purity of that portion of the molecule was unsuccessful. This approach should have given a product containing only four of the eight possible optical isomers, those having the ε-amino group in the L-configuration (presumably like tabtoxinine). Preparation of phthaloylglutamic acid α-methyl ester from phthaloyl-L-glutamic anhydride and sodium methoxide by the usual procedure gave a racemic product. Inverse addition of sodium methoxide to the anhydride gave a product with higher optical activity, but the desired α-ester was contaminated with a large proportion of the γ-ester and purification was considered too laborious for large-scale preparation. The yield of the desired product was very low.

In an earlier attempt to prepare HDAP, the α-ethyl-ε-methyl ester of β-keto-ε-phthalimidopimelic acid was prepared by the general method of Riegel and Lilienfeld[6] for the synthesis of β-keto esters. The acid chloride of phthaloyl-DL-glutamic acid α-methyl ester was condensed with the magnesium salt of malonic ester to give diethyl α-(4-carbomethoxy-4-phthalimidobutyryl)-malonate (IV) in excellent yield. Decarbethoxylation of this ester by pyrolysis gave the desired β-keto ester in fair yield. This β-keto ester was coupled with benzenediazonium chloride to give the methyl ethyl ester of α-phenylazo-β-keto-ε-phthalimidopimelate (V), but a satisfactory procedure for the reduction of this compound was not found. Procedures used in the preparation of β-phenylserine[7] and threonine[8] were not successful. In all of the methods tried, cleavage of the carbon chain occurred, giving glycine and glutamic acid as the ultimate products.



IV



V

Experimental

All melting points are uncorrected and were determined in capillary tubes unless otherwise stated. All evaporations were done under reduced pressure. We thank Mr. T. Bella of the Rockefeller Institute for elementary analyses and Mrs. E. Van Winkle for technical assistance.

2-Phenyl-5-oxazolone.—Since published procedures for the synthesis of this compound gave an unsatisfactory product, the following modified method, suggested by Dr. H. E. Carter, was used. A suspension of 100 g. of hippuric acid in 300 ml. of acetic anhydride was heated rapidly on the steam-bath, with efficient stirring, just until the solid was dissolved. The solution was concentrated rapidly in a flash evaporator with the bath temperature not over 45°. The residue was crystallized by addition of *t*-butyl alcohol as described.[4]

(6) B. Riegel and W. M. Lilienfeld, THIS JOURNAL, **67**, 1273 (1945).

(7) W. A. Bolhofer, *ibid.*, **74**, 5459 (1952).

(8) K. Pfister, C. A. Robinson, A. C. Shabica and M. Tishler, *ibid.*, **71**, 1101 (1949).

2-Phenyl-4-(DL-γ-phthalimido-γ-carbomethoxybutyryl)-5-oxazolone (I).—The acid chloride[3] from 29.1 g. of phthaloyl-DL-glutamic acid α-methyl ester, dissolved in an equal volume of ether, was added during 10 minutes to a stirred solution of 17.0 g. of 2-phenyl-5-oxazolone in 80 ml. of dry β-picoline. The temperature was held below 35°. After stirring for one hour longer, the solution was poured in a thin stream into a well-stirred mixture of cracked ice and 90 ml. of concentrated hydrochloric acid. The solid red product was collected on a filter and washed well with water.

The product was purified by dissolving in 700 ml. of ethyl acetate which was then washed once with water and once with 50 ml. of 5% sodium bicarbonate solution to remove hippuric acid. The product was then extracted by shaking with ten 250-ml. portions of 1% sodium bicarbonate solution. After washing once with ethyl acetate, the combined bicarbonate extracts were chilled by adding ice, overlayered with ethyl acetate, and acidified with hydrochloric acid. The aqueous layer was extracted twice more with ethyl acetate. The combined ethyl acetate solution was washed with water, dried over magnesium sulfate, and evaporated to yield 24.6 g. of a light yellow solid. Crystallization from benzene gave 11.6 g. of the desired product suitable for further use, m.p. 156–160°. One recrystallization gave the pure material, as lemon-yellow prisms, m.p. 160–161°.

Anal. Calcd. for $C_{23}H_{18}N_2O_7$: C, 63.60; H, 4.17; N, 6.44. Found: C, 63.93; H, 4.18; N, 6.21.

In chloroform solution the compound had an absorption maximum at 325 mμ, ε 24,300, and with ferric chloride in ethanol it gave an intense blue color.

Dimethyl α-Benzamido-β-keto-ε-phthalimidopimelate (II).—A solution of 5.8 g. of the oxazolone I in 40 ml. of absolute methanol was refluxed two hours, then concentrated to one-third volume and hexane added to the warm solution to incipient turbidity. After chilling, a nearly quantitative yield of colorless needles was obtained, m.p. 106–114°. It gave a deep burgundy color with ferric chloride in ethanol. Two recrystallizations from methanol raised the m.p. to 116–121°.

Anal. Calcd. for $C_{24}H_{22}N_2O_8$: C, 61.80; H, 4.76; N, 6.02. Found: C, 62.05; H, 4.77; N, 5.96.

Dimethyl α-Benzamido-β-hydroxy-ε-phthalimidopimelate, Isomer A.—A solution of 4.15 g. of the keto ester II in 50 ml. of methanol (warmed to dissolve and cooled to room temperature) was brought to *p*H 8 with 50% sodium hydroxide solution and treated slowly, with stirring, with a methanolic solution of 88 mg. of sodium borohydride. After standing for one hour at room temperature, the solution was acidified with hydrochloric acid, concentrated, and the residue partitioned between ethyl acetate and water. The ethyl acetate phase was washed with 1% sodium bicarbonate solution and water, dried over magnesium sulfate, and evaporated to a glass weighing 4.1 g. Hydrolysis of an aliquot by refluxing with 6 *N* hydrochloric acid for 8 hours and examination of the resulting amino acids by paper chromatography in *n*-propyl alcohol–water (2:1) showed the presence of HDAP contaminated with small amounts of glycine and glutamic acid.[9]

When a saturated solution of the crude hydroxy ester mixture in methanol–isopropyl ether (1:1) was allowed to stand at 4°, crystals eventually formed. One recrystallization from the same solvent pair gave 0.70 g. of colorless needles, m.p. 169–170°.

Anal. Calcd. for $C_{24}H_{24}N_2O_8$: C, 61.53; H, 5.16. Found: C, 61.48; H, 5.13.

Hydrogenation of the Keto Ester.—A suspension of 23.3 g. of dimethyl α-benzamido-β-keto-ε-phthalimidopimelate (II) in 125 ml. of methanol was hydrogenated over Raney nickel at 70° and an initial pressure of 1000 p.s.i. until uptake of hydrogen ceased (4 hours). The catalyst was filtered off and the solvent evaporated. The product was a glass which could not be crystallized from any solvent tried.

The crude hydroxy ester was hydrolyzed with refluxing 6 *N* hydrochloric acid for 8 hours. The acid was evaporated three times with ether. Benzoic acid and hexahydrophthalic

(9) The synthetic HDAP, although it contains four diastereoisomers, gave a single spot on paper chromatograms in all solvent systems tried including that of Rhuland, *et al.*,[10] which separates *meso* and L-diaminopimelic acid.

(10) L. E. Rhuland, E. Work, R. F. Denman and D. S. Hoare, THIS JOURNAL, **77**, 4844 (1955).

acid were obtained from the ether phase by evaporation of the solvent, and were identified by comparison with authentic samples on paper chromatograms. In descending *n*-butyl alcohol–88% formic acid–water (1:1:1) the acids moved thus: benzoic, R_f 0.91; hexahydrophthalic, R_f 0.81; reference, phthalic, R_f 0.75. The aqueous phase was evaporated to dryness repeatedly to remove hydrochloric acid. Paper chromatography of the residue showed principally HDAP, along with glycine and glutamic acid in amounts similar to those obtained when the keto ester was reduced with sodium borohydride.[9,10]

α,ε-Diamino-β-hydroxypimelic Acid, Isomer A.—The crystalline hydroxy ester obtained from the sodium borohydride reduction was hydrolyzed with refluxing 6 *N* hydrochloric acid for 8 hours. The cooled solution was filtered, and the filtrate was extracted once with ether and evaporated. The residue was twice redissolved in water and evaporated to dryness to remove excess hydrochloric acid. Analysis of the crystalline solid, after drying at 100° *in vacuo*, showed it to be a dihydrochloride. α,ε-Diaminopimelic acid, on the contrary, gives a monohydrochloride under these conditions.[11]

Anal. Calcd. for $C_7H_{16}Cl_2N_2O_5$: C, 30.12; H, 5.78. Found (corrected for 0.79% ash): C, 30.25; H, 5.92.

The free amino acid was prepared from the dihydrochloride by adsorption on Dowex-2 (hydroxide form) and elution with *M* acetic acid.[12] The eluate was evaporated and the residue recrystallized from water–ethanol.

Anal. Calcd. for $C_7H_{14}N_2O_5$: C, 40.77; H, 6.84; N, 13.59. Found: C, 40.84; H, 6.74; N, 13.60.

Chromatography on Whatman No. 1 paper gave R_f 0.12 in ascending *n*-propyl alcohol–water (2:1), R_f 0.11 in ascending phenol–water (5:1 by weight), R_f 0.21 in descending phenol saturated with 1% aqueous ammonium hydroxide, and R_f 0.22 in the system of Rhuland, *et al.*[10] Natural tabtoxinine run simultaneously had the same R_f values (*cf.* footnote 9).

HDAP isomer A gave a single peak at tube 130 when chromatographed on a 0.9 × 100 cm. column of Dowex-50 by the analytical procedure of Moore and Stein[5] (0.2 *M* citrate buffer, *p*H 3.42). In amino acid mixtures it emerged 10 tubes after glycine. A mixture of 0.3 mg. each of isomer A and tabtoxinine gave two peaks, at tubes 121 and 130.

α,ε-Bis-(2,4-dinitrophenylamino)-β-hydroxypimelic Acid (Di-DNP Isomer A).—A solution of 20 mg. of HDAP (isomer A) and 100 mg. of sodium bicarbonate in 5 ml. of water with a solution of 0.1 ml. of 2,4-dinitrofluorobenzene in 5 ml. of ethanol. After standing two hours at room temperature, the solution was concentrated to remove ethanol. The diluted aqueous solution was extracted three times with ether, acidified to *p*H 4.5 with dilute hydrochloric acid and extracted twice with ether to remove dinitrophenol, and then acidified to *p*H 1. Three extractions with ethyl acetate removed the product, which weighed 40 mg. after drying.

For purification, the product was dissolved in dilute sodium bicarbonate solution, chilled in an ice-bath, and precipitated with hydrochloric acid. Three washes with ice-water removed salt. After drying, the product melted at 128–130° (hot-stage). Paper chromatograms in descending *n*-butyl alcohol–ammonia[1] (run for 36 hours) gave R_f 0.18. On the same strip, di-DNP tabtoxinine had R_f 0.20. A mixture of the two substances gave two spots.

Anal. Calcd. for $C_{19}H_{18}N_6O_{13}$: C, 42.4; H, 3.4. Found: C, 42.3; H, 3.7.

Chromatographic Separation of DNP Mixed Isomers.—A sample of HDAP containing all four isomers was converted to the DNP derivative as above. On paper chromatograms developed as described, four spots appeared, R_f's 0.10, 0.16, 0.18 (isomer A) and 0.20. DNP glycine (R_f 0.28) and DNP glutamic acid (R_f 0.08), when present, did not interfere. Separation of DNP derivatives was unique, since benzoyl, 2,5-dinitrobenzoyl and 3,5-dinitrobenzoyl derivatives of the isomeric mixture moved as single spots.

Phthaloyl-L-glutamic Acid α- and γ-Monomethyl Esters.—A solution of 20 g. of phthaloyl-L-glutamic anhydride[13] in 250 ml. of dry dioxane was chilled in an ice-bath and an equimolar amount of sodium methoxide in methanol was added slowly with stirring. The solvent was evaporated, the gummy residue was dissolved in cold water, and the solution acidified with hydrochloric acid and extracted twice with chloroform. After drying and evaporation of the solvent, the crude product had $[\alpha]^{31}D$ −42° (*c* 3, dioxane). This material was fractionally recrystallized from ethyl acetate–hexane. The DL-α-ester, being less soluble, was removed in the first fractions. The crops having the highest specific rotation were combined, dissolved in ether and extracted with 5 portions of 2% sodium bicarbonate solution, each containing 0.2 equivalent of bicarbonate. The material from each separate fraction was recovered by acidification and extraction with ethyl acetate, and recrystallized from ethyl acetate–hexane.

The first fractions, containing the more acidic γ-ester, were combined and recrystallized to constant m.p. and specific rotation, to yield 2.7 g. of phthaloyl-L-glutamic acid γ-methyl ester, m.p. 135–137°, $[\alpha]^{33}D$ −47.1° (*c* 3, dioxane), *pK* 3.5.

Anal. Calcd. for $C_{14}H_{13}NO_6$: C, 57.74; H, 4.50; N, 4.81. Found: C, 57.81; H, 4.60; N, 4.72.

The later fractions, containing the less acidic phthaloyl-L-glutamic acid α-methyl ester, were purified similarly to yield 1.3 g., m.p. 138–140°, $[\alpha]^{33}D$ −52.6°, *pK* 4.8. Position of the ester was assigned on the basis of the *pK* value.[14]

Anal. Found: C, 57.78; H, 4.51; N, 4.76.

Diethyl α-(4-Carbomethoxy-4-phthalimidobutyryl)-malonate (IV).—By the general procedure of Riegel and Lilienfeld,[6] the magnesium derivative from 16 g. of diethyl malonate was condensed with the acid chloride[3] from 29.1 g. of phthaloyl-DL-glutamic acid α-methyl ester. The crude product, a viscous oil, weighed 44 g. A cold ether solution of the crude product was extracted 6 times with 250-ml. portions of cold 10% (w./v.) sodium carbonate solution. The yellow aqueous extracts were combined and acidified with hydrochloric acid while keeping the temperature low with cracked ice. The product was extracted into ether, washed with water, and dried. Evaporation gave 39.5 g. of a viscous oil which gave a red color with ferric chloride in ethanol.

For characterization, the 2,4-dinitrophenylhydrazone was prepared as lemon-yellow needles, m.p. 134–136°.

Anal. Calcd. for $C_{27}H_{27}N_5O_{12}$: C, 52.86; H, 4.44; N, 11.42. Found: C, 52.68; H, 4.47; N, 11.35.

β-Keto-ε-phthalimidopimelic Acid α-Ethyl-ε-methyl Ester.—The above acyl malonate IV (15 g.) was pyrolyzed in the presence of 0.4 g. of *p*-toluenesulfonic acid monohydrate by the general procedure of Riegel and Lilienfeld.[6] Purification of the product by their procedure gave 7.0 g. of a viscous oil; 5.5 g. of the acyl malonate was recovered unchanged. Distillation of the product at 0.3 mm. and a bath temperature of 225° gave 3.4 g. of a straw-colored oil, after a small forerun of diethyl malonate.

Anal. Calcd. for $C_{18}H_{19}NO_7$: C, 59.83; H, 5.30; N, 3.88. Found: C, 60.04; H, 5.51; N, 4.08.

The 2,4-dinitrophenylhydrazone was prepared by the usual procedure, m.p. 55–60°.

Anal. Calcd. for $C_{24}H_{23}N_5O_{10}$: C, 53.24; H, 4.28. Found: C, 53.34; H, 4.42.

α-Phenylazo-β-keto-ε-phthalimidopimelic Acid α-Ethyl-ε-methyl Ester (V).—By the procedure of Bolhofer,[7] 2.70 g. of β-keto-ε-phthalimidopimelic acid α-ethyl-ε-methyl ester was coupled with an equimolar amount of benzenediazonium chloride in buffered aqueous ethanol. The product was extracted from the diluted reaction mixture into chloroform, and the resulting solution was washed with dilute hydrochloric acid, 5% sodium carbonate and water. After drying over magnesium sulfate and evaporation, 3.0 g. of a red oil remained.

For characterization, the 2,4-dinitrophenylhydrazone was prepared and recrystallized from chloroform–hexane as red crystals, m.p. 180–182°.

Anal. Calcd. for $C_{30}H_{27}N_7O_{10}$: C, 55.81; H, 4.22; N, 15.18. Found: C, 55.69; H, 4.32; N, 15.27.

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(11) D. S. Hoare and E. Work, *Biochem. J.*, **61**, 562 (1955).

(12) A. Dreze, S. Moore and E. J. Bigwood, *Anal. Chim. Acta*, **11**, 554 (1954).

(13) F. E. King and D. A. A. Kidd, *J. Chem. Soc.*, 3315 (1949).

(14) W. J. le Quesne and G. T. Young, *ibid.*, 1954 (1950).