Synthesis and X-Ray Analysis of *cis*-3,4-Methylene-L-proline, the New Natural Amino Acid from Horse Chestnuts, and of Its Trans Isomer

Yasuo Fujimoto, Filadelfo Irreverre, Jean M. Karle, Isabella L. Karle, and Bernhard Witkop*

Contribution from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland 20014, and the Naval Research Laboratory, Washington, D. C. 20390.
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Abstract: The addition of carbene by the cuprous chloride catalyzed decomposition of diazomethane to 3,4-dehydro-L-proline led to a mixture of *cis*-3,4-methylene-L-proline (I) and *trans*-3,4-methylene-L-proline (II) in a ratio of 1:3.5. The cis acid I was identical with the natural amino acid isolated from seeds of *Aesculus parviflora* by a modified technique. Detailed X-ray analyses of the hydrochloride of I and of the free amino acid II gave complete bond distances, angles, and computer stereograms. The bicyclic system approaches a boat conformation both in the cis and in the trans acid, a result which confirms the evaluation of the nmr data. The pyrrolidine ring of I and II has all four carbons in a plane, while the nitrogen atom and the cyclopropyl carbon are displaced to the same side of the plane. In this respect the conformation of the hetero ring differs significantly from all other natural pyrrolidine amino acids.

The recent discovery of two cyclopropane amino acids, *cis*-3,4-methylene-L-proline and *cis*-α-(carboxycyclopropyl)glycine,[1] brings to six the number of naturally occurring amino acids which contain the cyclopropyl group.[2-12] Amongst these, hypoglycine, β-(methylenecyclopropyl)alanine, and its lower homolog, α-(methylenecyclopropyl)glycine, exhibit hypoglycemic activity.[3-5,13,14] Hypoglycine also affects the mitochondria of rat liver[15] and produces fetal abnormalities in rats.[16] Our continuing interest in antimetabolites of proline[17] and in inhibitors of proline hydroxylase[18] prompted us to synthesize the unique bicyclic structure of *cis*-3,4-methylene-L-proline and its diastereoisomer, *trans*-3,4-methylene-L-proline.

Addition of Carbene to 3,4-Dehydro-L-proline. The various synthetic methods available for the preparation of cyclopropyl compounds, *e.g.*, the Simmons–Smith reaction,[19] the reaction of dimethyloxosulfonium methylide with olefins,[20] the reaction of an olefin with photolytically produced methylene[21-23] or produced by catalytic decomposition of diazomethane,[24] all require 3,4-dehydro-L-proline[25] as starting material. When the latest variation of cyclopropane synthesis, *i.e.*, diethylzinc and methylene iodide,[26] was tried with the fully protected *N*-trifluoroacetyl-3,4-dehydro-L-proline methyl ester, no 3,4-methyleneproline was produced. However, cuprous chloride catalyzed decomposition of diazomethane[24] in neat *N*-trifluoroacetyl-3,4-dehydro-L-proline methyl ester yielded the desired cyclopropyl derivatives as shown in Scheme I.

The mixture of IV, V, and VI was saponified by 2.0 *N* methanolic alkali at room temperature for 1.5 hr, a treatment which removed both protecting groups. The amino acids were then desalted by passage through a column of Dowex 50W-X8 resin. The mixture of amino acids I, II, and III was accurately resolved by ion-exchange chromatography on Amberlite IR-120 in a 0.2 *N* citrate buffer, pH 3.17 (Figure 1). The synthetic cis compound was identical with the natural amino acid with regard to chromatographic behavior (tlc, paper-phase chromatography, and vapor-phase chromatography as the *N*-trifluoroacetyl-3,4-methyleneproline methyl ester), optical properties as well as ir and nmr spectra. The ratio of cis to trans amino acids formed in this reaction was 1:3.5.

A careful evaluation of the nmr data of natural *cis*-3,4-methylene-L-proline (Figure 2) has led to the assumption of boat conformation[27a] for the bicyclic system.

* Address correspondence to this author at the National Institute of Arthritis and Metabolic Diseases.

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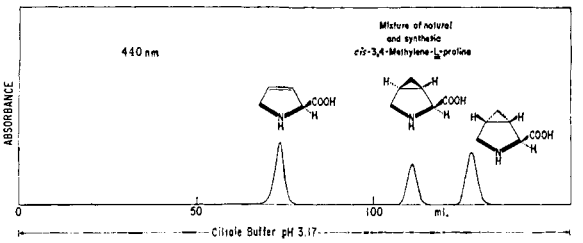
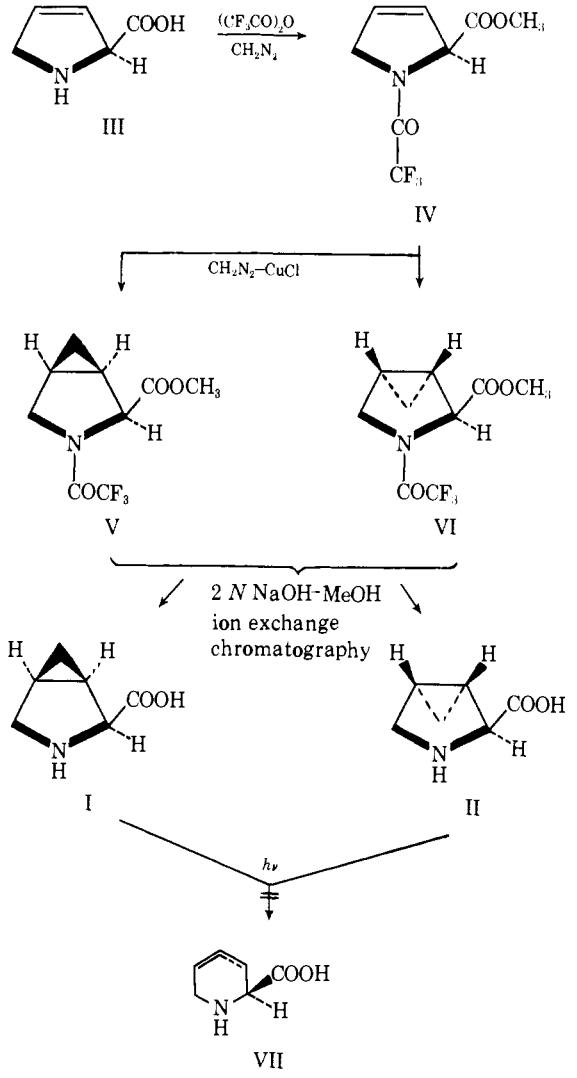


Figure 1. Position of the cyclic secondary amino acids on the automatic amino acid analyzer. The four amino acids were eluted from a UR-30 resin (Beckman, 0.9 × 56 cm) column with 0.2 *N* sodium citrate buffer, pH 3.17. The chromatogram was started at 32.5° and the temperature raised to 62.5° in 2 hr and 10 min. The values for the constant *C* in the standard calculation $H(W/C)$ = micromoles (*C* is the constant per micromole of ninhydrin-positive amino acid scanned at 440 mμ, *H* = height, and *W* = width of peak) are: 3,4-dehydro-L-proline, 4.7; natural *cis*-3,4-methylene-L-proline, 9.7; synthetic *cis*-3,4-methylene-L-proline, 9.7; *trans*-3,4-methylene-L-proline, 10.7.

Scheme I



Likewise, the nmr data of synthetic *trans*-3,4-methylene-L-proline (Figure 2) agree with the assumption of a boat conformation. These suggestions have been proved and confirmed by detailed X-ray crystallographic analysis.

The bicyclic structures of both *cis*- and *trans*-methylene-L-prolines are surprisingly stable to irradiation

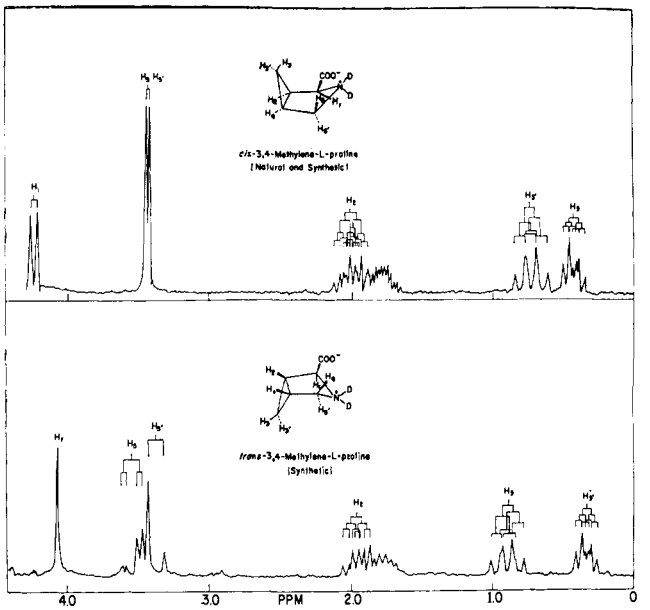


Figure 2. Nmr spectra of *cis*- and *trans*-3,4-methylene-L-prolines.

for 12 hr with a high-pressure mercury lamp (Western Quartz Products, Paso Robles, Calif.). Baikiain VII would be expected from methyleneproline by the collapse of the 3,4-endocyclic bond.[27b] However, both compounds gave recoveries of about 90–92%.

When the cis and trans compounds were heated at 110° in sealed tubes with constant boiling HCl for 24 hr, or with concentrated HCl at 110° for 3 hr, the recoveries for I were 73 and 85%, and for II, 69 and 81%, respectively. Besides the starting materials no ninhydrin-positive compounds were detected in the amino acid analyzer.

X-Ray Crystallography. Crystals of the free trans acid II and of the hydrochloride of the naturally occurring cis acid I were subjected to an X-ray analysis. Suitable crystals could not be grown for the free cis acid. Crystals of the hydrochloride of I formed regular dipyramids with good optical extinctions. Nevertheless they were not entirely single crystals but were composed of several slightly misaligned individuals. Many crystals were examined on the diffractometer before one was found that seemed to have a minimum of misalignment. Intensity data for both materials were collected with a four-circle automatic diffractometer using the θ–2θ scan technique with a $2.0° + 2\theta(\alpha_2) - 2\theta(\alpha_1)$ scan over 2θ. The intensity data were corrected for Lorentz and polarization factors and placed on an absolute scale by means of a *K* curve[28] and normalized structure factor magnitudes $|E|$ as well as structure magnitudes $|F|$ were derived. Cell parameters and other experimental data are listed in Table I.

Symbolic Addition Procedure. Phases for the free trans acid were derived directly from the normalized structure factor magnitudes by the symbolic addition procedure for noncentrosymmetric crystals.[29,30] The origin and enantiomorph were specified by the phase assignments: 015 ($+\pi/2$), 410 (0), 3 12 0 ($+\pi/2$), and 092 ($+\pi/2$). To implement the sum of angles formula

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Table I. Experimental Data for 3,4-Methylene-L-proline

	Trans	Cis
Mol formula	$C_6H_9NO_2 \cdot H_2O$	$C_6H_9NO_2 \cdot HCl \cdot H_2O$
Mol wt	145.16	181.63
Mp	248–250° dec	243–245° (base) 219–221° (HCl)
$[\alpha]^{20}D$ (water)	−94°	−131° (synthetic) −144° (natural)
Habit	Acicular c	Dipyramidal
Crystal size	~0.05 × 0.05 × 0.5 mm	0.6 × 0.6 × 0.7 mm
Space group	$P2_12_12_1$	$P2_12_12_1$
a	11.431 ± 0.004 Å	9.868 ± 0.003 Å
b	11.573 ± 0.004 Å	12.279 ± 0.004 Å
c	5.714 ± 0.002 Å	7.397 ± 0.003 Å
Z	4	4
Vol	755.9 Å^3	896.3 Å^3
Density (X-ray)	1.275 g/cm	1.346 g/cm
Radiation, λ	Cu Kα, 1.5418 Å	
No. of independent reflections measured	743	846

a symbol was assigned to represent the phase of the reflection 572. A value of zero for the unknown symbol led to the correct structure.

For the cis acid I, the chloride ion was located in a Patterson map and used as a partial structure[31] to obtain phases for a basic set to recycle and extend by means of the tangent formula.[32] The *E* map computed from this set of phases contained 11 maxima which corresponded to the chloride ion, the acid molecule, and a molecule of H_2O.

Coordinates and thermal parameters for each structure were refined by a full-matrix least-squares procedure. The function minimized was $\Sigma w(|F_o| - |F_c|)^2$ where $w = 0.5$ for $|F_o| = 0$, $w = 1.0$ for $|F_o| < 10$, and $w = 10.0/|F_o|$ for $|F_o| \geq 10.0$. Atomic scattering factors used were those listed in the "International Tables for X-Ray Crystallography." For the trans acid II, a difference map revealed all 11 hydrogen atoms whose coordinates are listed in Table II. Inclusion of the hydrogen atoms with constant parameters in the least-squares refinement resulted in an *R* factor of 4.7%.[33]

Table II. Approximate Coordinates for the Hydrogen Atoms in *trans*-3,4-Methylene-L-proline

	x	*y*	*z*
H(N1)	0.215	0.067	0.058
H(N2)	0.183	0.163	−0.125
H(2)	0.237	0.283	0.163
H(3)	0.037	0.312	0.375
H(4)	−0.120	0.188	0.208
H(51)	0.013	0.075	−0.175
H(52)	0.012	0.028	0.142
H(71)	−0.058	0.383	0.000
H(72)	0.050	0.325	−0.175
H(W1)	0.217	0.403	0.583
H(W2)	0.213	0.500	0.420

A difference map for the cis acid I indicated the positions for only five hydrogen atoms. In addition, there were a number of extraneous peaks in the map of the same order of magnitude as those ascribed to the hydrogen atoms which reflected the relatively poor quality of the crystal. The final *R* factor for the refinement in which constant parameters for the five hydrogen atoms were included was 8.9%.[33] Fractional coordinates and thermal parameters for both crystals are listed in Tables III and IV.

Stereochemistry of the Bicyclic System. The X-ray diffraction analysis confirms the structural formulas and establishes the conformations of the *cis*- and *trans*-3,4-methylene-L-prolines as illustrated in Figures 3 and 4. The feature of particular interest is the boat configuration of the six-membered ring in both the cis and trans acids. In each compound, using the numbering of Figures 5 and 6, the atoms C(2), C(3), C(4), and C(5) lie in a plane to within ±0.005 Å while the N and C(7) are displaced to the *same* side of the plane by 0.380 and 0.390 Å for the N atom and 1.213 and 1.247 Å for the C(7) atom in the trans and cis compounds, respectively. The dihedral angle between the plane of the three-membered ring and the plane of the four carbon atoms in the pyrrolidine ring is 111° in the trans compound II and 109.5° in the cis compound I.

The conformation of the pyrrolidine ring with the N atom out of the plane is different than that found in all other pyrrolidine-related amino acids whose structures have been studied. In L-proline,[34] L-hydroxyproline,[35] copper proline,[36] and the prolyl residues in tosylprolylhydroxyproline,[37] leucylprolylglycine,[38] and *p*-bromocarbobenzoxy-Gly-L-Pro-L-Leu-Gly(OH),[39] it is C(4) which is 0.26–0.60 Å out of the plane of the other four atoms. In natural 3,4-dihydroxy-L-proline,[40,41] on the other hand, C(3) is the atom which is out of the plane.

The carboxyl group is axial to the ring in the trans acid II and in an intermediate position between axial

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Table III. Fractional Coordinates and Thermal Parameters[a] for *trans*-3,4-Methylene-L-proline

	x	y	z	B_{11}	B_{22}	B_{33}	B_{12}	B_{13}	B_{23}
N	0.1573	0.1334	0.0177	4.20	2.69	1.31	0.62	0.18	0.07
C(2)	0.1681	0.2232	0.2041	4.10	2.45	1.33	0.51	−0.12	−0.18
C(3)	0.0476	0.2777	0.2155	4.89	3.24	2.03	1.40	−0.09	0.12
C(4)	−0.0364	0.1995	0.0920	3.91	4.73	3.10	0.50	−0.33	0.42
C(5)	0.0314	0.0973	0.0009	4.94	3.40	3.17	−0.59	−0.08	−0.30
C(6)	0.1995	0.1660	0.4374	3.89	3.02	1.62	0.61	0.28	0.30
C(7)	−0.0063	0.3149	−0.0116	4.75	3.86	3.45	1.45	−1.00	0.45
O(1)	0.2142	0.2346	0.6058	6.91	3.38	1.35	−0.76	−0.21	0.05
O(2)	0.2034	0.0594	0.4464	8.60	2.89	1.93	0.20	−0.91	0.25
W	0.2416	0.4719	0.5700	14.02	3.59	4.62	−0.96	−2.30	0.06
				Standard Deviations					
N	0.0003	0.0002	0.0005	0.12	0.10	0.07	0.10	0.11	0.10
C	0.0003	0.0003	0.0007	0.18	0.17	0.13	0.15	0.17	0.19
O	0.0003	0.0002	0.0004	0.17	0.10	0.07	0.12	0.12	0.10
W	0.0004	0.0002	0.0007	0.30	0.13	0.13	0.18	0.24	0.15

[a] The thermal parameters are expressed in the form $T = \exp[-1/4(B_{11}h^2a^{*2} + B_{22}k^2b^{*2} + B_{33}l^2c^{*2} + 2B_{12}hka^*b^* + 2B_{13}hla^*c^* + 2B_{23}klb^*c^*)]$.

Table IV. Fractional Coordinates and Thermal Parameters for *cis*-3,4-Methylene-L-proline Hydrochloride

	x	y	z	B_{11}	B_{22}	B_{33}	B_{12}	B_{13}	B_{23}
N	0.3838	0.0001	0.8522	2.67	6.09	3.04	0.30	0.06	0.15
C(2)	0.4980	0.0695	0.7862	2.63	5.97	3.22	−0.86	−0.25	−0.44
C(3)	0.6247	0.0018	0.8218	2.19	7.57	4.54	−0.01	−0.80	−0.29
C(4)	0.5871	−0.0885	0.9552	2.60	8.18	4.69	1.20	−0.92	−0.98
C(5)	0.4377	−0.0754	0.9962	3.33	6.82	5.44	0.90	−0.08	0.73
C(6)	0.4741	0.0959	0.5879	2.98	5.94	4.07	−0.73	0.23	−0.09
C(7)	0.6198	−0.1139	0.7543	3.84	9.23	5.20	1.87	−0.35	−2.34
O(1)	0.3679	0.0784	0.5149	3.77	9.87	4.55	−2.28	−1.57	1.77
O(2)	0.5767	0.1459	0.5140	3.46	10.45	3.96	−2.54	0.42	0.09
W	0.5181	0.2174	0.2059	5.05	20.18	3.69	−4.44	−0.11	2.28
Cl^-	0.2242	0.1830	0.0649	2.85	5.76	4.01	0.60	0.59	1.04
				Standard Deviations					
N	0.0007	0.0007	0.0010	0.27	0.39	0.32	0.28	0.26	0.28
C	0.0009	0.0009	0.0014	0.35	0.58	0.49	0.36	0.35	0.48
O	0.0006	0.0007	0.0010	0.29	0.50	0.33	0.32	0.26	0.35
W	0.0008	0.0011	0.0010	0.38	1.05	0.35	0.57	0.32	0.52
Cl^-	0.0002	0.0002	0.0003	0.08	0.10	0.10	0.08	0.08	0.09

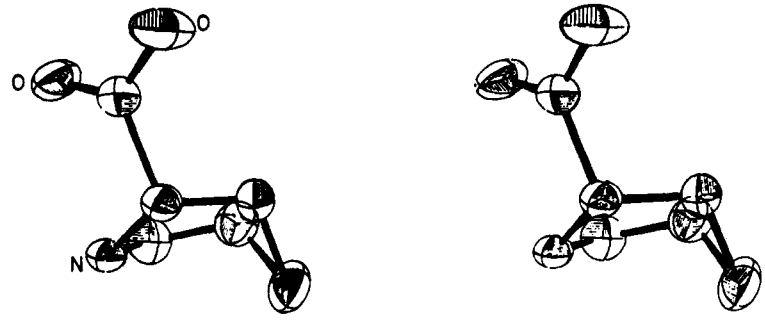


Figure 3. A stereodrawing of *trans*-3,4-methylene-L-proline. The ellipsoids are related to the thermal motion of the atoms and correspond to a 50% probability. The figure was drawn by a computer from a program by C. K. Johnson (Oak Ridge National Laboratory, Oak Ridge, Tenn.) and should be viewed with a three-dimensional viewer for printed stereophotographs.

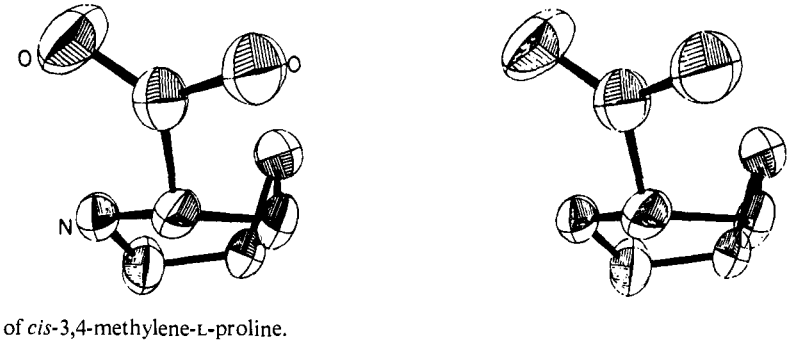


Figure 4. A stereodrawing of *cis*-3,4-methylene-L-proline.

and equatorial in the cis acid I. In each compound the N atom is near the plane containing the carboxyl group. The deviations of N from the plane are 0.07 and 0.21 Å in the trans and cis compounds, respectively, corre-

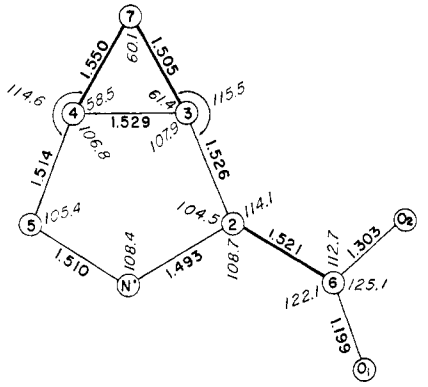


Figure 5. Bond lengths and angles of *cis*-3,4-methylene-L-proline (hydrochloride) identical with the natural product. The standard deviations for the bond lengths are 0.010–0.015 Å and for the bond angles ~0.9°.

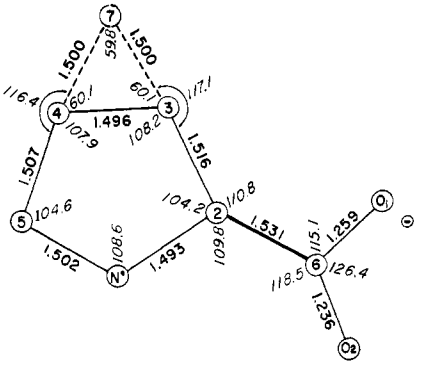


Figure 6. Bond lengths and angles of synthetic *trans*-3,4-methylene-L-proline. The standard deviations for the bond lengths are 0.005 Å and for the bond angles 0.3°.

sponding to torsion angles about the C(2)–C(6) bond of 3.5 and 9.5°.

Bond distances and angles are shown in Figures 5 and 6. The main difference in the two compounds are found in the carboxyl group. The free trans acid II is in the form of a zwitterion having a NH_2^+ group and a COO^- group in which there is little difference in the length of the two C–O bonds. On the other hand, the cis compound I exists as a cation in the crystal with a positive charge on the N atom and a –COOH group. In this case the C=O bond is more than 0.10 Å shorter than the C–OH bond. In the trans acid II the parameters for the moiety containing the N atom, the α and β carbon atoms, and the carboxyl ion agree very closely with the parameters derived by averaging the results of three-dimensional crystal-structure analyses of various amino acids.[42]

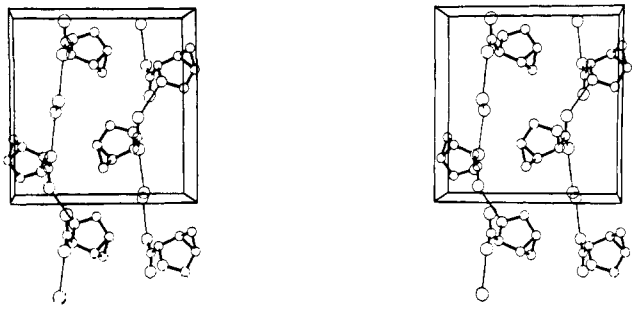


Figure 7. Stereodiagram showing the packing in the trans acid. The light lines represent hydrogen bonds. The directions of the axes are $a \rightarrow$, $b \uparrow$, and c out of the plane of the paper.

Organization of Crystal Lattice. Packing and hydrogen bonding for the trans acid are illustrated in Figures 7 and 8. There are four independent hydrogen bonds, listed in Table V, which link the acid molecules

Table V. Hydrogen Bonds

	Label	Donor	Acceptor	Symmetry operation on acceptor	Length, Å
trans-3,4-Methylene-L-proline[a]	A	N	O(2)	$1/2 - x, -y, -1/2 + z$	2.77
	B	N	O(1)	$x, y, -1 + z$	2.71
	C	W	O(1)	x, y, z	2.77
	D	W	W	$1/2 - x, 1 - y, 1/2 + z$	2.94
cis-3,4-Methylene-L-proline[b]	A	O(2)	W	x, y, z	2.51
	B	N	Cl^-	$x, y, 1 + z$	3.16
	C	N	Cl^-	$1/2 - x, -y, 1/2 + z$	3.27
	D	W	Cl^-	x, y, z	3.11
	E	W	Cl^-	$1/2 + x, 1/2 - y, -z$	3.11

[a] *Cf.* Figure 8. [b] *Cf.* Figure 9.

Parameters for the three-membered ring differ somewhat in the two compounds. Since the data were superior for the crystal of the trans compound, attention should be focused on the values shown in Figure 6. The three CCC angles are 60° and the three C–C bonds are 1.500 Å (within one standard deviation). These values can be compared to a C–C bond length of 1.510 Å in cyclopropane as determined by electron diffraction.[43] These values are smaller than the 1.53–1.54 Å usually observed for C–C bonds in *n*-alkanes.

(42) R. E. Marsh and J. Donohue, *Advan. Protein Chem.*, 22, 249 (1967).

(43) O. Bastiansen, F. N. Fritsch, and K. Hedberg, *Acta Crystallogr.*, 17, 538 (1964).

and the H_2O of crystallization into infinite sheets perpendicular to the *a* axis. Figure 8 illustrates a portion of one of these sheets. It is composed of single rows of H_2O molecules linked together by OH···O bonds, labeled D, interspersed by double rows of zwitterions linked by NH···O(1) and NH···O(2) bonds, labeled A and B. The rows of zwitterions and H_2O molecules are held together by OH···O bonds between the H_2O and C–O, labeled C.

The packing in *cis*-3,4-methylene-L-proline hydrochloride, illustrated in Figure 9, is dominated by the four hydrogen bonds to each Cl^- ion, labeled B, C, D, and E, and listed in Table V. The remaining hydrogen bond, A, between the OH of the –COOH group and the

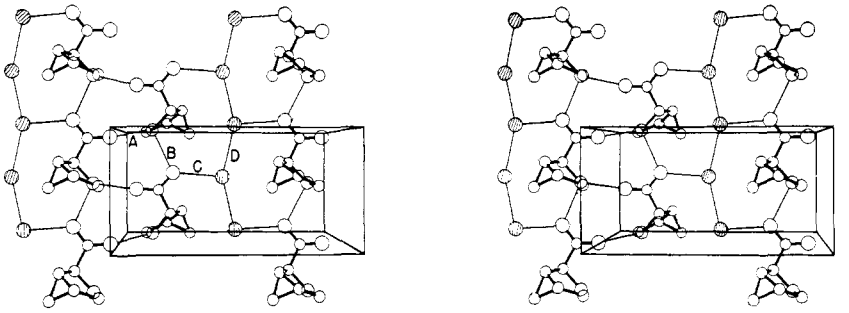


Figure 8. A view, with the axes rotated 90° from the orientation in Figure 7, showing the infinite sheets formed by hydrogen bonding. The axial directions are $b \rightarrow$, $c \uparrow$, and a out of the plane of the paper. The shaded molecules represent oxygen atoms of water of crystallization. A, B, C, and D signify the four independent hydrogen bonds (*cf.* Table V).

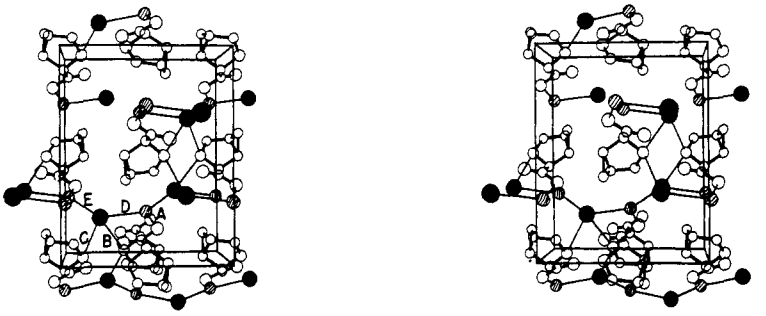
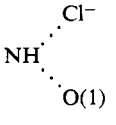


Figure 9. A stereodiagram of the packing of the *cis*-$C_6H_9NO_2 \cdot HCl \cdot H_2O$ crystal. One set of the five independent hydrogen bonds is labeled A, B, C, D, and E. The sizes of the circles depicting atoms increase in the order C, N, O, Cl. Axial directions are $a \rightarrow$, $b \uparrow$, and c out of the plane of the paper. The black circles signify Cl^- ions and the shaded circles are oxygen atoms of water of crystallization. The five independent hydrogen bonds A–E refer to Table V.

water molecule, has a length of only 2.51 Å, indicative of a very strong attraction. The three-dimensional bonding network is essentially composed of parallel Cl···HOH···Cl···HOH chains (bonds D and E), parallel to the a axis, with lateral bonds A, B, and C to the rows of organic molecules. There does not appear to be any hydrogen bonding between organic molecules. The distance between N of one molecule and O(1) of the neighboring molecule is 2.92 Å; however, the two protons on the N atom, assumed to be in tetrahedral positions with respect to the two C–N bonds, are directed toward two Cl^- ions and the geometry appears to be unfavorable for a bifurcated hydrogen bond



Under somewhat similar circumstances, such a bifurcated hydrogen bond appears to be possible in glycine hemihydrochloride[44] where the $-NH_3^+$ group has four near neighbors, three N···Cl at 3.13, 3.23, and 3.32 Å and one N···O at 2.90 Å.

Biochemical Consequences. There is a growing body of data on the stereochemical specificity of bacterial proline permease[45] on inhibitors of proline uptake and on competitive change of accumulated proline with proline analogs in various systems. The boat conformation of *cis*-3,4-methylene-L-proline must be connected with its properties as a powerful competitor for proline in several systems (*e.g.*, 85% inhibition for the permease system) while L-pipecolic acid, undoubtedly possessing a chair conformation, is inactive.

(44) T. Hahn and M. J. Buerger, *Z. Kristallogr., Krystallgeometrie, Kristallphys., Kristallchem.*, **108**, 419 (1957). See also J. Donohue, "Structural Chemistry and Molecular Biology," A. Rich and N. Davidson, Ed., W. H. Freeman, San Francisco, Calif., 1968, p 450.

(45) H. Tristram and S. Neale, *J. Gen. Microbiol.*, **50**, 121 (1968).

Experimental Section

Materials. The resin used for chromatography was Amberlite IR-120, particle size 47–65 μ. The column dimensions were 79 × 5.4 cm. The amount of resin was 1809 ml. Sodium citrate buffer, 0.2 *N*, pH 3.17, was used for elution and was pumped into the column with a Milton Roy mini-pump (Milton Roy Co., Philadelphia, Pa.). The automatic amino acid analyzer was from Phoenix Precision Instrument Co., Philadelphia, Pa.

The nmr spectra were obtained on 60-, 100-, and 220-MHz spectrometers (Varian Associates).

The 3,4-dehydro-L-proline (I), which was prepared according to Robertson and Witkop,[25] had an optical rotation, $[\alpha]^{20}D$ −318° (*c* 0.4, H_2O).

N-**Trifluoroacetyl-3,4-dehydro-L-proline Methyl Ester (IV).** To a mixture of 4.90 g of 3,4-dehydro-L-proline (III) and 30 ml of trifluoroacetic acid was added dropwise 7.0 ml of trifluoroacetic anhydride at −10°; it was stirred for 1 hr at room temperature. After evaporation of solvent *in vacuo*, the residual oil was dissolved in a small amount of methanol, and then treated with an excess of diazomethane to give *N*-trifluoroacetyl-3,4-dehydroproline methyl ester. When the evolution of N_2 gas had stopped, the reaction mixture was evaporated *in vacuo* and the residue was dissolved in ether. The ether solution was washed with saturated salt solution, dried over sodium sulfate, and evaporated under reduced pressure to give 8.0 g of *N*-trifluoroacetyl-3,4-dehydro-L-proline methyl ester (IV) as an oil: ir 5.68 (methyl ester), 5.88 (trifluoroacetyl), and 6.12 μ (double bond); nmr δ 3.75 (s, methyl proton), 4.58 (m, methylene proton), 5.26 (m, methine proton), 5.95 (m, olefinic proton).

cis- **and** *trans*-**3,4-Methylene-L-proline.** Excess diazomethane was bubbled through a magnetically stirred mixture of 4.5 g of *N*-trifluoroacetyl-3,4-dehydro-L-proline methyl ester and 0.5 g of anhydrous cuprous chloride at 0°[24] under nitrogen. At the end of the reaction, a small amount of ether was added and the cuprous

Table VI[a]

	R_f in solvents[b]										Distance,[c] mm	
	1	2	3	4	5	6	7	8	9	10		
											317	(proline as control)
Natural *cis*-3,4-methylene-L-proline	0.64	0.47	0.77	0.57	0.47	0.47	0.07	0.86	0.32	0.59	285	formic acid, pH 1.81
Synthetic *cis*-3,4-methylene-L-proline	0.64	0.47	0.77	0.57	0.47	0.47	0.07	0.86	0.32	0.59	285	4000 V, 160–218 mA
Synthetic *trans*-3,4-methylene-L-proline	0.68	0.59	0.77	0.62	0.51	0.52	0.10	0.79	0.36	0.45	279	2 hr, 15 min

[a] The tlc plates are Eastman Chromagram. [b] The solvents are: 1, methanol–pyridine–H_2O (20:1:5); 2, *n*-butyl alcohol–pyridine–H_2O (1:1:1); 3, ethanol–formic acid–H_2O (12:3:5); 4, *n*-propyl alcohol–formic acid–H_2O (75:5:20); 5, isopropyl alcohol–acetic acid–H_2O (75:1:24); 6, *n*-propyl alcohol–acetic acid–H_2O (15:1:4); 7, *tert*-amyl alcohol–acetic acid–H_2O (20:1:20); 8, chloroform–methanol–17% ammonia (2:2:1); 9, *n*-butyl alcohol–glacial acetic acid–H_2O (4:1:1); 10, *n*-propyl alcohol–34% ammonia (7:3). [c] High-voltage paper electrophoresis distance traveled toward the negative pole.

chloride was removed by filtration and washed with ether. The filtrate was concentrated under reduced pressure to give 4.5 g of an oil, which was dissolved in 40 ml of 2.0 *N* methanolic alkali (25:15) and stirred for 1.5 hr at room temperature. The mixture, after addition of 50 ml of water, was extracted twice with ether. The aqueous layer was acidified with 10% HCl and then freeze-dried. The residue was dissolved in a small amount of water and then absorbed on a column of Dowex 50W-X8 ion exchange resin (H^+ form). The resin was thoroughly washed with water to neutrality and then eluted with 2.0 *N* ammonia. The eluted fraction was evaporated to dryness under reduced pressure. The residue, which weighed 2.4 g (dried over P_2O_5), was dissolved in 10 ml of citrate buffer, pH 2.2. The automatic amino acid analyzer showed it to contain 10.6% *cis*-3,4-methyleneproline, 35.5% *trans*-3,4-methyleneproline, and 53.9% 3,4-dehydroproline.

Preparative Column Chromatography. One-half of the above solution was applied to a column of IR-120 as described previously. The pump was adjusted to deliver 106 ml/hr and the fraction collector adjusted to collect 8.0 ml/tube. Under these conditions, dehydro-L-proline was found in tubes 125–570, *cis*-3,4-methylene-L-proline in tubes 310–405, *trans*-3,4-methylene-L-proline in tubes 458–570. These cyclic amino acids were detected in the tubes by putting aliquots on a piece of filter paper and by developing the color with 0.25% ninhydrin in acetone. For more precise analysis and localization, especially in areas of overlap or tailing of the chromatogram, the amino acid analyzer and a small column, 0.9 × 25 cm, filled with 15 cm of A5 resin (Bio-Rad) was used with citrate buffer, 0.2 *N*, pH 3.17, as eluting agent.

The tubes containing the two diastereoisomeric methyleneprolines were pooled separately and desalted on a column of Dowex 50W-X8 in the hydrogen form. The amino acids which were eluted from the column with 7.0 *N* ammonia, after evaporation *in vacuo*, were recrystallized separately from alcohol–acetone–water (10:10:1). From four runs representing 4.8 g of the desalted mixture, 160 mg of *cis*-3,4-methylene-L-proline and 564 mg of *trans*-3,4-methylene-L-proline were obtained.

Synthetic *cis*-3,4-methylene-L-proline showed the following characteristics: $[\alpha]^{20}D$ −131° (*c* 1.0, H_2O); mp 235–245° (190–200°, crystalline transformation); ir 3430 (H–N^+<), 2300–2800 (–N^+–C<), and 1615 cm^{-1} ($-COO^-$); nmr (D_2O) (Figure 2) one-proton multiplet at δ 0.45 ($J_{3,2}$ = 4.4, $J_{3,4}$ = 4.5, $J_{3,3'}$ = 6.7 Hz, C-3 H), one-proton quartet at 0.76 ($J_{3',2}$ = 8.1, $J_{3',4}$ = 8.3 Hz, C-3′ H), one-proton multiplet at 1.80 ($J_{4,5}$ = 2.3 Hz, C-4 H), one-proton multiplet at 2.01 ($J_{2,1}$ = 4.5 Hz, C-2 H), one-proton doublet at 3.44 ($J_{5,4}$ = 2.3 Hz, C-5 H), one-proton doublet at 4.25 ($J_{1,2}$ = 4.5 Hz, C-1 H).

Anal. Calcd for $C_6H_9NO_2$: C, 56.68; H, 7.35; N, 11.02. Found: C, 56.16; H, 7.37; N, 11.26.

Synthetic *trans*-3,4-methylene-L-proline showed the following characteristics: $[\alpha]^{20}D$ −94° (*c* 1.0, H_2O); mp 245–250° (190–200°, crystalline transformation); ir 3435 (H–N^+<), 2400–2750 (>N^+–C<), 1620 ($-COO^-$), and 1460 cm^{-1}; nmr (D_2O) one-proton multiplet at δ 0.35 ($J_{3',2}$ = 4.4, $J_{3',4}$ = 4.5, $J_{3',3}$ = 6.7 Hz, C-3 H), one-proton quartet at 0.90 ($J_{3,2}$ = 8.1, $J_{3,4}$ = 8.3, $J_{3,3}$ = 6.7 Hz, C-3 H), one-proton multiplet at 1.77 ($J_{4,5}$ = 3.8 Hz, C-4 H), one-proton multiplet at 1.94 (C-2 H), one-proton doublet at 3.38 ($J_{5',5}$ = 11.3 Hz, C-5 H), one-proton quartet at 3.52 ($J_{5,4}$ = 3.8 Hz, C-5 H), one-proton singlet at 4.05 (C-1 H).

Anal. Calcd for $C_6H_9NO_2$: C, 56.68; H, 7.35; N, 11.02. Found: C, 56.26; H, 7.56; N, 10.44.

Isolation of Natural *cis*-3,4-Methylene-L-proline from the Seeds of *Aesculus parviflora*. In order to compare the natural amino acid directly with the synthetic compound 3,4-methylene-L-proline was isolated from fresh seeds of *Aesculus parviflora* by a slightly modified method.[1] The prepurified amino acid obtained from Dowex-1 X-8 column chromatography was obtained analytically pure by passage through a column of IR-120 with citrate buffer, pH 3.17, as eluting agent. The method parallels the separation technique used above. Extracts from 500 g of seeds yielded 722 mg of crystalline natural *cis*-3,4-methylene-L-proline; $[\alpha]^{20}D$ −144° (*c* 1.0, H_2O) (lit.[1] $[\alpha]^{29}D$ −132° (*c* 2.0, H_2O)). The ir and nmr spectra and the chromatographic mobility of the natural amino acid are the same as those of the synthetic cis compound (see Table VI).

***cis*-3,4-Methylene-L-proline Hydrochloride.** To 20 mg of the above natural *cis*-3,4-methylene-L-proline was added 1.0 ml of excess hydrochloric acid and the solution was evaporated to dryness *in vacuo* at room temperature. After drying in a vacuum desiccator over NaOH for several days, the hydrochloride was dissolved in a minimum amount of absolute alcohol, and a few drops of water was added until on warming the crystals went into solution. Ether was then added to beginning turbidity. After storage in the ice chest for several days the crystals were collected, washed with ether, and dried in a vacuum desiccator over P_2O_5 overnight. The colorless crystals had mp 219–221°. This hydrochloride was used for X-ray crystallographic studies.

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