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7. Supported by a grant from the National Research Council of Canada. We thank Monsanto (Canada) Ltd. for 1,2,dihydro-6-ethoxy-2,2,4-trimethylquinoline (Santoquin) and Hoffman-La Roche Ltd., Montreal, for *dl*-alpha-tocopheryl acetate used in the study.

11 March 1969

3,4-Dihydroxyproline: A New Amino Acid in Diatom Cell Walls

Abstract. *An analog of proline, 2,3-*cis*-3,4-*trans*-3,4-dihydroxy-*L*-proline, was found in the cell walls of the eight species of diatoms studied and was isolated from the proteinaceous material of the wall of* Navicula pelliculosa. *The properties of this substance are described; its structure was confirmed by nuclear magnetic resonance spectroscopy.*

The mature wall of the diatom is composed of a siliceous shell tightly enclosed by, and interlocked with, an organic casing (*1*) of an unusual and complex chemical structure. In addition to the known amino acids and a number of sugars, the cell wall contains a great many as yet unidentified compounds positive to the ninhydrin test, sugars and uronic acids (*2*) which constitute proteinaceous components, and a number of distinctive polysaccharides, several of which are sulfated (*2*). We describe here the isolation and characterization of one of the ninhydrin-positive compounds.

For the isolation of the compound, dried cell wall material (50 g) was hydrolyzed with 6*N* HCl in an atmosphere of nitrogen for 24 hours at 100°C. The hydrolyzate was evaporated at reduced pressure; the residue, dissolved in water, was passed through Dowex AGW 50-X4 ion-exchange column (hydrogen form, 20 to 50 mesh) and eluted with 2*N* aqueous ammonia. The eluate was concentrated at reduced pressure and passed through Dowex AG 2-X8 ion-exchange column (acetate form, 200 to 400 mesh) at 45°C; the column was washed with water and then with 0.1*N* acetic acid. Acidic amino acids were retained, and at this stage the eluate contained basic and neutral amino acids. These amino acids were separated by ion-exchange chromatography on Dowex AGW 50-X4 column (pyridinium form, 200 to 400 mesh) at 45°C, buffered at *p*H 3.1 with a solution (0.1*M*) of pyridine and formic acid (*p*H 3.1) (*3*). The new amino acid was eluted between methionine sulfoxide and threonine.

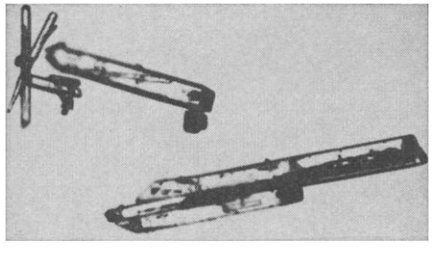

Fig. 1. Photomicrograph of crystals of 2,3-*cis*-3,4-*trans*-3,4-dihydroxy-L-proline (from aqueous ethanol) (× 146).

The eluate was concentrated under reduced pressure and the residue (45 mg), dissolved in aqueous ethanol, yielded 36 mg of crystals (fine prisms) (Fig. 1), melting at 262°C with decomposition. The specific rotation $[\alpha]_D^{20}$ was −61.2° at a concentration of 0.5 percent in H_2O (*4*). Analysis showed the values for $C_5H_9NO_4$ were: C, 40.81; H, 6.04; and N, 9.53 percent. The calculated values were: C, 40.82; H, 6.17; and N, 9.52 percent. As judged by conformity to the rule of Lutz-Jirgensons (*5*), the compound has L configuration.

Reaction with ninhydrin on paper gave a yellow color which turned brownish-yellow when heated over 100°C. When the paper was sprayed with isatin solution (*6*) a white spot appeared against the yellowish background, producing a white fluorescence under ultraviolet light. This reaction is similar to that of 3-substituted proline (*7*).

Thin-layer chromatography on cellulose sheets (*8*) in a number of solvents gave the following R_F values: solvent A, 0.27; solvent B, 0.25; solvent C, 0.24. In the amino acid analyzer (*9*) the peak due to the compound appeared 116 ml ahead of methionine sulfoxide; this position is similar to that of *trans*-3-hydroxyproline (*10*). The new amino acid showed a ninhydrin color at a maximum absorption of 440 nm, similar to that of proline-analog compounds.

Because a nitroso derivative, obtained by treatment with nitrous oxide, was converted to the original compound by hydrolysis (*11*), the new compound appears to be a cyclic imino acid.

The structure and stereochemistry of the new amino acid was elucidated by nuclear magnetic resonance (NMR) spectroscopy (*12*). The compound did not show a chemical shift contributed by the 3- or 4-methylene group in the pyrrolidine ring (Fig. 2) and hence appears to be a 3,4-dihydroxyproline. Comparison of the NMR spectrum with that of 3-hydroxyprolines (*13*) and 4-hydroxyprolines (*14*) allows no alternative assignment.

We concluded that, of the four isomers of 3,4-dihydroxyproline, the structure of the new amino acid is 2,3-*cis*-3,4-*trans*-3,4-dihydroxyproline (Fig. 3); two of the other isomers have been synthesized (*15*). This was confirmed by mass spectroscopy and x-ray diffraction analysis (*16*).

Analysis with paper chromatography (*17*) and the amino acid analyzer showed that the new compound is also present in acid hydrolyzates of the walls of seven marine diatom species of differing physiological characteristics and

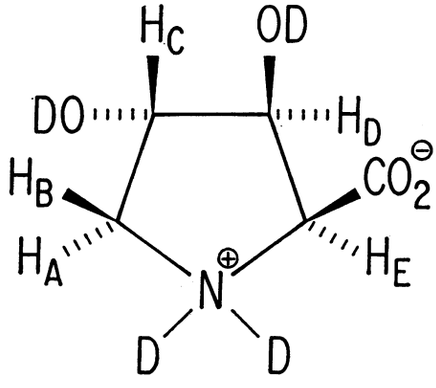

Fig. 2. Assignment of NMR data for 2,3-*cis*-3,4-*trans*-3,4-dihydroxyproline. H_A = 3.36 parts per million (1 proton, doublet) $J_{AB} = 13$ hz; $H_B = 3.72$ ppm (1 proton, double doublet) $J_{BC} = 3.5$; $H_C = 4.42$ ppm (1 proton, doublet) $J_{AC} \sim 0.7$; $H_D = 4.48$ ppm (1 proton, doublet) $J_{CD} \sim 1$; $H_E = 4.39$ ppm (1 proton, doublet) $J_{DE} = 4$.

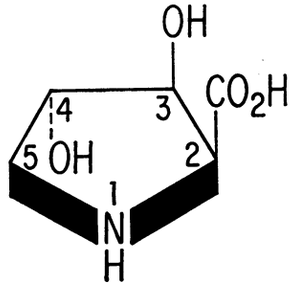

Fig. 3. Structure of 2,3-*cis*-3-4-*trans*-3,4-dihydroxyproline.

morphological complexity. These are *Navicula incerta, Nitzschia angularis, Nitzschia thermalis* (thermophile), *Nitzschia alba* (colorless), *Cylindrotheca fusiformis, Cyclotella cryptica,* and *Phaeodactylum tricornutum* (fusiformis and oval) (*18*).

The occurrence of 3,4-dihydroxyproline in the proteinaceous components of the diatom wall suggests that the new amino acid may be a determinant of the molecular structure of the organic matrix associated with silicification, as are hydroxyprolines in collagen.

TADASHI NAKAJIMA
B. E. VOLCANI
Scripps Institution of Oceanography, University of California, San Diego, La Jolla 92037

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2,3-cis-3,4-trans-3,4-Dihydroxy-L-proline: Mass Spectrometry and X-ray Analysis

Abstract. *Mass spectrometry showed the new amino acid from diatom cell walls to be a dihydroxyproline. X-ray analysis of the unmarked free amino acid by the use of the symbolic addition procedure revealed the complete three-dimensional structure as 2,3-*cis*-3,4-*trans*-3,4-dihydroxy-L-proline with carbon number 3 being 0.06 angstrom above the plane of the pyrrolidine ring.*

The preceding report (*1*) describes the isolation of a new amino acid from the cell walls of various diatoms. On the basis of nuclear magnetic resonance (NMR) spectrometry, mass spectrometry, and complete x-ray analysis, the structure of 2,3-*cis*,3,4-*trans*-3,4-dihydroxy-L-proline has been proposed.

The mass spectra of a synthetic mixture of the two *cis*-3,4-glycols obtained by permanganate oxidation of 3,4-dehydro-DL-proline (*2*) and of the new natural amino acid (*1*) were compared on a Hitachi RMU-6D mass spectrometer at 70 ev and 350° to 400°C. The spectra were virtually identical. Both compounds exhibited a large $M+1$ peak at m/e (mass to charge) = 148. The exact mass of this peak for the naturally occurring amino acid confirmed its identity as a 3,4-dihydroxyproline. Fragmentation of both compounds, by loss of the carboxyl group, led to a base peak at $m/e = 102$. Tentative formulations for the other major fragments at $m/e = 69$ and 87 are given in Fig. 1.

A single crystal of the new amino acid was subjected to an x-ray diffraction analysis. A total of 611 independent data were collected with Cu radia-

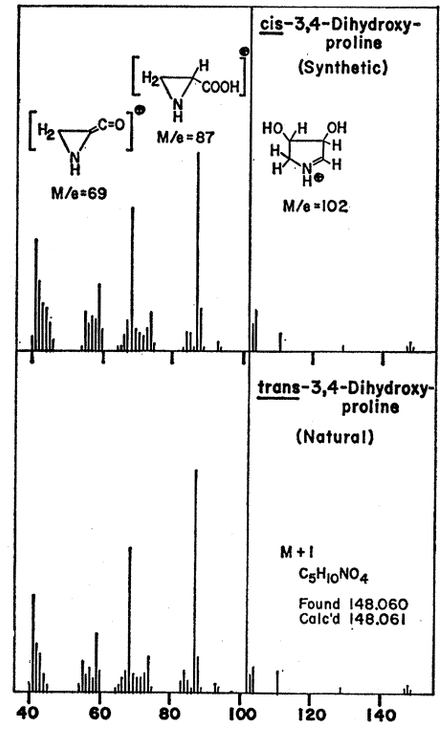

Fig. 1. Comparison of the mass spectra of the *cis*-glycols prepared from 3,4-dehydro-DL-proline by oxidation with permanganate (*1*) and of the *trans*-glycol of L-proline from diatom cell walls.

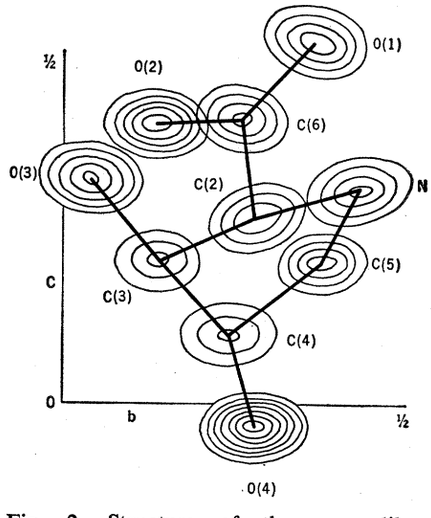

Fig. 2. Structure of the new dihydroxyproline from diatoms as determined by taking sections through maximum densities of the initial three-dimensional E-map computed with the phases directly derived from the intensities.

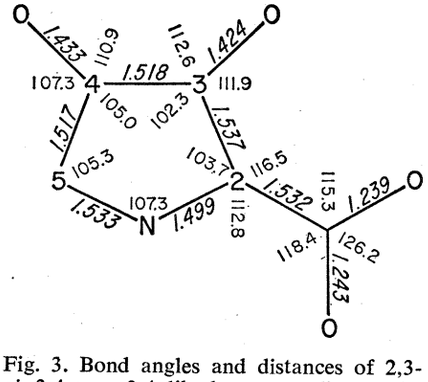

Fig. 3. Bond angles and distances of 2,3-*cis*-3,4-*trans*-3,4-dihydroxy-L-proline, the new natural amino acid from diatoms.