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Note

Double oxidation of D-xylose to D-*glycero*-pentos-2,3-diulose (2,3-diketo-D-xylose) by pyranose dehydrogenase from the mushroom *Agaricus bisporus*

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Abstract

Pyranose dehydrogenase purified to homogeneity from the mycelia of the basidiomycete fungus *Agaricus bisporus* catalyzed the oxidation of D-xylose at C-2 to D-*threo*-pentos-2-ulose (2-keto-D-xylose) and successively at C-3 to D-*glycero*-pentos-2,3-diulose (2,3-diketo-D-xylose) using 1,4-benzoquinone as an electron acceptor. The sites of oxidation were deduced from the spectroscopic analysis (MS, NMR) of the *N*,*N*-diphenylhydrazone derivatives of the reaction products.

Keywords: D-Xylose; 2-Keto-D-xylose; 2,3-Diketo-D-xylose; D-*threo*-Pentos-2-ulose; D-*glycero*-Pentos-2,3-diulose; Dicarbonyl sugars; Pyranose dehydrogenase; *Agaricus bisporus*

Transformations of common unprotected sugars (aldoses and ketoses) into compounds with applied potential for production of bulk chemicals, pharmaceuticals and various fine or specialty chemicals are gaining an increased interest [1]. Dicarbonyl sugar derivatives represent an example of such useful intermediates of which D-*arabino*-hexos-2-ulose (2-keto-D-glucose) and D-*lyxo*-hexos-2-ulose (2-keto-D-galactose) were proposed as key intermediates in the processes for conversion of D-glucose to D-fructose [2,3] and D-galactose to D-tagatose [4,5]. These and other aldosulose (ketoaldose) derivatives are conveniently available through regioselective oxidations catalyzed by the fungal enzymes pyranose 2-oxidase [6,7] and quinone-dependent pyranose dehydrogenase (PDH) [8]. Recently we showed that the latter enzyme purified from the basidiomycete *Agaricus bisporus* catalyzed successive C-2/C-3 (or C-3/C-2) dehydrogenation of D-glucose to D-*erythro*-hexos-2,3-diulose (2,3-diketo-D-glucose), while D-galactose was attacked only at C-2 to produce D-*lyxo*-hexos-2-ulose [9]. The

Abbreviations: DPH, diphenylhydrazone; PDH, pyranose dehydrogenase; MES, 2-(*N*-morpholino)ethanesulfonic acid.

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aim of this work was to demonstrate the oxidation pattern of *A. bisporus* PDH towards another of its preferred substrates, D-xylose, and to identify the corresponding reaction product(s).

D-Xylose derived from hemicelluloses serves, along with D-glucose and D-galactose, as a major carbon source utilized by the litter decomposing fungus *A. bisporus* in the natural environment. Interestingly, it is oxidized by PDH at least equally well as hexose constituents of lignocellulosics (116% activity relative to D-glucose [8]). Preliminary thin-layer chromatography (TLC) analyses of the D-xylose ($R_f = 0.31$) reaction product(s) indicated formation of the C-2 carbonyl derivative ($R_f = 0.23$) giving, on detection with diphenylamine–aniline reagent, a blue spot, which is characteristic of aldos-2-uloses (2-ketoaldoses) [10]. This product, however, accumulated in the PDH reaction mixtures only transiently and was further transformed into an end-product that was not detectable with the same reagent (not shown). An analogous reaction sequence for D-xylose was demonstrated by HPLC (Fig. 1). During the transformation time course, the intermediate **1** (Fig. 1, peak IV) was continually converted to **2** (peak I), which was eluted near the column void volume, similar to the double oxidation product of D-glucose described previously [9].

Dicarbonyl sugars adopt complex equilibria of different tautomeric forms in water solutions [11]. For the sake of simplicity, only the nonhydrated pyranoid forms participating in PDH reaction are shown in Fig. 3. Such mixtures might be characterized by NMR, but they are not useful for structure elucidation. To find the site(s) of oxidation of D-xylose by PDH purified to homogeneity, we used the derivatization of carbonyls with *N*,*N*-diphenylhydrazine (DPH) and studied the structure of the products.

Among the six isolated DPHs of biotransformation products, there were, according to the $[M + Na]^+$ ions observed in the FAB mass

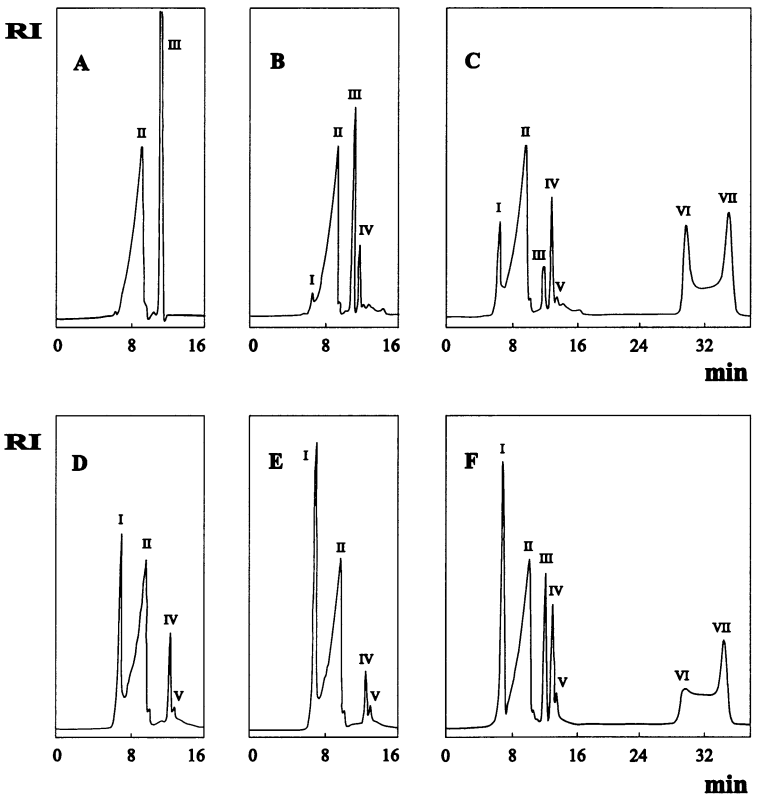

Fig. 1. (A)–(E) HPLC monitoring of D-xylose oxidation by PDH from *A. bisporus* at incubation times of 0, 10, 30, 90 and 240 min, respectively. Peaks: I, D-*glycero*-pentos-2,3-diulose; II, MES buffer; III, D-xylose; IV, D-*threo*-pentos-2-ulose (2-keto-D-xylose); V, hypothetical D-*erythro*-pentos-3-ulose (3-keto-D-xylose); VI, 1,4-benzoquinone; VII, hydroquinone; (F) Chromatogram showing composition of the PDH/D-xylose reaction mixture used for preparation and isolation of the spectroscopically well-defined *N*,*N*-diphenylhydrazone derivatives (Fig. 2). Reaction conditions are described in Section 1.

Table 1
^{1}H NMR data of hydrazone derivatives **4**–**9** (399.90 MHz, CD_3OD, 30 °C) [a]

Proton	**4**	**5**	**6**	**7**	**8**	**9**
H-1	6.578	6.638	7.036	6.013	6.631	7.030
$J_{1,2}$	*5.7*					
H-2	4.442					
$J_{2,3}$	*5.5*					
H-3	3.657	5.290	5.244		5.199	5.203
$J_{3,4}$	*3.1*	*2.1*	*2.9*			
$J_{3,4d}$					*3.5*	*3.7*
$J_{3,4u}$					*5.0*	*6.0*
H-4d	3.735	4.311	4.182	4.696	4.042	4.077
$J_{4d,4u}$					11.3	11.2
H-4u					3.956	3.958
$J_{4,5d}$	*5.2*	*7.0*	*6.0*	*4.3*		
$J_{4,5u}$	*6.4*	*6.4*	*6.6*	*7.4*		
H-5d	3.671	3.830	3.848	4.017		
$J_{5d,5u}$	*11.1*	*10.9*	*11.0*	*11.4*		
H-5u	3.623	3.695	3.737	3.870		

[a] Additional signals – **4**: 7.110 (4 H, m, *ortho*-Ph), 7.163 (2 H, m, *para*-Ph), 7.393 (4 H, m, *meta*-Ph); **5**: 7.235 (4 H, m, *ortho*-Ph), 7.344 (2 H, m, *para*-Ph), 7.506 (4 H, m, *meta*-Ph); **6**: 6.882 (4 H, m, *ortho*-Ph), 6.911 (4 H, m, *ortho*-Ph), 7.046 (2 H, m, *para*-Ph), 7.239 (2 H, m, *para*-Ph), 7.242 (4 H, m, *meta*-Ph), 7.386 (4 H, m, *meta*-Ph); **7**: 7.047 (4 H, m, *ortho*-Ph),7.070 (2 H, m, *para*-Ph), 7.143 (4 H, m, *ortho*-Ph), 7.249 (4 H, m, *meta*-Ph), 7.296 (2 H, m, *para*-Ph), 7.443 (4 H, m, *meta*-Ph); **8**: 7.231 (4 H, m, *ortho*-Ph), 7.359 (2 H, m, *para*-Ph), 7.518 (4 H, m, *meta*-Ph); **9**: 6.855 (4 H, m, *ortho*-Ph), 6.891 (4 H, m, *ortho*-Ph), 7.036 (2 H, m, *para*-Ph), 7.227 (2 H, m, *para*-Ph), 7.229 (4 H, m, *meta*-Ph), 7.377 (4 H, m, *meta*-Ph).

spectra, three mono- and three bis-DPH derivatives. The compound of M_r 316 containing a six-proton contiguous spin system in its ^{1}H NMR spectrum (Table 1) is therefore DPH-xylose (Fig. 2, **4**). The next two compounds, both exhibiting a contiguous four spin system (C-3–C-5) were mono-DPH and bis-DPH derivatives. Proton H-1 resonating as a singlet in these ^{1}H NMR spectra indicated an absence of protons at C-2. The former compound having a carbonyl group (δ_C 199.56) was assigned the structure **5**; the latter was its bis-DPH analogue **6**. The most interesting compound isolated was a bis-DPH derivative containing a –CH(OH)CH_2OH moiety and a free carbonyl. Heteronuclear coupling of the quaternary carbon carrying the second DPH group to both H-1 and H-5 unambiguously identifies it as C-3 (Fig. 4); the C=O is consequently at C-2 (Fig. 2, **7**). Therefore, the parent free sugar was D-*glycero*-pentos-2,3-diulose (2,3-diketo-D-xylose, Fig. 3, **2**). The remaining two isolated compounds were also a pair of mono- and bis-DPH derivatives, however, containing only four carbons originating from the substrate sugar. Again, the partial structure –CH(OH)CH_2OH was present in both of them; H-1 resonated as a singlet. The compound of M_r 284 containing a carbonyl (see Table 2) was therefore assigned the structure **8**, its bis-DPH counterpart is depicted as **9**. The presence of ions m/z 223 diagnostic for diphenylhydrazones having carbonyl at position 2 [10] observed in the mass spectra of **5**, **7**, and **8** confirms this deduction. Also, the chemical shift of H-1 in 1,2-bis-DPH derivatives **6** and **9** (7.036 and 7.070 ppm), different from those of remaining compounds

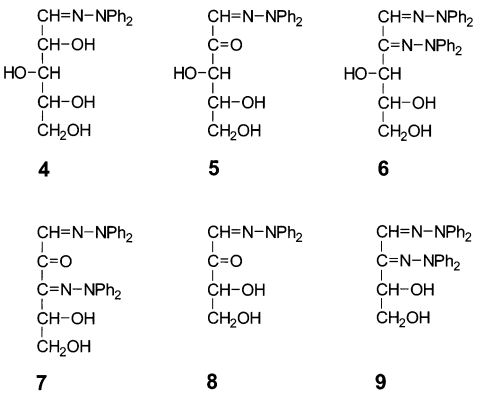

Fig. 2. Structures of diphenylhydrazone derivatives used for identification of the oxidation products of D-xylose by PDH (*A. bisporus*) and 1,4-benzoquinone as an electron acceptor.

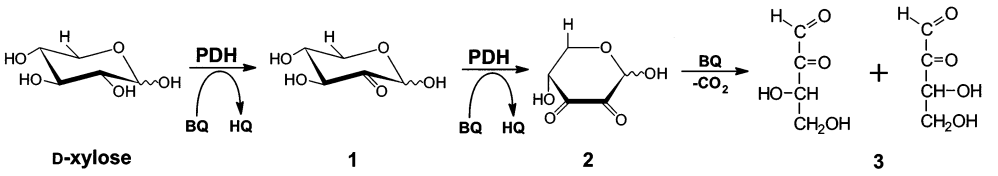

Fig. 3. Reaction sequence for oxidation of D-xylose by PDH from *A. bisporus*. D-Xylose is double oxidized in the sequence C-2→C-3 to diketopentose **2** (D-*glycero*-pentos-2,3-diulose), which is apparently susceptible to nonenzymatic oxidative decarboxylation resulting in **3** (D/L-*glycero*-tetros-2-ulose) via a keto/enol intermediate (not shown).

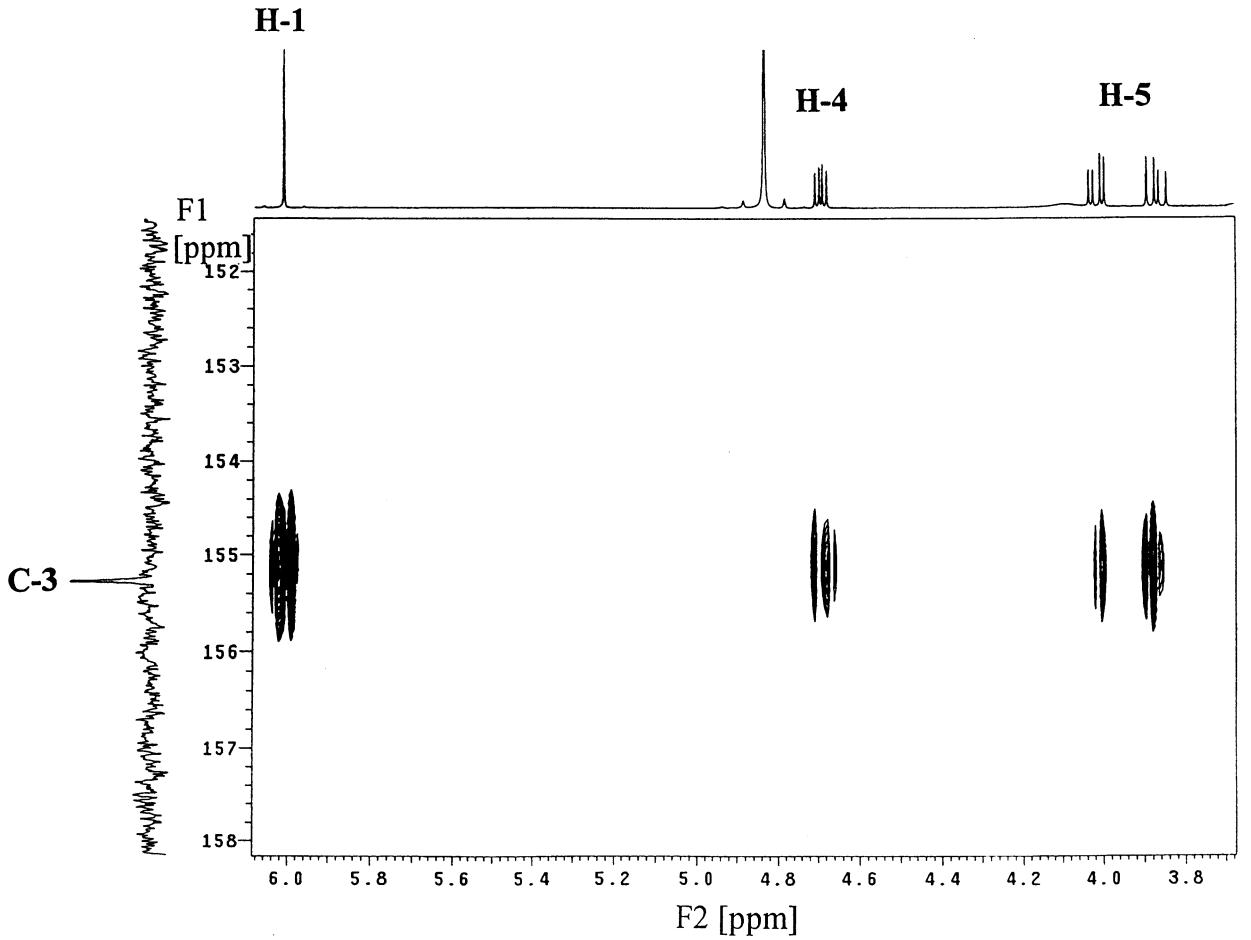

Fig. 4. Diagnostic HMBC correlations in compound **7**.

(6.0–6.6 ppm) is consistent with the assigned structures. In view of the findings on the reactivity of 3-ketoaldoses (e.g., susceptibility of 2-deoxy-3-keto-D-glucose to oxidative decomposition [7]), we suppose that minor hydrazone components **8** and **9** are derived from 2-ketotetrose **3** (D-*glycero*-tetros-2-ulose) formed nonenzymatically in the PDH reaction mixture upon oxidative decarboxylation of **2** (via an α,β-diketo-carboxylic acid) with molecular oxygen or benzoquinone as an oxidative agent.

Based on the results of HPLC analyses and the structures of free di- and tricarbonyl sugars deduced from the above identified hydrazone derivatives, we conclude that D-xylose is converted by PDH and 1,4-benzoquinone to ketoaldose **1**, which is subsequently oxidized at C-3 by the same enzyme to give diketoaldose **2** (Fig. 3). In contrast to the C-3 preferring primary oxidation of D-glucose, PDH activity at C-3 of D-xylose apparently does not play a significant role. Only the minor peak V may be indicative of trace amounts of the

primary C-3 oxidation product (Fig. 1). As shown previously, PDH exhibits the most pronounced regioselectivity towards D-galactose, which is only attacked at C-2 [9]. Dioxidation at C-2 and C-3 was recently described for pyranose oxidase (*Peniophora gigantea*) and 1,5-anhydro-D-glucitol as substrate with 1,5-anhydro-D-*erythro*-hex-2,3-diulose formed (via 1,5-anhydro-D-fructose) after prolonged reaction time [7]. Similar dual activity had also been reported for some alcohol oxidases, e.g., aryl alcohol oxidase [12]. In this case, however, the double oxidation took place at the same carbon, resulting in formation of the carboxylic group.

While ketopentose **1** (D-*threo*-pentos-2-ulose, 2-keto-D-xylose) has been known as a product of D-xylose oxidation by the related enzyme, pyranose 2-oxidase of different fungal origin [10,13,14], this is to our knowledge the first time that the tricarbonyl sugar derivative **2** was identified as a product of enzyme activity or organic synthesis. A search of the Chemical Abstracts database for double oxidation products of D-xylose other than D-*threo*-pent-2-ulosonic (2-keto-xylonic) acid was unsuccessful. Using PDH, D-xylose can be converted to **2** in yields approaching 80% (based on HPLC data). This work thus extends our previous reports [9,15,16] demonstrating attractive possibilities for pyranose oxidoreductases in the preparation of rare di- and tricarbonyl sugar derivatives. Nothing is so far known about the further metabolic fate of **2** in *A. bisporus*.

1. Experimental

Enzyme source and purification.—*A. bisporus* (Lange) Imbach, strain 306, obtained from the Culture Collection of Basidiomycetes (CCBAS) maintained at the Institute of Microbiology, Academy of Sciences of the Czech Republic, Prague, was grown stationary on a corn steep liquid medium [8] for 14 days. Intracellular PDH was purified to apparent homogeneity (16 U/mg protein) and assayed spectrophotometrically by measuring at 290 nm the reduction of 1,4-benzoquinone by D-glucose as described previously [8]. One unit of the enzyme activity oxidizes D-glucose at 1 μmol min^{-1} under the assay conditions.

PDH catalyzed oxidation of D-*xylose.*—Analytical transformations were performed aerobically in darkness at 30 °C under gentle stirring in a 10-mL Erlenmeyer flask containing D-xylose (10 mM, 7.5 mg), 1,4-benzoquinone (20 mM, 2 × 5.4 mg, the 2nd portion added after 15 min) and 6 U purified PDH in 10 mM MES buffer, pH 6.7 (5 mL). Time course of the reaction was followed by HPLC analysis of the samples (0.1 mL) withdrawn at time intervals, passed through Ultrafree-MC 30,000 NMWL Filter Units (Millipore, Bedford, MA, USA) to remove the enzyme protein and diluted ten times with water. The reaction mixture (40 mL) for semipreparative transformations (for 12 h at 30 °C) contained D-xylose (50 mM, 300 mg), 1,4-benzoquinone (100 mM, 2 × 204 mg, the 2nd portion added

Table 2
^{13}C NMR data of hydrazone derivatives **4**–**9** (100.57 MHz, CD_3OD, 30 °C)

Carbon	4	**5**	**6**	**7**	**8**	**9**
1	139.28	131.10	128.74	132.71	131.95	129.00
2	74.20	199.56	160.96	194.50	199.23	160.85
3	74.27	74.66	71.58	155.28	75.78	73.07
4	73.41	75.30	75.48	76.01	66.64	67.14
5	64.68	64.39	64.94	66.37		
ortho-Ph	123.82	124.00	123.76	125.70	123.71	123.52
meta-Ph	131.02	131.52	131.41	131.38	131.55	131.38
para-Ph	125.75	128.10	124.86	127.70	128.24	127.01
ipso-Ph	145.48	143.36	143.92	148.29	143.79	143.89
ortho-Ph			123.96	124.53		122.95
meta-Ph			130.56	130.31		130.55
para-Ph			127.02	125.78		124.85
ipso-Ph			150.40	148.29		150.38

after 4 h) and 13 U PDH in 50 mM MES, pH 6.7.

Chromatographic analysis of D-*xylose reaction product(s).*—TLC of the free sugars was conducted on microcrystalline cellulose sheets [9] using diphenylamine–aniline detection reagent [17]. Their hydrazone derivatives obtained from the PDH/D-xylose reaction mixture were separated on Silica Gel 60 F_{254} TLC aluminum sheets 20×20 (E. Merck, Darmstadt, Germany) using 30:1 $CHCl_3$–MeOH and detected under Vis (yellow streaks) or UV (**4**, **5**) light. HPLC was performed on a SP 8800 liquid chromatograph (Spectra Physics, San Jose, CA, USA) fitted with a refractive index detector, using Ostion LG KS 0800 Na^+ column 250×8 mm (Watrex, Prague, Czech Republic) eluted at 80 °C with deionized water (0.5 mL min^{-1}).

Preparation and identification of hydrazone derivatives of D-*xylose oxidation products.*—The mixture (40 mL) of D-xylose transformation products was passed through YM 30 membrane (Amicon, Danvers, MA, USA) and the ultrafiltrate was supplemented successively with the same volume of ethanol and 300 μL freshly distilled DPH (Koch-Light Lab., Colnbrook, UK), intensively stirred for 1 h and left for 1 day at rt. The solvent was removed by vacuum evaporation, and the residue obtained dissolved in 2 mL MeOH and subjected to TLC as above. Pale-yellow to orange streaks of six major hydrazones were excised, combined separately, and extracted into MeOH. Their further purification to substantial homogeneity was achieved by rechromatography using solvent systems given below.

Spectroscopy.—^1H and ^{13}C NMR spectra of isolated hydrazones (Tables 1 and 2) were measured on a Varian INOVA-400 spectrometer in CD_3OD at 30 °C. The residual solvent signals were used as an internal standard (δ_H 3.33, δ_C 49.3). Carbon signal multiplicity was determined by APT (Attached Proton Test). 2D NMR experiments (gCOSY, TOCSY, HMQC, and HMBC) and NOE difference were performed using manufacturer's software. The assignment given in Tables 1 and 2 is based on COSY, HMQC and HMBC experiments. Sugar protons and aromatic protons belonging to the same ring were found by COSY. Signals within the same benzene group were assigned using their characteristic multiplet pattern. The phenyl groups attached to C-1 were differentiated by NOE between H-1 and *ortho*-Ph protons. Quarternary carbons (C-2, C-3, C-*ipso*) were assigned by HMBC. Positive ion FAB mass spectra were recorded on a Finnigan MAT 95 double-focusing instrument using *m*-nitrobenzyl alcohol as a matrix. The saddle field FAB gun (Ion Tech, Teddington, UK) was operated at 2 mA current and 6 kV energy; xenon (1×10^{-5} bar) was used as a bombarding gas.

D-*xylose-1-(*N,N-*diphenylhydrazone)* (**4**). Compound of R_f 0.04, rechromatographed using 15:1 $CHCl_3$–MeOH–Silica Gel 60 F_{254} aluminum sheets (E. Merck), colorless syrup (36 mg). UV–Vis: λ_{max} (MeOH) 270, 282 nm. FABMS, *m*/*z* (% rel. int.): 339 (35, $[M + Na]^+$), 299 (7), 225 (32), 195 (4), 183 (3), 169 (28), 168 (100).

D-threo-*Pentos-2-ulose 1-(*N,N-*diphenylhydrazone)* (**5**). Compound of R_f 0.10, rechromatographed using 20:1 $CHCl_3$–MeOH–Silica Gel 60 F_{254} sheets, pale yellow syrup (38 mg). UV–Vis: λ_{max} (MeOH) 231, 286, 344 nm. FABMS, *m*/*z* (% rel. int.): 337 (18, $[M + Na]^+$), 315 (1, $[M + H]^+$), 297 (18), 285 (10), 223 (5), 195 (3), 169 (41), 168 (100).

D - threo - *Pentos - 2 - ulose 1,2 - bis(*N,N-*diphenylhydrazone)* (**6**). Compound of R_f 0.23, rechromatographed using 40:1 $CHCl_3$–MeOH–Silica Gel 60 foils, dark yellow syrup (22 mg). UV–Vis: λ_{max} (MeOH) 239, 299, 347, 390 nm. FABMS, *m*/*z* (% rel. int.): 503 (12, $[M + Na]^+$), 463 (2), 419 (4), 401 (3), 312 (10), 297 (13), 252 (4), 221 (3), 195 (4), 183 (3), 169 (54), 168 (100).

D-glycero-*Pentos-2,3-diulose 1,3-bis(*N,N-*diphenylhydrazone)* (7). Compound of R_f 0.39, rechromatographed using $CHCl_3$–Silica Gel 60 foils and crystallized from MeOH (yellow needles, 58 mg), mp 141–142 °C. UV–Vis: λ_{max} (MeOH) 241, 278, 362 nm. FABMS, *m*/*z* (% rel. int.): 501 (9, $[M + Na]^+$), 461 (4), 447 (1), 293 (3), 249 (2), 223 (13), 195 (3), 183 (3), 169 (32), 168 (100).

D-glycero-*Tetros-2-ulose 1-(*N,N-*diphenylhydrazone)* (**8**). Compound of R_f 0.26, rechromatographed using 40:1 $CHCl_3$–MeOH–Silica Gel 60 F_{254} foils, pale yellow syrup (18 mg). UV–Vis: λ_{max} (MeOH) 271, 344 nm.

FABMS, m/z (% rel. int.): 307 (100, $[M + Na]^+$), 285 (31, $[M + H]^+$), 223 (26), 195 (4), 183 (6), 169 (25), 168 (76).

D - glycero - *Tetros - 2 - ulose 1,2 - bis(*N,N-*diphenylhydrazone)* (**9**). Compound of R_f 0.54, rechromatographed using $CHCl_3$–Silica Gel 60 foils, orange solid (20 mg). UV–Vis: λ_{max} (MeOH) 239, 299, 345, 387 nm. FABMS, m/z (% rel. int.): 473 (4, $[M + Na]^+$), 419 (6), 401 (2), 282 (5), 252 (3), 221 (5), 195 (2), 183 (2), 169 (43), 168 (100).

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