Proc. Natl. Acad. Sci. USA
Vol. 77, No. 5, pp. 2370–2371, May 1980
Chemistry

2,3-*trans*-3,4-*trans*-3,4-Dihydroxy-L-proline: An amino acid in toxic peptides of *Amanita virosa* mushrooms

(mushroom toxins/virotoxins/dihydroxyproline)

A. BUKU*, H. FAULSTICH*†, T. WIELAND*, AND J. DABROWSKI‡

Max-Planck-Institut for Medical Research, *Abteilung Naturstoff-Chemie; and ‡Abteilung Organische Chemie, Jahnstrasse 29, 6900 Heidelberg, West Germany

Communicated by Bernhard Witkop, January 17, 1980

ABSTRACT Among the four possible stereoisomers of 3,4-dihydroxy-L-proline, 2,3-*trans*-3,4-*trans*-3,4-dihydroxy-L-proline (IV) had not been found in nature previously. It has now been detected as a component of virotoxins, toxic peptides of *Amanita virosa* mushrooms. Because periodate failed to effect an oxidative glycol splitting reaction, the two hydroxyl groups in positions 3 and 4 were expected to be in a *trans* configuration. Furthermore, the formation of a 4-lactone on treatment with acids pointed to the carboxyl group and the hydroxyl group at position 4 being in a *cis* configuration. These results are in agreement with structure IV only. Final proof for structure IV was given by NMR spectroscopy and direct comparison with the 2,3-*cis*-3,4-*trans*-3,4-dihydroxy-L-proline isomer.

Virotoxins are toxic components of the poisonous mushroom *Amanita virosa* (1), a white species (2) not very common in Europe but rather frequent in the United States.[§] After hydrolysis of a mixture of viroidin and viroisin with 70% (wt/vol) perchloric acid (4), among other amino acids a compound was isolated by ion-exchange chromatography that showed a yellow color reaction with ninhydrin, characteristic for α-imino acids. However, the compound was identical neither with proline nor with hydroxyproline or allohydroxyproline, amino acids that occur in toxic peptides of *A. phalloides* mushrooms (2). Therefore the unknown imino acid was isolated from a hydrolysate on a micropreparative scale.

EXPERIMENTAL PROCEDURES

Isolation of the Imino Acid. A solution of 100 mg of the virotoxin mixture in 5.0 ml of 70% perchloric acid was kept at 120°C for 2 hr (4). The hydrolysate was neutralized under cooling with 6 M KOH and stored at 0°C for 1 hr. The precipitate of $KClO_4$ was removed by centrifugation, and the brown supernatant was evaporated under reduced pressure.

For separation by ion-exchange chromatography the residue was dissolved in a small volume of pyridine/formic acid buffer at pH 3.1 (5) and passed at 55°C through a column of Amberlite IR-120/AS, 35–45 mesh, H^+-form, 1.1 cm in diameter and 40 cm high. The eluate was checked by ninhydrin reaction after thin-layer chromatography on silica gel with a mixture of ethanol and water in a volume ratio of 7:3. The fractions containing the imino acid after evaporation yielded 8.5 mg of the wanted substance. R_F with the 7:3 mixture: 0.52; reaction with ninhydrin: yellow; $[\alpha]_D^{26} = -45°$ (1% in water), less negative after addition of one drop of concentrated hydrochloric acid (6).

Analytical Reactions. *Dansyl derivative.* A few milligrams of the solid was dissolved in a small volume of sodium bicarbonate solution and reacted with a drop of dansyl chloride in acetone in the usual manner (e.g., see ref. 7). After extraction of unreacted dansyl chloride with ether, the solution was acidified (pH $\approx$ 2) and extracted with ethyl acetate. The organic solvent was evaporated, and the residue was subjected to mass spectrometry. The dansyl derivative exhibited a molecular ion M^+ with a mass-to-charge ratio of 380.93.

Oxidation with sodium periodate. A few milligrams of secoviroisin, a linear heptapeptide obtained from viroidin by mild acidolysis,[¶] was dissolved in a few drops of water. The neutral solution was mixed with a few drops of a 1% aqueous solution of $NaIO_4$ and after 2 hr freed from inorganic salt by chromatography on Sephadex LH-20 in 0.004 M NH_4OH as a solvent. The eluate fractions on total hydrolysis showed the unchanged imino acid as a component in thin-layer chromatography, as did a nonoxidized control.

Paper Electrophoresis. The imino acid isolated from viroidin hydrolysate (see above) migrated in buffer of pH 1.9 (acetic acid/formic acid) at 1000 V and 40 mA 97 mm toward the cathode in 90 min. A sample of 2,3-*cis*-3,4-*trans*-3,4-dihydroxyproline (5) under the same conditions migrated 108 mm. In a hydrolysate of viroidin, prepared with 6 M hydrochloric acid for 10 hr at 110°C, subjected to paper electrophoresis at pH 6.5 (pyridine/acetic acid) (where neutral amino acids do not move) a cathodic band was detected that gave a yellow coloration with ninhydrin.

RESULTS AND DISCUSSION

Structure Derivation from Analytical Data. The molecular weight of 380.93 for the dansyl compound corresponds to a molecular formula of $C_{17}H_{20}N_2O_6S$ (calc. 380.104), yielding $C_5H_9NO_4$ for the parent compound. This is in accordance with a dihydroxyproline.

Among the great number of possible stereoisomers of dihydroxyprolines, those containing one of the hydroxyl groups in the 2 or in the 5 position could be ruled out because such hemiaminal compounds would decompose under the conditions

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

† To whom reprint requests should be addressed.

§ As a historical footnote, the senior author (T.W.) was Fogarty Scholar in Residence at the National Institutes of Health in the fall of 1974. On a weekend visit to the mountain retreat of Bernhard Witkop near the Sleepy Creek Hunting Preserve, the specimens of *Amanita virosa* were discovered and collected; they were freeze-dried and shipped to Heidelberg. Allohydroxy-L-proline had been isolated from the hydrolysate of phalloidin in the laboratory of Heinrich Wieland (1877–1957) through Bernhard Witkop (3).

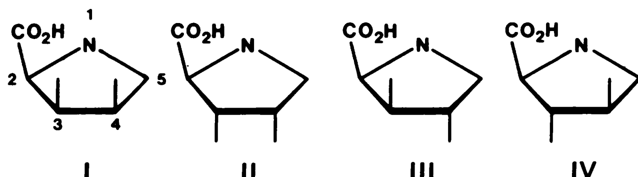
¶ H. Faulstich, A. Buku, H. Bodenmüller, and T. Wieland, unpublished.

Table 1. Proton chemical shifts (δ) and coupling constants (*J*) for 2,3-*cis*-3,4-*trans*-3,4-dihydroxy-L-proline (III) and 2,3-*trans*-3,4-*trans*-3,4-dihydroxy-L-proline (IV)

III				IV			
H	δ, ppm	*J*, Hz		H	δ, ppm	*J*, Hz	
2	3.972	2, 3 (*cis*)	4.0	2	4.066	2, 3 (*trans*)	1.5
3	4.054	3, 4 (*trans*)	1.3	3	4.520	2, 5b (long range)	0.7
4	4.008	3, 5a (long range)	0.6	4	4.306	3, 4 (*trans*)	1.3
5a	3.027	4, 5a (*trans*)	0.9	5a	3.603	3, 5b (long range)	1.1
5b	3.366	4, 5b (*cis*)	4.0	5b	3.519	4, 5a (*cis*)	3.7
		5a, 5b (*gem*)	12.9			4, 5b (*trans*)	1.4
						5a, 5b (*gem*)	13.2

Chemical shifts are measured from internal sodium 3-trimethylsilylpropionate. The spectra were measured in ^2H_2O (1.5 mg in 0.5 ml) at 298 K on a Bruker HX-360 NMR spectrometer, using 16,000 data points of the Bruker Aspect 2000 computer for 2200-Hz spectral width. A Lorentzian-to-Gaussian transformation (10–12) was applied for resolution enhancement.

of acidic hydrolysis. Rather, the hydroxyl groups had to be expected in positions 3 and 4, with four possible configurations (I–IV; a straight line in the formulas indicates a OH group) if only L-prolines are considered. The L configuration was established according to the rule of Lutz and Jirgenson (6) by finding a positive difference between the specific rotation values in hydrochloric acid and water.

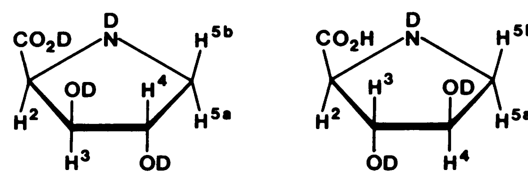


An exclusion of two from the four isomers could be made by the result of periodate oxidation. Glycol splitting with this reagent proceeds via a cyclic periodic diester and occurs only with *cis* glycols. Because this degradation experiment failed, the two *cis* glycols I and II could be ruled out.

An indication for structure IV as the correct one came from our observation that under the conditions of hydrolysis with 6 M hydrochloric acid a substance was formed that gave a yellow color reaction with ninhydrin and moving to the cathode in paper electrophoresis at nearly neutral pH. Because this behavior has been observed for *cis*-4-hydroxyproline, we favored structure IV over III, in which β-lactone formation involving the *cis*-3-hydroxyl is *a priori* less likely under these conditions [β-lactones of a *cis*-3-hydroxyproline derivative and analogues do exist (8)].

Of great value for definitively distinguishing between structures III and IV was an authentic sample of III, kindly provided by B. Volcani. This imino acid is a component of diatom cell wall protein and its structure had been established by x-ray analysis (9). As described in *Experimental Procedures*, we found on high-voltage paper electrophoresis at pH 1.9 that the unknown imino acid migrated 10% slower than compound III. Also, when compound III was subjected to the lactonization experiment mentioned above, no imino lactone was formed. Finally, compound III and the unknown differed in their ^1H NMR spectra, providing strong evidence that the unknown imino acid was not III but was its diastereomer.

Structural Proof by NMR Spectroscopy. Direct proof for structure IV was obtained by a complete analysis of the ^1H NMR spectrum, with the aid of decoupling experiments. The spectral parameters obtained at 360 MHz and listed in Table



D, deuterium

1 were confirmed by spectrum simulation using the Bruker SIMITE program (10) based on the LAOCN3 algorithm (11).

In compound III, the two *cis* vicinal coupling constants are characterized by magnitudes of 4.0 Hz, in contrast to those for the two *trans* vicinal couplings, which are characteristically smaller (0.9 and 1.3 Hz). The stereoisomer IV has only one *cis* vicinal coupling, and its value ($J_{4,5a}$, 3.7 Hz) is in harmony with the *cis* couplings in III. The other three vicinal couplings in IV must all be *trans*, and their observed magnitudes (1.3–1.5 Hz) confirm the 2,3-*trans*-3,4-*trans* relative configuration of our compound.

1. Faulstich, H., Georgopoulos, D., Bloching, M. & Wieland, T. (1974) *Z. Naturforsch.* **29c**, 86–88.
2. Wieland, T. (1968) *Science* **159**, 946–952.
3. Wieland, H. & Witkop, B. (1940) *Justus Liebigs Ann. Chem.* **543**, 171–183.
4. Galasinski, W., Gadek, A., Ratkiewicz, A. & Rzeczycki, W. (1978) *Anal. Biochem.* **85**, 550–555.
5. Nakajima, T. & Volcani, B. (1969) *Science* **164**, 1400–1401.
6. Lutz, O. & Jirgenson, B. (1931) *Ber. Dtsch. Chem. Ges.* **64**, 1221–1232.
7. Pataki, G. (1966) *Dünnschichtchromatographie in der Aminosäure- und Peptid-Chemie* (de Gruyter, Berlin), p. 167.
8. Philp, R. P. & Robertson, A. V. (1977) *Aust. J. Chem.* **30**, 123–130.
9. Karle, I. L., Daly J. W. & Witkop, B. (1969) *Science* **164**, 1401–1402.
10. Bruker Instruments (1978) *Bruker Aspect 2000 Software Manual* (Bruker, Karlsruhe-Rheinstetten, W. Germany).
11. Castellano, S. & Bothner-By, A. A. (1964) *J. Chem. Phys.* **41**, 3863–3869.
12. Ernst, R. R. (1966) in *Advances in Magnetic Resonance*, ed. Waugh, J. S. (Academic, New York), Vol. 2, pp. 1–135.