ELSEVIER

Carbohydrate Research 278 (1995) 59–70

CARBOHYDRATE RESEARCH

Conversion of D-glucose to D-*erythro*-hexos-2,3-diulose (2,3-diketo-D-glucose) by enzyme preparations from the basidiomycete *Oudemansiella mucida*

Jindřich Volc [a,*], Petr Sedmera [a], Vladimír Havlíček [a], Věra Přikrylová [a], Geoffrey Daniel [b]

[a] *Institute of Microbiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20 Prague 4, Czech Republic*

[b] *Department of Forest Products, Swedish University of Agricultural Sciences, Box 7008, S-750 07 Uppsala, Sweden*

Received 26 April 1995; accepted 28 June 1995

Abstract

Aerobic incubation of crude enzyme extracts from the white rot fungus *Oudemansiella mucida* with D-glucose resulted in transient accumulation of D-*arabino*-hexos-2-ulose (**1**, 2-keto-D-glucose) which was subsequently quantitatively converted into a new tricarbonyl sugar metabolite, D-*erythro*-hexos-2,3-diulose (**2**, 2,3-diketo-D-glucose). The latter end-product was derivatized with *N*,*N*-diphenylhydrazine; its structure was deduced from formulae of the four major hydrazones isolated from the reaction mixture using thin layer chromatography, and identified by nuclear magnetic resonance and mass spectroscopy. **2** was also obtained on incubations with chemically synthesized **1**, and with partially purified preparations of pyranose 2-oxidase. A hypothetical reaction scheme was proposed for two step oxidation of D-glucose to **2** by a single enzyme, pyranose 2-oxidase (EC 1.1.3.10).

Keywords: D-Glucose; D-*erythro*-Hexos-2,3-diulose; *Oudemansiella mucida*

Abbreviations: DMAB, 3-dimethylaminobenzoic acid; DTT, dithiothreitol; MBTH, 3-methyl-2-benzothiazolinone hydrazone-hydrochloride; PMSF, phenylmethylsulfonyl fluoride; P2O, pyranose 2-oxidase; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

* Corresponding author.

SSDI 0008-6215(95)00247-2

1. Introduction

The dicarbonyl monosaccharide D-*arabino*-hexos-2-ulose (**1**) plays an important role in sugar metabolism of numerous wood degrading basidiomycetes. **1** is produced by the enzyme pyranose 2-oxidase (P2O, glucose 2-oxidase, EC 1.1.3.10), which is expressed in both laboratory liquid cultures [1–4] and during wood decay under near-natural conditions [5]. P2O acts as a C-2 specific oxidase on several aldopyranoses [6] with D-glucose being the preferred substrate, and is considered a constituent of the lignin-olytic system of some white rot fungi as a major physiological source of hydrogen peroxide [5].

In our previous study on metabolism of glycosuloses (aldoketoses) by *Phanerochaete chrysosporium*, we have shown that **1** is transformed by this fungus into the β-pyrone antibiotic cortalcerone through double dehydration catalyzed by the enzyme aldos-2-ulose (pyranosone) dehydratase [7]. Initial dehydration of glycosuloses by this enzyme occurred at C-3 and C-4 resulting in formation of the corresponding 4-deoxy-glycos-2,3-diuloses, new enzymatically produced tricarbonyl sugar derivatives. Conversion of **1** into cortalcerone has also been reported for basidiomycete fungi *Polyporus obtusus* [8] and *Corticium caeruleum* [9].

The existence of an alternative metabolic pathway for **1** was apparent from our research on another white rotter, *Oudemansiella mucida*. This fungus failed to produce glycos-2-ulose dehydratase however, similarly to *P. chrysosporium*, was able to utilize **1** accumulated extracellularly in liquid cultures. The present paper describes oxidative conversion of D-glucose via **1** into another tricarbonyl sugar with P2O enzyme preparations of *O. mucida* and identification of this reaction product as D-*erythro*-hexos-2,3-diulose (**2**, 2,3-diketo-D-glucose) by NMR and MS spectroscopy of its well defined *N*,*N*-diphenylhydrazone derivatives.

2. Materials and methods

Organisms.—*Oudemansiella mucida* (Schrad.:Fr.) Hôhn., strain III (CCBAS 428) was obtained from the culture collection of basidiomycetes maintained at the Institute of Microbiology, Academy of Sciences of the Czech Republic, Prague. Submerged cultivation was performed on a reciprocal shaker (2 Hz) at 25°C in 500-mL flasks filled with 80 mL synthetic medium optimized for P2O production, consisting of 20 g glucose, 5 g powder cellulose CC41 (Whatman, Maidstone, UK), 2.5 g glutamic acid, 0.1 g glycine, 0.5 g ammonium tartrate, 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, and 0.25 g $CaCl_2$ per litre of distilled water, supplemented with salt solution (1 mL) and solution of vitamins (B_1, B_2, and B_6 25 mg/100 mL each) (0.2 mL). The salt solution contained: 1 g NaCl, 0.1 g $ZnSO_4 \cdot 7H_2O$, 0.1 g $MnCl_2$, 0.1 g H_3BO_3, 50 mg $FeCl_3$, 10 mg $CuSO_4 \cdot 5H_2O$, 10 mg KI, and 10 mg $CoCl_2$ per 100 mL. Medium pH was adjusted to 5.8 with NaOH. An agar slant culture was used to inoculate a primary shaker flask with a glucose-corn steep-based medium [4] and after sufficient growth a vegetative inoculum (4%) was prepared by mildly sterile homogenization (Ultra-Turrax homogenizer, IKA-Werk, Staufen, Germany) of the culture.

Basidiomycetes *Trametes versicolor* (L.:Fr.) Pilát (CCBAS 614) and *Phanerochaete chrysosporium* Burds. (CCBAS 571) were grown on glucose-corn steep medium [4].

Crude enzyme extracts and partial purification of P2O.—A 4-day mycelium of *O. mucida* was harvested by filtration, washed with distilled water, and, after removing excess water, suspended (30 g) in 80 mL of 25 mM TES buffer pH 7.0 containing 0.5 mM PMSF and 0.3 mM DTT. The suspension was supplemented with 6 g of powdered microcrystalline cellulose, homogenized under cooling in an Ultra-Turrax homogenizer (Shaft 18KG, full speed, 5 min) and centrifuged for 20 min at 10,000 *g*. The resulting supernatant was desalted by extensive diafiltration (YM 10 membrane; Amicon, Danvers, MA) using 10 mM TES, concentrated (3.3 mL, 34 U mL^{-1} P2O) and used as a crude extract in D-glucose transformation experiments. P2O from another portion of the crude extract was further partially purified by hydrophobic interaction chromatography on phenyl-Sepharose CL-4B (Pharmacia LKB, Uppsala, Sweden) as described previously [4] to yield a 6.4 times purified preparation (2.5 U mg^{-1} protein).

Enzyme assay.—P2O activity was assayed by spectrophotometric (590 nm) determination of H_2O_2 production using MBTH (Fluka Chemie, Buchs, Switzerland) + DMAB (Fluka) as the chromogen in the coupled peroxidase reaction [6]. The reaction mixture (2 mL total) contained 200 μmol Na phosphate pH 6.5, 100 μmol D-glucose, 5 μmol DMAB, 0.1 μmol MBTH, 85 nkat horseradish peroxidase and a suitable amount of P2O.

Transformation of D-glucose to a tricarbonyl product.—The reaction mixture (40 mL) consisted of 2 mmol D-glucose, 0.4 mmol TES · NaOH pH 7.0, 3.2 mL concentrated crude enzyme extract of *O. mucida* containing 109 U P2O and 6000 Sigma Units of catalase (Reanal, Budapest, Hungary). Incubation was performed aerobically under intensive stirring at 30°C; samples (0.3 mL) were withdrawn at 1-h intervals to monitor chromatographically the course of transformation. Before HPLC the samples were purified by passing through Ultrafree-MC 10,000 NMWL Filter Units (Millipore, Bedford, MA, USA), and diluted 4 times. In a further experiment D-glucose was replaced by 2 mmol synthetic **1** in the same reaction mixture. Controls included: (i) the glucose-containing reaction mixture incubated anaerobically under N_2; (ii) the enzyme blank reaction mixture containing **1** as a substrate incubated aerobically.

Chromatographic analysis of reaction products.—HPLC was performed on a SP 8880 liquid chromatograph (Spectra Physics, San Jose, CA, USA) fitted with an RI SE-61 refractive index detector (Shodex, Tokyo, Japan) using Ostion LG KS 0800 Ca^{2+} column 250 × 8 mm (Watrex, Prague, Czech Republic) eluted at 80°C with deionized water at a flow rate of 0.5 mL min^{-1}. The identity of peaks observed was determined in part by comparing retention times with those of known standards. Synthetic **1** was prepared via D-glucose phenylosazone [10].

Thin layer chromatography of free sugars was performed on Lucefol (Kavalier, Votice, Czech Republic) cellulose-coated foils using 140 : 100 : 33 : 80 *n*-butyl acetate–acetic acid–acetone–water. **1** was detected by diphenylamine–aniline–phosphoric acid reagent [11], with an intense blue colour indicative of aldos-2-uloses.

Isolation of the tricarbonyl reaction product from D-glucose.—The sugar transformation product obtained after 8-h incubation of the above reaction mixture was converted to a well defined derivative by reaction with *N*,*N*-diphenylhydrazine. The mixture (35

mL) was supplemented successively under stirring with the same volume of ethanol, 0.7 mL 99% acetic acid and 0.5 mL freshly distilled *N,N*-diphenylhydrazine (Koch-Light Lab., Colnbrook, UK) and stirred for another 3 h at room temperature. The solvents were vacuum evaporated, the dark residue obtained dissolved in 1 mL $CHCl_3$ and then subjected to preparative TLC on 20 Silufol (Kavalier) silica gel foils using 32:1 $CHCl_3$–ethanol. Yellow to orange streaks corresponding to four major hydrazone components (R_f 0.06, 0.16, and 0.25) were cut out, combined separately, and extracted with ethanol. These components were further purified by rechromatography and crystallization.

Identification of diphenylhydrazones of D-*glucose-derived tricarbonyl product.*—^1H and ^{13}C NMR spectra (399.95 and 100.577 MHz, respectively) of isolated *N,N*-diphenylhydrazones were recorded on a Varian VXR-400 spectrometer in $CDCl_3$ at 25°C. Tetramethylsilane or the residual solvent signal (δ_H 7.265, δ_C 77.00) were used as internal standards. All 2D NMR experiments (COSY, delayed-COSY [12], J-resolved, ROESY [13], and HECTOR) were performed using the manufacturer's software.

Positive-ion mass spectra were recorded on a Finnigan MAT 90 double sector instrument of BE geometry. Xenon (10^{-3} Pa) was used as the FAB gas in fast atom bombardment (FAB) ionization, the matrix was *m*-nitrobenzyl alcohol (Aldrich-Chemie, Steinheim, Germany) and magnetic calibration was performed with Ultramark 1600F (PCR Inc., USA) as standard. The standard saddle-field atom gun FAB11NF (Ion Tech, Teddington, UK) was operated at 2 mA and 8 kV energy. The products of unimolecular metastable decompositions (without collisional activation) in the first field-free region of the instrument were analysed by daughter ion linked scan: B/E constant; where B is the magnetic field and E is the electrostatic voltage, using the manufacturer's software. Some of the B/E spectra of hydrazone **3** were obtained under electron-impact conditions (ionizing energy 70 eV, source temperature 250°C, emission current 1 mA,

Table 1
^{13}C NMR data (chemical shift, multiplicity) of the hydrazone derivatives **3–6** (**3a, 3b**, anomers of **3**) used for identification of the D-glucose oxidation end-product

Carbon	Compound					
	3a [a]	**3b** [a]	**4**	**5**	**6** [b]	
1	92.09 d	92.20 d	130.26 d	129.44 d	127.40 d	126.01 d
2	144.56 s	144.52 s	190.98 s	188.13 s	102.67 s	198.26 s
3	201.17 s	200.23 s	101.96 s	141.62 s	150.63 s	140.01 s
4	78.61 d	81.24 d	73.99 d	67.88 d	75.20 d	73.66 d
5	73.16 d	73.84 d	71.10 d	73.23 d	73.31 d	70.96 d
6	62.70 t	62.08 t	73.66 t	63.91 t	62.90 t	73.56 t

[a] $CDCl_3 + CD_3OD$.

[b] Second column: primed moiety. Additional signals: **1a** 124.32 d (*ortho*-DPH); 130.10 d (*meta*-DPH); 127.87 d (*para*-DPH); 136.72 s (*ipso*-DPH); **1b**: 124.32 d (*ortho*-DPH); 130.10 d (*meta*-DPH); 127.87 d (*para*-DPH); 136.48 s (*ipso*-DPH); **2**: 122.65 d, 129.75 d (*ortho*-DPH); 129.75 d, 129.96 d (*meta*-DPH); 126.45 d (*para*-DPH); 145.03 s (*ipso*-DPH); **3**: 122.65 d (*ortho*-DPH); 129.75 d, 129.96 d (*meta*-DPH); 126.45 d (*para*-DPH); 145.03 s (*ipso*-DPH) (some signals missing due to exchange processes); **4**: 121.70 d, 122.43 d, 122.70 d, 123.94 d (*ortho*-DPH); 129.26 d, 129.39 d, 129.58 d, 129.88 d (*meta*-DPH); 125.02 d, 125.58 d, 125.91 d (double intensity) (*para*-DPH); 142.14 s, 142.52 s, 147.94 s (double intensity) (*ipso*-DPH).

Table 2
^{1}H NMR data (chemical shift, multiplicity) of the hydrazone derivatives **3–6** used for identification of the D-glucose oxidation end-product

H	**3a** [a]	**3b** [a]	**4**	**5**	**6** [b]	
1	4.718 s	4.669 s	6.814 s	8.051 s	6.884 s	6.831 s
4	4.297 d	4.220 d	4.904 dd	3.589 dd	5.390 d	5.044 d
5	3.895 ddd	3.927 ddd	4.397 m	3.851 dddd	4.163 dt	4.387 ddd
6	3.704 dd	3.736 dd	4.297 dd	3.307 dd	3.678 d	4.345 dd
	3.655 dd	3.720 dd	4.194 dd	3.551 dd	3.678 d	4.191 dd

[a] $CDCl_3 + CD_3OD$.
[b] Second column: primed moiety. Additional signals: **1a** + **1b**: 7.269 m (*ortho*-DPH); 7.323 m (*para*- and *meta*-DPH); 7.417 m (*meta*-DPH); **2**: 2.806 d (5-OH); 3.150 d (4-OH); 6.073 s (3-OH); 7.143 m (*ortho*-DPH); 7.300 m (*para*-DPH); 7.468 m (*meta*-DPH); **3**: 1.843 m (6-OH); 2.446 d (5-OH); 4.672 d (4-OH); 6.952 m (*ortho*-DPH); 7.252 m (*para*-DPH); 7.258 m (*para*-DPH); 7.276 m (*ortho*-DPH); 7.327 m (*meta*-DPH); 7.479 m (*meta*-DPH); **4**: 6.795 m (*ortho*-DPH); 6.865 m (*ortho*-DPH); 7.048 m (*ortho*- + *para*-DPH); 7.066–7.332 m (*meta*- + *para*-DPH).

acceleration voltage 5 kV, direct inlet). The NMR data of hydrazones **3–6** below are shown in Tables 1 to 3, respectively.

Hydrazone **3**: Compound of R_f 0.06, crystallized from ethanol as pale yellow fine needles (22 mg), mp 164–167°C, decomp. IR (KBr): ν_{max} 3459, 3390, 3130, 1719 cm^{-1}. FABMS *m/z* (rel int): 343 $[M + H]^+$, (daughter ions of the protonated molecule) 325 (100), 307 (2), 282 (2), 265 (2), 251 (1), 224 (6), 223 (12), 222 (5), 195 (1), 184 (3), 183 (4), 169 (5), 168 (4).

Hydrazone **4**: Compound of R_f 0.16, accompanying **5** during chromatography, separated from **5** by fraction crystallization from ethanol (pale-yellow needles, 11 mg), mp 113–116°C, decomp. IR (KBr): ν_{max} 3463, 3385, 3299, 1672w, 1613 cm^{-1}. FABMS, *m/z* (rel int): 343 $[M + H]^+$, 325 (100), 225 (2), 224 (8), 223 (2), 195 (7), 183 (1), 169 (3), 168 (3).

Hydrazone **5**: Compound of R_f 0.16, crystallized from ethanol (yellow needles, 75 mg), mp 119–120°C. IR (KBr): ν_{max} 3601, 3418, 3343, 1657, 1638w cm^{-1}. FABMS, *m/z* (rel int): 509 $[M + H]^+$, 491 (100), 448 (10), 392 (3), 363 (2), 340 (3), 325 (3), 285 (27), 284 (19), 224 (1), 223 (5), 183 (2), 169 (4).

Table 3
Proton–proton coupling constants [Hz] of compounds **3–6**

(*i*,*j*)	**3a**	**3b**	**4** [a]	**5** [b]	**6** [c]	
4,5	4.3	3.2	5.4	8.6	4.9	5.5
5,6d	6.3	6.2	4.4	3.4	5.2	4.7
5,6u	5.3	6.5	1.9	5.6	5.2	1.6
−6d,6u	−11.8	−11.4	−10.2	−11.4	− [d]	−9.9

[a] *J*(4,OH) 9.6, *J*(5,OH) 7.6.
[b] *J*(4,OH) 11.0, *J*(5,OH) 6.5, *J*(6,OH) not resolved.
[c] Second column: primed moiety.
[d] Not observed because of magnetic equivalence.

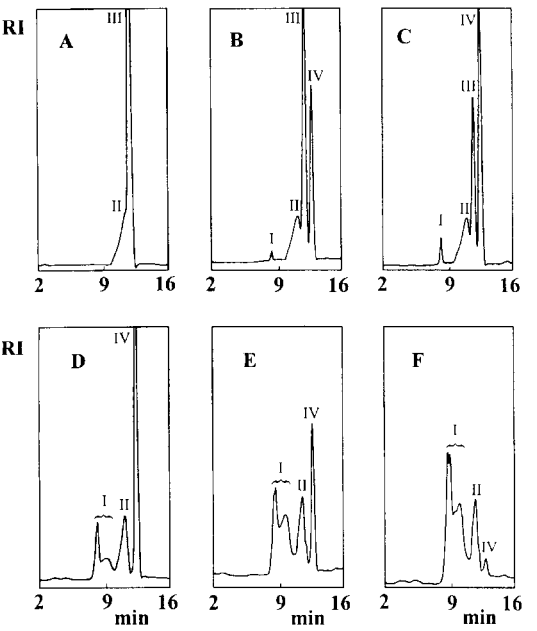

Fig. 1. HPLC monitoring of the enzymatic oxidation of D-glucose to D-*erythro*-hexos-2,3-diulose via D-*arabino*-hexos-2-ulose by crude extracts of the fungus *Oudemansiella mucida* at incubation times of (A) 0, (B) 1, (C) 2, (D) 4, (E) 6, and (F) 8 h. Reaction and assay conditions are described in Materials and methods. Peaks: I, D-*erythro*-hexos-2,3-diulose and its isoforms; II, TES (buffer); III, D-glucose; IV, D-*arabino*-hexos-2-ulose.

Hydrazone **6**: Compound of R_f 0.25, rechromatographed on Silica Gel 60 coated HPTLC-Alufolien (Merck, Darmstadt, Germany) using 120:1 $CHCl_3$–ethanol, orange microcrystals from ethanol, 105 mg, mp 75–77°C, decomp. IR (KBr): ν_{max} 3383, 3337, 1670 cm^{-1}. FABMS, *m/z* (rel int): 1017 $[M + H]^+$, 999 (18), 848 (17), 679 (28), 531 (36), 509 (100); *m/z* 509: 491 (61), 448 (2), 340 (100), 325 (23), 280 (3), 223 (1), 222 (3), 195 (0.5), 183 (3), 169 (7).

3. Results and discussion

Evidence for enzymatic oxidation of D-arabino-*hexos-2-ulose*.—Desalted crude enzyme extracts of *O. mucida* were used to study oxidative metabolism of D-*arabino*-hexos-2-ulose (**1**), the characteristic sugar intermediate produced during wood decay by this fungus [5]. **1** was prepared in situ as a product of the crude extract pyranose 2-oxidase (P2O) acting on D-glucose in the transformation system used (see Materials and methods). TLC analysis of reaction products during the time course of D-glucose (grey spot, R_f 0.22) transformation revealed that **1** (blue spot, R_f 0.29) accumulated transiently with maximum at 4 h, and was completely converted to the tricarbonyl end-product (**2**) of the same R_f (yellow spot) after 10-h incubation (not shown). By

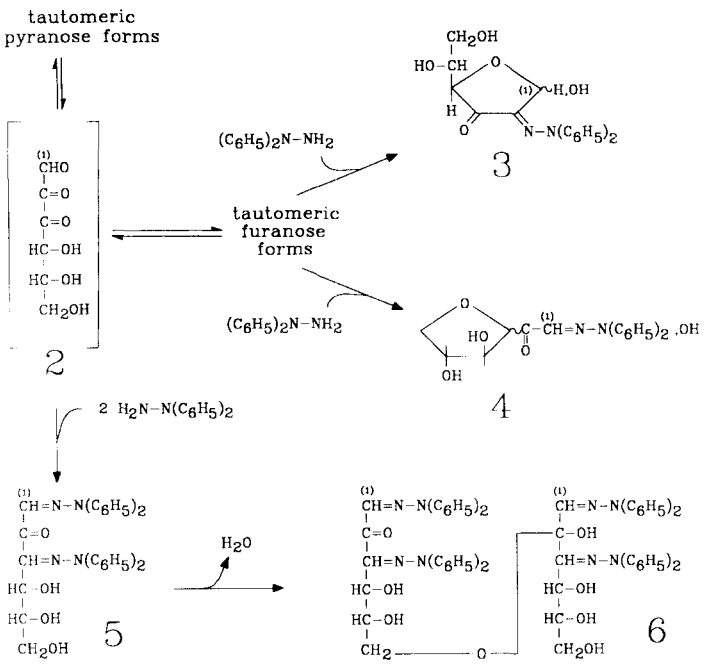

Fig. 2. Hydrazone derivatives used for identification of the oxidation end-product of D-glucose by enzyme preparations from the basidiomycete *Oudemansiella mucida*. **2**, the structure of D-*erythro*-hexos-2,3-diulose deduced for the identified end-product; **3**, bis(*N*,*N*-diphenylhydrazone) of the product linear form; **4** and **5**, *N*,*N*-diphenylhydrazones of the product furanose cyclic forms (1 → 4 and 3 → 6, respectively); **6**, a dimer of **3** (2 → 6′ hemiacetal bond).

analogy the intermediary role of **1** in conversion of D-glucose to **2** was demonstrated by HPLC analysis (Fig. 1). The peak of **1** which was maximal at 4 h is only a trace at 8 h, while a complex of peaks develop gradually at retention times of 8.00 to 9.54 min, corresponding to major isoforms (carbonyl groups could be hydrated as well) and dimers of **2** as indicated by stabilized structures of their isolated *N*,*N*-diphenylhydrazine derivatives (Fig. 2). Chromatograms show good resolution of corresponding mono-, di- (**1**), and tri-carbonyl (**2**) monosaccharide components on the Ostion Ca^{2+} ion exchange column under the HPLC conditions used (Fig. 1C). TES buffer was chosen because its peak (11.48 min) did not interfere with analysis of the D-glucose oxidation products. It was important to thoroughly deionize the crude enzyme extract (YM10 Amicon membrane) since peaks corresponding to salts in the sample reaction mixture coincided with those of **2**. Deionization using OH^- anion exchangers was not possible because of the rapid decomposition of both **1** and **2**.

The same HPLC pattern of the oxidation end-product was obtained when synthetic **1** was used as the initial substrate in the above reaction mixture, or when partially purified (hydrophobic interaction chromatography) P2O was substituted for the crude extract (not shown). The anaerobic control experiment (i) (see Materials and methods) did not afford any detectable **2**; similarly, the enzyme blank aerobic control experiment (ii) did not

reveal **2** nor a significant non-enzymatic decomposition of **1**. The enzyme synthesis of **2** from D-glucose was also demonstrated (HPLC) with crude extracts from the basidiomycete fungi *Trametes versicolor* and *Phanerochaete chrysosporium* using the same reaction conditions; aldos-2-ulose dehydratase activity in the crude extract of *P. chrysosporium* was completely inhibited by 2 mM Cu^{2+} added as cupric acetate [7] (not shown).

Spectroscopic structural analyses of hydrazone derivatives.—Chromatographic analyses showed that D-glucose reacted with the crude enzyme extract of *O. mucida*, containing P2O as the primary oxidase activity (see Materials and methods), in at least two oxidation steps. In order to avoid difficulties with isolation and analysis of free di- and tri-carbonyl sugars, the reaction products were derivatized with *N,N*-diphenylhydrazine, major hydrazones isolated by preparative TLC, crystallized and spectroscopically characterized.

According to $[M + 1]^+$ ions observed in the positive-ion FAB mass spectra, two compounds isolated from the reaction mixture (Fig. 2, compounds **3** and **4**) were mono-DPH derivatives. The former is a mixture (55 : 45) of two related compounds (^1H and ^{13}C NMR, Tables 1–3). Carbon signals at 92.09 and 92.20 ppm, directly coupled (^1J 177.8 and 178.4 Hz, respectively) to proton singlets resonating at 4.718 and 4.669 ppm, were assigned to underivatized anomeric carbons C-1 of the –OCHO– type. ^{13}C NMR spectrum (Table 1) shows that both compounds contain one keto group, two oxymethines, and one oxymethylene. COSY spectra revealed the presence of two systems $-CH(O-)CH(OH)CH_2OH$. Therefore, C-4 is joined to H-1 via an oxygen bridge so that the molecule contains a furan ring. This deduction is supported by a doublet (5 Hz) splitting of the C-1 signal of the minor isomer observed in the proton-coupled ^{13}C NMR spectrum and by a cross-peak between H-1 and H-4 of the major isomer found in the ROESY [13] spectrum. Carbonyl signals of both forms display three fine splittings in the above mentioned proton-coupled ^{13}C NMR spectrum; that is consistent with free carbonyl at position 3 only (two ^3J and one ^2J, whereas for 2-keto only one ^3J and one ^2J are possible). Another argument is provided by the daughter spectrum (EI, *B/E* scan) of the ion *m/z* 252 containing carbon atoms C-1 to C-3. This ion might be either $^+O{\equiv}C\text{-}C(CH{\cdot}OH){=}N\text{-}N(C_6H_5)_2$ or $(C_6H_5)_2N\text{-}N{=}C^+\text{-}CO\text{-}CH{\cdot}OH$. The *B/E* spectrum contains ions *m/z* 252 (60%, precursor ion), 251 (100), 224 (0.5), and 223 (1). The cation–radical *m/z* 252 loses predominantly a hydrogen radical to give the cation *m/z* 251 stabilized by a conjugated double bond system. The elimination of CO leading to the *m/z* 224 ion is much easier in the former of the two proposed structures. Thus, this compound is described by formula **3** (Fig. 2).

The second mono-DPH derivative contains a keto group, one –OCO– type carbon, a derivatized C-1 (–CH=N–), and one OCH_2 (Table 1). Three OH groups (two secondary and one tertiary) were observed in the ^1H NMR spectrum (Table 2, footnote). COSY experiment revealed a fragment $-OCH_2CH(OH)CH(OH)C_q$ (where C_q means a quaternary carbon). A long-range coupling between the tertiary OH and H-4 was detected by delayed-COSY [12]. The carbon chemical shift of the OCH_2 group suggests its involvement in the ether bridge formation. Therefore, the furanose structure **4** is proposed for this compound. The presence of an ion *m/z* 223 in the FAB daughter spectrum of the $[M + H]^+$ ion *m/z* 343 supports this conclusion.

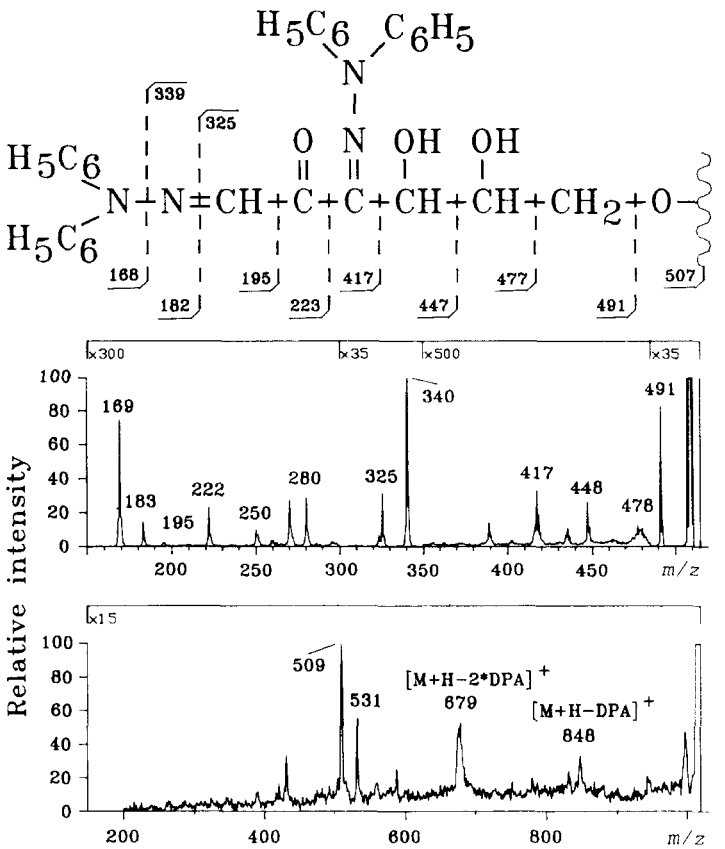

Fig. 3. Daughter mass spectrum of the protonated molecule of the identified sugar derivative dimer **6** (Fig. 2, bottom) and daughter mass spectrum of the fragment ion m/z 509 (top). The origin of individual ions is illustrated above the spectra.

A further isolated component of the reaction mixture (Fig. 2, **5**) is a bis-DPH derivative (FAB: $[M + H]^+$ m/z 509). According to NMR, the molecule contains a derivatized C-1 (δ_H 8.051 s, δ_C 129.44 d), a conjugated keto group, and a partial structure –CH(OH)CH(OH)CH_2OH (COSY). The free keto group was placed at C-2 on the basis of the m/z 222 ion observed in the FAB daughter ion scan (see the scheme at the top of Fig. 3).

The last isolated hydrazone (Fig. 2, **6**) exhibits a $[M + H]^+$ ion m/z 1017 in its FAB spectrum. Consecutive losses of diphenylamine units and the breaking of the molecule into two m/z 509 species were observed in the daughter spectrum of this ion (Fig. 3, bottom). Further sequencing of the m/z 509 ion (Fig. 3, top figure and scheme above) affords the primary structure for one half of the molecule. However, the NMR spectra indicate that the molecule is not symmetric. It contains two derivatized C-1s (δ_H, δ_C) but only one keto group, and one sp^3-hybridized –OCO– carbon. Signals of two different quaternary –C=N– type carbons are also present in the spectra together with

carbon and proton signals due to the eight phenyl groups. Two partial structures $-CH(O-)CH(O-)CH_2O-$ were found by COSY. The heteronuclear correlation was then achieved by $^{1}H,^{13}C$-COSY (HETCOR). The CH_2O group exhibiting the larger carbon shift evidently forms an ether bridge. According to a HETCOR experiment optimized for the detection of small (^{2}J and ^{3}J) couplings, the cross-peak between δ_C 102.67 and δ_H 4.345) means that this bridge ends with a quarternary carbon atom of the hemiacetal type. Similarly as described above, both keto groups were placed at C-2 because of ions *m/z* 223 and 222 observed in the daughter spectra of the half-molecule ion *m/z* 509. The assignments of C-1, -2, -3, -1′, -2′, and -3′ were accomplished by the same long-range HETCOR experiments using their couplings to H-1 and H-1′ (Tables 1 and 2). The resulting molecule **6** is a dimer of compound **5** containing a 2 → 6′ hemiacetal bond.

Identification of D-arabino-*hexos-2-ulose oxidation product.*—Structures of all the above hydrazones **3**–**6**, derived from the end product of D-glucose oxidation by enzyme extracts of *O. mucida*, have a common property, oxidized C-2 and C-3 of the sugar moiety. The free carbonyl group at C-2 in all these structures and deduced carbonyls at C-1 and C-3 are consistent with identification of the parent (non-derivatized) sugar as D-*erythro*-hexos-2,3-diulose (2,3-diketo-D-glucose) in an open (Fig. 2, structure **2**) or furanose cyclic form corresponding to structures **3** and **4** (Fig. 2). D-*erythro* Configuration results from stereochemistry of the parent sugar.

To our knowledge, this enzymatically prepared compound containing three adjacent carbonyl groups has not previously been reported as a sugar metabolite in any organism. Also we have failed to find any reference to synthetic **2** in the literature. Interestingly, we did not succeed in detecting a product corresponding to the primary oxidation at C-3 of D-glucose.

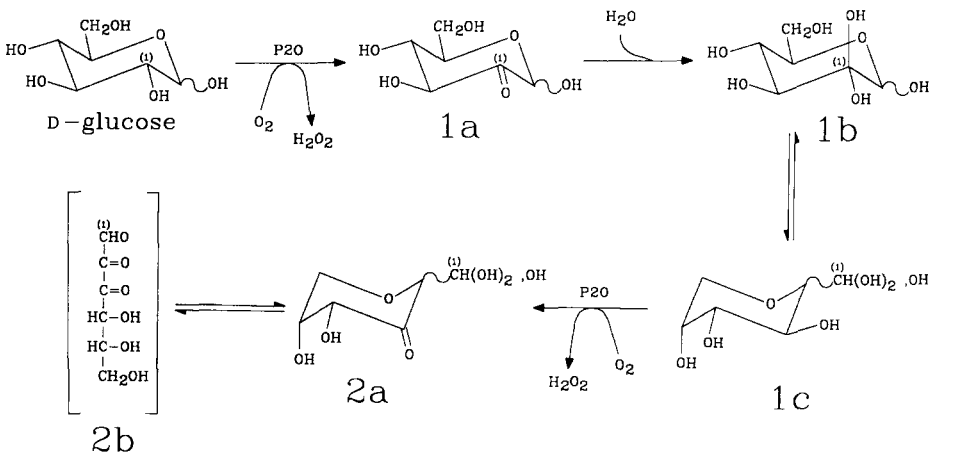

Fig. 4. Hypothetical pathway for 2-step oxidation of D-glucose to D-*erythro*-hexos-2,3-diulose (2,3-diketo-D-glucose) by enzyme preparations from the fungus *Oudemansiella mucida*. C-1 in each molecule and corresponding chemical rearrangements are indicated. **1a, 1b, 1c**, D-*arabino*-hexos-2-ulose, C-2 hydrated, and recyclized, respectively; **2a, 2b**, D-*erythro*-hexos-2,3-diulose in cyclic hemiacetal, and linear form, respectively; P2O, pyranose 2-oxidase.

Enzyme aspects of D-erythro-*hexos-2,3-diulose biosynthesis.*—As evidenced by HPLC (Fig. 1) the first step of conversion of D-glucose to **2** is C-2 oxidation to intermediate **1** catalysed by P2O, a well documented enzyme reaction by some wood decay fungi [1–5]. Nothing is so far known about **1**-utilizing enzyme catalysing the oxygen dependent second step of the transformation, C-3 oxidation of the aldosulose. Positive transformation with the partially purified P2O preparation may indicate that only one enzyme, i.e., P2O, is responsible for the double oxidation of D-glucose. Considering that after establishment of isoform equilibrium **1** can assume a pyranose form sterically compatible with P2O structural requirements for substrate recognition, we hypothesize a reaction mechanism for P2O catalysed production of **2** (Fig. 4) where, on recycling, P2O product **1** can serve as substrate of the same enzyme. Interestingly in this connection, we have demonstrated C-3 oxidation of 2-deoxy-D-*arabino*-hexose by enzyme preparations of the same fungus (unpublished observations). Further, oxidase activity staining (chloronaphthol as chromogen) on isoelectric focusing agarose gels (pH 3.5–9.5) gave bands of the same mobility, detected separately with D-glucose and with **1** as substrates (not shown). Analogy could be also found with aldos-2-ulose dehydratase, its apparent ability to catalyse two subsequent dehydrations of the same initial substrate (**1**) [7,9]. On the other hand, the isolated hydrazones **4** and **5** suggest domination of isoforms with a furanose ring. To verify our scheme (Fig. 4), it is necessary to conduct further experiments using highly purified P2O.

Our findings may be of significance in relation to the Cetus biotechnological process for production of crystalline D-fructose from D-glucose via **1** [14]. The authors recognized an aldosulose-utilizing enzyme, aldos-2-ulose dehydratase, which was responsible for contamination by cortalcerone and its decomposition products of **1** produced in the first P2O catalysed step [8,14]. We have now shown that another aldosulose-utilizing activity, perhaps a P2O-related one, may interfere with quantitative conversion of D-glucose to **1**, i.e., **1** is not an end-product of the conversion even by enzyme preparations of aldos-2-ulose dehydratase deficient fungi. The biological activities of **2** are still unknown.

4. Conclusions

(1) D-*arabino*-Hexos-2-ulose (2-keto-D-glucose) is not an end oxidation product of D-glucose by pyranose 2-oxidase (P2O) containing preparations of wood-degrading basidiomycete fungi, as thought earlier [8]. Enzyme extracts of *Oudemansiella mucida*, *Trametes versicolor*, and *Phanerochaete chrysosporium* catalyse a second step oxidation of the substrate resulting in production of a tricarbonyl sugar derivative identified as D-*erythro*-hexos-2,3-diulose (2,3-diketo-D-glucose).

(2) The existence of O_2-dependent enzyme mechanisms for successive oxidation of D-glucose at C-2 and C-3, yielding 2 mol H_2O_2 per 1 mol substrate, supports our findings [5] that P2O plays an important physiological role as a major source of H_2O_2 in the ligninolytic systems of P2O-positive wood rot fungi.

(3) The C-3 oxidation described here represents the fifth type of enzyme conversion recognized so far for D-*arabino*-hexos-2-ulose, besides C-1 oxidation by glucose 1-

oxidase [15] (EC 1.1.3.4), C-3/C-4 dehydration by aldos-2-ulose (pyranosone) dehydratase [7], NADPH-dependent reduction by an aldos-2-ulose (osone) reductase [16] (the product not yet identified), and C-6 phosphorylation by homogenates of rat adipose tissue [17].

Acknowledgements

We thank Padmanabh Dwivedi, postgraduate UNESCO course participant, India, for electrophoretic analysis. This work was supported by grants 204/94/1191 from the Grant Agency of the Czech Republic, and 620421 from the Grant Agency—Academy of Sciences of the Czech Republic.

References

[1] Y. Machida and T. Nakanishi, *Agric. Biol. Chem.*, 48 (1984) 2463–2470.
[2] H.-J. Danneel, E. Rôssner, A. Zeeck, and F. Giffhorn, *Eur. J. Biochem.*, 214 (1993) 795–802.
[3] K.-S. Shin, H.-D. Youn, Y.-H. Han, S.-O. Kang, and Y.C. Hah, *Eur. J. Biochem.*, 215 (1993) 747–752.
[4] J. Volc, E. Kubátová, P. Sedmera, G. Daniel, and J. Gabriel, *Arch. Microbiol.*, 156 (1991) 297–301.
[5] G. Daniel, J. Volc, and E. Kubátová, *Appl. Environ. Microbiol.*, 60 (1994) 2524–2532.
[6] J. Volc and K.-E. Eriksson, *Methods Enzymol.*, 161 (1988) 316–322.
[7] J. Gabriel, J. Volc, P. Sedmera, G. Daniel, and E. Kubátová, *Arch. Microbiol.*, 160 (1993) 27–34.
[8] K. Koths, R. Halenbeck, and M. Moreland, *Carbohydr. Res.*, 232 (1992) 59–75.
[9] R. Baute, M.-A. Baute, and G. Deffieux, *Phytochemistry*, 26 (1987) 1395–1397.
[10] S. Bayne, *Methods Carbohydr. Chem.*, 2 (1963) 421–423.
[11] J. Kocourek, M. Tichá, and J. Koštíř, *J. Chromatogr.*, 24 (1966) 117–124.
[12] A. Bax and R. Freeman, *J. Magn. Reson.*, 44 (1981) 542–561.
[13] H. Kessler, C. Griesinger, R. Kerssebaum, K. Wagner, and R.R. Ernst, *J. Am. Chem. Soc.*, 109 (1987) 607–609.
[14] K.E. Koths, R.F. Halenbeck, and P.M. Fernandes, U.S. Patent 4,569,910, 11 February 1986.
[15] J. Geigert, S.L. Neidleman, D.S. Hirano, B. Wolf, and B.M. Panschar, *Carbohydr. Res.*, 113 (1983) 163–165.
[16] K. Uehara and M. Takeda, *J. Biochem.*, 56 (1964) 48–56.
[17] A. Hernandes and A. Sols, *Biochem. J.*, 86 (1963) 166–172.