solvent was removed by distillation *in vacuo*. The temperature of the reaction mixture was not allowed to rise above 40°. The dark residues from four such runs were poured into 800 cc. of ice-water. The mixture was stirred for about 15 min. and filtered by suction. The orange-red filtrate was extracted with six 150-cc. portions of ether. The ethereal extracts were washed with water and with excess aqueous sodium bicarbonate. After a final washing with water and drying over anhydrous sodium sulfate, the solvent was evaporated and the red oily residue was distilled *in vacuo*. Three fractions were taken: 1, b.p. 109–116° (0.3 mm.), 1.52 g. of yellow oil which almost completely crystallized on seeding; 2, b.p. 118–122° (0.3 mm.), 3.88 g. of yellow oil which crystallized completely on seeding; and 3, b.p. 122–140° (0.3 mm.), 2.49 g. of viscous red oil in which a small amount of crystals formed. Fractions 1 and 2 were combined and crystallized from ether–pentane as colorless prismatic crystals (3.75 g.) melting at 75–76° (reported[24] 81°). The material was analytically pure; $\lambda_{max}^{C_2H_5OH}$ 245 mμ (4.16); $\lambda_{max}^{CHCl_3}$ 5.74, 6.00, 6.13 μ.

The black tar which separated when the reaction mixture was poured into water and which constituted the major portion of the organic reaction product was dissolved in ethyl acetate, washed with water, 0.1 *N* NaOH until alkaline (the alkaline solutions were deeply colored) and again with water. The dried solution on evaporation left 12 g. of a thick black tarry mass. A 5-g. portion of this tar was subjected to systematic chromatography on alumina. Infrared spectra were recorded for selected fractions. These data showed that the benzene eluates contained some of VIII contaminated with other materials. The chloroform and methanol fractions of the chromatogram gave no crystalline or identifiable materials.

5,7-Diacetoxytetralin (XII).—When a solution of 0.5 g. of VIII in 2 cc. of acetic anhydride was mixed with a solution of 2 drops of concd. sulfuric acid in 0.2 cc. of acetic anhydride, an immediate exothermic reaction occurred. After standing for 30 min. the slightly discolored reaction mixture was poured into ice-water. The product was extracted with ether in the usual manner. The analytical sample was distilled at 0.1 mm. and a bath temperature of 175–185°; reported[49] b.p. 196° (15 mm.), m.p. 39–40°. The diacetate of 5,8-dihydroxytetralin (m.p. 179–180°) melts at 188°.

5-Acetoxy-7-hydroxytetralin (XI).—When 800 mg. of VIII was dissolved in 5 cc. of freshly distilled boron trifluoride etherate, the initially clear colorless solution became progressively darker and slightly warm. After standing overnight the dark solution was poured into 100 cc. of water and extracted with ether in the usual manner. A red-brown oily residue (572 mg., 71% yield) remained on evaporation of the ether. On standing, needle-like crystals formed in the oil. Crystallization of the crude product from benzene–pentane gave 356 mg. of red crystalline material which was sublimed *in vacuo* at 0.3 mm. and a bath temperature of 110–115°. The colorless crystalline sublimate, on recrystallization from benzene–pentane, afforded long silky needles, m.p. 113–114°; $\lambda_{max}^{CHCl_3}$ 2.80, 3.04, 5.66, 6.19 μ.

Anal. Calcd. for $C_{12}H_{14}O_3$: C, 69.88; H, 6.84. Found: C, 69.99; H, 6.93.

5,7-Dihydroxytetralin (XV).—A solution of 400 mg. of crude XI in 15 cc. of methanol and 20 cc. of 2 *N* hydrochloric acid was refluxed for 2 hr., diluted with 200 cc. of water and extracted with three 100-cc. portions of ether. The dried ethereal extracts were evaporated and the resulting oily residue was taken up in about 10 cc. of benzene. On addition of pentane and cooling, 297 mg. of brown crystals was obtained. Sublimation *in vacuo* (0.3 mm., bath temp. 160°) gave a pale-yellow solid sublimate which crystallized from benzene as a colorless crystalline powder, m.p. 119–120° (reported[49] 122°). Similarly XV from XII was a colorless solid melting at 120–121°, m.m.p. with XV from XI was 119–120°. Both compounds were analytically pure.

The infrared spectra were identical in all respects; λ_{max}^{CHCl} 2.79, 3.00, 6.17, 6.26 μ.

Acknowledgment.—The technical assistance and skillful coöperation of Marie Johnson and (in part) C. Donaher and A. Moore are gratefully acknowledged.

(49) G. Schroeter, K. Erzberger and L. Passavant, *Ber.*, **71**, 1040 (1938).

BETHESDA 14, MARYLAND

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

Studies on Hydroxyproline

BY ARTHUR A. PATCHETT AND BERNHARD WITKOP

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The N-carbobenzyloxy derivatives II and IV of natural hydroxy-L-proline (I), as well as of allohydroxy-D- (III) and -L-proline, have been prepared in a crystalline form and oxidized to N-carbobenzyloxy-4-keto-L- and -D-proline (V and VII). The stereospecific reduction of N-carbobenzyloxy-4-keto-L-proline with sodium borohydride led in good yield to the allohydroxy-L-proline derivative. 4-Keto-L-proline hydrobromide (VI) was prepared and investigated. The N-acetyl- and N-carbobenzyloxyallohydroxy-D- and -L-proline lactones (XIV, XV) were obtained by an internal displacement reaction, by dehydration of N-acylallohydroxyprolines with *p*-tolylsulfonyl chloride in pyridine and by the use of N,N′-dicyclohexylcarbodiimide. The solvolysis of the N-acetyl XIV as well as of the free lactone (XVI, hydrobromide) in aqueous buffer of varying *p*H was investigated. By opening of the lactones with esters of amino acids, peptides of allohydroxy-D- and -L-prolines were prepared. The rate of hydrolysis of allohydroxy-L-proline lactone hydrobromide (XVI) was followed by the mutarotation of its aqueous solution. The application of Hudson's rule confirmed the D-G configuration for C(4) in allohydroxy-L-proline. The general applicability of nucleophilic displacement reactions by suitable anions for the preparation of 4-substituted proline derivatives from the 4-tosylates was explored using sodium methyl mercaptide. The two diastereoisomeric 4-methylmercapto-L-prolines (XX and XVII) were prepared. The conversion of hydroxy-L-proline and of allohydroxy-D-proline into their betaines XXI and XXIII under non-epimerizing conditions led to conclusive stereochemical assignments for betonicine (XXI) and turicine (XXIII). The easy base-catalyzed epimerization of these betaines at room temperature casts some doubt upon the natural occurrence of turicine.

The most abundant protein in the body, collagen, is chemically distinguished from all other proteins by its high content of hydroxyproline.[1,2] The need for derivatives, analogs and antimetabolites of hydroxyproline for testing in collagen-producing tissue cultures[3] prompted the following study.

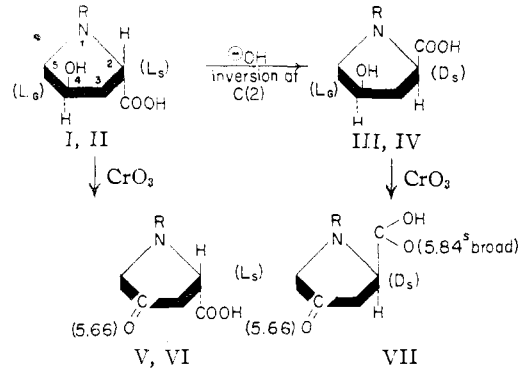
4-Ketoproline (VI) Hydrobromide.—The starting materials in this investigation were the carbobenzyloxy derivatives of natural and allohydroxy-

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

(2) "Nature and Structure of Collagen," edited by J. T. Randall and S. F. Jackson, Academic Press, Inc., New York, N. Y., 1953.

(3) The results on this collaborative project with Prof. F. C. Steward and Drs. S. Udenfriend and C. Mitoma will be published separately.

proline which were obtained crystalline for the first time.[4]



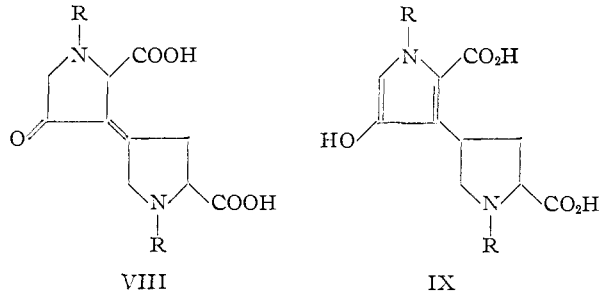
I, II III, IV V, VI VII

I, R = H: L-Hydroxyproline (natural) $[\alpha]_D$ −76.3°
II, R = $OCOCH_2C_6H_5$: m.p. 106–107°, $[\alpha]_D$ −72°
III, R = H: Allohydroxy-D-proline $[\alpha]_D$ +58.6°
IV, R = $OCOCH_2C_6H_5$: m.p. 111°, $[\alpha]_D$ +26.3°
V, R = $OCOCH_2C_6H_5$: m.p. 101–102°, $[\alpha]_D$ +18.5°
VI, R = H (hydrobromide) $[\alpha]_D$ −41°
VII, R = $OCOCH_2C_6H_5$: m.p. 100–101°, $[\alpha]_D$ −19.4°

The first objective was the preparation of the 4-keto-D- and L-prolines by oxidation of suitable derivatives of the corresponding amino acids.[5] Numerous oxidations of N-acetylhydroxyproline and of its methyl ester were carried out by Dr. T. Beiler (unpublished experiments). They led to various keto derivatives which were all difficult to handle and seemed to be unstable. The oxidation of the N-carbobenzyloxyamino acids II and IV with chromic acid in acetone was more successful and led to the two keto compounds V and VII in 70% yield. These compounds proved to be rather sensitive to base, and it is thought that some of the early synthetic attempts to obtain 4-ketoproline derivatives failed because of this lability. The action of *N* NaOH at room temperature for one hour on VII yielded amorphous material, m.p. 112–117°, $[\alpha]_D$ +25.4° with an approximate doubling of molecular weight. The loss in the base condensation products of the ketone band at 5.66 μ and its ultraviolet spectrum (λ_{max} 259 mμ, ϵ 9,500)[6] are consistent with formulation of the reaction as a base-catalyzed aldol condensation.

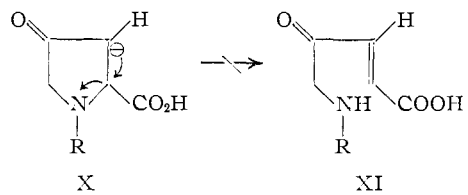
More vigorous base treatment of the amorphous "aldol condensation products" (4*N* NaOH at 100° for one hour) removed the carbobenzyloxy groups and led to water-soluble material which was positive to the Ehrlich test. Conversion of an aldol product such as VIII (R = $C_6H_5CH_2OCO$) to IX (R = H) is just one possibility.

The catalytic decarbobenzyloxylation of V, which did not proceed well in methanol, led in glacial acetic acid to a colored product whose analysis agreed with a product formed from two molecules of 4-keto-L-proline with the loss of water. Fortunately, 4-keto-L-proline could be obtained as its hydrobromide by the action on V of glacial acetic acid saturated with hydrogen bromide. Solutions of 4-ketoproline hydrobromide were readily oxidized by Fehling reagent and on base treatment gradually darkened, accompanied by a decline in rotation



VIII IX

toward 0°. The action of base on the carbobenzyloxy derivative V of 4-keto-L-proline could be followed by rotation with more ease than with the free compound since coloration developed much more slowly. The fact that base treatment of carbobenzyloxy derivative caused a mutarotation with increasingly negative rotation is consistent with the assumption that base would be unlikely to promote β-elimination and ring opening in the sense X → XI.



X XI

Similar systems such as 1-methyl-3-pyrrolidone[7] and its quaternary salt[8] are not known to undergo β-aminoketone cleavage with ring opening.[9] However, both 1-methyl-3-piperidone and 1-methyl-3-pyrrolidone are easily oxidized by ammoniacal silver nitrate and Fehling solution and in this respect they behave as α-aminoketones. The ease of oxidation of DL-4-ketoproline has been commented on previously.[5] In general, 3-pyrrolidones with an unsubstituted imino group such as VI, are less stable than the N-alkyl analogs and cannot be prepared[10] by Dieckmann cyclization.[11]

The action of dilute base or influenza virus on mucoproteins results in the formation of pyrrole-2-carboxylic acid. As expected, dilute aqueous sodium carbonate on VI did not lead to pyrrole-2-carboxylic acid. 4-Hydroxy-Δ^3-pyrroline-2-carboxylic acid need not be considered any longer as the "pyrrole precursor," in agreement with the conclusions of Gottschalk.[12]

4-Keto-L-proline (VI) was inactive in an enzymatic system of a soil bacterium adapted to utilize hydroxy-L-proline as its only source of nitro-

(4) *Cf.* F. H. Carpenter and D. T. Gish, THIS JOURNAL, **74**, 3818 (1952).

(5) A ring synthetic method for the preparation of DL-4-ketoproline has been reported in the meantime by R. Kuhn and G. Osswald, *Ber.*, **89**, 1423 (1956).

(6) *Cf.* Cyclopentylidenecyclopentanone, λ_{max} 255 (ϵ 16,000); J. M. Conia, *Bull. soc. chim.*, 945 (1954).

(7) E. A. Prill and S. M. McElvain, THIS JOURNAL, **55**, 1233 (1933).

(8) C. Mannich and Th. Gollasch, *Ber.*, **61**, 263 (1928).

(9) *Cf.* W. R. Vaughan, *J. Org. Chem.*, **20**, 1613, 1619 (1955).

(10) L. Ruzicka and C. F. Seidel, *Helv. Chim. Acta*, **5**, 715 (1922).

(11) N. J. Leonard, F. E. Fischer, E. Barthel, S. Figueras and W. C. Wildman, THIS JOURNAL, **72**, 2371 (1951).

(12) A. Gottschalk, *Biochem. J.*, **61**, 298 (1955); *Nature*, **174**, 652 (1954). We are greatly indebted to Dr. A. Gottschalk for exchange of information and for a sample of pyrrole-2-carboxylic acid. *Cf.* E. Klenk, *Angew. Chem.*, **68**, 349 (1956).

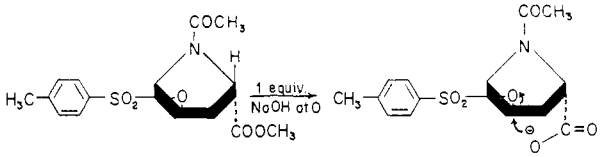
gen and carbon.[13] Further, it was without cytoactivity.[14]

The reduction of N-carbobenzyloxy-4-keto-L-proline (V) with sodium borohydride is stereospecific; hydrolysis and ion-exchange analysis showed that the reduction leads practically quantitatively to the carbobenzyloxy derivative of allohydroxy-L-proline. This conversion from N-carbobenzyloxyhydroxy-L-proline (II) to N-carbobenzyloxyallohydroxy-L-proline proceeds in a 50% over-all yield and is of considerable preparative value. The methyl ester of N-carbobenzyloxyhydroxy-L-proline can be subjected to the same oxidation–reduction sequence. No ion-exchange analysis was performed but direct crystallization gave the allo-L derivative, an indication that the steric course of the reduction is not strongly dependent on the presence of a free carboxyl group. The reduction of an analogous nitrogen-free system, such as 3-ketocyclopentanecarboxylic acid, is apparently not known.[15]

The hydroxyl group of hydroxyproline is thought to play an important role in stabilizing the collagen structure by hydrogen bond formation with an adjacent amide carbonyl group.[16] Obviously, a keto group at the 4-position of a proline residue could not function in this same manner. However, of greater interest is the possibility that such keto groups might lead to cross-linking condensations on the peptide level as they apparently do in self-condensation reactions in the N-carbobenzyloxy derivatives. The application of the oxidation and reduction reactions on the gelatin level would be an obvious extension although little selectivity could be anticipated. Some work has already been reported on "oxypolygelatin" formed by the cross-linking of gelatin with glyoxal followed by oxidation with H_2O_2[17] and on the selective acetylation of the hydroxyl groups in gelatin so as to study the physical properties of a gelatin modified at the hydroxyl.[18]

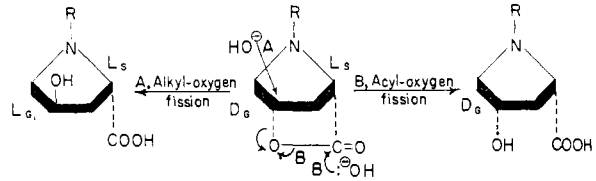
The keto derivatives of hydroxyproline and 5-hydroxypipecolic acid are of further interest, since they bear resemblance to δ-ketolysine whose ε-diazo derivative occurs in nature and has carcinostatic activity.[19]

Allohydroxy-D- and -L-proline Lactone.—When N - acetyl - O - *p* - tolylsulfonylhydroxy - L - proline (XIII) prepared from the ester XII[20] was refluxed in methyl ethyl ketone in the presence of anhydrous potassium carbonate the carboxylate anion displaced the toluenesulfonate ion in an internal SN2 reaction leading to the long-sought lactone of N-acetylallohydroxy-L-proline (XIV) with inversion of configuration at C(4). The assignments made in XII, XIII and XIV follow from the facts that hydroxy-L-proline belongs to the natural LS-series and that the hydroxyl group is *trans* to the carboxyl.[20]



XII XIII

1 hr. (40%) | K_2CO_3 anhydrous reflux in MeCOEt



XIV, R = CH_3CO
XV, R = $COOCH_2C_6H_5$
XVI, R = H

From these data one can relate the configuration at C(4) in hydroxy-L-proline to L-glyceraldehyde and in the lactone XIV to D-glyceraldehyde. If this be so then, according to Hudson's rule,[21] the difference of rotation $[\alpha]_D$ (lactone) − $[\alpha]_D$ (N-acetylallohydroxy-L-proline) should be positive: $+61.1° - (-91.5°) = +152.6°$. This confirmation of assignments with the aid of Hudson's rule was also obtained with the homologous lactone of 5-allohydroxy-L-pipecolic acid.[22]

A superior method leading to the N-carbobenzyloxy lactone XV and its D-enantiomer in up to 87% yield was found in the action of *p*-tolylsulfonyl chloride on N-carbobenzyloxyallohydroxy-L- (and D-)proline in anhydrous pyridine.[23] Apparently, mixed anhydride and lactone formations proceed at a faster rate than tosylation of the secondary alcohol.

Dicyclohexylcarbodiimide[24] in dry methylene chloride was also used successfully to convert N-carbobenzyloxyallohydroxyproline into its lactone. This lactonizing procedure is convenient but did not rival the yield of the tosyl chloride–pyridine method.

The opening of the lactone XV with an α-amino acid ester gave, after decarbobenzyloxylation and hydrolysis, the dipeptide. The preparation of allohydroxy-L- and -D-prolylglycine is described in the Experimental part. In view of the ready lactonization of N-carbobenzyloxyallohydroxyproline with dicyclohexylcarbodiimide, it seems likely that coupling procedures for allohydroxyproline peptides may proceed in part through the lactone as intermediate. The formation of peptide links by

(13) We are obligated to Dr. Elijah Adams, Department of Pharmacology, New York University, for carrying out these tests; *cf. Federation Proc.*, **15**, 209 (1956).

(14) We are thankful to Professor F. Bergel, Chester Beatty Institute, London, for this information.

(15) *Cf.* N. G. Gaylord, "Reduction with Complex Metal Hydrides," Interscience Publishers, Inc., New York, N. Y., 1956, pp. 124–321.

(16) Ref. 1, pp. 134–140.

(17) D. H. Campbell, J. B. Koepfli, L. Pauling, N. Abrahamsen, W. Dandliker, G. A. Feigen, F. Lanni and A. LeRosen, *Texas Repts. Biol. and Med.*, **9**, 235 (1951); D. H. Campbell, J. B. Koepfli and L. Pauling, U. S. 2,591,133 (April 1, 1952); *C. A.*, **46**, 6797 (1952).

(18) J. Bello and J. R. Vinograd, THIS JOURNAL, **78**, 1369 (1956).

(19) D. A. Clarke, *et al.*, American Chemical Society Meeting, Dallas, Texas, April 8–13, 1956, Abstracts, p. 12-M.

(20) A. Neuberger, *J. Chem. Soc.*, 429 (1945); C. A. Hudson and A. Neuberger, *J. Org. Chem.*, **15**, 24 (1950).

(21) W. Klyne, *Chem. and Ind.*, 1198 (1954).

(22) B. Witkop and C. M. Foltz, THIS JOURNAL, **79**, 192 (1957).

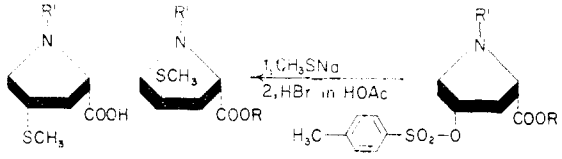
(23) J. H. Brewster and C. H. Kucera, *ibid.*, **77**, 4564 (1955); J. H. Brewster and C. J. Ciotti, *ibid.*, **77**, 6214 (1955).

(24) *Cf.* S. C. Sheehan, M. Goodman and G. P. Hess, *ibid.*, **78**, 1367 (1956); H. G. Khorana, *Chem. and Ind.*, 1087 (1955).

amino acid reactions with lactones[25a] and thiolactones[25b] has been described.

The hydrobromide of the free lactone XVI which was prepared from XV with hydrogen bromide in glacial acetic acid, mutarotated in water to the open acid over a period of 2.5 hours. This instability of a γ-lactone in respect to its hydroxy acid is normally not found with nitrogen-free bicyclo[2,2,1]lactones.[26] After solvolysis of XIV and XVI in aqueous buffer solutions of various *p*H a careful analysis by column chromatography showed the absence of hydroxy-L-proline and excludes, as expected, opening by alkyl-oxygen fission. More drastic conditions, such as sodium acetate in the melt, led to complete racemization of the molecule.

SN2 Displacements on O-Tosyl Derivatives of (Allo)Hydroxyprolines.—The *internal* SN2 displacement of a tosylate anion by a free carboxyl group led from the normal to the allohydroxy-L-proline series *via* the lactone. For *external* SN2 displacements in the normal series the carboxyl group was esterified to avoid lactonization and any possible shielding effects of the carboxylate anion. The key compound N-carbobenzyloxy-O-*p*-tolylsulfonylhydroxy-L-proline methyl ester was obtained crystalline. By the use of sodium methyl mercaptide, 4-allomethylmercapto-L-proline (XVII) was synthesized. For displacement reactions in the *allo*-series, leading to derivatives of natural hydroxyproline, the free N-carbobenzyloxy-O-*p*-tolylsulfonylallohydroxy-L-proline (XIX, m.p. 101.5°) was employed. The latter was obtained crystalline from the methyl ester (XVIII, m.p. 138–139°) which was made from

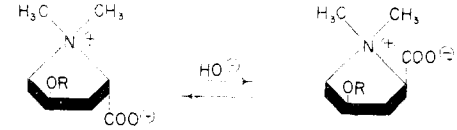


XVII, R′ = H XX, R = R′ = H
XVIII, R = CH_3, R′ = Cbz
XIX, R = H, R′ = Cbz

N-carbobenzyloxyallohydroxy-L-proline, now easily available by sodium borohydride reduction of the 4-keto derivative. The over-all yield to 4-methylmercapto-L-proline (XX) hydrobromide was excellent. Experiments have been started which show that the free thiols and their disulfides can be obtained by this route.

The 4-thiomethylprolines XVII and XX have no activity in the enzyme system which condenses methionine with ATP to yield "active methionine" and three moles of inorganic phosphate.[26a]

Betaine Analogs of Hydroxyproline.—Several betaine analogs of hydroxyproline were needed in the testing program. In addition, it was inviting to establish the stereochemistry of the known hydroxyproline betaines, betonicine and turicine.[27] Solution of the latter problem was possible through the use of non-epimerizing methylating conditions on hydroxyprolines of known stereochemistry. The early syntheses[28] of these betaines utilized hot base and methyl iodide with the result that a nearly 1:1 ratio of turicine and betonicine was obtained from hydroxy-L-proline. It was recognized that the basic conditions were undesirable but an attempt to circumvent them was unsuccessful. In our work, diazomethane[29] was at first used for the preparation of the betaines but this was soon abandoned in favor of the reaction of methyl iodide with the silver salts of the amino acids[30] in methanol at room temperature. In this manner, only betonicine (XXI) was isolated from hydroxy-L-proline and only turicine (XXIII) from allohydroxy-D-proline.



XXI, R = H XXIII, R = H
XXII, R = $COCH_3$

Starting with pure betonicine or turicine the base-catalyzed epimerization at C(2) proceeded under mild conditions to yield a 60:40 mixture (by rotation) of betonicine (XXI) and turicine (XXIII). This may be compared with the 1:1 mixture[31] of hydroxyproline and allohydroxyproline obtained when hydroxyproline is heated in baryta. Apparently, there is little difference in thermodynamic stability between *cis*- and *trans*-1,3-disubstituted rings of this type.

The easy base-catalyzed epimerization raises some doubts about the natural occurrence of turicine (XXIII), the betaine related to allohydroxy-D-proline. With the natural occurrence of the L-form of allohydroxyproline,[32] the betaine of the D-form is unexpected. We suspect that turicine (XXIII) from plant sources arises from betonicine (XXI) during isolation. If turicine truly is naturally occurring then it is our view that its biosynthesis proceeds not through a D-amino acid but through betonicine which may then be epimerized in the plant. It may be noted that the stereochemistry of the *cis*- and *trans*-3-hydroxystachydrines in *Courbonia virgata*[33] presents similar problems which have not yet been settled. The betaine of hydroxypipecolic acid which we have synthesized (see Experimental part) has yet to be isolated from natural sources. The betaines, including the acetyl derivative of betonicine, are being studied in the acetylcholinesterase system by Dr. S. L. Friess. Betonicine, though not turicine, is a competitive inhibitor

(25) (a) E. T. Stiller, S. A. Harris, S. Finkelstein, J. C. Keresztesy and K. Folkers, THIS JOURNAL, **62**, 1785 (1940); (b) R. Benesch and R. E. Benesch, *ibid.*, **78**, 1597 (1956).

(26) R. Fittig and L. Woringer, *Ann.*, **227**, 10 (1885); W. Treibs, *Ber.*, **64**, 2545 (1931); R. Stoermer, H. Starck and H. E. Anker, *ibid.*, **70**, 483 (1937); C. Djerassi, E. Farkas, L. H. Liu and G. H. Thomas, THIS JOURNAL, **77**, 5330 (1955).

(26a) We are greatly indebted to Dr. G. L. Cantoni for this information.

(27) No assignments are made in "The Alkaloids," by Manske and Holmes, Vol. I, Academic Press, Inc., New York, N. Y., 1949, p. 103–104. "The Merck Index," Sixth Edition, Rahway, 1952, p. 983, describes turicine as related to *d*-hydroxyproline.

(28) A. Kung, *Z. physiol. Chem.*, **85**, 217 (1913); J. A. Goodson and H. W. B. Clewer, *J. Chem. Soc.*, **115**, 923 (1919).

(29) R. Kuhn and W. Brydowna, *Ber.*, **70**, 1333 (1937).

(30) J. W. Cornforth and A. J. Henry, *J. Chem. Soc.*, 602 (1952).

(31) D. S. Robinson and J. P. Greenstein, *J. Biol. Chem.*, **195**, 383 (1951); H. Leuchs and K. Bohrmann, *Ber.*, **52**, 2086 (1919).

(32) A. N. Radhakrishnan and K. V. Giri, *Biochem. J.*, **58**, 57 (1954).

(33) J. W. Cornforth and A. J. Henry, *ibid.*, 597 (1952).

of the same order of magnitude as choline itself. A discussion of the various activities of these compounds in terms of the active site requirements of the enzyme will be presented elsewhere.

Experimental[34]

N-Carbobenzyloxyhydroxy-L-proline (II).—Hydroxy-L-proline (I, 5.00 g.) was dissolved in 70 cc. of 4 *N* sodium hydroxide and the solution was cooled in an ice–salt bath to −10°. Benzyl chloroformate (8.5 cc.) was added slowly with magnetic stirring to this alkaline solution during the course of 15 minutes. The reaction mixture was stirred for an additional half hour at −5° and finally was extracted with ether and the ether layer discarded. The derivative was precipitated as an oil by addition of concentrated hydrochloric acid to the aqueous solution cooled to 0°. The oil was extracted into ethyl acetate and washed with water and concentrated salt solution. After drying and removal of the ethyl acetate, the resulting oil was crystallized from ethyl acetate and petroleum ether (b.p. 65°) to yield 5.23 g. (52%) of crystals, m.p. 106–107°, $[\alpha]^{20}D$ −72° (*c* 1.0, in chloroform).

Anal. Calcd. for $C_{13}H_{15}NO_5$: C, 58.86; H, 5.70; N, 5.28. Found: C, 58.98; H, 5.74; N, 5.10.

N-Carbobenzyloxyallohydroxy-D-proline (IV).—Allohydroxy-D-proline (III, 5.00 g.) was allowed to react with 8.5 cc. of benzyl chloroformate under the same conditions as described for the preparation of the N-carbobenzyloxyhydroxy-L-proline. The crude oily carbobenzyloxy compound was crystallized from ethyl acetate and petroleum ether (b.p. 65°) to yield 4.08 g. (40%) of N-carbobenzyloxyallohydroxy-D-proline (IV), m.p. 110–111°. A recrystallized sample had m.p. 110.5–111.5°, $[\alpha]^{20}D$ +26.3°.

Anal. Calcd. for $C_{13}H_{15}NO_5$: C, 58.86; H, 5.70; N, 5.28. Found: C, 59.05; H, 5.82; N, 5.46.

N-Carbobenzyloxy-4-keto-L-proline (V.)—N-Carbobenzyloxyhydroxy-L-proline (II, 7.58 g.), was dissolved in 420 cc. of reagent acetone. This solution was stirred magnetically and to it was added 29 cc. of approximately 8 *N* chromic acid in sulfuric acid[35] during a period of about five minutes. Stirring was continued for an additional 30 minutes and then the excess oxidant destroyed by the addition of 5 cc. of methanol. Most of the chromium salts were removed by filtration with the aid of Hy-Flo Supercel. The acetone solution was diluted with chloroform and extracted several times with concentrated salt solution, dried and evaporated. The N-carbobenzyloxyketo-L-proline (V) was crystallized from ether–petroleum ether (b.p. 65°) to give 5.08 g. (68%) of colorless crystals, m.p. 99–101°. The analytical sample had m.p. 101–102°, $[\alpha]D$ +18.5° (*c* 1.0, in chloroform), $\lambda_{max}^{CHCl_3}$ 5.66, 5.84 μ.

Anal. Calcd. for $C_{13}H_{13}NO_5$: C, 59.31; H, 4.98; N, 5.32. Found: C, 59.48; H, 5.13; N, 5.14.

N-Carbobenzyloxyketo-D-proline (VII).—Carbobenzyloxyallohydroxy-D-proline (IV, 0.79 g.) was converted into the oily N-carbobenzyloxyketo-D-proline (VII) as described above. Recrystallization from ether–petroleum ether (b.p. 65°) afforded 0.522 g. (66.5%) of colorless crystals, m.p. 98.5–99.5°. The analytical sample had m.p. 99–100°, $[\alpha]^{20}D$ −19.4° (*c* 1.0, in chloroform), $\lambda_{max}^{CHCl_3}$ 5.66, 5.84 μ.

Anal. Calcd. for $C_{13}H_{13}NO_5$: C, 59.31; H, 4.98; N, 5.32. Found: C, 59.59; H, 5.20; N, 5.18.

Action of Base on N-Carbobenzyloxyketo-D- and -L-proline.—N-Carbobenzyloxyketo-D-proline (VII, 0.30 g.) was dissolved under nitrogen in 4 cc. of *N* NaOH. After one hour at room temperature, the slightly yellow solution was filtered and acidified with concentrated HCl at 0°. A white solid was precipitated which was centrifuged, washed over with distilled water, redissolved in 1 cc. of *N* NaOH and again precipitated at 0° with concentrated HCl. It was centrifuged, washed several times with distilled water and dried. The material could not be crystallized and its physical data were rather variable. The following are representative: m.p. 112–117°; $[\alpha]D$ +25.4° (*c* 1.0, in chloroform); molecular weight 445 (method of Signer in chloroform); λ_{max} 259 mμ (ε 9,500).[36] The infrared spectrum differed from the starting material in the loss of the (C=O) absorption at 5.66 μ and in the presence of a stronger, broad band at 5.85 μ (in chloroform). 0.200 g. of this material was heated in 4 *N* NaOH at 100° for one hour. The reddish colored water solution formed under these conditions gave a positive Ehrlich test.

The rotation of N-carbobenzyloxyketo-L-proline (II) in *N* NaOH was found to change with time: $[\alpha]^{20}D$ −84° (initial reading); −158° (after one hour); −179° (after 4 hours); −164° (after 15 hours). The yellow color which gradually developed in basic solution rendered this last reading rather inaccurate.

The analytical figures for three different preparations of the amorphous base product suggest a condensation of two molecules of carbobenzyloxyketoproline with partial loss of one molecule of water.

Anal. Calcd. for $C_{26}H_{24}N_2O_9$: C, 61.41; H, 4.76; N, 5.51. Found: C, 61.29, 60.23, 60.14; H, 4.85, 4.86, 5.03; N, 5.35, 5.42, 5.36.

4-Keto-L-proline (VI) Hydrobromide.—N-Carbobenzyloxyketo-L-proline (V, 1.00 g.) was placed in a 40-cc. centrifuge tube and treated for one hour with 5 cc. of a saturated solution of hydrogen bromide in glacial acetic acid. At the end of this time, 30 cc. of anhydrous ether was added and the precipitated keto-L-proline (VI) hydrobromide was centrifuged, the ether decanted, and the tube immediately transferred to a vacuum desiccator. Keto-L-proline hydrobromide of this purity had m.p. 154–156° (decomposition to a froth). The infrared (KBr pellet) displayed λ_{max} 5.65, 5.81 μ. The aqueous solution was characterized by λ_{max}. 276 mμ (ε 27) and $[\alpha]^{20}D$ −41° (*c* 1.0, in water), and it readily reduced Fehling solution. In *N* NaOH ketoproline hydrobromide mutarotated with darkening of the solution. No accurate readings could be taken after two hours. The recorded values in this solvent were: $[\alpha]^{20}D$ −83.5 (initial reading); −47.3° (after one hour); −32.9° (after 2 hours).

No pyrrole-2-carboxylic acid could be detected when ketoproline hydrobromide (0.02 g.) was heated 10 minutes at 100° in 2 cc. of *N* Na_2CO_3, acidified and chromatographed on paper with a butanol–acetic acid system. An authentic sample of pyrrole-2-carboxylic acid, supplied by Dr. A. Gottshalk, was used as a marker.

Anal. Calcd. for $C_5H_7NO_3 \cdot HBr$: C, 28.59; H, 3.84; N, 6.67. Found: C, 28.75; H, 4.13; N, 6.75.

Hydrogenolysis of N-Carbobenzyloxyketo-L-proline (V).—The carbobenzyloxy group was removed from N-carbobenzyloxyketo-L-proline (V, 0.5 g.) by hydrogenation in 10 cc. of glacial acetic acid at atmospheric pressure with 10% palladium on charcoal. As the hydrogenation proceeded the solution turned deep bluish-green. After the catalyst had been filtered off, the addition of an excess of ether precipitated 0.223 g. of a light green solid which gradually became brown in the air. This material displayed no well-defined melting point and the determination of its rotation was impossible because of the deep green color of its aqueous solutions. An ultraviolet spectrum of such a solution displayed $\lambda_{max}^{H_2O}$ at 346 mμ. The infrared spectrum in Nujol showed bands at 5.65, 5.84 and 6.19 μ. The analytical figures approximated two molecules of ketoproline minus one molecule of water.

Anal. Calcd. for $C_{10}H_{12}N_2O_5$: C, 50.00; H, 5.04; N, 11.66. Found: C, 49.99; H, 5.31; N, 11.51.

An attempt to hydrogenate N-carbobenzyloxyketo-L-proline (0.5 g.) in methanol (15 cc.) in the presence of only one drop of glacial acetic acid resulted in an incomplete uptake of hydrogen.

N-Carbobenzyloxyallohydroxy-L-proline by Stereospecific Reduction of N-Carbobenzyloxyketo-L-proline (V).—A solution of sodium borohydride (0.575 g.) in 2 cc. of water was added to a solution of N-carbobenzyloxyketo-L-proline (1.00 g.) in 30 cc. of methanol at 0°. The mixture was left overnight at +5° and the methanol was then removed *in vacuo*. The residue was treated with 15 cc. of 3 *N* NaOH at room temperature for 30 minutes. After the solution had been cooled to 0°, it was acidified with concentrated HCl and extracted with ethyl acetate. The ethyl acetate layer was

(34) All melting points are corrected. The analytical and rotational data were obtained by Dr. W. C. Alford and associates, Analytical Service Laboratory of the National Institutes of Health. We are indebted to Mrs. Anne Wright for the determination of the ultraviolet spectra.

(35) P. Bladon, J. M. Fabian, H. B. Henbest, H. P. Koch and G. W. Wood, *J. Chem. Soc.*, 2407 (1951).

(36) The value for ε was calculated using a molecular weight of 445.

washed twice with concentrated salt solution and dried over $MgSO_4$. A slight amount of yellow color was removed from the solution with the aid of Darco. The ethyl acetate solution was concentrated and petroleum ether (b.p. 65°) was added to the hot solution until a cloudiness persisted. In this manner, 0.781 g. (77%) of N-carbobenzyloxyallohydroxy-L-proline was obtained, m.p. 108–110°. One more crystallization yielded 0.757 g. of colorless crystals, m.p. 110–111°; $[\alpha]^{20}D$ −23.7° (*c* 1.0, in chloroform).

Anal. Calcd. for $C_{13}H_{15}NO_5$: C, 58.86; H, 5.70; N, 5.28. Found: C, 58.88; H, 5.72; N, 4.96.

N-Acetyl-O-*p*-tolylsulfonylhydroxy-L-proline Methyl Ester (XII).—*p*-Toluenesulfonyl chloride (2.12 g.) was dissolved in 4.0 cc. of dry pyridine and cooled in an ice–salt bath. To it was added finely powdered N-acetylhydroxy-L-proline methyl ester (2.00 g.). The mixture was allowed to warm to room temperature with stirring until all was in solution and was then left overnight at +5°. At the end of this time the reaction mixture was poured into 30.4 cc. of *N* HCl at 0°. The resulting suspension was allowed to solidify by standing at +5° for one day. The colorless crystals were collected and washed with a little cold water. After drying, they weighed 2.76 g. Recrystallization from ether yielded 2.12 g. (58%) of N-acetyl-O-*p*-tolylsulfonylhydroxy-L-proline methyl ester, m.p. 71–73°; lit. 60°.[20]

Anal. Calcd. for $C_{15}H_{19}NO_6S$: C, 52.77; H, 5.61; Found: C, 52.77; H, 5.61.

N-Acetyl-O-*p*-tolylsulfonylhydroxy-L-proline (XIII).—N-Acetyl-O-*p*-tolylsulfonylhydroxy-L-proline methyl ester (XII, 1.08 g.) was dissolved in 2.1 cc. of methanol and the solution cooled to 0°. To this was added sodium hydroxide (0.133 g.) dissolved in 3.0 cc. of water. The mixture was allowed to stand at +5° overnight and then 3.6 cc. of *N* HCl was added. The acidified mixture was kept at 0° for 15 minutes after which the crystalline mass of the acid XIII was collected and dried. The yield was 1.02 g. (99%), m.p. 166.5–168°. A sample recrystallized from acetone melted at 168.5–170°; lit. 181–2°.[20]

Anal. Calcd. for $C_{14}H_{17}NO_6S$: C, 51.36; H, 5.24; N, 4.28. Found: C, 51.61; H, 5.40; N, 4.41.

N-Acetylallohydroxy-L-proline Lactone (XIV).—N-Acetyl-O-*p*-tolylsulfonylhydroxy-L-proline (XIII, 0.373 g.) and anhydrous potassium carbonate (0.185 g.) were added to 90 cc. of redistilled methyl ethyl ketone and this mixture was refluxed for one hour with exclusion of moisture. Magnesium sulfate was added and the solution was filtered. The methyl ethyl ketone solution was evaporated to a small volume from which 72 mg. (41%) of the lactone XIV crystallized, m.p. 88–94°. The constants of the analytical sample prepared by two crystallizations from methyl ethyl ketone–cyclohexane were: m.p. 99–101°, $[\alpha]D$ +61.1° (*c* 1.0, in chloroform), $\lambda_{max}^{CHCl_3}$ 5.56, 6.01 μ.

Anal. Calcd. for $C_7H_9NO_3$: C, 54.19; H, 5.85; N, 9.03. Found: C, 54.31; H, 5.67; N, 9.01.

Solvolysis Studies with N-Acetylallohydroxy-L-proline Lactone. (XIV). (A) In 6 *N* Hydrochloric Acid.—The lactone XIV (10 mg.) was refluxed with 5 cc. of 6 *N* hydrochloric acid for three hours. The solution was then evaporated to dryness *in vacuo*. Approximately 2 mg. of the product was chromatographed on a 50 × 0.9 cm. column of Dowex-50 with a citrate buffer of *p*H 3.30. Methionine-L-sulfoxide was used as a marker and fractions of 1 cc. were collected. The ninhydrin reagent that was employed was prepared according to the directions of Moore and Stein[37] and the general procedure was that of Piez.[38] The analysis of the hydrolysate showed that allohydroxyproline constituted at least 95% of the hydroxyprolines formed in the solvolysis.

(B) In *p*H 7.0 Buffer.—The lactone XIV (10 mg.) was refluxed for two days with a 0.1 molar phosphate buffer of *p*H 7.0. Then 5 cc. of concentrated HCl was added and refluxing was continued for an additional three hours. Evaporation of the solution and ion-exchange analysis of the products were carried out as above. The hydroxyprolines produced in this solvolysis contained at least 95% allohydroxyproline.

A duplicate solvolysis was conducted in which the phosphate buffer solution was taken to dryness *in vacuo* after refluxing for two days. The residue so obtained was dried thoroughly and extracted with 0.5 cc. of chloroform. An infrared spectrum of this chloroform solution demonstrated that solvolysis of the lactone had been completed at the end of the two-day period. The minimum time for the solvolysis was not determined.

(C) In *p*H 9.3 Buffer.—N-Acetylallohydroxy-L-proline lactone (XIV, 10 mg.) was refluxed for ten hours with 5 cc. of a 0.1 *M* carbonate buffer of *p*H 9.3. It was then acidified with 5 cc. of concentrated HCl and reflux was continued for three hours. Ion-exchange analysis of the products conducted as above demonstrated that at least 95% of the hydroxyprolines produced was allohydroxyproline.

N-Carbobenzyloxyallohydroxy-L-proline Lactone (XV). (A) By the Action of N,N′-Dicyclohexylcarbodiimide.—N-Carbobenzyloxyallohydroxy-L-proline (0.500 g.) was dissolved in 25 cc. of methylene chloride. Some anhydrous magnesium sulfate was added to this solution followed after 0.5 hour by N,N′-dicyclohexylcarbodiimide (0.41 g.). Four hours later 15 cc. of ethyl acetate was added, the solution filtered and the filtrate evaporated to dryness at room temperature *in vacuo*. Crystallization of the residual oil from ethyl acetate and petroleum ether (b.p. 65°) afforded 0.198 g. (43%) of N-carbobenzyloxyallohydroxy-L-proline lactone (XV), m.p. 94–102°. Two additional crystallizations gave an analytical sample of m.p. 102–103°.

Anal. Calcd. for $C_{13}H_{13}NO_4$: C, 63.15; H, 5.30; N, 5.67. Found: C, 62.88; H, 5.40; N, 5.51.

(B) **By the Action of *p*-Tolylsulfonyl Chloride in Pyridine.**—A solution of 2.18 g. of N-carbobenzyloxyallohydroxy-L-proline in 4.35 cc. of anhydrous pyridine was cooled in an ice-bath. To this was added *p*-tolylsulfonyl chloride (1.75 g.) in 4.35 cc. of anhydrous pyridine. The reaction mixture was left overnight at +5° and was then poured into 70 cc. of 2 *N* HCl which was cooled in an ice–salt bath. The reaction flask was washed out with an additional 1 cc. of pyridine. After the addition of the reaction mixture to the dilute acid the oily suspension containing the crude lactone solidified on vigorous scratching. The crystalline lactone was collected, washed several times with water and dried. It weighed 1.78 g. (87.5%) and had m.p. 97–102°. Recrystallization from ethyl acetate–petroleum ether (b.p. 65°) afforded N-carbobenzyloxyallohydroxy-L-proline lactone (XV, 1.16 g.), m.p. 102–103°, $[\alpha]^{20}D$ +33.6° (*c* 1.0, in chloroform), $\lambda_{max}^{CHCl_3}$ 5.56, 5.86 μ.

N-Carbobenzyloxyallohydroxy-D-proline Lactone.—This compound was prepared from N-carbobenzyloxyallohydroxy-D-proline (IV) by the tosyl chloride–pyridine procedure as described above for the L-enantiomer. The pure D-lactone showed these constants: m.p. 102–103° and $[\alpha]D$ −30.9° (*c* 1.0, in chloroform).

Anal. Calcd. for $C_{13}H_{13}NO_4$: C, 63.15; H, 5.30; N, 5.67. Found: C, 63.03; H, 5.48; N, 5.71.

Allohydroxy-L-proline Lactone (XVI) Hydrobromide.—N-Carbobenzyloxyallohydroxy-L-proline (XV, 0.200 g.) was treated in a 12-cc. centrifuge tube with 1 cc. of a saturated solution of hydrogen bromide in glacial acetic acid. After an hour at room temperature the crystalline precipitate of the lactone hydrobromide was centrifuged, washed once with 1 cc. of glacial acetic acid, and then washed with 5 cc. of anhydrous ether. In this manner, 0.110 g. (70%) of allohydroxy-L-proline lactone (XVI) hydrobromide was obtained, m.p. 193–194° dec., λ_{max}^{Nujol} 5.58 μ.

Anal. Calcd. for $C_5H_7NO_2 \cdot HBr$: C, 30.95; H, 4.16; N, 7.22. Found: C, 31.09; H, 4.23; N, 7.17.

Allohydroxy-D-proline Lactone Hydrobromide.—This compound was prepared from N-carbobenzyloxyallohydroxy-D-proline lactone in the manner described above for the L-enantiomer. A sample of allohydroxy-D-proline lactone hydrobromide had m.p. 188° (with dec.) and λ_{max}^{Nujol} 5.58 μ. The lactone dissolved instantly in water. A constant rotation of $[\alpha]D$ +23° is obtained within 2.5 hours by a 1% solution of the hydrobromide in distilled water at room temperature.

Anal. Calcd. for $C_5H_7NO_2 \cdot HBr$: C, 30.95; H, 4.16; N, 7.22. Found: C, 30.50, 30.66; H, 4.25, 4.26; N, 7.52.

The Solvolysis of Allohydroxy-L-proline Lactone (XVI) Hydrobromide at *p*H 7.02—Allohydroxy-L-proline lactone hydrobromide (10 mg., m.p. 193–194°) was dissolved in 2.5 cc. of a 0.1 *M* phosphate buffer of *p*H 7.02. This solution was allowed to stand at room temperature for four hours and

(37) S. Moore and W. H. Stein, *J. Biol. Chem.*, **211**, 907 (1954).

(38) K. A. Piez, *ibid.*, **207**, 77–80 (1954).

then evaporated to dryness *in vacuo* with the aid of a rotating evaporator. The bath temperature during this concentration did not exceed 35°. The residual material which was left after this evaporation was dissolved in 0.7 cc. of *N* HCl and 0.2 cc. of this solution was developed on an ion-exchange column of Dowex-50 with a citrate buffer of *p*H 3.30, as described above. The analysis demonstrated that of the hydroxyproline products, at least 95% was allohydroxyproline.

Allohydroxy-L-prolylglycine.—N-Carbobenzyloxyallohydroxy-L-proline lactone (0.300 g.), glycine ethyl ester hydrochloride (0.186 g.) and anhydrous magnesium sulfate (0.1 g.) were added to 15 cc. of anhydrous benzene (dried over sodium). The free glycine ethyl ester was liberated by addition of 0.4 cc. of dry triethylamine and the mixture was then refluxed for 1 day with exclusion of moisture. At the end of this period the solution was diluted with ethyl acetate and washed with dilute HCl, water containing 1 cc. of *N* NaOH, and finally with a saturated sodium chloride solution. When the dried solution ($MgSO_4$) was evaporated, 0.361 g. of oil was obtained. The crude N-carbobenzyloxyallohydroxy-L-prolylglycine ethyl ester was saponified in 5 cc. of methanol with a total of 4 cc. of *N* NaOH added in two portions over one hour. At the end of this time the solution was acidified, concentrated to half the volume, diluted with ethyl acetate and washed with a saturated sodium chloride solution. After drying and removal of solvent, 0.194 g. of oil was obtained from which the carbobenzyloxy group was removed by hydrogenation with a 10% palladium–charcoal catalyst using as solvent 15 cc. of methanol containing one drop of glacial acetic acid. Crystallization from ethanol and water yielded 0.050 g. (20%) of allohydroxy-L-prolylglycine monohydrate, m.p. 209–211°. A recrystallized sample showed m.p. 214–216°, $[\alpha]^{20}D$ −11.1° (*c* 1.0, in water) (reported[39] −19.8°).

Anal. Calcd. for $C_7H_{12}N_2O_4 \cdot H_2O$: C, 40.77; H, 6.84; N, 13.59. Found: C, 40.54; H, 6.87; N, 13.82.

Allohydroxy-D-prolylglycine.—Dry glycine ethyl ester (0.138 g.) was dissolved in 5 cc. of tetrahydrofuran which had been dried by distillation from lithium aluminum hydride. Anhydrous magnesium sulfate was added to this solution followed by N-carbobenzyloxyallohydroxy-D-proline lactone (0.300 g.). This solution was left at room temperature for 20 hours whereupon it was diluted with ethyl acetate and washed with *N* HCl, 5% $NaHCO_3$ and a saturated sodium chloride solution. The dried solution was evaporated to an oil weighing 0.369 g. which was saponified and converted to the free dipeptide in the manner described above for the L-peptide. The yield of crystalline material of m.p. 215–218° was 0.072 g. (32%), $[\alpha]^{20}D$ +11.3° (*c* 1.0, in water).

Anal. Calcd. for $C_7H_{12}N_2O_4 \cdot H_2O$: C, 40.77; H, 6.84; N, 13.59. Found: C, 40.82; H, 6.96; N, 13.48.

N-Carbobenzyloxy-O-*p*-tolylsulfonylhydroxy-L-proline Methyl Ester.—A solution of 4.00 g. of N-carbobenzyloxyhydroxy-L-proline in 50 cc. of dry dioxane was treated at 0° with ethereal diazomethane until the yellow color persisted. The excess diazomethane was destroyed with glacial acetic acid and the resulting solution was dried for several hours with anhydrous magnesium sulfate. Removal of the solvent left the N-carbobenzyloxyhydroxy-L-proline methyl ester as an oil which was dissolved in 9.5 cc. of anhydrous pyridine and cooled in an ice–salt bath. A solution of 3.55 g. of *p*-tolylsulfonyl chloride in 4.7 cc. of anhydrous pyridine was added to this cooled solution and the resulting mixture was left for 3 days at −5°. At the end of this time 80 cc. of ice-cold 2 *N* HCl was added to precipitate the N-carbobenzyloxy-O-*p*-tolylsulfonylhydroxy-L-proline methyl ester as a gum which was crystallized from methanol and water to yield 4.04 g. (62%) of crystals, m.p. 70–80°. Another crystallization yielded 2.95 g., m.p. 74–77°. The analytical sample of N-carbobenzyloxy-O-*p*-tolylsulfonylhydroxy-L-proline methyl ester showed m.p. 76–78° and $[\alpha]^{20}D$ −32.4° (*c* 1.0, in methanol).

Anal. Calcd. for $C_{21}H_{23}NO_7S$: C, 58.19; H, 5.35; N, 3.23. Found: C, 58.43; H, 5.47; N, 3.15.

N-Carbobenzyloxy-O-*p*-tolylsulfonylallohydroxy-L-proline Methyl Ester (XVIII).—N-Carbobenzyloxyallohydroxy-L-proline (4.00 g.) was esterified with diazomethane as described above for the normal-L-diastereoisomer. The dried oily N-carbobenzyloxyallohydroxy-L-proline methyl ester was dissolved in 14 cc. of dry pyridine, cooled in an ice–salt bath and to it was added a solution of *p*-tolylsulfonyl chloride (6.20 g.) in 7 cc. of dry pyridine. This mixture was seeded with the product and left for 3 days at −5°. The crude methyl ester was precipitated at the end of this time with 120 cc. of cold 2 *N* HCl. After standing at −5° for 4 hours, it was collected and dried. In this manner 10.42 g. (87%) of crystalline material was obtained, m.p. 126–132°. Recrystallization from dioxane and methanol afforded an analytical sample of the ester, m.p. 138–139°, $[\alpha]^{20}D$ −25.4° (*c* 1.0, in chloroform).

Anal. Calcd. for $C_{21}H_{23}NO_7S$: C, 58.19; H, 5.35; N, 3.23. Found: C, 58.26; H, 5.45; N, 3.23.

N-Carbobenzyloxy-O-*p*-tolylsulfonylallohydroxy-L-proline (XIX).—The saponification of the methyl ester XVIII (3.00 g.) occurred smoothly overnight in a solution of 30 cc. of methanol and 45 cc. of dioxane containing 2.7 cc. of 4 *N* NaOH. The reaction mixture was worked up as described for similar saponifications reported above. Crystallization from methanol and water afforded 2.51 g. (83%) of the monohydrate of the acid XIX, m.p. 100–101.5°, $[\alpha]D$ −20.0° (*c* 1.0, in methanol).

Anal. Calcd. for $C_{20}H_{21}NO_7S \cdot H_2O$: C, 54.91; H, 5.30; N, 3.20. Found: C, 55.16; H, 5.25; N, 3.40.

Examples of Nucleophilic Displacement Reactions on the Tosylates of the N-Carbobenzyloxy Normal and Allohydroxy-L-prolines. (A) 4-Methylmercapto-L-proline (XX) Hydrobromide.—N-Carbobenzyloxy-O-*p*-tolylsulfonylallohydroxy-L-proline (XIX, 0.500 g.) was dried by azeotropic distillation with benzene and dissolved in 4 cc. of absolute ethanol and 4 cc. of absolute acetone. Sodium methyl mercaptide (0.334 g.) was added and this mixture was refluxed for one hour with exclusion of moisture. At the end of this period, most of the solvent was removed *in vacuo*. After addition of *N* HCl, the product was extracted into ethyl acetate. The ethyl acetate solution was washed with water, dried with $MgSO_4$ and taken to dryness leaving the crude N-carbobenzyloxy-4-methylmercapto-L-proline (XX, R = $OCOCH_2C_6H_5$) as an oil. The carbobenzyloxy group was removed from this material with acetic acid saturated with hydrogen bromide and containing 7.5% thiophenol. Precipitation with ether afforded 0.237 g. (82%) of tan colored 4-methylmercapto-L-proline (XX, R = H) hydrobromide, m.p. 165–168°. The analytical sample from ethanol and ether formed colorless needles and showed m.p. 170–172° $[\alpha]^{20}D$ −24.0° (*c* 1.0, in water).

Anal. Calcd. for $C_6H_{11}NO_2S \cdot HBr$: C, 29.76; H, 5.00; N, 5.79; S, 13.24. Found: C, 29.83; H, 4.97; N, 5.86; S, 13.18.

(B) 4-Allomethylmercapto-L-proline (XVII).—N-Carbobenzyloxy-O-*p*-tolylsulfonylhydroxy-L-proline methyl ester (0.500 g.) and sodium methyl mercaptide (0.322 g.) reacted together for two hours in a refluxing mixture of 4 cc. of anhydrous acetone and 4 cc. of absolute ethanol. Most of the solvent was removed *in vacuo*, several cc. of 2 *N* HCl were added and concentration was continued *in vacuo* at room temperature until the reaction product separated as an oil. The crude N-carbobenzyloxy-4-allomethylmercapto-L-proline methyl ester was taken up in ethyl acetate and washed with a saturated sodium chloride solution. After removal of the solvent, the ester was saponified overnight in 7.5 cc. of dioxane and 5 cc. of methanol with 0.5 cc. of 4 *N* NaOH. Concentration, acidification and extraction into ethyl acetate afforded 0.335 g. of oily N-carbobenzyloxy-4-allomethylmercapto-L-proline. After thorough drying, this oil was treated with 2.5 cc. of the glacial acetic acid solution containing HBr and thiophenol as described above. After 1 hour at room temperature an oily hydrobromide of 4-allomethylmercapto-L-proline was precipitated by the addition of anhydrous ether. Since this material resisted crystallization, the free amino acid was prepared by passing the hydrobromide over a short column of Dowex-acetate. The eluent was *N* acetic acid. In this manner 0.047 g. (25%) of 4-allomethylmercapto-L-proline was obtained as colorless needles, m.p. 230–232°. The analytical sample had m.p. 243–244°.

Anal. Calcd. for $C_6H_{11}NO_2S$: C, 44.69; H, 6.88; N, 8.69; S, 19.89. Found: C, 44.76; H, 6.77; N, 8.32; S, 19.78.

(39) N. C. Davies and E. Adams, *Arch. Biochem. and Biophys.*, **57**, 301 (1955).

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.

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

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The Configuration of 5-Hydroxypipecolic Acid from Dates

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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.

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

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