Betaine of Hydroxy-L-proline (XXI, Betonicine).—Hydroxy-L-proline (2.00 g.) was added to a suspension of 4 g. of silver oxide in 5 cc. of water. After 3 hours at room temperature the silver salt had formed and half of the water was removed *in vacuo* and 40 cc. of methanol and 2 cc. of methyl iodide were added. The solution warmed up immediately and silver iodide formed. The mixture remained at room temperature overnight. After that 1.5 cc. of methyl iodide was added. The mixture was refluxed for 3 hours, filtered and taken to dryness *in vacuo*. Trituration with acetone and ethanol yielded a crystalline residue weighing 1.11 g. (46%), $[\alpha]^{20}D$ −37.6° (*c* 1.0, in water). A recrystallized sample of betonicine from ethanol had m.p. 252–253° and $[\alpha]^{20}D$ −34.2° (*c* 1.0, in water); lit. m.p. 243° and $[\alpha]^{21}D$ −37° (*c* 4.8, in water).[28] The initial rotation of a 1% solution in *N* NaOH was −36.0° (*c* 1.0, in water). After next reading (after 18 hours) it was 0.0° and after 24 hours it was still 0.0°.

Anal. Calcd. for $C_7H_{13}NO_3$: C, 52.81; H, 8.23; N, 8.80. Found: C, 52.83; H, 8.35; N, 8.58.

O-Acetylbetonicine Hydrochloride (XXII).—O-Acetylhydroxy-L-proline (0.66 g.), prepared according to Sakami and Toennies[40] was dissolved in 1.25 cc. of water and treated with 1 g. of silver oxide. After 3 hours, 10 cc. of methanol and 0.5 cc. of methyl iodide were added at 0°. This mixture was shaken at room temperature for 1 hour whereupon 0.4 cc. of methyl iodide was added and shaking was continued for one additional hour. Filtration, evaporation of solvent and trituration of the residual oil with acetone, ethanol and ether afforded 0.12 g. of crystalline material which was largely betonicine. The mother liquor was taken to dryness and the oil so obtained was converted to its hydrochloride with hydrogen chloride gas in ethyl acetate. After several crystallizations at room temperature from water, ethanol and ethyl acetate, acetylbetonicine hydrochloride was obtained, m.p. 200–201°.

Anal. Calcd. for $C_9H_{15}NO_4 \cdot HCl$: C, 45.48; H, 6.79; N, 5.89. Found: C, 45.36; H, 6.94; N, 5.66.

Betaine of Allohydroxy-D-proline (XXIII, Turicine).—Silver oxide (4.00 g.) was added to allohydroxy-D-proline (2.00 g.) dissolved in 2.5 cc. of water. After 3 hours 15 cc. of methanol and 2 cc. of methyl iodide were added to the suspension of the silver salt. The mixture was agitated at room temperature for 4 hours, 1.5 cc. of methyl iodide was added and shaking was continued for an additional 3 hours. Filtration, evaporation of the solvent *in vacuo* and trituration with acetone and ethanol afforded 2.35 g. (97%) of turicine of m.p. 252° and $[\alpha]^{20}D$ +35.1° (*c* 1.0, in water). A recrystallized sample from water, ethanol and ethyl acetate had m.p. 259–260° and $[\alpha]^{20}D$ +37.8° (*c* 1.0, in water); lit. m.p. 249° and $[\alpha]^{21}D$ +36° (*c* 0.5, in water).[28] A mutarotation study of a 1% solution of turicine in *N* NaOH at 20° gave the following values; $[\alpha]D$ initial, +51.1°; after 3 hours, +29.2°; after 5.5 hours, +20.6°; and after 22 hours, 0.0°.

Anal. Calcd. for $C_7H_{13}NO_3$: C, 52.81; H, 8.23; N, 8.80. Found: C, 52.56; H, 8.30; N, 8.80.

Betaine of 5-Hydroxy-L-pipecolic Acid.—The methylation of 5-hydroxy-L-pipecolic acid (0.300 g.) at room temperature with silver oxide and methyl iodide according to the procedure for O-acetylbetonicine hydrochloride gave on trituration with acetone and ethanol, the betaine (0.275 g., 77%), m.p. 265° dec. A recrystallized sample from water, ethanol and ethyl acetate showed m.p. 267–268° dec., $[\alpha]^{20}D$ −13.9° (*c* 1.0, in water).

Anal. Calcd. for $C_8H_{15}NO_3$: C, 55.47; H, 8.73; N, 8.09. Found: C, 55.73; H, 8.43; N, 7.83.

BETHESDA, MARYLAND

(40) Sakami and Toennies, *J. Biol. Chem.*, **144**, 203 (1942).

[CONTRIBUTION FROM THE NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES, NATIONAL INSTITUTES OF HEALTH PUBLIC HEALTH SERVICE]

The Configuration of 5-Hydroxypipecolic Acid from Dates

BY BERNHARD WITKOP AND CALVIN M. FOLTZ

RECEIVED JULY 9, 1956

5-Hydroxypipecolic acid (I) was isolated on a preparative scale from the fruits of *Phoenix dactylifera*. The mixture of the free amino acids was treated with nitrous oxides which deaminated the primary amino acids and converted the secondary amino acids to the ether-soluble N-nitroso acids. The latter were hydrolyzed to the secondary amino acids and separated on a column of Dowex-50 ion exchange resin. The oxidation of N-carbobenzyloxy-5-hydroxy-L-pipecolic acid (II) with chromium trioxide in sulfuric acid yielded the 5-keto compound III which was reduced with sodium borohydride to give, on treatment with acetic anhydride, the lactone of N-carbobenzyloxy-5-allohydroxy-L-pipecolic acid (VI). The hydrobromide of the free lactone VII opened up in water with mutarotation to give the salt of 5-allohydroxy-L-pipecolic acid (V). The formation of the lactone in the allo series established the *cis*-relationship of the functional groups and the application of the rule of Lutz and Jirgensons made possible the complete steric assignments to natural and allo-5-hydroxypipecolic acids. Hudson's lactone rule was applied to the mutarotation of the lactone VII and found to be valid and applicable. The formation of the two racemic amino acids (I and V) was observed in the internal opening of diethyl 2-(3′,4′-epoxybutyl)-2-formamidomalonate (IX), after removal of the formyl group by base, together with the five-membered isomers XI and XII.

The configuration of δ-hydroxy-L-lysine,[1] an important building stone of collagen,[2] is not known. Its cyclization to the two diastereoisomeric 5-hydroxypipecolic acids has been achieved[3] in the same manner as the conversion of the γ-hydroxyornithines to the normal and allo-hydroxyprolines.[4] This cyclization, done on a preparative scale, would allow of the exact steric correlation of the two asymmetric centers carrying the hydroxyl of δ-hydroxylysine and 5-hydroxypipecolic acid. As a step in this direction, this paper describes the configuration of 5-hydroxy-L-pipecolic acid, a simple method of isolation, from dates, and the synthetic formation of the two diastereoisomeric 5-hydroxy-D,L-pipecolic acids.

Isolation.—The colorful history of the more or less simultaneous discovery of 5-hydroxypipecolic acid in various plants (Rhodesian Teak,[5] dates[5] *Rhapis excelsa*,[6] Acacia[6]) and in different laboratories has been presented by F. C. Steward.[5] In this investigation dates served as a source for the isolation of the new amino acid on a scale of several grams. After separation from the sugars the secondary amino acids were separated from the primary amino acids by reaction in aqueous

(1) *Cf.* W. S. Fones, THIS JOURNAL, **75**, 4865 (1953).

(2) *Cf.* K. H. Gustavson, "The Chemistry and Reactivity of Collagen," Academic Press, Inc., New York, N. Y., 1956.

(3) L. A. Cohen, F. Irreverre, K. A. Piez, B. Witkop and H. L. Wolff, *Science*, **123**, 842 (1956).

(4) B. Witkop and Th. Beiler, THIS JOURNAL, **78**, 2882 (1956).

(5) N. Grobbelaar, J. K. Pollard and F. C. Steward, *Nature*, **175**, 703 (1955).

(6) A. I. Virtanen and S. Kari, *Acta Chem. Scand.*, **8**, 1290 (1954).

TABLE I

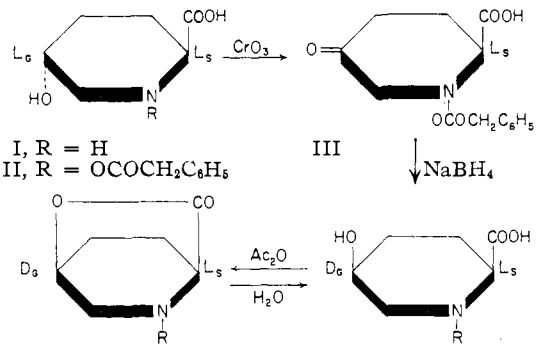
ROTATIONS OF THE FREE NATURAL AND ALLO-5-HYDROXYPIPECOLIC ACIDS AND HYDROXYPROLINES AND THEIR SALTS

Compound	$[\alpha]^{20}D$ free amino acid	c, H_2O	$[\alpha]^{20}D$ salt	$\Delta[\alpha]^{20}D$
5-Hydroxy-L-pipecolic acid	−23.1°	1.0	−10.9°(hydrochloride in H_2O, c 0.9)	+12.2°
5-Allohydroxy-L-pipecolic acid	−31.1	1.0	− 7.4 (hydrobromide in H_2O, c 1.0)	+23.7
Hydroxy-L-proline	−75.2	1.0	−47.3 (in 1 N HCl, c 1.3)	+27.9
Allohydroxy-L-proline	−58.1	2.6	−15.7 (in 1 N HCl, c 1.4)	+42.4

solution with the nitrous oxides generated from nitrite and acid. The nitrosamino acids were extracted into ether and reconverted to the free imino acids by short refluxing in hydrochloric acid.[7] The resulting mixture was fractionated on a large column of Dowex-50 ion exchange resin. The elution with 1.5 N hydrochloric acid was followed by spot testing on paper. From 15 pounds of dates, 6.5 g. of pure 5-hydroxy-L-pipecolic acid was obtained.

Stereochemistry.—The L-configuration for the α-carbon atom is supported by the more positive (or less negative) optical rotation of the salts compared with the free amino acids (empirical rule of Lutz and Jirgensons[8]) as Table I shows for the four related hydroxy-L-amino acids.

The steric relations of the two functional groups



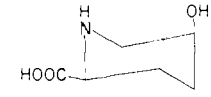
I, R = H
II, R = $OCOCH_2C_6H_5$

VI, R = $OCOCH_2C_6H_5$
VII, R = H

IV, R = $OCOCH_2C_6H_5$
V, R = H

were established in the same fashion as in the hydroxyproline series.[9,10] The amino acid I was converted into its readily-crystallized N-carbobenzyloxy derivative II. The secondary alcoholic hydroxyl group was oxidized to a keto group with chromium trioxide in aqueous sulfuric acid. The ketoacid III could not be crystallized and seemed to deteriorate on manipulation, an observation reminiscent of the behavior of 4-ketoproline[9,10] and its derivatives and of 5-ketopiperidine-2-carboxylic acid.[11] Sodium borohydride reduced III to N-carbobenzyloxy-5-allohydroxy-L-pipecolic acid (IV) which did not crystallize. Catalytic debenzylation led to the allohydroxy acid which was assayed for purity on a two-dimensional paper chromatogram using a basic ternary solvent system.[12] The occurrence of a large new spot next to 5-hydroxypipecolic acid run on the same strip, and the absence of starting material in the reduction product showed the stereospecificity of the reducing process. It may be recalled that N-carbobenzyloxy-4-keto-D- and L-proline are reduced by sodium borohydride to allohydroxy-D- and L-proline in almost quantitative yield.[9]

The reduction of III to IV introduces an axial hydroxyl as shown in VIII, disregarding the other chair form with an equatorial COOH and any boat forms. Axial, rather than equatorial, hydroxyls are formed in increasing amounts in the sodium borohydride reductions of ketones with increasing hindrance.[13] Such hindrance in the case of III might be brought about by a complex of boron with the functional groups of one or two molecules of the keto acid. The remarkable stereospecificity of the reduction of III and similar 4-substituted ketones might well decrease with diminishing polarity of the substituent *para* to the carbonyl.[14]



VIII

That in the reduced allo compound IV the hydroxyl was on the same side of the ring as the carboxyl was shown by lactonization which was brought about readily by brief reaction of IV with acetic anhydride at room temperature; II did not react with acetic anhydride.[15] The lactone VI crystallized after removal of the acetic anhydride and was recrystallized from methanol. The rotation in methanol was $[\alpha]^{20}D$ −6.3°. After the addition of one equivalent of sodium hydroxide the rotation decreased rapidly to $[\alpha]^{20}D$ −39.3°. Applying Hudson's lactone rule[16] one finds $\Delta[\alpha]D$

(7) This procedure was suggested by the separation of secondary amines from primary and tertiary amines with the aid of nitrous acid (*cf.* Houben-Weyl, "Die Methoden der Organischen Chemie," Vol. IV, Leipzig, 1941, p. 575). Its application to mixtures of primary and secondary amino acids on a preparative scale should be of considerable use.

(8) O. Lutz and B. Jirgensons, *Ber.*, **63**, 448 (1930); **64**, 1221 (1931); **65**, 784 (1932); *cf.* the comprehensive contribution of M. Winitz, S. M. Birnbaum and J. P. Greenstein, THIS JOURNAL, **77**, 716 (1955).

(9) A. A. Patchett and B. Witkop, *ibid.*, **79**, 185 (1957).

(10) *Cf.* B. Witkop, Recent work on Naturally Occurring Nitrogen Heterocyclic Compounds, Special Publication No. 3, London, The Chemical Society, Burlington House, W. 1, 1955, pp. 60–81.

(11) F. E. King, T. J. King and A. J. Warwick, *J. Chem. Soc.*, 3590 (1950).

(12) F. Irreverre and W. Martin, *Anal. Chem.*, **26**, 257 (1954).

(13) For reading references and a comprehensive discussion see W. G. Dauben, G. J. Fonken and D. S. Noyce, THIS JOURNAL, **78**, 2579 (1956).

(14) With a non-polar 4-methyl group in an isocyclic system, cyclohexanone, the reduction with lithium aluminum hydride furnishes 19% of the *cis*- and 81% of the *trans*-4-methyl-cyclohexanol [D. S. Noyce and D. B. Denney, *ibid.*, **72**, 5743 (1950)] and with $NaBH_4$, 25% *cis* and 75% *trans* (in CH_3OH) and 40% *cis* and 60% *trans* (in pyridine).[13]

(15) Epimerization at C(2), which occurs easily with α-acylamino acids and acetic anhydride [J. L. O'Brien and C. Niemann, *ibid.*, **72**, 5348 (1950)] is not observed with α-N-carbobenzyloxyamino acids, not even with acetic anhydride in the presence of sodium hydroxide, since ring-closure to an oxazolone cannot happen [M. B. North and G. T. Young, *Chemistry & Industry*, 1597 (1955)].

(16) γ- or δ- (sugar) lactones in which the carbon carrying the hydroxyl group is related to D-glyceraldehyde are dextrorotatory or $\Delta[\alpha]D$ lactone acid is positive; *cf.* W. Klyne, *ibid.*, 1198 (1954).

lactone VI $-[\alpha]_D$ open acid IV or sodium salt: $-6.3° - (-39.3°) = +33.0°$. The positive value for this difference confirms the D$_G$ configuration of allo-5-hydroxypipecolic acid (V), its lactone VII and the corresponding N-carbobenzyloxy derivatives IV and VI. The configuration of the carbon carrying the hydroxyl group in natural or *trans*-5-hydroxypipecolic acid is that of L-glyceraldehyde just as in natural hydroxy-L-proline. The sequence of elution from Dowex-50, namely, first natural then allo (see Fig. 1), is the same for 5-hydroxypipecolic acid as for hydroxyproline and has been taken as indirect evidence for the *trans* arrangement of the functional groups in I.[10]

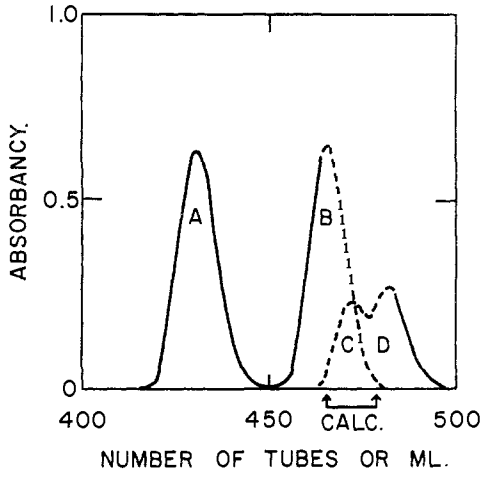


Fig. 1.—Analysis on Dowex 50 of cyclization mixture from alkali treatment of diethyl 2-(3',4'-epoxybutyl)-2-formamidomalonate. Peaks A and B are probably the diastereoisomeric 2-hydroxymethylprolines (measured at 375 mμ) and peaks C and D are normal and allohydroxy-D,L-pipecolic acid (measured at 350 mμ). The dotted parts of the curves are calculated.

For the debenzylation of the N-carbobenzyloxy-lactone VI hydrogen bromide in glacial acetic acid rather than the catalytic method was used. Pure preparations of VI gave directly the beautifully crystalline hydrobromide of the lactone VII. Paper chromatograms of preparations from the debenzylation of less pure VI showed the presence of glutamic acid, a well-known oxidation product of 5-hydroxypipecolic acid,[6] possibly a small amount of baikiain[11] resulting from dehydration of IV (or II) and of two further spots indicative of norvaline and of norleucine.

Rapid lactone opening, as detected by change of rotation, is observed with the lactone VII hydrobromide in aqueous solution. This lactone instability, even under acidic conditions, is greater than normally found in non-nitrogenous γ- or δ-lactones,[17] even bicyclic ones. An important factor affecting the equilibrium VII·HBr $\rightleftarrows$ V·HBr is that the latter as an amino acid hydrobromide is in equilibrium with its zwitterion. This view is supported by the much greater stability of the N-carbobenzyloxy lactone VI which, for example, is recrystallizable from methanol. The greater instability of VII·HBr compared with ordinary γ- or δ-lactones is unlikely to be caused by increased strain in the nitrogen-containing lactone ring, since the substitution of >NH for $>CH_2$ is generally considered to cause only minor conformational changes.[18]

The total configuration of 5-hydroxy-L-pipecolic acid was established by making C(2) the absolute reference point (Lutz–Jirgensons rule) for C(5) whose relative position was deduced from the lactone VII. With these two centers fixed a model was available to test the applicability of Hudson's rule. Just as in the case of the lactones of allohydroxy-D and -L-proline the rule was obeyed. This extension of Hudson's rule to γ- and δ-hydroxyamino acids is a useful new method and has been applied to δ-hydroxy-L-lysine.

Synthesis.—The starting material for the synthesis of 5-hydroxypipecolic acid was diethyl 2-(3',4'-epoxybutyl)-2-formamidomalonate (IX)[19] obtained by perbenzoic acid oxidation of diethyl 2-(3'-butenyl)-2-formamidomalonate. δ-Hydroxylysine was obtained by opening of the epoxide with ammonia.[19] The *internal* opening of the epoxide by the amino group of X was visualized as a straightforward reaction (arrows B) leading to I and V. An excess of *N* alkali at 37° for 3 days converted IX into X which was decarboxylated by refluxing in acid solution. The reaction mixture was assayed on a 150-cm. column of Dowex-50 using 0.15% ninhydrin solution in glacial acetic acid and λ_{max} 350 mμ, according to the procedure developed for the analysis of 5-hydroxypipecolic acid by Piez.[20] Figure 1 shows the results. The two smaller peaks are those of normal and allohydroxy-D,L-pipecolic acid whose identities were independently established by paper chromatography. The other two peaks belong to the two secondary amino acids (presumably the diastereoisomers XI and XII) which gave an orange color with ninhydrin and which were not deaminated by nitrous acid. Pathway A is apparently favored over pathway B. An analogous case is the *alkali-catalyzed* cyclization of unsaturated tertiary amines in the conessine series to yield quaternary pyrrolidines rather than piperidines.[21] It may be recalled in this connection that a hydroxymethylproline has recently been isolated from the wood of apple trees.[22] A third compound present in the cyclization mixture gave a blue color with ninhydrin, was deaminated by nitrous acid and behaved like serine on paper chromatography. It is probably the glycol, δ,ϵ-dihydroxy-α-aminocaproic acid. The approximate yields were 10% of I + V, 30–40% of XI + XII and 50–60% of a hydroxyamino acid, presumably the glycol.

The open-chain analog of 5-hydroxypipecolic acid (I) is *erythro*-δ-hydroxy-L-lysine (XIII), and of the allo isomer V the *threo* diastereoisomer XIV. There seems to be a preponderance for D$_G$-config-

(17) *Cf.* L. N. Owen and P. A. Robins, *J. Chem. Soc.*, 326 (1949); N. R. Campbell and J. H. Hunt, *ibid.*, 1379 (1950); K. Fisher, W. H. Perkin, *ibid.*, 93, 1884 (1908).

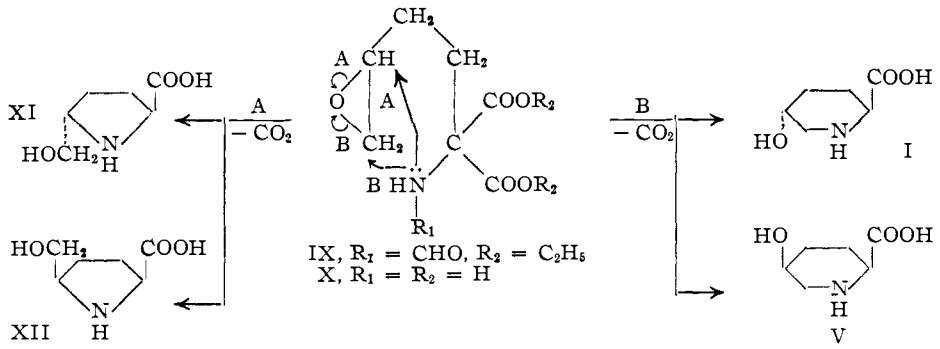
(18) D. H. R. Barton and R. C. Cookson, *Quarterly Reviews*, **10**, 72 (1956).

(19) J. R. Weisiger, *J. Biol. Chem.*, **186**, 591 (1950).

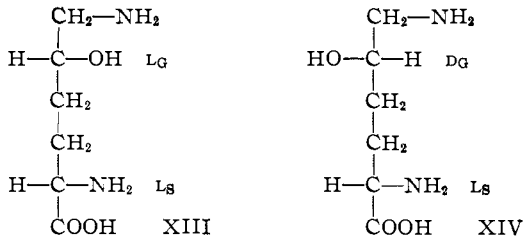
(20) K. Piez, F. Irreverre and H. L. Wolff, *ibid.*, December (1956).

(21) R. D. Haworth, ref. 10, pp. 6–7.

(22) F. Urbach, *Nature*, **175**, 171 (1955).



urations of natural hydroxyamino acids as in L-threonine, a *threo* compound[23] and D-sphingo-



sine[24] and natural (−)-ephedrine,[25] both *erythro* compounds. On the other hand, natural hydroxy-L-proline has the L-configuration at C(4)[10] and would be related to allothreonine, whereas natural allohydroxy-L-proline would be the threonine type. The application of Hudson's lactone rule on the newly prepared α,ϵ-dibenzoyl lactone in the normal hydroxylysine series points to XIII as the correct structure for δ-hydroxy-L-lysine from collagen.[26]

The betaine of 5-hydroxy-L-pipecolic acid whose preparation is described in the preceding paper[9] furnished additional evidence for the *trans* arrangement of the two functional groups. The betaine of hydroxy-L-proline was half as good an inhibitor as its lower homolog, betonicine, in the enzymatic hydrolysis of acetylcholine with cholinesterase. This activity still is fivefold the inhibitory power of choline. A *cis*-disubstituted betaine would have been without action, just as D- or L-turicine.[27]

Experimental[28]

Isolation of 5-Hydroxy-L-pipecolic Acid from Dates. A. On a 2-Pound Scale.—The pericarps of two pounds of dates (Cal-Trop. Brand) were cut into one-fourth inch segments. The slices were mixed with 70% aqueous ethanol in a Waring blendor. The resulting mixture was centrifuged, the supernatant liquid decanted, the solids suspended in fresh 70% ethanol and the mixture centrifuged and the supernatant liquid decanted. In this way, two pounds of dates yielded a brown sirupy extract. The extract was passed through a column (2.8 × 42 cm.) of Dowex-50 in the acid form. Six liters of distilled water was required to wash the column free of sugars (negative Linde–Molisch test). The amino acids were eluted from the column with *N* ammonium hydroxide solution. The first 480 ml. of effluent, collected in 16 fractions, gave a negative test for amino acids with ninhydrin. The next 24 fractions gave positive tests for amino acids with ninhydrin; combination of these fractions yielded 730 ml. of a brown solution which was concentrated at reduced pressure under nitrogen to a volume of 140 ml. Fourteen ml. of concentrated hydrochloric acid was added to the solution and oxides of nitrogen, generated from sodium nitrite with hydrochloric acid, in a stream of nitrogen were bubbled into the mixture until the test for amino acids with ninhydrin was negative. Additional water (300 ml.) and concentrated hydrochloric acid (30 ml.) were added during the treatment to control frothing. The mixture was concentrated to 250 ml. at reduced pressure and extracted four times with 500 ml. of ether. The ethereal extracts were combined and evaporated to yield a deep-yellow viscous liquid which was a mixture containing the nitrosation product of the secondary amino acids. This residue was dissolved in water to make 50 ml. of solution. Fifty ml. of concentrated hydrochloric acid was added and the mixture boiled under reflux for 15 minutes and then concentrated at reduced pressure. The residue was dissolved in water to make 20 ml. and the solution passed through a column (2.8 × 44 cm.) of Dowex-50 in the acid form. The column was eluted with 1.5 *N* hydrochloric acid; 255 5-ml. fractions followed by 50 7-ml. fractions were collected. The course of the elution was followed by means of spot tests on paper with ninhydrin. The tests revealed the presence of 5-hydroxy-L-pipecolic acid in fractions 111–138 and of proline in tubes 194–240. The fractions containing the 5-hydroxy-L-pipecolic acid were combined, concentrated to a volume of several ml. and allowed to stand at 4°. The crystals of the hydrochloride which formed were collected and washed with 80% aqueous ethanol. There was obtained 1.08 g. of colorless crystals, m.p. 210–215° dec., $[\alpha]^{20}D$ −10.9° (*c* 0.92%, H_2O). Paper chromatographic comparison of this amino acid with 5-hydroxy-L-pipecolic acid isolated without the nitrosation step, using *t*-amyl alcohol–2,4-lutidine, established that this was the natural acid uncontaminated by the allo acid.

Major bands of the infrared absorption spectrum (Nujol mull); 3.15m, 5.73s, 6.27w, 6.85s, 7.13m, 7.27m, 7.48w, 7.87w, 8.05m, 8.40s, 9.28s, 9.99m, 10.70m, 10.80m, 11.15w, 11.68m.

B. On a 15-Pound Scale.—Fifteen pounds of dates was extracted with 70% aqueous ethanol and freed of sugars and other non-cationic substances in the way described. The concentrated aqueous solution of amino acids had a volume of 250 ml. To it there was added 41.4 g. of sodium nitrite followed by 60 ml. of concentrated hydrochloric acid. The mixture was allowed to stand overnight at room temperature; then an additional 10 g. of sodium nitrite and 15 ml. of concentrated hydrochloric acid were added to the mixture. An hour later the ninhydrin test for amino acids was negative and excess free nitrous acid was present. The mixture was extracted with 12 l. of peroxide-free ether. The residue from the evaporated ethereal extracts was dissolved in water to make 275 ml. of solution. To this solution 4 g. of urea and 200 ml. of concentrated hydrochloric acid were added. The resulting mixture was heated below the boiling temperature for 50 minutes and then boiled under reflux for 30 minutes. The 5-hydroxypipecolic acid was isolated from the mixture as it had been in the previous isolation. The hydrochloride was passed through a Dowex-50 column in the acid form and the free amino acid eluted with *N* ammonium hydroxide solution. Ethanol and ace-

(23) C. E. Meyer and W. C. Rose, *J. Biol. Chem.*, **115**, 721 (1936).

(24) J. Kiss, G. Fodor and D. Barifi, *Helv. Chim. Acta*, **37**, 1471 (1954).

(25) *Cf.* B. Witkop and C. M. Foltz, THIS JOURNAL, **79**, 197 (1957).

(26) B. Witkop, *Experientia*, in press.

(27) S. L. Friess, A. A. Patchett and B. Witkop, THIS JOURNAL, in press.

(28) All melting points are corrected (Kofler block), all boiling points are uncorrected. The analyses were performed by Dr. W. C. Alford and his associates.

tone were added to the concentrated aqueous solution to bring about crystallization. The crystals of the free amino acid were collected and washed with cold 80% aqueous ethanol, yielding 6.57 g., m.p. 235° dec., $[\alpha]^{20}D$ −23.1° (*c* 1.0, H_2O). Comparison of this preparation by m.p., m.m.p., infrared spectrum and paper chromatography with an authentic sample of natural 5-hydroxy-L-pipecolic acid, kindly provided by Prof. A. Virtanen, established that this was in all respects the pure natural amino acid.

Major bands of the infrared absorption spectrum (Nujol mull): 3.07s, 3.15m, 6.23vs, 6.30vs, 6.83vs, 6.88vs, 7.25vs, 7.48s, 7.60s, 7.72m, 8.08s, 8.90m, 9.01w, 9.47m, 9.66m, 9.73m, 10.21m, 11.15m, 11.45w.

N-Carbobenzyloxy-5-hydroxy-L-pipecolic Acid (II).—A solution of 3.0 g. of 5-hydroxy-L-pipecolic acid in 20.7 ml. of *N* sodium hydroxide solution was cooled in an ice-bath and treated dropwise with 3.52 g. of benzyl chloroformate and 20.7 ml. of *N* sodium hydroxide solution during a period of 10 minutes. The mixture was stirred an additional 2 hours and extracted twice with 30 ml. of ether. The aqueous solution was acidified to congo red with 6 *N* hydrochloric acid. Scratching induced crystallization of the oil which separated. The crystals were collected, washed twice with cold water and dried over phosphorus pentoxide to yield 2.70 g. (46.8%) of colorless crystals, m.p. 149–151°, $[\alpha]^{20}D$ −17.9° (1.0% in acetone). Recrystallization from acetone–ligroin (66–68°) afforded the analytical sample, m.p. 150–152°.

Anal. Calcd. for $C_{14}H_{17}O_5N$: C, 60.20; H, 6.14; N, 5.02. Found: C, 60.34; H, 6.27; N, 4.74.

Major bands of the infrared absorption spectrum (chloroform): 2.80w, 2.90w, 5.88vs, 6.18w, 7.13m, 7.39w, 7.61w, 8.75w, 8.94m, 9.66w, 9.90w, 10.20w, 11.09vw.

Oxidation of N-Carbobenzyloxy-5-hydroxy-L-pipecolic Acid by Chromic Acid and Reduction by Sodium Borohydride.—A solution of 1.0 g. of N-carbobenzyloxy-5-hydroxy-L-pipecolic acid (II) in 40 ml. of acetone was treated dropwise at room temperature with 3.6 ml. of 8 *N* solution of chromium trioxide in diluted sulfuric acid over a period of 5 minutes,[9,29] stirring was continued 1.5 hr., 2 ml. of methanol added to destroy excess oxidant and the mixture taken up with 30 ml. of water and 200 ml. of ether. The aqueous solution was extracted twice additionally with ether. The ethereal extracts were washed three times with water, combined, dried and freed of ether on a steam-bath and in a stream of nitrogen. The residue was a pale yellow, viscous liquid. In a preliminary run unsuccessful attempts to crystallize this oxidation product had resulted in extensive decomposition of the product. The amorphous keto compound was dissolved in 30 ml. of methanol and treated in an ice-bath with a solution of 0.54 g. of sodium borohydride in 2 ml. of water. After 45 minutes in the ice-bath and 3 hr. at room temperature the methanol was distilled from the mixture at reduced pressure. The residue was treated with 15 ml. of 3 *N* sodium hydroxide solution. After 1.5 hr. at room temperature the mixture was acidified to *p*H 2, 15 ml. of water added to it and the mixture extracted 3 times with ether. The ethereal extracts were washed 3 times with water, combined, dried and freed of ether on a steam-bath. The residue was 0.85 g. of a vitreous solid which could not be crystallized.

Ninety mg. of the crude product was dissolved in 15 ml. of methanol and debenzylated by hydrogenation over 0.2 g. of palladium–charcoal (10%). When the calculated amount of hydrogen had been consumed, the mixture was filtered and the filtrate free of solvent at reduced pressure. The residue was a vitreous solid. Paper chromatographic comparison of the crude product with natural 5-hydroxy-L-pipecolic acid, using *t*-amyl alcohol–2,4-lutidine, indicated that the product was 5-allohydroxy-L-pipecolic acid accompanied by a small amount of glutamic acid. This observation was confirmed by a two-dimensional paper chromatogram.

An attempt to debenzylate and lactonize the crude sodium borohydride reduction product in one step in a preliminary experiment in which attempts had been made to crystallize the intermediate oxidation product did not yield the lactone. A two-dimensional chromatogram of the crude product using a basic ternary solvent system revealed the presence of a small amount of allo-5-hydroxypipecolic acid accompanied by glutamic acid and possibly baikiain, norvaline and norleucine.

Lactone VI of N-Carbobenzyloxy-5-allohydroxy-L-pipecolic Acid. A. By Dehydration with Oxalic Acid and Sublimation.—A mixture of 0.2 g. of crude N-carbobenzyloxy-5-allohydroxy-L-pipecolic acid (IV) and 0.2 g. of anhydrous oxalic acid was sublimed at 105° and 2 mm. The sublimate was taken up in chloroform and water and the chloroform solution washed with 1% sodium hydroxide solution and water, dried and evaporated at reduced pressure. Crystallization of the residue from methanol yielded the lactone, m.p. 104–105°. Careful recrystallization from methanol afforded the analytical sample, m.p. 105–106°.

Anal. Calcd. for $C_{14}H_{15}NO_4$: C, 64.36; H, 5.79. Found: C, 64.46; H, 5.73.

Major infrared bands (chloroform): no bands in OH–NH region, 5.65vs (six-membered lactone carbonyl), 5.86vs (carbonyl of carbobenzyloxy group), 6.91w, 7.10vs, 7.37s, 7.41s, 7.45s, 7.61m, 7.75vs, 8.82vs, 9.02vs, 9.43vs, 9.78m.

B. By Dehydration with Acetic Anhydride.—A mixture of 0.7 g. of the crude N-carbobenzyloxy-5-allohydroxy-L-pipecolic acid with 5 ml. of acetic anhydride was allowed to evaporate at reduced pressure over sodium hydroxide. The vitreous residue crystallized from methanol to yield the lactone VI, m.p. 104–105°. The lactone VI could be recrystallized from methanol or cyclohexane–acetone. Recrystallization from methanol yielded the analytical sample, m.p. 106–107°, $[\alpha]^{20}D$ −6.3° (*c* 1.5%, CH_3OH).

Anal. Calcd. for $C_{14}H_{15}NO_4$: C, 64.36; H, 5.79; N, 5.36. Found: C, 64.87, 64.16; H, 5.79, 5.73; N, 5.40.

Opening of the N-Carbobenzyloxylactone.—The solution of 75 mg. of N-carbobenzyloxylactone in methanol on the addition of one equivalent of methanolic sodium hydroxide showed a rotation of $[\alpha]^{20}D$ −39.3°. Acidification with concd. HCl did not change this value. The difference in rotations between lactone and salt (or acid) is −6.3 − (−39.3) = +33°.

5-Allohydroxy-L-pipecolic Acid Lactone Hydrobromide.—Two-hundred mg. of N-carbobenzyloxy-5-allohydroxy-L-pipecolic acid lactone was suspended in 2 ml. of a saturated solution of anhydrous hydrogen bromide in glacial acetic acid with cooling in an ice-bath. When the evolution of carbon dioxide had subsided, the reaction mixture was left for 20 minutes at room temperature. During this time crystallization of the lactone hydrobromide began. A two-fold excess of ether was added and the colorless crystalline precipitate collected and washed well with ether. The product was 110 mg. of rectangular platelets, m.p. 228–231° dec. In aqueous solution the rotation taken after 1 hr. was already the final value, $[\alpha]^{20}D$ −7.4° (*c* 0.9). This value did not change on standing and is the rotation of the hydrobromide of 5-allohydroxy-L-pipecolic acid whose isolation is described below.

Anal. Calcd. for $C_6H_9NO_2 \cdot HBr$: C, 34.64; H, 4.84; N, 6.73. Found: C, 34.60; H, 4.90; N, 6.64.

Major bands in the infrared spectrum (KBr window): 3.93, 4.08, 4.19 (ammonium bands), 5.65vs (carbonyl of six-membered lactone), 5.70 (carbonyl of carboxyl), 6.36m, 6.88 m, 7.21m, 7.30w, 7.39vw, 7.66vw, 7.77m, 8.0m, 8.18s, 8.50vw, 8.6m, 9.18, 9.23, 9.29 (triplet of medium intensity), 9.63w, 9.85s, 9.94s.

5-Allohydroxy-L-pipecolic Acid Hydrobromide.—The aqueous solution of 85 mg. of lactone hydrobromide on concentration gave colorless crystals, m.p. 205–207°, which no longer had a lactone band in the infrared spectrum. The hydrobromide of the open acid did not revert to the lactone on renewed treatment with glacial acetic acid saturated with hydrogen bromide.

Anal. Calcd. for $C_6H_{11}NO_3 \cdot HBr$: C, 31.87; H, 5.35. Found: C, 31.64; H, 5.14.

Major bands in the infrared spectrum (Nujol): 2.88 (sharp and narrow OH), 5.76s (carboxyl), 6.45m, 6.89s, 6.84vs, 7.26s, 7.60w, 7.73w, 7.92w, 8.10m, 8.16m, 8.34m, 8.86w, 9.39w, 9.67m, 10.11m, 10.5m, 10.77w, 10.97n, 11.74w.

5-Allohydroxy-L-pipecolic Acid.—The aqueous solution of 70 mg. of the above hydrobromide was filtered through a column of Dowex-50 in the acetate form. The crystalline residue of the main elution on recrystallization from water–ethanol (with 2 drops of acetone to induce crystallization) gave clusters of slim needles, m.p. 255–258° dec. (subliming

(29) P. Bladon, J. M. Fabian, H. B. Henbest, H. P. Koch and G. W. Wood, *J. Chem. Soc.*, 2402 (1951).

at 220–240° to rosettes of needles, 245° sintering), $[\alpha]^{20}D$ −31.1 ± 0.2 (*c* 0.8%, H_2O).

Anal. Calcd. for $C_6H_{11}NO_3$: C, 49.64; H, 7.64; N, 9.65. Found: C, 49.44; H, 7.69; N, 9.46.

Synthesis of the Two Diastereoisomers of 5-Hydroxy-D,L-pipecolic Acid. Diethyl 2-(3′-Butenyl)-2-formamidomalonate.[19]—Diethyl aminomalonate was prepared by hydrogenating diethyl isonitrosomalonate at 1500 p.s.i. using 10% palladium–charcoal,[30] b.p. 93–98° (2.5 mm.), 85.5% and formylated in toluene with 90% formic acid[31] to yield diethyl formamidomalonate, b.p. 145–150° (3.25 mm.), m.p. 47–49°, 75% yield. To a solution of one equivalent of sodium ethoxide in 50 ml. of absolute ethanol there was added 17.4 g. of diethyl formamidomalonate. The mixture was heated to gentle boiling under reflux and then 11.6 g. of 3-butenyl bromide (b.p. 98–102°, yield 74% from 3-buten-1-ol[32]) was added slowly. Using essentially the directions of Weisiger, there was obtained, after one recrystallization of the crude product from ethanol, 10.2 g. (46.2%) of colorless crystals, m.p. 94–96°. Two additional recrystallizations from aqueous ethanol yielded the analytical sample, m.p. 96–97° (reported m.p.[18,33] 84°).

Anal. Calcd. for $C_{12}H_{19}O_5N$: C, 56.02; H, 7.44; N, 5.46. Found: C, 55.98; H, 7.59; N, 5.46.

Diethyl 2-(3′,4′-Epoxybutyl)-2-formamidomalonate (IX).—By the use of perbenzoic rather than peracetic acid[19] a simpler procedure and higher yields were made possible. A solution of 5.14 g. (0.02 mole) of diethyl 2-(3′-butenyl)-2-formamidomalonate in chloroform was treated with a chloroform solution of 0.022 mole of perbenzoic acid. After 4 days at 4° titrations indicated that the reaction was 98.3% complete. At this point 50 ml. of water containing several drops of phenolphthalein was added to the chloroform solution of the reaction products. The mixture was treated dropwise with 5 *N* sodium hydroxide solution until the aqueous phase was alkaline. The chloroform solution then was washed with water, dried over sodium sulfate and freed of chloroform at reduced pressure. The residue was 4.8 g. of a pale yellow liquid which crystallized on standing, m.p. 73–78°. Recrystallization of the crude product from acetone–ligroin (66–68°) and benzene–ligroin (66–68°) effected little purification so it was subjected to a four-funnel countercurrent distribution using 20 ml. of benzene to 100 ml. of water–methanol (1:1). Concentration of the combined water–methanol phases at reduced pressure and recrystallization of the residue from benzene–ligroin (66–68°) yielded 2.76 g. (50.6%) of epoxide, m.p. 79–84° (reported[1] 75°). Three recrystallizations from benzene–ligroin (66–68°) yielded the analytical sample, m.p. 78.5–83.0°.

(30) Roche Products Ltd., A. Cohen and J. A. Silk, British Patent 611,600, Nov. 1, 1948; *C. A.*, **43**, 3445 (1949).

(31) "Organic Syntheses," Coll. Vol. III, John Wiley and Sons, Inc., New York, N. Y., 1955, p. 590.

(32) R. P. Linstead and H. N. Rydon, *J. Chem. Soc.*, 1955 (1934).

(33) Dr. Weisiger kindly supplied us with a sample of the compound which was recrystallized from chloroform; it had m.p. 95–96°.

Anal. Calcd. for $C_{12}H_{19}O_6N$: C, 52.74; H, 7.01; N, 5.13. Found: C, 52.53; H, 7.18; N, 5.19.

5-Hydroxypipecolic Acid (I).—A solution of 0.1 g. of diethyl 2-(3′,4′-epoxybutyl)-2-formamidomalonate (IX) in 10 ml. of *N* sodium hydroxide solution was allowed to stand at 37° for 3 days. Ten ml. of concentrated hydrochloric acid then was added to the solution. The mixture was boiled under reflux for 5 hr. and then evaporated to dryness at reduced pressure. Two 10-ml. portions of ethanol were added to the residue and evaporated. The residue was dissolved in 10 ml. of water and the resulting solution was adjusted to *p*H 6.5 with ammonia water and filtered from a small amount of an amorphous solid. A two-dimensional paper chromatogram of the solution prepared with 2,4-lutidine–*t*-amyl alcohol and *n*-butyl alcohol–acetone–ammonia indicated the presence of two pairs of diastereoisomeric secondary amino acids and a primary amino acid. One pair with ninhydrin gave a yellow color which exhibited a brick-red fluorescence in the ultraviolet light; these two secondary amino acids presumably were the *cis*- and *trans*-5-hydroxymethylprolines (XI and XII). The other pair gave a violet color with ninhydrin which exhibited a bright red fluorescence in ultraviolet light. Paper chromatographic comparison with natural 5-hydroxypipecolic acid isolated from dates indicated that the amino acids were normal and allo-5-hydroxy-D,L-pipecolic acid (racemes of I and V). The remaining amino acid gave a blue color with ninhydrin, was deaminated with nitrous acid, behaved like serine on paper chromatography and presumably was 5,6-dihydroxy-2-aminocaproic acid. The relative intensities of the colors produced with ninhydrin indicated that the non-cyclic amino acid was much more abundant in the crude product than either of the cyclic amino acids and the 5-hydroxypipecolic acids were the least abundant. The crude product was analyzed on a 150-cm. column of Dowex-50 using 0.15% ninhydrin solution in glacial acetic acid and measuring the absorption at 350 and at 375 mμ. The results of the analysis are shown in Fig. 1. The two smaller peaks, C and D, are those of normal and allo-5-hydroxy-D,L-pipecolic acids, respectively, and the two larger peaks presumably are those of the diastereoisomeric 5-hydroxymethylprolines (XI and XII). In another attempt at synthesis a solution of 0.1 g. of diethyl 2-(3′,4′-epoxybutyl)-2-formamidomalonate (IX) in 5 ml. of a 0.42 *N* solution of anhydrous hydrogen chloride in ether was allowed to stand at room temperature for 3 hr. The ether was then evaporated in a stream of nitrogen. Ten ml. of 0.5 *N* barium hydroxide solution was added to the residue and the mixture boiled under reflux for 3 hr. Ten ml. of concd. hydrochloric acid then was added and the mixture boiled under reflux for 3 hr. Silver sulfate was added and the mixture digested on a hot-plate and filtered. The filtrate was adjusted to *p*H 6 with ammonia water and filtered. A two-dimensional paper chromatogram of the solution of products indicated that the same products were present in approximately the same relative amounts as were present in the previous synthesis.

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[CONTRIBUTION FROM THE NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES, NATIONAL INSTITUTES OF HEALTH]

Studies on the Stereochemistry of Ephedrine and ψ-Ephedrine*

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The epoxides obtained from the quaternary bases of ephedrine, *i.e.*, L-*erythro*-1-phenyl-1-hydroxy-2-methylaminopropane, and of ψ-ephedrine, *i.e.*, L-*threo*-1-phenyl-1-hydroxy-2-methylaminopropane, are D-*threo*-1-phenyl-1,2-epoxypropane (III) and D-*erythro*-1-phenyl-1,2-epoxypropane (IV). The Walden inversion occurring in the epoxide formation at the carbon which loses the trimethylamine group, was proved by catalytic reduction of III and IV. Both epoxides yielded the same D-*glycero*-1-phenyl-2-hydroxypropane (V) characterized as the *p*-toluenesulfonate. Acid-catalyzed hydrolytic ring opening of the epoxides III and IV yielded as major products D-*erythro*-1-phenyl-1,2-propanediol (VI), characterized as the dibenzoate, *m*.p. 95.5–97°, and D-*threo*-1,2-propanediol VII, *m*.p. 62°, $[\alpha]D$ −60°, dibenzoate m.p. 89.5–91°.

The Hofmann degradation of the quaternary salts of ephedrine leads to optically active oxides of β-methylstyrene. Although this reaction was discovered in 1902,[1,2] the steps and products of this

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(1) E. R. Miller, *Arch. Pharm.*, **240**, 481 (1902); H. Emde, *ibid.*, **244**, 241 (1906); P. Rabe, *Ber.*, **43**, 884, 2622 (1910); **44**, 824 (1911).

(2) E. Schmidt, *Arch. Pharm.*, **249**, 305 (1911).