A Reinvestigation of the Synthesis and Properties of *trans*-4,5-Dehydrolysine

Alvie L. Davis,[1a] Michael B. Cavitt,[1a] Tommy J. McCord,[1a] Paul E. Vickrey,[1b] and William Shive[1b]

Contribution from the Department of Chemistry, Abilene Christian College, Abilene, Texas, and the Clayton Foundation Biochemical Institute and the Department of Chemistry, The University of Texas, Austin, Texas.
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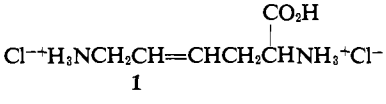
Abstract: *trans*-4,5-Dehydro-DL-lysine dihydrochloride was synthesized by the hydrolysis of ethyl *trans*-2-acetamido-2-carboethoxy-6-phthalimido-4-hexenoate in refluxing concentrated HCl and found to possess a marked resistance to lactonization under acidic conditions. Notwithstanding a recent claim to the contrary that this product is a diastereomeric mixture of 4-hydroxy-DL-lysine lactone dihydrochlorides, different lines of evidence based on structural, chromatographic, spectral (ir and nmr), and microbiological studies are presented to reaffirm the correctness of the dehydrolysine structure as originally formulated.

In 1961, the synthesis and microbiological activities of *cis*- and *trans*-4,5-dehydrolysines were first reported.[2] The structures of these two isomeric dehydrolysines were confirmed by elemental analyses and by catalytic hydrogenation to lysine, which was identified by paper chromatography and quantitative microbiological assay. The trans isomer in contrast to the cis isomer was highly active in inhibiting the growth of several lactobacilli, and these growth inhibitions were reversed competititively by lysine.

Some 10 years later, this method of synthesis was used by Christner and Rosenbloom[3] to obtain *trans*-4,5-dehydro-DL-lysine dihydrochloride (**1**) with homogeneity greater than 99.9% in order to demonstrate that it was incorporated into collagen. The resulting abnormal collagen contained much less 5-hydroxylysine and glycosylated 5-hydroxylysine than the natural collagen.[3]



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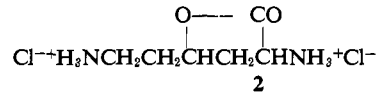

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Despite the seemingly strong evidence for the structure of **1** based upon the results of the biochemical studies, Hider and John[4] have recently asserted that our reported procedure involving an 18-hr hydrolysis of ethyl *trans*-2-acetamido-2-carboethoxy-6-phthalimido-4-hexenoate (**3**) in refluxing concentrated HCl does not produce **1** but instead forms a diastereoisomeric mixture of 4-hydroxy-DL-lysine lactone dihydrochlorides (**2**). From a rate study of the hydrolysis of **3** in refluxing 20% HCl, it is claimed that a product isolated in high yield after 4 hr of hydrolysis is **1** and that the 18-hr hydrolysis converts **1** almost quantitatively to **2**. The physical data and the synthesis of **2** by a different method appear to confirm its identification.[4]

However, the experimental results obtained with our preparations of *cis*- and *trans*-4,5-dehydrolysines cannot be ascribed to a lactone structure. For example, unlike the dehydrolysines, neither 4-hydroxylysines nor their corresponding lactones would be expected to yield lysine on catalytic hydrogenation.

In view of the apparent discrepancy that now exists between our experimental results and those obtained by Hider and John, we decided to reinvestigate our original preparation of **1** and to study its physical, chemical, and biological properties more extensively. Thus, the present paper describes the results of this further study of **1** and reaffirms our earlier work on its method of synthesis and its structural formulation.

No difficulty was experienced in performing the condensation reactions of *trans*-1,4-dichloro-2-butene with potassium phthalimide, followed by ethyl sodioacetamidomalonate since *N*-*trans*-(4-chloro-2-butenyl)-phthalimide and **3** were obtained in good yields as described in the Experimental Section. Thus, in contrast to the report of Hider and John,[4] the key intermediate, **3**, may be prepared conveniently without the necessity of changing the order of the condensation reactions.

Our method of hydrolyisis of **3** in refluxing concentrated HCl for 18 hr led to the formation of a single crystalline product. Even though the melting point of this product was higher than that reported previously,[2] its chromatographic R_f values, microbiological activities, and ir spectrum were identical with those of our original preparation. In particular, its carbonyl absorption appeared at the same frequency (1765 cm^{-1}) as that reported by Hider and John[4] for their 18-hr hydrolysis product using 20% HCl.

The structure of **1** for this product was confirmed again by hydrogenation to lysine and by ozonolysis to glycine and aspartic acid. In these chemical studies, lysine was isolated from the former reaction as the dihydrochloride salt which was identified by comparing its ir spectrum with that of an authentic sample; however, glycine and aspartic acid were identified in the ozonolysis mixture by paper chromatography in two different solvent systems.

(1) (a) Abilene Christian College; (b) The University of Texas.
(2) A. L. Davis, C. G. Skinner, and W. Shive, *J. Amer. Chem. Soc.*, **83**, 2279 (1961).
(3) P. J. Christner and J. Rosenbloom, *J. Biol. Chem.*, **246**, 7551 (1971).
(4) R. C. Hider and D. I. John, *J. Chem. Soc., Perkin Trans. 1*, 1825 (1972).

On examination of the ir spectra of **1** and related compounds, the dihydrochloride and monohydrochloride salts of *trans*-4,5-dehydrolysine, lysine, and ornithine show carbonyl absorptions at 1765, 1735, and 1745 cm^{-1} and 1625, 1625, and 1635 cm^{-1}, respectively. Thus, in accord with the spectral correlations of other investigators,[5,6] the carbonyl absorption frequencies of the dihydrochlorides of these diamino acids are higher than their corresponding monohydrochlorides because of the un-ionized carboxyl of the former compounds.

Unlike the general effect of diamino acid hydrochlorides on carbonyl absorption behavior, the ν(C=O) of saturated γ-lactones appear normally in the 1760–1780 cm^{-1} range independent of salt formation of their amino-substituted derivatives. For example, *threo*-4-hydroxy-L-lysine lactone dihydrochloride,[7] *erythro*-4-hydroxy-L-lysine lactone dihydrochloride,[7] and L-homoserine lactone hydrochloride show their carbonyl absorptions at 1783, 1775, and 1776 cm^{-1}, respectively.

It follows that the un-ionized carboxyl of the dihydrochloride salt of *trans*-4,5-dehydrolysine appears abnormal in its absorption at 1765 cm^{-1} which slightly overlaps the normal frequency range of γ-lactones. However, when this dihydrochloride was converted to its monohydrochloride salt, the shift in carbonyl absorption frequency to 1625 cm^{-1} which is quite characteristic for the ionized carboxyl of a diaminomonocarboxylic acid monohydrochloride provides evidence for the diaminomonocarboxylic acid structure of **1** in spite of its rather anomalous carbonyl absorption.

Moreover, the proton magnetic resonance data as recorded in the Experimental Section are consistent with the assignment of the 4,5-dehydrolysine structure (**1**) and do not correspond to a lactone structure. The spectra in D_2O provide the best data for decoupling experiments, and these results confirm unequivocally the structure of the 18-hr hydrolysis product as **1**.

In addition to these studies, the chromatographic R_f values of **1** were determined in four different solvents and its microbiological activities were examined in *Leuconostoc dextranicum* 8086. These properties were compared with those of *erythro*- and *threo*-4-hydroxy-L-lysine lactone dihydrochlorides and *threo*-4-hydroxy-L-lysine hydrochloride[7] under the same experimental conditions.

Although no chromatographic data were given, Hider and John[4] reported that the R_f values of the 18-hr hydrolysis product were identical with those of *threo*-4-hydroxy-L-lysine lactone dihydrochloride. Contrary to their report, paper chromatograms in 65% pyridine showed that the 0.33 R_f value of *trans*-4,5-dehydrolysine differs markedly from the 0.74 and 0.70 values of *erythro*- and *threo*-4-hydroxy-L-lysine lactones, respectively.

Again, our preparation of **1** was found to inhibit the growth of *L. dextranicum* at a concentration level of 0.2 μg/ml, and this growth inhibition is reversed competitively by lysine over a broad range of concentrations. This preparation of the racemic form of **1** is 30 times more effective than either *threo*-4-hydroxy-L-lysine or its lactone and 50 times more effective than *erythro*-4-hydroxy-L-lysine lactone as a growth inhibitor of *L. dextranicum*. Since only the L form of amino acids would be expected to be biologically active, it is apparent that the growth-inhibiting properties of **1** cannot be accounted for on the basis of those exhibited by the lactones.

The cumulative evidence based on these physical, chemical, and microbiological studies substantiates the structural formulation of our 18-hr acid hydrolysis product as *trans*-4,5-dehydro-DL-lysine dihydrochloride and refutes the recent claim that the product prepared by our synthetic procedure is a diastereomeric mixture of 4-hydroxy-DL-lysine lactone dihydrochlorides.

The general metabolic importance of *trans*-4,5-dehydro-DL-lysine stems from its use as a lysine antagonist for inhibition studies[2] and its incorporation into proteins.[3,8] Consequently, this reinvestigative study is of considerable importance to these early biochemical studies since the results were interpreted on the basis of the dehydrolysine structure.

Experimental Section

General. Melting points were determined on a Thomas-Hoover capillary melting apparatus and are uncorrected. Infrared spectra were recorded on a Beckman Model IR-10 spectrophotometer (KBr) and were calibrated with polystyrene film. Nmr spectra were recorded on a Varian Model A60 spectrometer at 60 MHz unless otherwise stated. Chemical shifts are given in τ units downfield from an internal TMS standard. Ozonolysis was performed with a OREC Model 341 ozonator. Microanalyses were performed by M-H-W Laboratories, Garden City, Mich.

Microbiological Assays. For *L. dextranicum* 8086 a similar assay procedure was used as described previously.[2] In all assays the amount of growth was determined photometrically at 625 mμ with a Bausch and Lomb spectrophotometer in terms of absorbance readings of the turbid culture medium against a blank of uninoculated medium set at 0 absorbance.

***N*-(*trans*-4-Chloro-2-butenyl)phthalimide.** This procedure was patterned after a previously reported method[2] for the conversion of *trans*-1,4-dibromo-2-butene to *N*-(*trans*-4-bromo-2-butenyl)-phthalimide with some modifications. To 32.9 g of *trans*-1,4-dichloro-2-butene was added in small increments 23.5 g of potassium phthalimide with frequent shaking. During the addition period and for an additional 2 hr the reaction mxture was heated in an oil bath at 145–150°. The reaction mixture was cooled and extracted with 700 ml of ether. The ether layer was separated and then reduced in volume to yield a precipitate. The latter was filtered, recrystallized from ethanol, and dried to yield 18.2 g (61%) of product, mp 100–103°.

Anal. Calcd for $C_{12}H_{10}ClNO_2$: C, 61.16; H, 4.28; N, 5.94. Found: C, 61.27; H, 4.08; N, 6.19.

Ethyl *trans*-2-Acetamido-2-carboethoxy-6-phthalimido-4-hexenoate (3). In a manner similar to that previously reported[2] for this compound, a 9.6-g sample of *N*-(*trans*-4-chloro-2-butenyl)-phthalimide was reacted with an equivalent amount of ethyl sodioacetamidomalonate in 100 ml of Mg-dried ethanol under reflux for 2 hr. The NaCl was removed by filtration, and the filtrate was stored at −15° for 30 min to form a precipitate. Recrystallization from ethanol of the latter precipitate gave 12.9 g (76%) of product, mp 127–129° (lit.[2] mp 126–127°).

***trans*-2,6-Diamino-4-hexenoic Acid (*trans*-4,5-Dehydrolysine) Dihydrochloride (1).** In a manner similar to that previously reported,[2] a mixture of 3.5 g of **3** and 25 ml of concentrated hydrochloric acid was heated under reflux for 18 hr. Paper chromatography studies of the reaction mixture in *n*-BuOH–AcOH–H_2O (3:1:1) revealed strong ninhydrin spots (R_f values of 0.65 and 0.14) after 5 hr reflux, and only the lower R_f value (0.14) after 18 hr reflux. The reaction mixture was chilled at 2–4° overnight, the

(5) L. J. Bellamy, "The Infra-red Spectra of Complex Molecules," 2nd ed, Wiley, New York, N. Y., 1957, p 242.

(6) J. P. Greenstein and M. Wintz, "Chemistry of the Amino Acids," Vol. II, Wiley, New York, N. Y., 1961, p 1714.

(7) N. Izumiya, Y. Futjita, F. Irreverre, and B. Witkop, *Biochemistry*, **4**, 2501 (1965).

(8) E. M. Lansford, Jr., N. M. Lee, and W. Shive, *Arch. Biochem. Biophys.*, **119**, 272 (1967).

phthalic acid was removed by filtration, and the filtrate was reduced to dryness *in vacuo*. Absolute ethanol (10 ml) was added to the residue and evaporated *in vacuo* to remove the excess HCl. Addition of another 10 ml of ethanol and filtration gave 1.59 g (87%) of **1**, mp 201–205°.[9] When spotted on paper, **1** gave a yellow ninhydrin reaction which gradually darkened to purple on standing as observed for our former product.[2] The rate of color change appears to decrease with increasing concentrations of **1**. When the paper spotted with **1** was treated with pyridine followed by spraying with ninhydrin, a greenish-grey spot appeared initially which turned to purple on standing. The ir spectrum showed major absorption bands at 3000 (broad), 1765, 1480, 1450, 1400, 1190, 1120, and 975 cm^{-1}; nmr (D_2O) τ 4.07 (m, 2 H (H^a)), 5.75 (t, J = 6 Hz, 1 H (H^b)), 6.30 (m, 2 H (H^c)), 7.20 (m, 2 H (H^d)); (CF_3COOH) τ 2.30 (3 H), 2.72 (3 H), 3.80 (2 H), 5.35 (1 H), 6.01 (2 H), 6.85 (2 H).

The structure, $H_2NCH^c_2CH^a{=}CH^aCH^d_2CH^b(NH_2)COOH \cdot 2HCl$, was confirmed by decoupling (100 MHz, D_2O). Irradiation at τ 4.07 (H^a) produced the following: τ 5.75 (H^b, t), 6.30 (H^c, s), 7.20 (H^d, d). Irradiation at τ 5.75 (H^b) gave τ 4.07 (H^a, m), 6.30 (H^c, m), 7.20 (H^d,d). Irradiation at τ 6.30 (H^c) gave τ 4.07 (H^a, m), 7.20 (H^d, m). Irradiation at τ 7.20 (H^d) gave τ 4.07 (H^a, m), 5.75 (H^b, s) 6.30 (H^c, m).

Anal. Calcd for $C_6H_{12}N_2O_2 \cdot 2HCl$: C, 33.19; H, 6.50; N, 12.90. Found: C, 32.97; H, 6.47; N, 12.76.

***trans*-4,5-Dehydrolysine Monohydrochloride.** A 100-mg sample of **1** in 5 ml of 95% ethanol was passed through a column of 1.0 g of alumina (Alcoa F-20). The column was eluted with 150 ml of 95% ethanol and the eluent of approximately pH 6 was reduced in volume *in vacuo* to about 25 ml and precipitation occurred. Filtration and drying of the resulting precipitate gave 32 mg of product, mp 238–239°, ν_{max}^{KBr} 1625 cm^{-1} (acid, C=O).

Anal. Calcd for $C_6H_{12}N_2O_2 \cdot HCl$: C, 39.89; H, 7.25; N, 15.51. Found: C, 39.64; H, 7.43; N, 15.34.

Catalytic Hydrogenation of *trans*-4,5-Dehydrolysine. A 25-mg sample of **1** in 10 ml of H_2O was hydrogenated at 3.52 kg/cm^2 H_2 pressure in the presence of Pd black for 1 hr. The catalyst was removed by filtration, and the volume of the solution was reduced *in vacuo* to dryness. The resulting residue was washed with a small amount of ethanol and filtered to give 20 mg of product. The latter compound was unequivocally identified by melting point, R_f values, and ir spectral analysis as lysine dihydrochloride with an authentic sample prepared by treatment of lysine hydrochloride with HCl.

As a control experiment, *threo*-4-hydroxy-L-lysine lactone dihydrochloride was subjected to the same conditions of catalytic hydrogenation. Paper chromatograms of this hydrogenation mixture in *n*-BuOH–AcOH–H_2O (3:1:1) and 65% pyridine showed no detectable lysine.

Ozonolysis of *trans*-4,5-Dehydrolysine. A 20-mg sample of **1** in a solution of 10 ml of glacial acetic acid and 3 ml of H_2O was treated with a gaseous mixture of ozone–oxygen (approximately 40% O_3:60% O_2) for a period of 1 hr. Then, 0.5 ml of concentrated HCl was added, and the solution was heated on a steam cone for an additional hour Paper chromatograms of the reaction mixture gave R_f values of 0.40 and 0.28 in PhOH saturated with H_2O and 0.33 and 0.17 in *t*-BuOH–MeCOEt–H_2O–NH_4OH (40:30:20:10) corresponding to glycine and aspartic acid, respectively.

Chromatographic and Biological Data. The *trans*-4,5-dehydrolysine was compared with related compounds in terms of activity in inhibiting growth of *L. dextranicum* 8086 and R_f values on paper chromatograms developed in four different solvents. The data in Table I were obtained.

Table I

Compd	Inhibitory activity μg/ml[a]	R_f[b] A	B	C	D
trans-4,5-Dehydro-DL-lysine·2HCl	0.2	0.14	0.33	0.47	0.25
threo-4-Hydroxy-L-lysine lactone·2HCl	6.0	0.10	0.70	0.48	0.26
erythro-4-Hydroxy-L-lysine lactone·2HCl	10.0	0.09	0.74	0.48	0.23
threo-4-Hydroxy-L-lysine·HCl	6.0	0.09	0.28	0.44	0.22
DL-Lysine·2HCl		0.13	0.26	0.51	0.29

[a] Minimal amount of compound required for complete inhibition of growth of *L. dextranicum* in a medium devoid of lysine. [b] Solvent systems: A, *n*-BuOH–AcOH–H_2O (3:1:1); B, 65% pyridine; C, PhOH saturated with H_2O; D, *t*-BuOH–MeCOEt–H_2O–NH_4OH (40:30:20:10).

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(9) The final stage of the work-up procedure of the original preparation was different and gave a less crystalline product, mp 180–185°.