Phytochemistry, Vol. 26, No. 5, pp. 1391–1393, 1987.
Printed in Great Britain.

0031-9422/87 $3.00 + 0.00

1,5-D-ANHYDROFRUCTOSE, THE PRECURSOR OF THE PYRONE MICROTHECIN IN *MORCHELLA VULGARIS*

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(*Received* 12 *August* 1986)

Key Word Index—*Morchella vulgaris*; fungi; morels; microthecin; cortalcerone; pyrones; 1,5-anhydro-D-fructose; carbohydrates; enzymatic activity; bioconversions.

Abstract—The carbohydrate which, in various Discomycetes, is enzymatically converted under plasmolytic conditions to the antibiotic pyrone microthecin was isolated from a strain of *Morchella vulgaris* and identified as 1,5-D-anhydrofructose from X-ray analysis of its oxime.

INTRODUCTION

In previous articles [1–3], we showed that several fungi produce, when 'activated' by plasmolytic treatments (e.g. freezing–thawing), the closely related pyrones cortalcerone (1) or microthecin (2), which were isolated from *Corticium caeruleum* [3] and *Morchella costata* [2], respectively. Cortalcerone arises through enzymatic dehydration of D-glucosone (D-arabino-2-hexosulose) [4], but experiments showed that the precursor of microthecin was not D-fructopyranose, as we had anticipated on the basis of structural resemblance to a possible 2,6-cyclized form of D-glucosone [2]. Isolation of the actual precursor was therefore undertaken.

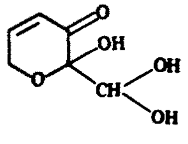
RESULTS AND DISCUSSION

Enzymatic conversion of the precursor to microthecin during extraction steps could be inhibited by 0.01 M EDTA. However, it proved more advantageous to carry out extraction on a strain which did not produce microthecin, although it synthesized its precursor; such strains were detected (either as mycelia or fruit bodies) during a screening of various Discomycetes species for microthecin production [unpublished results]. We chose a microthecin-deficient strain of *M. vulgaris*, whose mycelium is easy to cultivate on agar.

Fractionation of a mycelium extract (see Experimental section) yielded a white, amorphous, water-soluble, hygroscopic, reducing substance, which could be transformed *in vitro* into microthecin by an enzyme extract of a microthecin-producing fungus such as *M. costata*. Data from mass spectrometry and elemental analysis ($[M]^+$ m/z 162 for $C_6H_{10}O_5$) were consistent with an anhydrohexose. The IR spectrum (in KBr) exhibited broad bands, but indicated the presence of OH ($3400\ cm^{-1}$) and CO ($1730\ cm^{-1}$) groups. However, ^{13}C NMR in D_2O showed only chemical shifts between 60 and 100 ppm, which precluded the presence of CO or C=C; since two degrees of unsaturation are indicated by the empirical formula, it follows that the compound has a bicyclic structure in aqueous solution and exists as the carbonyl form in the solid state. Data from ^1H NMR did not elucidate the steric configuration of the three OH groups, the presence of which was indicated by the empirical formula and ^{13}C NMR data, which showed three hydrogens bound to non-C atoms. Derivatization of the precursor was therefore undertaken in order to obtain a crystalline compound suitable for X-ray analysis.

Acetylation gave an oily substance, but an oxime was obtained as single crystals which were studied by X-ray crystallography (Tables 1 and 2). The six-membered pyranose ring adopts a chair conformation: atoms C-1, C-3, C-4 and O-7 are in a single plane, C-2 and C-5 being above and below the plane, respectively (Fig. 1a and b). X-Ray analysis did not elucidate the absolute configuration of the molecule, but by comparison of its optical rotation with that of the same oxime obtained by synthesis from a D-sugar by Lichtenthaler *et al.* [5], we concluded that our oxime—and consequently the precursor itself—also had the D configuration. Therefore, the precursor formula should be **3** or **4** according to the usual convention for pyranoses. In aqueous solution, the adopted bicyclic form should be, as shown by molecular models, the 2,6-cyclized compound **5a**; the equivalent formula **5b** shows that this compound is 1,5-β-D-anhydrofructose, a carbohydrate which had been previously obtained from its peracylated oxime by Lichtenthaler *et al.*, but has never been reported, as far as we know, as a natural product.

Comparison of its structure with those of D-glucosone and D-xylosone allows us to propose a satisfactory theory



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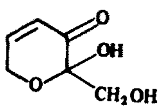

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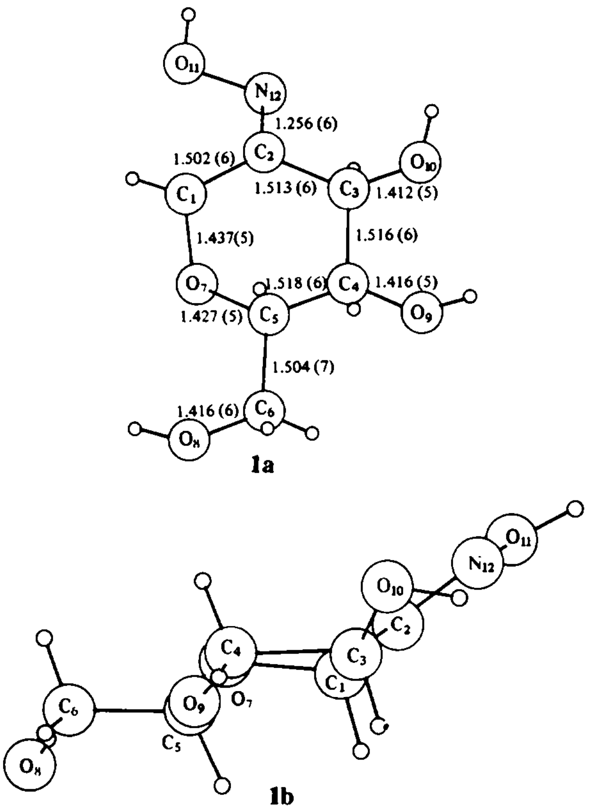
Table 1. Mean-plane and distances to the plane. Equation of the plane: $0.8687X - 0.4904Y + 0.0703Z = 4.4999$

Atoms	Distances to the plane
Atoms constituting the plane	
C-1	−0.031 (5)
C-3	0.029 (5)
C-4	−0.031 (4)
O-7	0.033 (3)
Other atoms	
C-2	0.529 (4)
C-5	−0.697 (4)
C-6	−0.716 (5)
O-9	−0.761 (4)
O-10	0.820 (4)
N-12	1.316 (4)

Table 2. Atomic coordinates ($\times 10^4$) and equivalent isotropic temperature factors $B_{eq} = 4/3 \sum_i \sum_j \beta_{ij} Q_i Q_j$

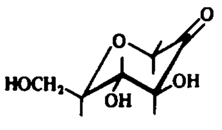
Atoms	X	Y	Z	$B_{eq}(A^2)$
C-1	5441 (7)	−1434 (5)	1100 (4)	3.6 (2)
C-2	5734 (5)	−2395 (5)	12 (5)	2.9 (1)
C-3	5621 (6)	−1685 (4)	−1240 (4)	3.2 (2)
C-4	6500 (6)	−248 (4)	−1250 (4)	3.0 (2)
C-5	5992 (6)	628 (4)	−131 (4)	3.2 (2)
C-6	6902 (7)	2034 (4)	−74 (5)	4.0 (2)
O-7	6414 (4)	−150 (3)	959 (2)	3.3 (1)
O-8	6399 (5)	2856 (3)	960 (3)	4.2 (1)
O-9	6067 (5)	515 (3)	−2333 (3)	3.9 (1)
O-11	6022 (5)	−4268 (3)	1243 (3)	4.2 (1)
N-12	5994 (5)	−3715 (4)	38 (4)	3.4 (1)



1a

1b

Fig. 1. Projection on the mean-plane of the molecule showing the numbering of atoms, the interatomic distances (a) and the cross-section of the molecule (b).



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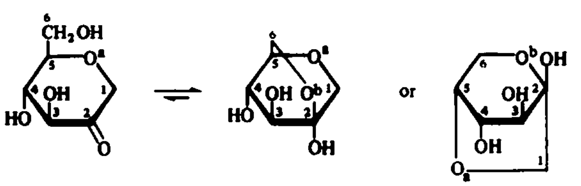

4 5a 5b

for the biogenetic pathway to cortalcerone and microthecin, which will be reported in the next paper of this series.

EXPERIMENTAL

Microthecin precursor (*1,5-anhydro-D-fructose*). Mycelia of a microthecin-less strain of *M. vulgaris* were grown at 25°, as previously described for *M. costata* [2], until they were 10–15 days old, then harvested and frozen. 20 mycelia were macerated for 2–3 hr at room temp. in 200 ml H_2O. The filtrate was concd under red. pres. to 10 ml and 60 ml MeOH added; the resulting ppt. was centrifuged and washed twice with MeOH which was pooled with the supernatant; a new ppt. was discarded and the MeOH liquor was concd to 5 ml of a syrupy liquid which was added to 100 ml MeOH. The resulting pasty material was triturated repeatedly with MeOH, and evapn of the combined MeOH liquors yielded a white solid which was dissolved in MeOH to give a 250 mg/ml soln. This liquid was streaked on prep. 20 × 20 cm silica gel F plates (0.8 ml per plate) which were developed with $CHCl_3$–MeOH (7:3). Bands containing microthecin precursor were detected with triphenyltetrazolium reagent and could also be visually detected as white, matt streaks; they were pooled, extracted with the same solvent and evapd. The aq. soln of the residue was filtered on a 0.22 μm filter. Lyophilization yielded 200 mg of a white amorphous material which was chromatographed on Sephadex G 10 in a 16 × 950 mm column (sample: 50 mg in 0.5 ml H_2O; eluent: H_2O; flow rate: 7 ml/hr; temp.: 15°; fraction size: 2 ml/tube; fraction monitoring by TLC as described below). The precursor eluted in fractions 42–48, that were concd and lyophilized, which yielded *ca* 35 mg of a white amorphous solid, $[\alpha]_D^{23} = -40°$ (0.5, H_2O). Found: C, 44.21; H, 6.3; O, 49.22. Calc for $C_6H_{10}O_5$: C, 44.44; H, 6.17; O, 49.38%. CIMS (CH_4, probe) 70 eV, *m/z* (rel. int.): 163 $[M+H]^+$ (55), 145 $[M+H-H_2O]^+$ (100), 127 $[M+H-2H_2O]^+$ (63), 85 $[M+H-H_2O-CH_2CO]^+$ (66). ^{13}C NMR(D_2O): δ95.3 (*s*), 83.3 (*d*), 79.6 (*d*), 74.4 (*t*), 71.7 (*d*), 63.9 (*t*).

TLC of precursor. Its R_f was 0.5 on Macherey–Nagel Sil/G UV_{254} silica gel plates with $CHCl_3$–MeOH (7:3). The best reagent for detection was anisaldehyde [3], which gave a blue colour. Enzymatic detection could also be carried out on TLC plate by spraying an enzyme extract (see below) to convert the precursor into microthecin, then phenylhydrazine [1] to detect the latter (orange-red colour).

Semi-purified enzyme extract of M. costata. Mycelia grown as previously described [2] and frozen were thawed and homogenized in the minimal vol. (*ca* 5 ml/mycelium) of 0.05 M NaPi buffer, pH 6. The homogenate was centrifuged at 20000 *g* for 30 min. and the supernatant mixed with polyethylene glycol 6000 (3 g per 10 ml). The resulting ppt. was recovered by a similar centrifugation and resuspended in 0.05 M NaPi buffer, pH 6, made 0.2 M with NaCl (0.5 ml per mycelium). Insoluble material was discarded by a new centrifugation, using the same conditions, and the supernatant was PEG-ppted (1 g per 10 ml). The ppt. was recovered by centrifugation as above and taken up by the same NaPi buffer, pH 6 (*ca* 0.25 ml per mycelium). A 5-fold dilution in H_2O was used as the spray reagent.

Oxime of microthecin precursor. Precursor (900 mg) and 375 mg of hydroxylamine hydrochloride in 25 ml of pyridine were reacted at 24° for 24 hr, then added to 20 ml of toluene–EtOH (3:1) and evapd under red. pres. to give an oily residue which was taken up in 2 × 10 ml of toluene. This soln was evapd again until pyridine was eliminated. The resulting pasty residue was triturated with 20 ml $CHCl_3$–MeOH (7:3); insoluble material was filtered out, the filtrate concd to 10 ml and chromatographed on 20 × 20 cm prep. silica gel plates (Merck 7747) with $CHCl_3$–MeOH (7:3). A band at R_f 0.5 was detected by its brown colour with the anisaldehyde reagent (this band did not reduce triphenyltetrazolium, contrary to the precursor). Pooled bands were eluted with $CHCl_3$–MeOH (1:1) and the eluate evapd under red. pres. to give a slightly amber-coloured oil which was dissolved in 3 ml MeOH; slow evapn afforded a crystalline material which was recrystallized from the same solvent, yielding small prisms (220 mg), mp 155–157°, $[\alpha]_D^{23} = -43°$ (H_2O, 2). Found: C, 40.47; H, 6.29; N, 7.83; O, 44.85. Calc. for $C_6H_{11}NO_5$: C, 40.67; H, 6.21; N, 7.90; O, 45.19%. IR ν_{max}^{KBr} cm^{-1}: 3400 (O–H), 1620 (C=N), 1470, 1190, 1150, 990 (N–O).

X-Ray analysis of oxime. Single crystals were grown by slow crystallization from MeOH. The crystal chosen for intensity measurements had the dimensions 0.25 × 0.18 × 0.13 mm. The symmetry was orthorhombic, space group non-centrosymmetric $p2_12_12_1$ with $a = 7.882$ (3), $b = 9.384$ (5), $c = 10.832$ (6) Å; $d_{obs} = 1.4598$; $d_{calc} = 1.469$ g/cm^3 for $Z = 4$ ($M_r = 177.15$). Intensity data were collected on a CAD4 Enraf-Nonius diffractometer (monochromated CuKα radiation, $\lambda = 1.54178$ Å, and corrected for Lorentz and polarization effects. No absorption correction was necessary ($\mu = 10.8$ cm^{-1}). A total of 812 independent reflections with $\theta \leqslant 65°$ was measured, of which 708 were considered as observed $[I \geqslant 3\sigma(I)]$ and were used in the refinement. The structure was solved by direct methods using MULTAN 80 [6] and refined by block diagonal-matrix least-squares methods with anisotropic temperature factors for the non-H atoms, and isotropic ones for H-atoms (found in a difference Fourier map). The final $R = 0.051$, $wR = 0.073$ ($w = 1$ if $|F_0| < P$, $P = [F_0^2(\max)/10]^{1/2}$, $w = (P/F_0)^2$ if $|F_0| > P$), $S = 1.263$ (708 reflections, 153 parameters). The scattering factors for the O, N and C atoms were from ref. [7], and for the H atoms from [8]. Calculations were performed on a Mini 6-92, CII-Honeywell Bull computer. A list of the observed and calculated structure factors, anisotropic thermal parameters and atomic coordinates for the H-atoms was deposited at the Cambridge Crystallographic Data Centre.

Acknowledgements—The authors thank A. Soriano and G. Fondeville for technical assistance.

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