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α,ε-Diamino-β-hydroxypimelic Acid. II. Configuration of the Isomers

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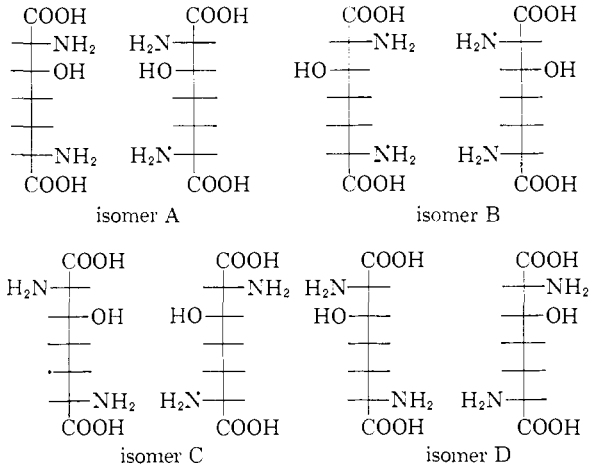
The four DL-diastereomers of α,ε-diamino-β-hydroxypimelic acid have been isolated in pure form, and the total configuration of each isomer has been determined. Certain isomers were interconverted *via* the oxazolines derived from the α-benzoyl-ε-phthalyl dimethyl ester. The relative configuration of the α-amino and β-hydroxyl was determined by converting a mixture of all four isomers to the two *threo* isomers by equilibration of the oxazolines. The relative configuration of the two amino groups was determined by converting one isomer to α,ε-diaminopimelic acid of known configuration.

The first paper of this series[1] described the synthesis of α,ε-diamino-β-hydroxypimelic acid (HDAP) and isolation of one of the four possible DL-diastereomers. In the present paper, the preparation of the other three diastereomers and the establishment of the total configuration of each of the isomers[2] are described. The evidence presented here indicates that the configuration of the isomers of HDAP is that shown in Table I.

TABLE I

CONFIGURATION OF HDAP ISOMERS (FISCHER PROJECTION)



In order to carry out separation and characterization of these four diastereomers, analytical methods able to distinguish the four were necessary. Two such methods that were used in this work were the column chromatography on Dowex-50 cation exchange resin of the HDAP amino acids, and the paper chromatography of the N,N′-bis-2,4-dinitrophenyl (di-DNP) derivatives. Paper chromatography of the di-DNP derivatives was the only method found that would distinguish all four of the isomers; development in a system of 1-butanol and ammonia gave the R_f's shown in Table II. Ion exchange column chromatography was helpful in that it was able to distinguish the two *erythro* isomers from the two *threo* isomers, although it would not separate all four isomers.

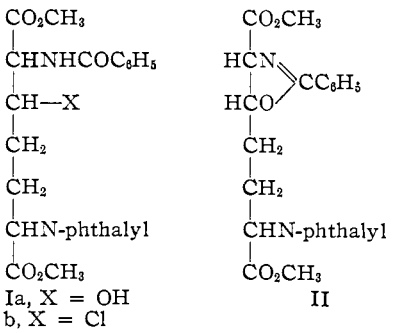
The key intermediate in the isolation of the isomers of HDAP was dimethyl- α-benzamido-β-hydroxy-ε-phthalimidopimelate (Ia) ("hydroxy ester"), the synthesis of which was described in part I. It was ultimately possible to crystallize two of the isomers of this compound in pure form from the synthetic product containing all four isomers. These two were arbitrarily designated A and D, and usually amounted to 30 and 15%, respectively, of the total product. The other two isomers, called B and C, were obtained by inversion of the β-carbon of isomers A and D. This isomerization was carried out by treating the isomerically pure hydroxy ester Ia with thionyl chloride to form 2-phenyl-4-carbomethoxy-5-(γ-carbomethoxy-γ-phthalimidopropionyl)-oxazoline (II), and subsequent hydrolysis of the oxazoline with acid. It was shown[3] by the elegant work of Elliott on the threonine isomers that this procedure caused inversion of the β-carbon without any effect on the configuration of the α-carbon. Application of this procedure to isomer A hydroxy ester gave a new isomer of HDAP, called isomer B. Paper chromatography of DNP derivatives showed that A and B were different isomers, and that the conversion of A to B was complete. DNP isomer B had the same R_f as one of the other components of the synthetic mixture of isomers. This showed that isomer B was present in the original synthetic mixture. In the same manner, isomer D hydroxy ester was con-

TABLE II

PROPERTIES OF THE HDAP ISOMERS

Isomer	Dowex-50 tube no.[a]	Rf of DNP[b]	M.p. of hydroxy ester, °C.	M.p. of amino acid, °C.[c]	α,β-Config.	Amino config.
A	130	0.18	169–170	278	*Erythro*	*Meso*
B	121	.10	.....	239	*Threo*	*Meso*
C	122	.16	.....	260	*Threo*	DD, LL
D	130	.20	149–150	244	*Erythro*	DD, LL

[a] Tubes of 1 ml. of collected from 100 × 0.9 cm. columns run by the procedure of Moore and Stein, ref. 4. [b] On paper chromatograms developed in 1-butanol–ammonia (see Experimental). [c] With decomposition.



(1) J. M. Stewart and D. W. Woolley, THIS JOURNAL, **78**, 5336 (1956).

(2) Throughout this paper "isomer" will refer to a DL-pair, one of the four possible diastereomeric racemates of HDAP. A single letter, *e.g.* "isomer A," designates not only the amino acid but also all derivatives having this same configuration.

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

verted to isomer C amino acid, making available all four of the DL-diastereomers in pure crystalline condition. The four isomeric amino acids differed somewhat in solubility and ease of crystallization, and one of them (isomer D) crystallized from water–ethanol in hydrated form. The infrared spectra of the four isomers were different.

In order to establish the complete configuration of the isomers of HDAP, two sets of data must be determined: (1) the relative configuration of the α-amino and β-hydroxy groups, whether *threo* or *erythro*, and (2) the relative configuration of the two amino groups, whether DD, LL or DL, LD (*meso*). The relative configuration of the α,β-carbons was established by applying another finding from the threonine work. Elliott found that the mixture of *cis*- and *trans*-oxazolines derived from a mixture of N-benzoylthreonine methyl ester and N-benzoylallothreonine methyl ester could be equilibrated with base to a product which was exclusively the *trans*-oxazoline, which upon acid hydrolysis gave only threonine. This procedure was successful because the *trans*-oxazoline, which led to threonine (*threo* configuration) on hydrolysis, was thermodynamically much more stable that the hindered *cis*-oxazoline, which yielded allothreonine (*erythro* configuration) on hydrolysis. Application of this procedure to a mixture of all four isomers of HDAP (containing two *threo* and two *erythro* isomers) should lead to a product containing only the two *threo* isomers. That this method was indeed applicable to HDAP and gave the expected results was shown by the presence of only two isomers (R_f of DNP's, 0.10 and 0.16) in the product obtained by treatment of the synthetic hydroxy ester mixture of isomers (R_f of DNP's 0.10, 0.16, 0.18, 0.20) first with thionyl chloride to form the oxazolines, then with sodium methoxide in methanol to equilibrate the 4-carbon of the oxazolines, followed by acid hydrolysis. These two *threo* isomers were identical to isomers B and C, and were the same as the two isomers obtained by thionyl chloride inversion of the crystalline hydroxy esters A and D. It is not surprising that the two esters which crystallized from the original reaction mixture were those having the *erythro* configuration, because *erythro* compounds are generally less soluble than the corresponding *threo* isomers.

The relative configuration of the amino groups was established by removing the hydroxyl from HDAP, and comparing the resulting α,ϵ-diaminopimelic acid with samples of known configuration. Early attempts to remove the hydroxyl by means of phosphorus–hydriodic acid reduction were unsuccessful, since this reagent was found to cause epimerization. All four isomers of HDAP gave the same mixture of *meso*- and DL-diaminopimelic acid by this method. The hydroxyl of HDAP was successfully removed without epimerization by conversion to the chloro derivative, followed by hydrogenolysis of the chlorine. When the hydrochloride of the oxazoline II (from isomer A) was heated, dimethyl α-benzamido-β-chloro-ϵ-phthalimidopimelate (Ib) was formed in good yield. This type of transformation has been studied for similar compounds,[4,5] and has been found to proceed with inversion of the β-carbon without affecting the configuration of the α-carbon. The chlorine was hydrogenolyzed with Raney nickel, and the protecting groups removed by acid hydrolysis. The diaminopimelic acid (DAP) thus formed was characterized by paper chromatography and bioassay, and found to be exclusively the *meso* isomer. This result established that HDAP isomer A (and also isomer B, which differs from A only in the configuration of its hydroxyl-bearing carbon) has its amino groups in the *meso* configuration (one D and one L within the same molecule). Isomer C must then have the DD-configuration of one enantiomorph and LL-configuration of the other. Isomer D also has the DD–LL-amino group configuration. The total configuration of all of the isomers of HDAP must be that shown in Table I.

Three attempts to establish the configuration of the amino groups by means of the action of stereospecific enzymes were unsuccessful. These involved the use of the D-amino acid oxidase of *Neurospora crassa*,[6] the diaminopimelic acid decarboxylase of *Aerobacter aerogenes*,[7] and hog kidney amidase. The first two of these enzymes did not attack HDAP. The experiments with hog kidney amidase were modeled after the successful characterization of the isomers of diaminopimelic acid by Work, *et al.*[8] If HDAP, isomer A, had the *meso*-group configuration, action of this enzyme on the DL-isomer A diamide should yield solely the monoamide, while if the amino configuration were DD, LL, the product should consist of equal parts of diamide and free amino acid. An attempt to prepare HDAP diamide isomer A, by the usual method of making the ester hydrochloride and treating that with ethanolic ammonia was unsuccessful. The product, upon analysis, was shown to contain other isomers than A, *i.e.*, epimerization had occurred during the synthesis, making this route unsatisfactory for the preparation of a sterically pure product. The method ultimately chosen for synthesis of HDAP diamide involved ammonolysis of the mixed anhydride prepared from dicarbobenzoxy HDAP, isomer A, and ethyl chloroformate. Subsequent hydrogenolysis of the carbobenzoxy groups yielded HDAP diamide, which was shown to be exclusively isomer A by hydrolysis of an aliquot, conversion to the DNP derivative, and paper chromatography of the latter. When the HDAP isomer A diamide thus prepared was incubated with the renal acylase preparation, it was slowly hydrolyzed to a mixture of equal parts of the monoamide and the free amino acid. This unexpected result was due to the spontaneous hydrolysis of the diamide to monoamide under the conditions of the assay. Since the non-enzymic hydrolysis was not stereospecific, HDAP of either of the possible configurations would have led to the same product, making the result equivocal.

Although the structure of the HDAP monoamide obtained by spontaneous hydrolysis of the diamide

(4) E. M. Fry, *J. Org. Chem.*, **15**, 802 (1950).

(5) J. Sicher and M. Pankova, *Coll. Czech. Chem. Comm.*, **20**, 1409 (1955).

(6) A. E. Bender and H. A. Krebs, *Biochem. J.*, **46**, 210 (1950).

(7) D. L. Dewey, D. S. Hoare and E. Work, *ibid.*, **58**, 523 (1954).

(8) E. Work, S. M. Birnbaum, M. Winitz and J. P. Greenstein, THIS JOURNAL, **77**, 1916 (1955).

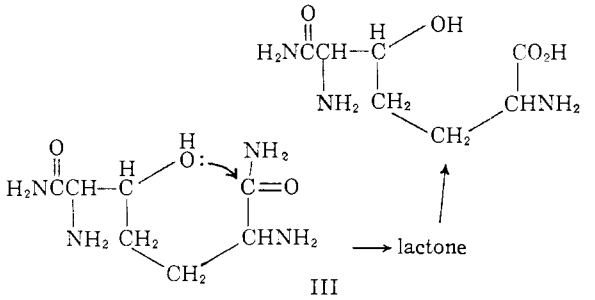
TABLE III

PRINCIPAL INFRARED BANDS OF HDAP ISOMERS AND TABTOXININE[a]

Cpd.	OH	AA I	CO_2^-	AA II	CO_2^-		OH			
HDAP A	2.98s	6.14s	6.37s	6.60s	7.06s	7.38s	7.60s	8.53m	8.70m	
HDAP B	2.97s	6.06s	6.22s	6.55s	7.20s	7.38s	7.48s	8.60m	8.80m	
HDAP C	2.99s	6.08s	6.30s	6.53s	7.01s	7.37w	7.48s	8.61s	8.74s	
	3.04s									
HDAP D	2.98s	6.14s	6.30s	6.50s	7.08s	7.35m	7.51s	8.54w	8.65w	
Tabtoxinine	3.03s	6.06s	6.30vs	6.73s	7.05s	7.40s	7.62s	8.46s	....	
	3.11s									
				9.45m	9.58s	11.35s	....	12.00m	12.54m	12.85vw
				9.40m	9.56w	11.30m	....	11.92w	12.42m	12.86s
				9.48m	9.85s	11.25w	11.56s	....	12.43m	12.98s
				9.40m	9.52s	11.45w	11.74s	12.05w	....	12.85vw
				9.32s	9.54w	11.42w	11.72w	11.98w	12.33m	12.57m

[a] s = strong, m = medium, w = weak, vw = very weak.

was not determined, it was most likely that shown in III, having the free ε-carboxyl. Such a compound could easily be formed by the N–O shift shown, for which precedents are known. For example, 3,5-dihydroxy-3,4-dimethylvaleramide was found[9] to lactonize with elimination of ammonia even upon standing under anhydrous conditions.



III

The original purpose for undertaking the work described in this paper was to confirm the structure deduced by degradation for tabtoxinine, an amino acid from the toxin of *Pseudomonas tabaci*.[10] Tabtoxinine was found to be extremely similar to HDAP, and mixtures of the two were not separated by any of the many paper chromatography systems tried. More stringent comparison, however, revealed slight differences. Tabtoxinine emerged from Dowex-50 columns in tube 121, and its DNP derivative, when chromatographed on paper in butanol–ammonia, moved with an R_f of 0.20. By comparison with Table II, it will be seen that although these data for tabtoxinine, considered individually, correspond with certain isomers of HDAP, when taken together, they do not match exactly with any one isomer. The DNP derivatives of tabtoxinine and HDAP isomer D had the same R_f, but tabtoxinine and isomer D were separated on Dowex-50 columns. There were also significant differences in the infrared spectra. The actual structure of tabtoxinine must then be sought in some structural isomer of HDAP.

Acknowledgment.—The author wishes to thank Dr. D. W. Woolley for his continued interest and many helpful discussions throughout this work, and Dr. Herbert Jaffe for much painstaking work in obtaining and interpreting the infrared spectra. We thank Miss Jeanice Cooper for technical assistance and Mr. T. Bella for microanalyses.

(9) J. M. Stewart and D. W. Woolley, THIS JOURNAL, **81**, 4951 (1959).

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

Experimental[11]

α,ε-Diamino-β-hydroxypimelic acid, isomer A, and its derivatives were prepared as described in Part I.[1]

Chromatography of HDAP Isomers on Dowex-50.—Behavior of the isomers of HDAP on Dowex-50 × 8 cation exchange resin columns was examined by the use of the analytical procedure of Moore and Stein.[12] Columns 100 × 0.9 cm. were developed with *p*H 3.42 citrate buffer at 37°. Fractions of 1 ml. were collected. Positions of the effluent peaks of the HDAP isomers are given in Table II. The same column and the same batch of buffer were used for all of the isomers, so that slight differences in experimental conditions did not confuse the results. Mixtures of all of the possible pairs of isomers were also run. Isomers A and D could be separated from isomers B and C, but not from each other. Isomers B and C could not be separated from each other. Mixtures of all four isomers were not satisfactorily separated. The separation was not improved by running the column at *p*H 3.25.

Infrared Spectra of HDAP and Tabtoxinine.—Infrared spectra were obtained with a Perkin–Elmer model 21 spectrophotometer with sodium chloride optics. Samples were pressed in potassium bromide pellets. Principal bands of the isomers of HDAP and of tabtoxinine (as the free amino acids) are given in Table III.

α, ε-Bis-(2,4-dinitrophenylamino)-β-hydroxypimelic Acids (Di-DNP Isomers B, C and D).—HDAP isomers B, C and D were each converted to the DNP derivative by the procedure described in Part I for isomer A. The purified DNP isomers were chromatographed on paper in descending 1-butanol–water–concd. ammonium hydroxide (20:19:1 by volume). The R_f values are given in Table II. By allowing the chromatograms to run for 36 hours, the isomers were all clearly separated.

Preparation of Isomer B by Isomerization of Isomer A.—A solution of 500 mg. of crystalline dimethyl α-benzamido-β-hydroxy-ε-phthalimidopimelate (isomer A)[1] in 2 ml. of cold thionyl chloride was allowed to stand for 1 hour at room temperature and was then evaporated. The residue was twice dissolved in chloroform and evaporated to remove residual thionyl chloride, and then hydrolyzed by refluxing for 8 hours with 6 *N* hydrochloric acid. The amino acid was purified by the same method used for isomer A;[1] isomer B could be crystallized from water–ethanol only with difficulty, as it showed a tendency to separate as an oil.

Anal. Calcd. for $C_7H_{14}N_2O_5$: C, 40.77; H, 6.84; N, 13.59. Found: C, 40.61; H, 7.10; N, 13.27.

(11) All melting points were taken in capillaries and are uncorrected. All evaporations were done under reduced pressure.

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

On paper chromatograms, isomer B was not resolved from isomer A in any of the solvent systems tried (the three systems of ref. 1; *sec*-butyl alcohol–water–concd. ammonium hydroxide (25:9:1); *sec*-butyl alcohol–88% formic acid–water (100:20:13.3); phenol saturated with 0.1 *N* hydrochloric acid; the systems of Shaw and Fox[13] and Drell[14]). On analytical columns of Dowex-50, isomer B emerged at tube 121, and mixtures of isomers A and B were resolved. Paper chromatography of DNP isomer B showed no contamination with isomer A.

Dimethyl α-Benzamido-β-hydroxy-ε-phthalimidopimelate, Isomer D.—During one crystallization of the isomer A hydroxy ester from the crude product, it was observed that the needles of isomer A were contaminated with small prisms. The mother liquor, when seeded with these prisms, gave a crop (usually about 15% yield) of material which after recrystallization from methanol or benzene had m.p. 149.5–150° (mixed m.p. with isomer A ester, 141–153°).

Anal. Calcd. for $C_{24}H_{24}N_2O_8$: C, 61.53; H, 5.16; N, 5.98. Found: C, 61.53; H, 5.15; N, 5.81.

α,ε-Diamino-β-hydroxypimelic Acid, Isomer D.—Hydrolysis of the isomer D hydroxy ester and purification of the resulting amino acid by the usual procedure[1] gave a product which showed a single spot on paper chromatograms. The amino acid was recrystallized from water–ethanol. Unlike the other three isomers of HDAP, which could be satisfactorily dried by heating *in vacuo* at 100° for 3 hours, isomer D required heating at 110° for 5 hours.

Anal. Calcd. for $C_7H_{14}N_2O_5$: C, 40.77; H, 6.84; N, 13.59. Found: C, 40.69, H. 6.92; N, 13.46.

α,ε-Diamino-β-hydroxypimelic Acid, Isomer C.—By the same procedure used for the preparation of isomer B, the isomer D hydroxy ester was isomerized with thionyl chloride, and the product hydrolyzed and purified as before. Recrystallization from water removed traces of isomer B and gave a sterically pure product.

Anal. Calcd. for $C_7H_{14}N_2O_5$: C, 40.77; H, 6.84; N, 13.59. Found: C, 40.83; H, 6.85; N, 13.75.

Preparation of the Two *threo* Isomers (Isomers B and C) by Isomerization of the Total Crude Product.—A solution of 5.0 g. of dimethyl α-benzamido-β-hydroxy-ε-phthalimidopimelate (crude product either from sodium borohydride reduction or hydrogenation,[1] containing all four isomers) in 15 ml. of cold thionyl chloride was allowed to stand at room temperature for 2 hours, and the solvent was then evaporated. The residue was twice dissolved in dry chloroform and evaporated to remove excess thionyl chloride, and then dissolved in 50 ml. of absolute methanol. The solution was made strongly alkaline with sodium methoxide, was allowed to stand for 15 minutes, and was then acidified with hydrochloric acid and concentrated to remove methanol. Hydrolysis of the product and purification of the amino acids were effected as described for the other isomers. Paper chromatograms showed only HDAP. On the Dowex-50 column, the product gave one peak in tube 122, the position of isomers B and C.

Di-DNP Derivative of the Mixed *threo* Isomers.—A sample of the above mixture of the two *threo* isomers of HDAP was treated with fluorodinitrobenzene by the previously described procedure.[1] On paper chromatograms (descending, 1-butanol–ammonia) this product gave two spots, R_f 0.10 and 0.16. Chromatography of mixtures of this product with pure DNP-isomers confirmed the identity of these spots with isomers B and C and their difference from isomers A and D.

Phosphorus–Hydriodic Acid Reaction of HDAP.—Each isomer of HDAP was reduced by heating a mixture of 25 mg. of the amino acid, 200 mg. of red phosphorus and 1.5 ml. of hydriodic acid (sp. gr. 1.7) in a sealed tube at 120° for 8 hours. After the tube had been cooled and opened, its contents were evaporated to dryness repeatedly with addition of water after each evaporation. The resulting solution was brought to *p*H 6.5 by stirring with well-washed anion exchange resin Amberlite IR-4B and then filtered and concentrated to 3 ml. The amino acids were analyzed by two-dimensional paper chromatography, the first direction in phenol–water (5:1, by weight),[15] and the second direction (after washing the paper free of phenol with ether) in the system of Rhuland, *et al.*[16] Each isomer of HDAP gave both *meso*- and DL-DAP, and the ratio of *meso* to DL was the same in the product from each of the four isomers of HDAP.

Dimethyl α-Benzamido-β-chloro-ε-phthalimidopimelate, from Isomer A.—Dimethyl α-benzamido-β-hydroxy-ε-phthalimidopimelate, isomer A (1.0 g.), was dissolved in 5 ml. of thionyl chloride, and the solution allowed to stand at room temperature overnight. Excess thionyl chloride was evaporated and the last traces removed by dissolving the residue in ethyl acetate and again evaporating. The residue, the oxazoline hydrochloride, was a friable solid, and gave a precipitate in the cold with alcoholic silver nitrate. The oxazoline hydrochloride was isomerized to the β-chloro ester by refluxing for 1 hour in dioxane. The chloro ester was recrystallized from ethyl acetate–hexane, and had m.p. 109–110°. It gave a precipitate with alcoholic silver nitrate only when boiled.

Anal. Calcd. for $C_{24}H_{23}ClN_2O_7$: C, 59.25; H, 4.76; N, 5.76; Cl, 7.28. Found: C, 59.14; H, 5.06; N, 5.89; Cl, 7.12.

Diaminopimelic Acid by Hydrogenolysis of Chloro Ester.—Commercial Raney nickel was washed thoroughly with water to remove alkali, and was then washed with ethanol. Dimethyl α-benzamido-β-chloro-ε-phthalimidopimelate (25 mg.) was dissolved in ethanol and the solution refluxed for 6 hours with 2 g. of the washed Raney catalyst. After removal of the catalyst and solvent, the product was hydrolyzed by refluxing for 8 hours with 6 *N* hydrochloric acid. Solvent was removed by evaporation, and traces of nickel were precipitated from ammoniacal solution with hydrogen sulfide. The remaining amino acid solution was desalted on Dowex-2[17] and analyzed by paper chromatography in the two systems described above. The only spot present was that of *meso*-DAP. The identity of *meso*-DAP was also confirmed by Dr. Charles Gilvarg, who kindly performed a specific bioassay with a DAP-requiring mutant (173–25) of *Escherichia coli*. This assay distinguished between *meso*- and L-DAP.

HDAP Diamide, *via* Ester.—Esterification of HDAP (isomer A) by refluxing with ethanolic hydrogen chloride gave a product which was shown by paper electrophoresis (*p*H 5.0) to be a mixture of diester, monoester (or lactone) and amino acid. Repetition of the procedure did not improve the yield of diester. The crude product was treated directly with ethanolic ammonia, and the diamide isolated by passage through a column of Amberlite IRC-50 cation exchange resin.[8] When a sample of the diamide was hydrolyzed with hydrochloric acid, converted to the DNP derivative and the latter chromatographed on paper, spots were seen corresponding not only to isomer A, but also to isomers B and C.

N,N′-Dicarbobenzoxy HDAP Lactone, Isomer A.—A solution of 4.2 g. of HDAP isomer A in 50 ml. of 1.2 *N* sodium hydroxide solution was chilled in ice–salt and treated with 7.8 g. of carbobenzoxy chloride and 40 ml. of 1.2 *N* sodium hydroxide solution in portions during 15 minutes with very vigorous stirring. The solution was stirred for an additional 30 minutes in the cold, and for 1 hour while warming to room temperature. After two extractions with ether to remove unreacted carbobenzoxy chloride, the solution was acidified with hydrochloric acid and extracted three times with ethyl acetate. The ethyl acetate solution was washed with water, dried over magnesium sulfate and evaporated, leaving 7.2 g. of a colorless solid. When the product was crystallized from ethyl acetate–hexane, there was removed first 1.2 g. of an unidentified by-product, m.p. 133–136°, then 5.5 g. of the desired product. Further recrystallization of the latter from ethyl acetate gave a pure compound, m.p. 151–153°. Potentiometric titration of the pure compound revealed one acid group and one lactone which opened readily in alkali and reclosed upon back-titration with acid.

Anal. Calcd. for $C_{23}H_{24}N_2O_8$: C, 60.52; H, 5.30; N, 6.14. Found: C, 60.52; H, 5.49; N, 6.39.

N,N′-Dicarbobenzoxy HDAP Diamide, Isomer A.—A solution of 1.14 g. (2.5 mmoles) of dicarbobenzoxy HDAP lactone in 20 ml. of peroxide-free tetrahydrofuran was treated with 45 mg. (2.5 mmoles) of water and 0.50 g. (5 mmoles) of triethylamine and allowed to stand at room temperature for

(13) K. N. F. Shaw and S. W. Fox, THIS JOURNAL, **75**, 3421 (1953).

(14) W. Drell, *ibid.*, **77**, 5429 (1955).

(15) Suggested by Dr. Charles Gilvarg.

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

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

1 hour to open the lactone. The solution was then chilled to −15°, and 0.54 g. (5% excess over 5 mmoles) of ethyl chloroformate was added, with shaking. After it had stood for 5 minutes in the cold, the solution was saturated with dry ammonia and then allowed to warm to room temperature during 1 hour. The solvent was evaporated, and the residue broken up and shaken well with a mixture of ethyl acetate and 1% aqueous sodium bicarbonate. The insoluble material (1.05 g.) was filtered off, washed well with ethyl acetate and water, and dried. The product was insoluble in hot ethyl acetate, and only very slightly soluble in hot dioxane or tetrahydrofuran, but it was quite soluble in the latter two solvents when they contained some water. For purification the product was dissolved in hot 90% tetrahydrofuran, filtered, and caused to crystallize by addition of more water and chilling; needles m.p. 207–209° dec. For analysis, it was necessary to dry the compound for 7 hours at 100°.

Anal. Calcd. for $C_{23}H_{28}N_4O_7$: C, 58.46; H, 5.97; N, 11.86. Found: C, 58.46; H, 6.06; N, 11.92.

Hydroxydiaminopimelic Diamide Diacetate, Isomer A.—A solution of 0.5 g. of N,N'-dicarbobenzoxy HDAP diamide, isomer A, in 25 ml. of acetic acid was shaken for 19 hours with 0.15 g. of 5% palladium-on-carbon catalyst under a hydrogen pressure of 45 p.s.i. The catalyst was removed by filtration, and the solvent evaporated. Last traces of acetic acid were removed by three evaporations from ethanol solution, leaving a colorless glass. Paper electrophoresis at *p*H 5 showed HDAP diamide, essentially free of contamination by any other ninhydrin-positive substances. The product was dissolved in water, filtered to remove some insoluble material, and lyophilyzed, leaving a hygroscopic glass.

Anal. Calcd. for $C_{11}H_{24}N_4O_7$: C, 40.70; H, 7.45; N, 17.25. Found: C, 40.71; H, 7.90; N, 16.40.

A sample was hydrolyzed for 3 hours in refluxing 6 *N* hydrochloric acid. Electrophoresis now showed only amino acid. After removal of hydrochloric acid, the amino acid was converted to the DNP derivative by the usual procedure. Paper chromatography of the latter revealed only one substance, having the same R_f as DNP isomer A run alongside.

Behavior of HDAP Diamide toward Hog Kidney Amidase.—Hog kidney amidase was prepared according to the directions of Birnbaum,[18] and assayed for activity with L-leucinamide. This enzyme preparation hydrolyzed 260 μmoles of leucinamide/mg. protein/hour when incubated in "Tris" buffer, *p*H 8, at 37°. The course of the hydrolysis was followed by paper electrophoresis of aliquots which had been acidified with *N* acetic acid and heated for 10 minutes at 100° to inactivate the enzyme. HDAP diamide, when incubated with the enzyme under these conditions, was hydrolyzed at the rate of 0.36 μmole/mg. protein/hour, and the ultimate product was a mixture of equal parts of monoamide and free amino acid. Without enzyme, the diamide hydrolyzed at the rate of 0.10 μmole/hour to the monoamide, which was stable.

Behavior of HDAP toward the DAP Decarboxylase of *Aerobacter aerogenes*.—The isomers of HDAP were tested with the crude DAP decarboxylase of *A. aerogenes*[19] by the qualitative procedure of Dewey, *et al.*[7] Paper electrophoresis of the assay tubes after inactivation of the enzyme showed that while both *meso*- and L-DAP were decarboxylated to lysine, HDAP was not decarboxylated.

Behavior of HDAP toward the D-Amino Acid Oxidase of *Neurospora crassa*.—*N. crassa*, strain 25a,[20] was grown in the medium of Bender and Krebs,[6] and the crude D-amino acid oxidase was prepared from the mycelium by their procedure. The enzyme was assayed by incubation with substrate in pyrophosphate buffer, *p*H 8.4, and examination of the resulting mixture by paper chromatography. While D-alanine and D-glutamic acid were oxidized, none of the isomers of HDAP was attacked by this enzyme.

(18) S. M. Birnbaum, in S. P. Colowick and N. O. Kaplan, "Methods In Enzymology," Vol. II, Academic Press, Inc., New York, N. Y., 1955, p. 397.

(19) Freeze-dried cells of *A. aerogenes*, rich in this enzyme, kindly supplied by Dr. H. T. Huang of Chas. Pfizer and Co.

(20) We thank Dr. E. L. Tatum for this culture.

[CONTRIBUTION FROM THE ORGANIC CHEMICAL RESEARCH SECTION, LEDERLE LABORATORIES DIVISION, AMERICAN CYANAMID CO., PEARL RIVER, N. Y.]

Total Synthesis of Tetracyclines. IV. Synthesis of an Anhydrotetracycline Derivative

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Synthetic 8-chloro-1,2,3,4-tetrahydro-5-methoxy-4-oxo-2-naphthaleneacetic acid (14) has been converted in seven steps to the *syn* isomer (20) of methyl 5-benzyloxy-8-chloro-1,2,3,4,4a,9,9a,10-octahydro-4,10-dioxo-2-anthraceneacetate. Subsequent elaboration of this intermediate into biologically active (±)-dedimethylamino-12a-deoxy-6-demethylanhydro-7-chlorotetracycline (39), and comparison of the latter with a sample of dextrorotatory 39 derived by degradation are described.

The yellow crystalline antibiotic Aureomycin,[1] isolated by Duggar in 1948 from the actinomycete *Streptomyces aureofaciens*,[2] was the first example of an important class of naturally occurring antibacterial substances.[3] The recognition of its powerful activity against a broad spectrum of pathogenic microörganisms, followed in 1950 by the isolation of a similar substance, Terramycin,[4] from *Streptomyces rimosus*,[5] stimulated intense efforts toward the structure elucidation of these complex natural products. Success was achieved in 1952, when a brilliant investigation involving alkaline, acidic and reductive degradation and full use of spectrophotometric and pK_a determinations culminated in the establishment of Terramycin as 5-hydroxytetracycline (1).[6] Research upon aureomycin led in turn to announcement of its structure as 7-chlorotetracycline (2).[7] Subsequent investigations have resulted in the identification of a number of

(1) Aureomycin is the registered trademark of the American Cyanamid Co. for the antibiotic chlorotetracycline.

(2) B. M. Duggar, *Ann. N. Y. Acad. Sci.*, **51**, 177 (1948); U. S. Patent 2,482,055.

(3) A concise review of the chemistry of the principal members of this group of antibiotics is given by P. Regna in "Antibiotics: Their Chemistry and Non-Medical Uses," H. Goldberg ed., D. Van Nostrand Co., Inc., Princeton, N. J., 1959, pp. 77–96.

(4) Terramycin is the registered trademark of Charles Pfizer and Co. for the antibiotic oxytetracycline.

(5) A. C. Finlay, G. L. Hobby, S. Y. P'an, P. P. Regna, J. B. Routien, D. B. Seeley, G. M. Shull, B. A. Sobin, I. A. Solomons, J. W. Vinson and J. H. Kane, *Science*, **111**, 85 (1950).

(6) (a) F. A. Hochstein, C. R. Stephens, L. H. Conover, P. P. Regna, R. Pasternack, K. J. Brunings and R. B. Woodward, THIS JOURNAL, **74**, 3708 (1952); (b) F. A. Hochstein, C. R. Stephens, L. H. Conover, P. P. Regna, R. Pasternack, P. N. Gordon, F. J. Pilgrim, K. J. Brunings and R. B. Woodward, *ibid.*, **75**, 5455 (1953).

(7) (a) C. R. Stephens, L. H. Conover, F. A. Hochstein, P. P. Regna, F. J. Pilgrim, K. J. Brunings and R. B. Woodward, *ibid.*, **74**, 4976 (1952); (b) C. W. Waller, B. L. Hutchings, R. W. Broschard, A. A. Goldman, C. F. Wolf and J. H. Williams, *ibid.*, **74**, 4981 (1952); (c) C. R. Stephens, L. H. Conover, F. A. Hochstein, W. T. Moreland, P. P. Regna, F. J. Pilgrim, K. J. Brunings and R. B. Woodward, *ibid.*, **76**, 3568 (1954).