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One-Step Synthesis and Enzymatic Resolution of *cis*- and *trans*-3-Hydroxyproline

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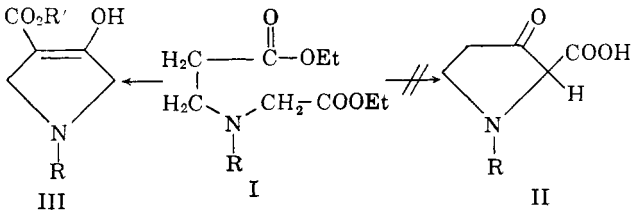
A simple one-step synthesis of 3-hydroxyproline consists in the Michael addition–Aldol condensation of the metabisulfite addition product of acrolein with aminomalonic acid. The ratio of *cis*- and *trans*-hydroxyprolines, as assayed by column and vapor phase chromatography, depends on the nature and pH of the buffer system, which also affects the stereochemistry of decarboxylation of the intermediate 3-hydroxy-2,2-dicarboxypyrrolidine. Enzymatic resolution of the amides of *trans*- and *cis*-3-hydroxy-DL-prolines with leucine amino peptidase gave *trans*- and *cis*-3-hydroxy-L-proline identical with the natural amino acids isolated from sponge and telomycin, respectively.

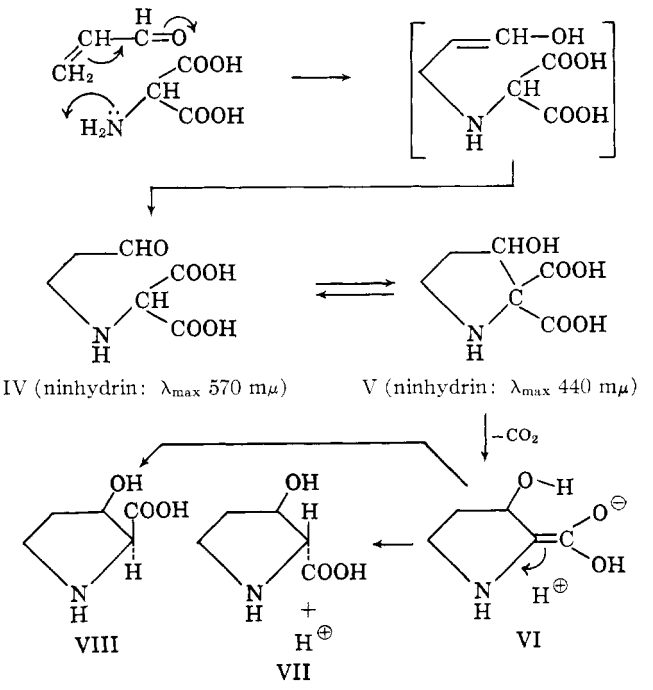
trans-3-Hydroxy-DL-proline has been synthesized by stereospecific hydroboration of 3,4-dehydro-DL-proline.[2] In addition, two nonstereospecific syntheses have been reported which lead to mixtures of *cis*- and *trans*-3-hydroxy-DL-prolines separable by column chromatography[3] or *via* fractional crystallization of the copper salts.[4] The attempted synthesis of 3-ketoproline (II) *via* the addition product I of glycine (or hippuric acid[3]) to ethyl acrylate and subsequent



III I II

Dieckmann condensation leads to the wrong isomer III with the carboxyl function in the 4-position.[5] However, the homologous 3-ketopyrrolidine-2-acetic acid is accessible by this route.[6] The somewhat cumbersome preparation of 3,4-dehydroproline[7] made an alternate synthesis from simple starting materials desirable. Such a synthesis was found in the "one-step" condensation of aminomalonic acid with the "bisulfite addition product of acrolein." Numerous variations of the reaction conditions and the starting materials were necessary to obtain acceptable yields of 3-hydroxyproline (Table I). Acrolein itself was too reactive a partner in this condensation. Sodium bisulfite, $NaHSO_3$, which is known to add to the double bond of acrolein,[8] could not be used in the one-step synthesis. Only the use of sodium metabisulfite (pyrosulfite), $Na_2S_2O_5$, gave good yields of 3-hydroxyproline. The "bisulfite complex" acts probably as a depot from which active acrolein is released in a controlled fashion. Glycine, instead of aminomalonic acid, was too unreactive. This "one-step" synthesis, comparable in principle to the reaction used in the new synthesis of pyrroles,[9] consists of a Michael addition and an aldol condensation (IV → V). The processes are probably not concerted and the latter reaction is reversible to judge from the existence of an intermediate with the properties of IV which precedes V on the amino acid analyzer (Fig. 1.). A second intermediate V was isolable by electrophoresis (pH 6.5, Fig. 2) when the temperature of the condensation reaction was kept below 45°. Combined yield of the two 3-hydroxyprolines VIII and VII was 40% and their ratio 1:1 when the condensation was carried out in pyridine buffer (pH 5.5–6.5) at 55–60°.

The decarboxylation of the malonic acid intermediate V should lead to an enolate ion VI[10] which then would add a proton to give either *cis*- (VIII) or *trans*-3-hydroxyproline (VII). *Prima facie*, one would expect little stereoselectivity in the addition of this proton to the enolate VI, and the ratios of *cis*- and *trans*-3-hydroxyprolines initially showed little variation from 1:1. However, slight changes in pH and buffer system led to significant variations (Table I). The replacement of the pyridine (pH 6.5) by potassium acetate buffer (pH 4.5) led to twice as much *cis* as *trans* product *in the one-step synthesis*. However, when the *purified intermediate* V was left in acetate buffer (pH 3.3), spontaneous decarboxylation at room temperature occurred to produce 67% *trans*- and 33% *cis*-3-hydroxyproline (Table II). Apparently factors such as the presence or absence of a charge on the ring nitrogen, the possible existence of hydrogen bridges between the hydroxyl and/or imino groups and the carboxyls of V, the geometry of the enolate ion IX, and hydrogen bonding in the final products VII and VIII all contribute in a



IV (ninhydrin: λ_{max} 570 mμ) V (ninhydrin: λ_{max} 440 mμ)

VIII VII VI

(1) Associate in the Visiting Program of the USPHS, 1962.

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(5) L. A. Cohen and W. M. Jones, unpublished results.

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(7) A. V. Robertson and B. Witkop, *J. Am. Chem. Soc.*, **84**, 1697 (1962).

(8) *Cf.* J. Houben and T. Weyl, "Methoden der Organischen Chemie," Vol. VII, Stuttgart, 1954, Pt. 1, p. 485.

(9) A. H. Jackson, G. W. Kenner, and W. G. Terry, *Tetrahedron Letters*, **No. 20**, 921 (1962). Acrolein has been used for a ring synthetic approach to proline in which diethyl N-carbobenzyloxyaminomalonateun dergoes C,C-Michael addition and the resultant aldehyde adds the NH to yield diethyl N-Cbz-Δ^4-pyrrolidine-2,2-dicarboxylate (A. Mauger, Chester Beatty Research Institute, London, private communication).

(10) F. H. Westheimer and W. A. Jones, *J. Am. Chem. Soc.*, **63**, 3283 (1941).

TABLE I

ONE-STEP SYNTHESIS OF 3-HYDROXYPROLINE

Run	Reaction condition	Reaction time, hr.	Temp., °C.	Yield, % *trans*	Yield, % *cis*	Remarks
1	AMA[a] on Dowex 1[b] + acrolein	0.5	20	1.5	2.3	Molar ratio of AMA:acrolein = 1:5
2	AMA, acrolein adduct,[c] NaOAc	1.0	100	3.3	5.8	AMA:acrolein = 1:1
3	AMA, acrolein adduct, pyridine	20[c]	60 20	9	9	AMA:acrolein = 1:1
4	AMA, acrolein adduct, pyridine	25	60	23	20	After 20 hr. the addition of acrolein adduct and pyridine was repeated
5	AMA, pyridine, acrolein adduct	7	60	19	17	After 3 hr. the addition of acrolein adduct was repeated
6	AMA, KOAc, acrolein adduct	41	60	6.3	12.0	After 18 hr. the addition of acrolein adduct was repeated
7	AMA, pyridine, acrolein adduct	14	60	22	20	After 4 hr. the addition of acrolein adduct was repeated
8	AMA, pyridine, acrolein adduct	12	55	18	15	...
9	AMA, pyridine, acrolein adduct	12	55	15.5	16	...

[a] AMA: aminomalonic acid. [b] A considerable amount of a compound, which gave a yellow color with ninhydrin, was present in the Dowex-1-catalyzed condensations, but not in runs 2–9 (Fig. 1). [c] The concentration of each reactant was: AMA (0.01 mole), pyridine (0.05 mole), acrolein (0.02 mole), and sodium metabisulfite (0.02 mole) in water (60 ml.). However, in run 4 the ratio of AMA:acrolein adduct was 1:3 and in runs 5, 6, and 7, 1:4 after the second addition of acrolein adduct.

TABLE II

EFFECT OF BUFFERS IN THE STEREOCHEMISTRY OF DECARBOXYLATION OF 3-HYDROXYPYRROLIDINE-2,2-DICARBOXYLIC ACID

Buffer system	Molar ratio of 3-hydroxyprolines *trans*	Molar ratio of 3-hydroxyprolines *cis*	Remarks
pH 3.3 in 0.2 *M* acetate buffer	2	1	Spontaneous decarboxylation at room temp. for 4 days
pH 8.8 in 0.2 *M* pyridine buffer	1.5	1	

manner, still to be determined, to the preferential formation of *cis* and *trans* product, respectively. The influence of metal ions is now the object of a separate study.

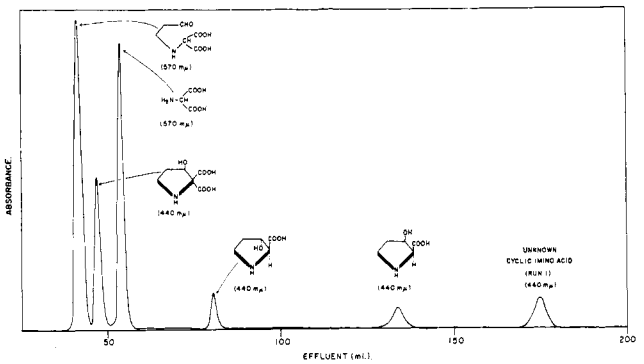

Fig. 1.—Position of the cyclic secondary amino acids on the automatic amino acid analyzer under standard conditions (ref. 2). The figures in parentheses refer to λ_{max} of the ninhydrin coloration. The first two peaks, belonging to two highly unstable intermediates, disappear in the course of the further purification, a process which is matched by a corresponding rise of the peaks for *cis*- and *trans*-3-hydroxyproline.

The synthetic *cis*- and *trans*-3-hydroxy-DL-prolines were resolved by enzymatic hydrolysis of the amides of the two isomers, either separately or in a mixture, with leucine aminopeptidase. The resolved amino acids were separated and isolated by ion-exchange technique. The individual *cis*- and *trans*-3-hydroxy-L-prolines had the same optical and physical constants as the corresponding natural amino acids from collagen, sponge, and telomycin.

Experimental

Gas Chromatographic Separation and Detection of Dehydroproline and Hydroxyproline Derivatives.—For a rapid analysis of position and diastereoisomers of hydroxyprolines in reaction mixtures, gas chromatography of N-carbobenzyloxy, N-carbobenzyloxy-O-acetyl, N-carbobenzyloxy-O-trifluoro-acetyl, N,O-diacetyl, and N,O-bistrifluoroacetyl derivatives was investigated (Table III). Among these, the use of the N,O-diacetyl derivatives (prepared by a modification of the published method[11]), the N,O-bistrifluoroacetyl and the N-Cbz-O-trifluoroacetyl derivatives made possible an accurate separation of *cis* and *trans* isomers.

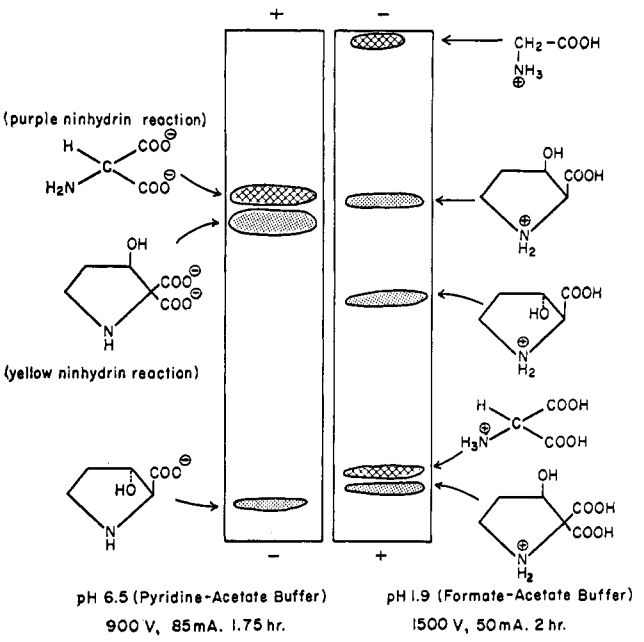

Fig. 2.—Electrophoretic purification of 3-hydroxy-2,2-dicarboxypyrrolidine.

Aminomalonic Acid.—Malonic acid (10.4 g.), suspended in 200 ml. of ether, was cooled in an ice bath and treated with 16 g. of bromine under vigorous stirring. Bromine was consumed rapidly and the crystals of the starting material disappeared. Ether was removed under reduced pressure at room temperature and the resulting crystalline mass recrystallized from acetone and benzene (1:5) to give 11 g. of white crystalline monobromomalonic acid (m.p. 113°),[12] which was very hygroscopic and kept in a tightly stoppered bottle under refrigeration. Bromomalonic acid, 6.0 g., was dissolved in 20 ml. of methanol and 5 ml. of water, treated with 50 ml. of methanolic ammonia (10%) at room temperature, and then heated at 40° for 10 min. After the addition of another 10 ml. of water, the solution was allowed to stand at room temperature for 2 days. The crystalline (acidic) aminonium salt (9.1 g.) of aminomalonic acid was collected, washed with methanol, and converted to free aminomalonic acid (5.6 g., m.p. 109°) by ion-exchange technique or by the addition of one equivalent of HCl.

One-Step Synthesis of 3-Hydroxyprolines.—To a solution of sodium metabisulfite (2.2 g. as $Na_2S_2O_5$) in water (30 ml.) was added freshly distilled acrolein (1.4 ml.) below 30° under stirring. After 45 min. aminomalonic acid (1.19 g.) dissolved in water (30 ml.) and pyridine (4 ml.) were added to the above solution and

(11) D. E. Johnson, S. J. Scott, and A. Meister, *Anal. Chem.*, **33**, 669 (1961).

(12) M. Conrad and H. Reinbach, *Ber.*, **35**, 1813 (1902).

TABLE III

ANALYSIS OF *cis*- AND *trans*-3- AND -4-HYDROXYPROLINE DERIVATIVES BY VAPOR PHASE CHROMATOGRAPHY

Conditions	Compound	Retention time, min.
2% QF-1, gaschrom A, 210°, 170 × 0.4 cm., argon, 10 p.s.i.	N-Cbz-$\Delta^{3,4}$-Dehydroproline methyl ester	1.5
	N-Cbz-*trans*-3-Hydroxyproline methyl ester	3.3
	N-Cbz-*cis*-3-Hydroxyproline methyl ester	3.8
1% SE-30, gaschrom P, 187°, 170 × 0.4 cm., N_2, 24 ml./min.	N-Cbz-O-Acetyl derivatives of	
	trans-3-Hydroxyproline methyl ester	9.0
	cis-3-Hydroxyproline methyl ester	10.1
	4-Hydroxyproline methyl ester	10.3
	Allo-4-hydroxyproline methyl ester	11.2
1% SE-30, gaschrom P, 187°, 170 × 0.4 cm., N_2, 24 ml./min.	Carbobenzyloxy-O-trifluoroacetyl derivatives of	
	trans-3-Hydroxyproline methyl ester	3.8
	cis-3-Hydroxyproline methyl ester	4.8
	4-Hydroxyproline methyl ester	4.4
	Allo-4-hydroxyproline methyl ester	5.2
2% NGS[a] gaschrom P, 192°, 170 × 0.4 cm., argon, 10 p.s.i.	N-Acetylproline methyl ester	1.2
	N,O-Diacetyl-*trans*-3-hydroxyproline methyl ester	5.0
	N,O-Diacetyl-*cis*-3-hydroxyproline methyl ester	6.3
	N,O-Diacetyl-allo-4-hydroxyproline methyl ester	7.5
2% NGS[a] gaschrom P, 170 × 0.4 cm., N_2, 33 ml./min.	N,O-Bis-trifluoroacetyl derivatives of	
	trans-3-Hydroxyproline methyl ester	5.7 (138°)
		4.0 (150°)
	cis-3-Hydroxyproline methyl ester	12.4 (139°)
		6.4 (150°)
	4-Hydroxyproline methyl ester	7.6 (143°)
	Allo-4-hydroxyproline methyl ester[b]	13.5 (143°)

[a] NGS: neopentylglycol succinate. [b] The treatment of allo-4-hydroxyproline with trifluoroacetic anhydride probably does not cause lactonization to judge from the comparable retention times of the two 3-hydroxyprolines.

the mixture was allowed to stand at 60° for 4 hr., when another equal amount of acrolein–bisulfite adduct in solution (30 ml.), prepared as described above, was added and the mixture kept at 60° for 10 hr. In this particular instance analysis of the reaction mixture by the automatic amino acid analyzer showed the composition

trans-3-Hydroxyproline	21.6%	Aminomalonic acid	9.0%
cis-3-Hydroxyproline	19.7%	Glycine	10.0%

Variations of the conditions of which the above is a representative example gave variations in yields and ratios of *cis*- and *trans*-3-hydroxyprolines as summarized in Table I.

No yield of 3-hydroxyproline was obtained in experiments where sodium bisulfite ($NaHSO_3$) instead of $Na_2S_2O_5$ was used. The analysis of products in this case was by electrophoresis.

The separation of *cis*- and *trans*-3-hydroxy-DL-proline by preparative column chromatography has been described in the preceding paper.[2]

Preparation of 3-Hydroxypyrrolidine-2,2-dicarboxylic Acid.—At an early stage (3–4 hr.) in the one-step synthesis an aliquot of the reaction mixture was subjected to paper electrophoresis at pH 1.9, followed by elution with water. The dicarboxylic intermediate stayed closest to the anodic area which was used as the starting line. The spot containing the intermediate was eluted with water and the solution was again subjected to electrophoresis at pH 6.5. In the neutral system the acidic dicarboxy intermediate migrated almost as far from the cathode as aminomalonic acid, from which it was easily distinguished by the yellow (instead of purple) ninhydrin color. A band corresponding to the intermediate dicarboxylic acid was cut out and immediately extracted with the respective buffer; the eluted buffer solution was kept at room temperature for 4 days during which time spontaneous decarboxylation took place (Table II).

Enzymatic Resolution of *trans*-3-Hydroxy-DL-proline Amide.—The incubation mixture in a total volume of 40 ml. contained: *trans*-3-hydroxy-DL-proline amide (prepared by aminolysis of N-Cbz-ester (m.p. 80–81°)), 262 μmoles; $MgCl_2$, 0.05 *M*; $MnCl_2$, 0.005 *M*; Tris buffer, pH 8.0, 0.05 *M*; leucine aminopeptidase (Worthington, C_l value = 13), 5.0 mg. The final mixture after the addition of 0.5 ml. of toluene was incubated at 37° for 24 hr. Analysis of the reaction mixture by a Phoenix automatic amino acid analyzer showed 128 μmoles (98% of theory) of 3-hydroxyproline. The remainder after adding 6 ml. of 40% trichloroacetic acid was centrifuged. The supernate was extracted with ether. The aqueous phase was adjusted to pH 3 and desalted on Dowex-50W (H^+). The amino acids were eluted from the column with 7% ammonia and the latter removed *in vacuo* at 30°. The residue was dissolved in water and passed through a column of Dowex-1 (OH^-) which retained the *trans*-3-hydroxy-L-proline and permitted elution of the unhydrolyzed *trans*-3-hydroxy-D-proline amide. The L-compound was eluted from the column with 2.0 *N* acetic acid and the solution evaporated to dryness *in vacuo*. It was redissolved in water, decolorized with charcoal, and concentrated to a small volume (0.3 ml.). After the addition of 10 ml. of absolute ethanol and after standing at −10° for 2 days the crystals were collected and dried *in vacuo* over P_2O_5. Two enzymatic resolutions yielded 14 mg. of crytalline *trans*-3-hydroxy-L-proline, $[\alpha]^{20}D$ −17.9 ± 4.5° (*c* 0.34 in water), identical in all respects with natural 3-hydroxy-L-proline from sponge.

3-*cis*-Hydroxy-DL-proline Amide.—3-*cis*-Hydroxy-DL-proline (149 mg.) was dissolved in aqueous sodium carbonate solution and carbobenzyloxylated in the usual manner. The sirupy carbobenzyloxy derivative was converted to the methyl ester by the action of diazomethane in dimethoxyethane. N-Carbobenzoxy-3-*cis*-hydroxy-DL-proline methyl ester was treated with ammonia in methanol (15 ml.) for 6 days at room temperature. Then the solvent and excess ammonia were removed by evaporation and a sirupy material was dissolved in ethyl aceate. By dilution with petroleum ether fine crystals (32 mg.) were obtained. Recrystallization from ethyl acetate gave 24 mg. of analytically pure N-carbobenzyloxyamide, m.p. 166–167°.

Anal. Calcd. $C_{13}H_{16}O_4N_2$: C, 58.86; H, 6.21; N, 10.52. Found: C, 59.07; H, 6.10; N, 10.60.

From the mother liquor the starting ester (292 mg.) was recovered and was repeatedly subjected to ammonolysis as described above. This process increassed the total yield to 152 mg. (52%).

N-Carbobenzyloxy-3-*cis*-hydroxy-DL-proline amide (120 mg.) was dissolved in 66% methanol containing a few drops of glacial acetic acid and was hydrogenated on 10% Pd–C for 1 hr. The catalyst was removed by filtration over Celite and the filtrate was evaporated to dryness. The noncrystalline amide, purified by ion-exchange technique (Amberlite IRC 50 and Dowex 1 X 8), was used directly for the enzymatic resolution.

Enzymatic Resolution of *cis*-3-Hydroxy-DL-proline Amide.—A sample of *cis*-3-hydroxy-DL-proline amide (15 mg.) was incubated with leucine amidopeptidase and the hydrolyzed amino acid separated from the amide following the same procedure as described above. There was obtained 4 mg. of crystalline *cis*-3-hydroxy-L-proline, $[\alpha]^{20}D$ −99.0 ± 10.0° (*c* 0.20 in water), identical in all respects with the amino acid of the same rotation from the antibiotic telomycin.