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Carbohydrate Research 310 (1998) 151–156

CARBOHYDRATE RESEARCH

Note

C-2 and C-3 oxidation of D-Glc, and C-2 oxidation of D-Gal by pyranose dehydrogenase from *Agaricus bisporus*

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Received 16 March 1998; accepted 13 June 1998

Abstract

The quinone-dependent sugar oxidoreductase, pyranose dehydrogenase purified from the basidiomycete *Agaricus bisporus* catalyzed C-2 and C-3 oxidation of D-Glc to D-*arabino*-hexos-2-ulose (2-keto-D-Glc) and, preferentially, D-*ribo*-hexos-3-ulose (3-keto-D-Glc). The two aldoketoses accumulated only transiently in the reaction mixture being continually converted to the same end-product, D-*erythro*-hexos-2,3-diulose (2,3-diketo-D-Glc). D-Gal was oxidized by pyranose dehydrogenase and 1,4-benzoquinone exclusively at C-2 to produce D-*lyxo*-hexos-2-ulose (2-keto-D-Gal). Structures of the enzyme reaction products were deduced from their *N*,*N*-diphenylhydrazone derivatives.

Keywords: 2-Keto-D-Glc, 3-Keto-D-Glc, 2,3-Diketo-D-Glc, 2-Keto-D-Gal, D-*arabino*-Hexos-2-ulose, D-*ribo*-Hexos-3-ulose, D-*erythro*-Hexos-2,3-diulose, D-*lyxo*-Hexos-2-ulose, Pyranose dehydrogenase, *Agaricus bisporus*

Characteristic for wood degrading fungi seems to be an increasingly growing family of pyranose oxidases (EC 1.1.3.10), enzymes catalyzing the C-2 oxidation of several aldoses [1,2]. Recently, we reported a novel dicarbonyl sugar-producing oxidoreductase, the quinone dependent pyranose dehydrogenase (PDH) isolated from the fungus *Agaricus bisporus* [3]. With 1,4-benzoquinone as an electron acceptor, this flavoglycoprotein catalyzes oxidation of numerous monosaccharides and oligosaccharides. The time course TLC analysis of D-Glc transformation by PDH revealed that next to a product of R_f 0.28 (blue spot), identified as D-*arabino*-hexos-2-ulose (**1**) [3], another sugar derivative, **2** (R_f 0.40, brownish yellow spot) was formed simultaneously with **1**. Both products accumulated only transiently in the reaction mixture and disappeared after prolonged incubation. We have now identified the second of the two products and clarified the metabolic fate of both the products. With respect to the complexity of the PDH reaction

Abbreviations: PDH, pyranose dehydrogenase, DPH, diphenylhydrazone.

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0008-6215/98/$—see front matter
PII S0008-6215(98)00151-7

mixture with D-Glc (several products in tautomeric equilibrium), spectroscopic examination of their well defined hydrazone derivatives was used to determine the position of carbonyl group(s).

The multiplicity of PDH/glucose oxidation products was confirmed by HPLC analyses demonstrating that peak of **1** (IV, $T = 12.25$ min) was followed by a further two peaks (V, VI) of unknown product(s), probably partially separated tautomeric forms of **2** [Fig. 1(B)–(D)]. Peak VII seemed to correspond to a decomposition product because it was strongly repressed in conversions at 4 °C or at lower pHs. The total area of the product peaks IV–VII at maximum was significantly less than that of starting Glc, indicating that the primary products were continually transformed into the end-product which co-eluted substantially with buffer peak I, thereby increasing its area. In control experiments where **1** was substituted for Glc in the same PDH reaction mixture, it was completely utilized, while in the PDH blank mixture **1** was not consumed after overnight incubation (not shown).

FAB MS together with ^{1}H and ^{13}C NMR spectra showed that the reaction products isolated as

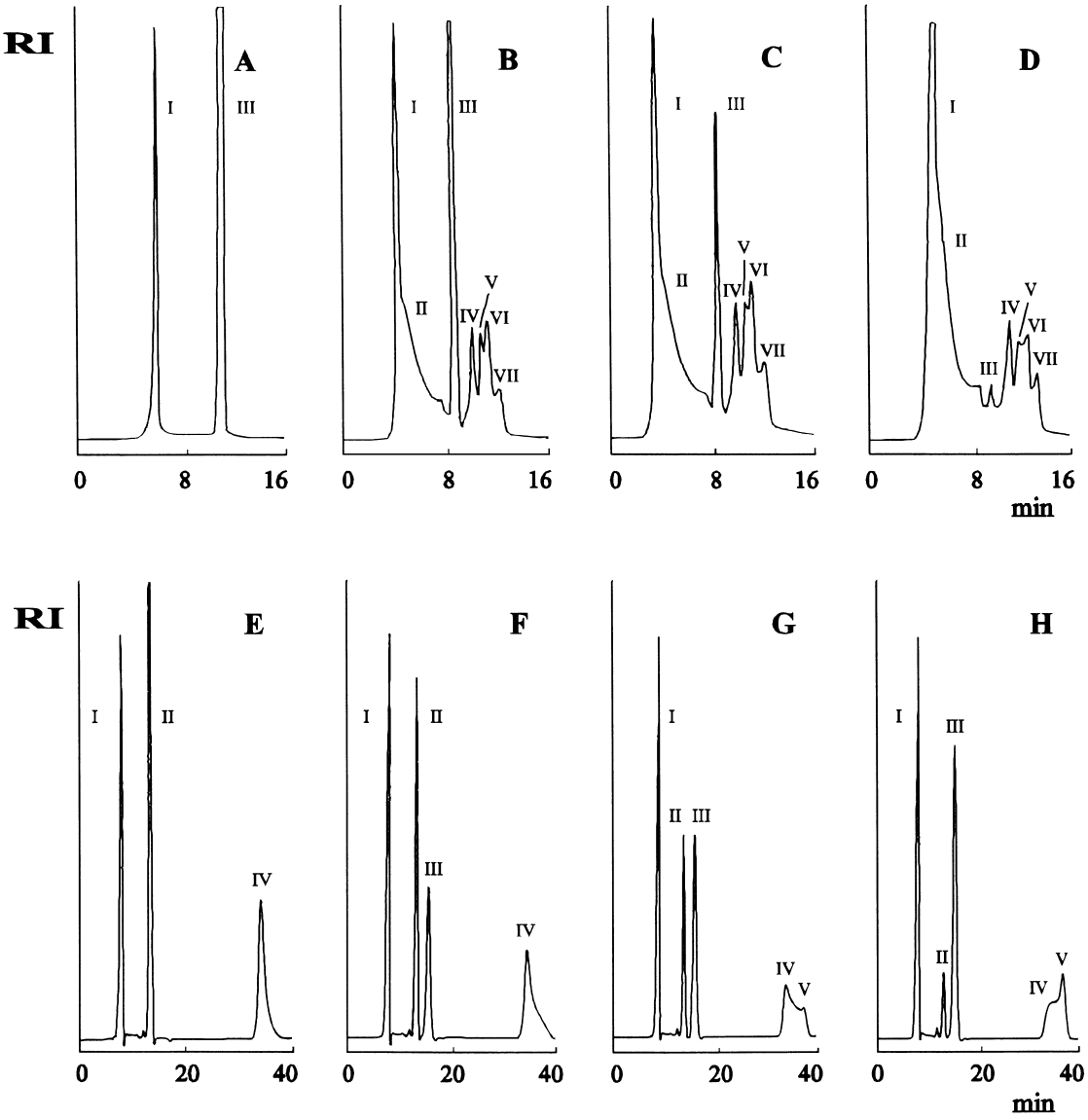

Fig. 1. (A)–(D) HPLC monitoring of D-Glc oxidation to corresponding hexosuloses by pyranose dehydrogenase at incubation times of 0, 0.5, 2 and 4 h, respectively. Peaks: I, citrate buffer; II, D-*erythro*-hexos-2,3-diulose; III, D-Glc; IV, D-*arabino*-hexos-2-ulose; V,VI, D-*ribo*-hexos-3-ulose (tautomeric forms); VII, unknown, probably decomposition product; (E)–(H) monitoring of D-Gal oxidation at times of 0, 20, 40 and 90 min, respectively. Peaks: I, citrate buffer; II, D-Gal; III, D-*lyxo*-hexos-2-ulose; IV, 1,4-benzoquinone; V, hydroquinone. Reaction conditions are described in Experimental.

N,*N*-diphenylhydrazone (DPH) derivatives were three bis-DPH (**5**, **6**, **7**) and one mono-DPH derivative (**4**). AB and ABCD spin systems found in ^{1}H NMR spectra of **4** (Tables 1 and 2) indicate that C-3 carries no hydrogen. The only carbon signal assignable to this atom (Table 3, a singlet at 104.92 ppm) implies an acetal or hemiacetal group. As C-6 resonates downfield with respect to free sugars, it forms a 6→3 bond completing a furanose ring (**4**, Fig. 2). Further supporting evidence is provided by the elimination of the side chain with hydrogen transfer in FAB MS (Fig. 2) producing the *m*/*z* 226 ion. Therefore, the parent free sugar was D-*ribo*-hexos-3-ulose (**2**, 3-keto-D-Glc, Fig. 3). Singlet H-1 and an $(OCH)_3CH_2OH$ moiety present in **5** identifies it as bis-DPH of D-*arabino*-hexos-2-ulose (**1**, 2-keto-D-Glc). This is in accord with previous identification of one of the PDH/D-Glc reaction products as **1** through its peracetylated 1-mono-DPH derivative [3]. Singlet H-1 and four-spin system of H-4 to H-6 in ^{1}H NMR spectra of **6** and **7** suggest that these compounds are derived from D-*erythro*-hexos-2,3-diulose (**3**, Fig. 3). The position of the free carbonyl was inferred from mass spectral fragmentation (Fig. 2) and from the examination of fine structure of multiplets of the respective quaternary carbon atoms in proton-coupled ^{13}C NMR spectra. The tricarbonyl sugar **3** was recently identified as a new sugar metabolite resulting from oxidation of **1** by another fungal enzyme, pyranose 2-oxidase [4]. On HPLC, **3** in the PDH reaction mixture had the same retention time [Fig. 1(B)–(D), II, co-eluting substantially with buffer peak] as authentic **3** giving a broad peak of its tautomeric forms.

Table 1
^{1}H NMR data (chemical shift, multiplicity) of hydrazone derivatives **4**–**8**

Proton	**4**	**5**	**8**	**6**	**7**
1	6.640 d	7.017 s	6.679 s	8.009 s	6.422 s
2	4.287 d	—	—	—	—
3	—	5.546 d	5.393 d	—	—
4	4.063 d	4.091 dd	3.985 dd	3.490 d	5.448 d
5	4.160 ddd	3.951 ddd	3.915 ddd	3.757 ddd	4.177 ddd
6u	3.813 dd	3.752 dd	3.637 dd	3.130 dd	3.671 dd
6d	3.900 dd	3.942 dd	3.656 dd	3.470 dd	3.741 dd

Table 2
Proton–proton coupling constants [Hz] of hydrazones **4**–**8**

J(i,j)	**4**	**5**	**8**	**6**	**7**
4,5	5.5	8.1	1.8	8.8	5.1
5,6u	5.0	7.0	6.7	6.0	6.4
5,6d	3.7	3.2	6.0	3.3	4.5
6u,6d	−9.5	−11.9	−11.0	−11.5	−11.4

Additional couplings: **4**, $J_{1,2}$ = 5.6 Hz; **5**, $J_{3,4}$ = 1.4 Hz; **8**, $J_{3,4}$ = 7.4 Hz.

Table 3
^{13}C NMR data (chemical shift, multiplicity) of hydrazones **4**–**8**

Carbon	**4**	**5**	**8**	**6**	**7**
1	138.04 d	128.41 d	133.51 d	128.83 d	128.33 d
2	75.75 d	161.21 s	201.16 s	188.31 s	143.25 s
3	104.92 s	70.66 d	73.32 d	145.03 s	201.14 s
4	72.15 d	75.06 d	73.55 d	67.73 d	76.14 d
5	72.07 d	73.39 d	72.40 d	72.97 d	75.33 d
6	72.88 t	65.61 t	64.68 t	63.71 t	63.97 t

Based on the above deduced structures of free di- and tricarbonyl sugars and results of TLC and HPLC analyses, we conclude that D-Glc is with PDH and 1,4-benzoquinone simultaneously converted to aldoketoses **1** and **2** which are subsequently oxidized by the same enzyme to the identical end-product, aldodiketose **3** (Fig. 3). Thus, PDH acts alternatively at D-Glc C-2 and C-3, whether or not one of these positions adjacent to the site of attack bears a ketonic carbonyl group. Time course analyses of the PDH reaction showed that the molar ratio of the two aldoketoses varied depending on the individual enzyme preparation and reaction time. Under conditions corresponding to Fig. 1(C) it was ca. 1:3 for **1** and **2**, respectively, during the time of their maximum accumulation. Generally, compound **2** was initially accumulated faster, suggesting that position C-3 is the principle site of Glc oxidation. The molar ratio of **1** and **2** was, however, affected by the relative rate of their consumption during oxidation to **3**.

Dehydrogenation of another PDH preferred substrate, D-Gal, resulted in almost quantitative conversion into a single product [Fig. 1(F)–(H)]. Its chromatographic behaviour corresponded to that of authentic D-*lyxo*-hexos-2-ulose. Consistently, H-1 of its mono-DPH derivative (**8**, Fig. 2) resonates as a singlet, C-3 to C-6 form a contiguous spin system and C-2 is free (Table 3). Consequently, PDH catalyses dehydrogenation of D-Gal exclusively at C-2.

Oxidation of D-Glc to **2** has been earlier reported for another microbial enzyme, D-glucoside 3-dehydrogenase (EC 1.1.99.13) isolated from *Agrobacterium tumefaciens* and *Flavobacterium saccharophilum*, and characterized as an 85 and 66 kDa iron-sulfur flavoprotein, respectively [5,6]. Similar to PDH, this enzyme has high affinity for a

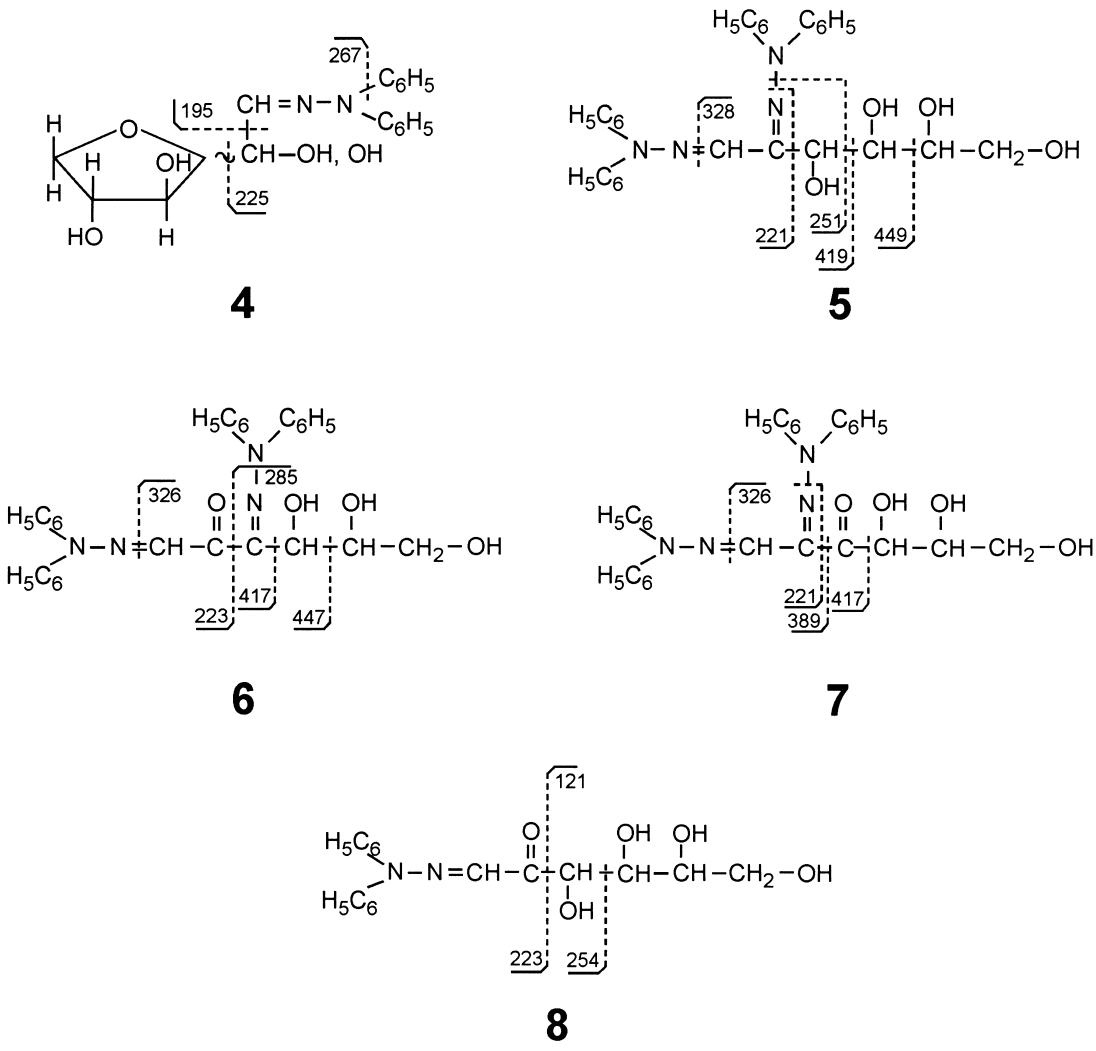

Fig. 2. Structures and FAB MS fragmentation of hydrazone derivatives used for identification of the oxidation products of D-Glc and D-Gal by pyranose dehydrogenase.

variety of oligomeric sugars and glucosides but, in contrast, is much more selective for monosaccharides and oxidizes D-Gal at C-3. To our knowledge, there has been no earlier report of a fungal oxidoreductase acting at C-3 of D-Glc. On the other hand, C-2 oxidation of D-Gal is a common catalytic feature of PDH (monomeric extracellular 79 kDa protein [3]) with pyranose 2-oxidase, typically a tetrameric intracellular ca. 300 kDa protein of fungal origin [7,8].

Considering the above demonstrated lower specificity of the *Agaricus's* sugar oxidoreductase for the site of action on D-Glc (dehydrogenation at both C-2 and C-3, the latter prevailing), we propose pyranose dehydrogenase (PDH) as a more suitable trivial name for this enzyme compared with the name pyranose 2-dehydrogenase (P2D) we used in our preliminary report on the enzyme [3].

1. Experimental

Enzyme source and purification.—Agaricus bisporus (Lange) Imbach, strain U3, obtained from the culture collection at Horticulture Research International (Wellesbourne, UK) was grown stationary on a liquid medium [3] for 14 days. Pyranose dehydrogenase (PDH) was purified from mycelial extracts to apparent homogeneity (16 U/mg protein) and assayed by monitoring the reduction of 1,4-benzoquinone to hydroquinone ($\varepsilon_{290} = 2.3\ mM^{-1}\ cm^{-1}$) by Glc [3]. One U of the enzyme activity oxidizes D-Glc at $1\ \mu mol\ min^{-1}$ under the assay conditions.

PDH catalyzed oxidation of D-*Glc and* D-*Gal.*—The reaction mixture (20 mL) for semipreparative transformations contained D-Glc [Fig. 1(A)–(B)] or D-Gal (180 mg, 50 mM), 1,4-benzoquinone [(Sigma,

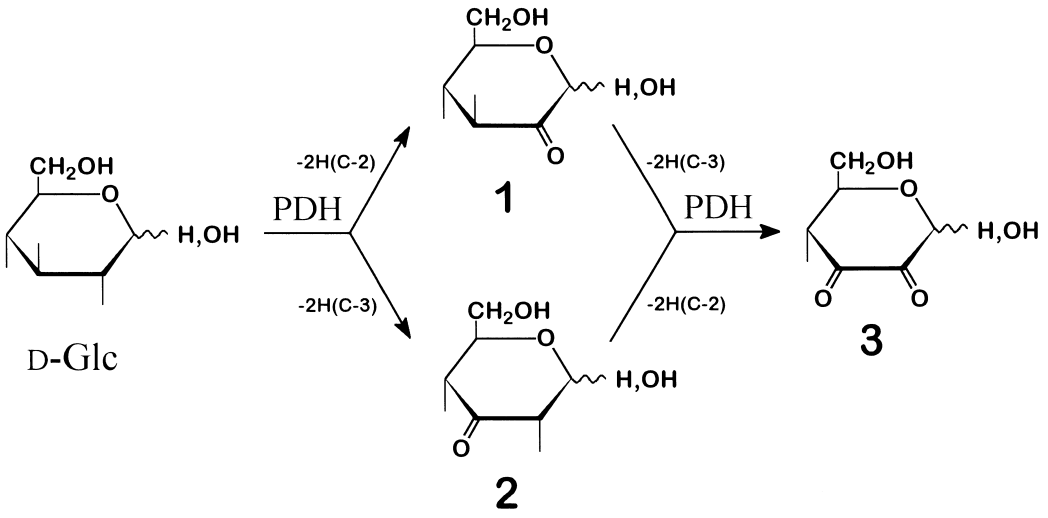

Fig. 3. The proposed scheme for two step oxidation of D-Glc to D-*erythro*-hexos-2,3-diulose (**3**, 2,3-diketoGlc) via D-*arabino*-hexos-2-ulose (**1**, 2-ketoGlc) and D-*ribo*-hexos-3-ulose (**2**, 3-ketoGlc), by pyranose dehydrogenase from *Agaricus bisporus*.

St. Louis, MO) 108 mg, 50 mM] and PDH (10 U) in 10 mM Na citrate, pH 5.5. Aerobic incubations proceeded under gentle stirring in darkness at 30 °C for 2 h. Samples (0.1 mL) withdrawn at time intervals for HPLC analyses were purified by passing through Ultrafree-MC 30,000 NMWL Filter Units (Millipore, Bedford, MA) and diluted 10 times with water. Analytical scale conversions [Fig. 1(E)–(H), Gal conversion] were followed in reaction mixtures (5 mL) consisting of the respective sugar (10 mM), 1,4-benzoquinone (10 mM) and PDH (1 U) in 5 mM Na citrate, pH 5.5.

Chromatographic analysis of sugar oxidation products.—TLC of free sugars was conducted on SigmaCell Type 100 (Sigma) cellulose sheets using 140:100:33:80 1-BuOAc–AcOH–Me_2CO–H_2O and detection by diphenylamine reagent [9]. Standard D-*lyxo*-hexos-2-ulose and D-*arabino*-hexos-2-ulose were prepared enzymatically using pyranose oxidase from *Oudemansiella mucida* [10]. Aldosulose hydrazones were separated on Silica Gel 60 F_{254} TLC aluminium sheets (Merck, Darmstadt, Germany) using 12:1 $CHCl_3$–MeOH. HPLC was performed on a SP 8800 liquid chromatograph (Spectra Physics, San Jose, CA) fitted with a refractive index detector, using Ostion LG KS 0800 Na^+ (D-Glc oxidation products) or Ostion LG KS 0800 Ca^{2+} (D-Gal) 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}.

Derivatization and identification of D-*Glc and* D-*Gal oxidation products.*—The mixtures (20 mL) of sugar semipreparative transformation products were each supplemented successively with the same volume of ethanol, 1 mL 99% acetic acid and 270 μL freshly distilled *N*,*N*-diphenylhydrazine (Koch-Light Lab., Colnbrook, UK), stirred for 1 h and left overnight at 25 °C. The dark residue obtained after vacuum evaporation of the solvents was dissolved in 1.5 mL ethanol and subjected to TLC as above. Pale-yellow to orange streaks of four major hydrazones (R_f 0.13, 0.25, 0.39 and 0.41) derived from D-Glc oxidation products were cut out, combined separately, and extracted into methanol. The individual components were further purified by rechromatography to substantial homogeneity. The hydrazone of the D-Gal oxidation product (R_f 0.10, one major component) crystallized directly from the reaction mixture at −15 °C.

^1H and ^{13}C NMR data (400 and 100 MHz, respectively) of isolated hydrazones (Tables 1–3) were recorded on a Varian INOVA-400 spectrometer in CD_3OD at 30 °C. Residual solvent signals (δ_H 3.33, δ_C 49.3) were used as internal standards. 2D NMR experiments (COSY, ROESY, HETCOR) were performed using the manufacturer's software. Positive-ion FAB mass spectra were recorded on a Finnigan MAT 95 double-focusing instrument (Finnigan MAT, Bremen, Germany). The matrix was *m*-nitrobenzyl alcohol (Aldrich-Chemie, Steinheim, Germany) and the calibration was performed with Ultramark 1600F (PCR Inc., USA) as a standard. The products of CIDs in the first field-free region of the instrument were analysed by daughter-ion linked scan (B/E constant) using the manufacturer's software.

D-ribo-*Hexos-3-ulose-(3,6) 1-(*N,N-*diphenylhydrazone)* (**4**). Compound of R_f 0.13, obtained as a pale-yellow sirup after rechromatography on Silica Gel 60 F_{254} coated HPTLC aluminium sheets (Merck) in 40:3 $CHCl_3$–MeOH (24 mg). UV/VIS: λ_{max} (MeOH) 271, 416 nm. FAB MS, *m/z* (% rel. int.): 345 $[M+H]^+$, (daughter ions of the protonated molecule) 327 (100), 267 (3), 226 (12), 195 (1), 184 (1), 168 (3).

D-arabino-*Hexos-2-ulose 1,2-bis(*N,N-*diphenylhydrazone)* (**5**). Compound of R_f 0.25, rechromatographed using 40:1 $CHCl_3$–MeOH/20 Silufol (Kavalier, Votice, Czech Republic) silica gel foils, dark yellow solid (17 mg). UV/VIS: λ_{max} (MeOH) 234, 295, 346, 411 nm. FAB MS, *m/z* (% rel. int.): 511 $[M+H]^+$, 493 (20), 449 (5), 419 (52), 342 (56), 327 (100), 252 (20), 223 (1), 195 (2), 169 (8).

D-erythro-*Hexos-2,3-diulose 1,2-bis(*N,N-*diphenylhydrazone)* (**6**). Compound of R_f 0.39, rechromatographed on Silica Gel 60 TLC aluminium sheets (Merck) in 20:1 $CHCl_3$–MeOH and crystallized from methanol (yellow needles, 8 mg), mp 118–119 °C. UV/VIS: λ_{max} (MeOH) 250, 413 nm. FAB MS, *m/z* (% rel. int.): 509 $[M+H]^+$, 491 (100), 461 (2), 447 (100), 418 (1), 392 (1), 360 (1), 325 (2), 285 (8), 250 (1), 223 (2), 184 (1), 169 (2).

D-erythro-*Hexos-2,3-diulose 1,3-bis(*N,N-*diphenylhydrazone)* (**7**). Compound of R_f 0.41, purified on Silica Gel 60 sheets (Merck) in 40:1 $CHCl_3$–MeOH (orange sirup, 66 mg). UV/VIS: λ_{max} (MeOH) 237, 288, 412 nm. FAB MS, *m/z* (% rel. int.): 509 $[M+H]^+$, 491 (100), 417 (1), 390 (1), 340 (62), 325 (17), 323 (9), 280 (3), 222 (2), 195 (1), 183 (2), 169 (7).

D-lyxo-*Hexos-2-ulose 1-(*N,N-*diphenylhydrazone)* (**8**). Compound of R_f 0.10, recrystallized twice from ethanol, pale-yellow fine needels (109 mg), m.p. 148–149 °C, UV/VIS: λ_{max} (MeOH) 229, 348, 412 nm. FAB MS, *m/z* (% rel.int.): 345 $[M+H]^+$, 327 (100), 254 (10), 223 (16), 183 (7), 169 (24), 168 (20), 121 (2).

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