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# Conversion of D-Galactose to D-threo-Hexos-2,3-diulose by Fungal Pyranose Oxidase

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# ABSTRACT

Pyranose oxidase (pyranose:O<sub>2</sub> 2-oxidoreductase, EC 1.1.3.10) purified from mycelia of the basidiomycete fungi *Trametes versicolor* and *Oudemansiella mucida* catalyzed oxidation of D-galactose successively at C-2 and C-3 to D-*threo*-hexos-2,3-diulose (2,3-dehydro-D-galactose, 2,3-diketo-D-galactose) in the yields up to 80%. The sites of oxidation were deduced from structures of the *N*,*N*-diphenylhydrazone derivatives of the reaction products. Under the reaction conditions used, the diulose was susceptible to non-enzymatic oxidative decarboxylation to D-*threo*-pentos-2-ulose (2-dehydro-D-xylose) in yields of 5-10%.

*Key Words:* D-Galactose; D-*lyxo*-Hexos-2-ulose; D-*threo*-Hexos-2,3-diulose; Pyranose dehydrogenase; Pyranose oxidase; *Trametes versicolor; Oudemansiella mucida.* 

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## **INTRODUCTION**

Highly reactive carbonyl sugar derivatives are gaining in importance in carbohydrate chemistry as intermediates or synthons in procedures leading to useful products and rare sugars.<sup>[1]</sup> This is now also due to their easier accessibility through enzymatic conversions of common sugars by employing stereoselective sugar oxidoreductases, such as fungal pyranose oxidase (P2O, pyranose:O<sub>2</sub> 2-oxidoreductase, EC 1.1.3.10)<sup>[2]</sup> and pyranose dehydrogenase.<sup>[3]</sup> These compounds have high potential applicability as intermediates in the isomerizations of aldoses to ketoses.<sup>[2]</sup> P2O shows a broad substrate tolerance, acting on C-2 equatorial hydroxyls of some aldopyranoses.<sup>[2,4]</sup> Its activity at C-3 of several substrates has also been recognized.<sup>[5,6]</sup> P2Os, so far characterized from different sources, oxidize D-galactose with activities ranging from 0.9 to 8.8% relative to preferred D-glucose (100%).<sup>[7-9]</sup> The corresponding reaction product was identified as D-lyxo-hexos-2-ulose (Scheme 1, 1; 2-dehydro-D-galactose).<sup>[4,10]</sup> This compound is attractive due to its intermediate role in the proposed (chemo)enzymatic processes for production of D-tagatose from D-galactose.<sup>[10,11]</sup> However, our previous observations indicated that P2O of some fungi may exhibit modified catalytic properties towards D-galactose, resulting in its further transformation.

In this paper we demonstrate that P2O purified from *Trametes versicolor* and *Oudemansiella mucida* converts D-galactose, upon prolonged incubations, to D-*threo*-hexos-2,3-diulose (Scheme 1, 2; 2,3-didehydro-D-galactose), a new sugar derivative with three adjacent carbonyl groups. Its structure was determined spectroscopically using the corresponding diphenylhydrazone derivatives.

### **RESULTS AND DISCUSSION**

### Time Course of **D**-Galactose Oxidation

The HPLC analyses of sugar components during the course of D-galactose transformation carried out on an analytical scale using homogeneous P2O from *T. versicolor* revealed that **1** (Figure 1, peak III, T=15.85 min) accumulated in the reaction mixture only transiently being in turn converted to a final product (peak I, T=7.95 min). The latter compound was eluted near the column void volume as observed earlier for the end product of D-glucose double oxidation.<sup>[12]</sup> The similar chromatographic pattern was also obtained for products of D-galactose transformation by P2O partially purified from mycelia of *O. mucida* (not shown). These enzyme activities were in contrast with high C-2 regioselectivity of P2O from *T. multicolor*<sup>[11]</sup> and pyranose dehydrogenase, a quinone-dependent flavoglycoprotein from *Agaricus bisporus*,<sup>[12]</sup> which oxidized D-galactose almost quantitatively to **1**. The enzyme source-dependent variability in catalytic regioselectivity has already been demonstrated before with pyranose dehydrogenase and D-glucose.<sup>[13]</sup>

#### **Structure Elucidation**

To determine spectroscopically the site of the second oxidation of D-galactose by P2O, the products in the semipreparative reaction mixture were treated with



*Figure 1.* (A)–(E) HPLC monitoring of D-galactose oxidation by pyranose 2-oxidase from *T. versicolor* at incubation times of 0, 1.5, 4 and 20 h, respectively. Peaks: I, D-*threo*-hexos-2,3-diulose; II, D-galactose; III, D-*lyxo*-hexos-2-ulose; the peak I at (A) corresponds to the salt originating from the enzyme preparations added to the reaction mixture.



*Figure 2.* Structures and FAB-MS fragmentation of N,N-diphenylhydrazone derivatives **4**–**9** used for identification of the oxidation products of D-galactose by pyranose oxidase.

N,N-diphenylhydrazine and the diphenylhydrazone (DPH) compounds formed were isolated using semipreparative TLC. This step substantially simplified the NMR measurements by converting multiple (bi)cyclic isomers of the individual free oxidation products to the mostly 'linear' structures, the only source of diversity being then the extent of derivatization of carbonyl groups.

The compound displaying  $[M+H]^+$  ion at m/z 345, containing one DPH moiety, one carbonyl group, and a contiguous five-spin system - $[CH(OH)]_3CH_2OH$  was assigned the structure of mono-DPH derivative (**4**, Figure 2) of D-*lyxo*-hexos-2-ulose. The next compound with  $[M+H]^+$  at m/z 511, contained two DPH units and a similar five-spin system. It was therefore assigned the structure of bis-DPH derivative (**5**) of D-*lyxo*-hexos-2-ulose. Both compounds **6** and **7** exhibited  $[M+H]^+$  ions at m/z 509, contained two DPH units each, one free carbonyl per molecule, and a contiguous four-spin system in their <sup>1</sup>H NMR spectra (Table 1). The localization of the free carbonyl group (Table 2) was determined by HMBC: at C-3 in **6** and at C-2 in **7**. These two compounds were DPH derivatives of the parent D-*threo*-hexos-2,3-diulose (**2**, Scheme 1). The compound exhibiting  $[M+H]^+$  ion at m/z 675 contained three DPH units, no free carbonyl, and a similar proton spin system to those described above, clearly consistent

Proton/J(i,j)	4	5	6	7	8	9
H-1	6.681	7.095	6.450	6.030	6.107	7.038
H-3	5.406	5.173	_	_	_	5.244
$J_{3.4}$	7.2	8.0				2.9
H-4	4.018	4.306	5.440	4.698	4.966	4.181
$J_{4,5}$	1.8	1.7	2.1	4.1	2.7	6.6 6.0*
H-5	3.908	4.114	4.266	4.077	4.178	3.736 3.847
$J_{5.6d}$	6.0	6.4	6.4	5.5	6.2	11.0**
$J_{5.6u}$	6.6	6.4	6.7	6.4	6.2	
H-6u	3.634	3.728	3.679	3.700	3.795	
H-6d	3.667	3.728	3.774	3.824	3.885	
J <sub>6d,6u</sub>	11.0	_	11.0	11.1	11.0	

*Table 1.* <sup>1</sup>H NMR data of compounds 4-9 (399.91 MHz, CD<sub>3</sub>OD, 30°C).

Additional signals - **4**: 7.245 (4 H, m, *ortho*-Ph), 7.340 (2 H, m, *para*-Ph), 7.499 (4 H, m, *meta*-Ph); **5**: 6.896 (4 H, m, *ortho*-Ph<sub>2</sub>), 6.915 (4 H, m, *ortho*-Ph<sub>1</sub>), 7.037 (2 H, m, *para*-Ph<sub>1</sub>), 7.220 (4 H, m, *meta*-Ph<sub>1</sub>), 7.237 (4 H, m, *meta*-Ph<sub>2</sub>), **6**: 6.823 (4 H, m, *ortho*-Ph<sub>1</sub>), 7.086 (4 H, m, *ortho*-Ph<sub>2</sub>), 7.169 (2 H, m, *para*-Ph<sub>1</sub>), 7.194 (2 H, m, *para*-Ph<sub>2</sub>), 7.312 (4 H, m, *meta*-Ph<sub>2</sub>), 7.333 (4 H, m, *meta*-Ph<sub>1</sub>); **7**: 7.069 (4 H, m, *ortho*-Ph<sub>1</sub>), 7.086 (2 H, m, *para*-Ph<sub>1</sub>), 7.165 (4 H, m, *ortho*-Ph<sub>2</sub>), 7.249 (2 H, m, *para*-Ph<sub>2</sub>), 7.266 (4 H, m, *meta*-Ph<sub>1</sub>), 7.381 (4 H, m, *meta*-Ph<sub>2</sub>); **8**: 6.696 (4 H, m, *ortho*-Ph<sub>2</sub>), 6.728 (4 H, m, *ortho*-Ph<sub>1</sub>), 7.022 (2 H, m, *para*-Ph<sub>3</sub>), 7.037 (2 H, m, *para*-Ph<sub>2</sub>), 7.054 (4 H, m, *ortho*-Ph<sub>3</sub>), 7.166 (2 H, m, *para*-Ph<sub>1</sub>), 7.169 (4 H, m, *meta*-Ph<sub>2</sub>), 7.204 (4 H, m, *meta*-Ph<sub>3</sub>), 7.281 (4 H, m, *meta*-Ph<sub>1</sub>); **9**: 6.887 (4 H, m, *ortho*-Ph<sub>2</sub>), 6.915 (4 H, m, *ortho*-Ph<sub>1</sub>), 7.048 (2 H, m, *para*-Ph<sub>2</sub>), 7.237 (2 H, m, *para*-Ph<sub>1</sub>), 7.243 (4 H, m, *meta*-Ph<sub>7</sub>), 7.390 (4 H, m, *meta*-Ph<sub>1</sub>). \* $J_{4 5m}$ .

\*\*J<sub>5d,5u</sub>; d, downfield; u, upfield.

Carbon	4	5	6	7	8	9
1	133.46	130.19	128.31	134.42	129.28	128.76
2	201.05	162.91	141.82	194.46	143.90	161.01
3	73.52	73.05	201.04	155.45	161.54	71.58
4	73.57	74.08	75.41	75.04	75.91	75.47
5	72.40	72.37	74.87	74.49	74.46	64.95
6	64.68	65.14	64.60	64.45	64.57	

Table 2.  $^{13}$ C NMR data of compounds 4–9 (100.57 MHz, CD<sub>3</sub>OD, 30°C).

Chemical shifts of free keto group are given in bold.

Additional signals -4: 123.86 d (4 C, *ortho*-Ph), 128.09 d (2 C, *para*-Ph), 131.44 d (4 C, *meta*-Ph), 143.82 s (2 C, *ipso*-Ph); **5**: 123.09 d (4 C, *ortho*-Ph<sub>2</sub>), 123.88 d (4 C, *ortho*-Ph<sub>1</sub>), 124.76 d (2 C, *ortho*-Ph<sub>2</sub>), 126.91 d (2 C, *para*-Ph<sub>1</sub>), 130.49 d (4 C, *meta*-Ph<sub>2</sub>), 131.34 d (4 C, *meta*-Ph<sub>1</sub>), 144.06 s (2 C, *ipso*-Ph<sub>1</sub>), 150.38 s (2 C, *ipso*-Ph<sub>2</sub>); **6**: 123.95 d (4 C, *ortho*-Ph<sub>1</sub>), 124.19 d (4 C, *ortho*-Ph<sub>2</sub>), 126.49 d (2 C, *para*-Ph<sub>1</sub>), 127.21 d (2 C, *para*-Ph<sub>2</sub>), 130.86 d (4 C, *meta*-Ph<sub>2</sub>), 131.01 d (4 C, *meta*-Ph<sub>1</sub>), 144.06 s (2 C, *ipso*-Ph<sub>1</sub>), 127.21 d (2 C, *para*-Ph<sub>2</sub>), 130.86 d (4 C, *meta*-Ph<sub>2</sub>), 131.01 d (4 C, *meta*-Ph<sub>1</sub>), 144.06 s (2 C, *ipso*-Ph<sub>1</sub>), 147.71 s (2 C, *ipso*-Ph<sub>2</sub>); **7**: 124.31 d (4 C, *ortho*-Ph<sub>2</sub>), 124.54 d (4 C, *ortho*-Ph<sub>1</sub>), 125.82 d (2 C, *para*-Ph<sub>1</sub>), 127.82 d (2 C, *para*-Ph<sub>2</sub>), 130.34 d (4 C, *meta*-Ph<sub>1</sub>), 131.47 d (4 C, *meta*-Ph<sub>2</sub>), 148.37 s (4 C, *ipso*-Ph); **8**: 123.50 d (4 C, *ortho*-Ph<sub>3</sub>), 123.66 d (4 C, *ortho*-Ph<sub>2</sub>), 123.99 d (4 C, *ortho*-Ph<sub>1</sub>), 125.02 d (2 C, *para*-Ph<sub>3</sub>), 125.91 d (2 C, *para*-Ph<sub>2</sub>), 126.72 d (2 C, *para*-Ph<sub>1</sub>), 130.22 d (4 C, *meta*-Ph<sub>3</sub>), 130.64 d (4 C, meta-Ph<sub>2</sub>), 131.13 d (4 C, *meta*-Ph<sub>1</sub>), 148.20 s (2 C, *ipso*-Ph<sub>1</sub>), 148.78 s (2 C, *ipso*-Ph<sub>2</sub>), 127.03 d (2 C, *para*-Ph<sub>1</sub>), 130.56 d (4 C, *meta*-Ph<sub>2</sub>), 123.74 d (4 C, ortho-Ph<sub>1</sub>), 124.87 d (2 C, para-Ph<sub>2</sub>), 127.03 d (2 C, *para*-Ph<sub>1</sub>), 130.56 d (4 C, *meta*-Ph<sub>2</sub>), 131.41 d (4 C, *meta*-Ph<sub>1</sub>), 144.80 s (2 C, *ipso*-Ph<sub>1</sub>), 150.42 s (2 C, *ipso*-Ph<sub>2</sub>).

with structure 8 (Figure 2), a tris-DPH derivative of 2. The last reaction product of  $[M+H]^+$  ion m/z 481 contained two DPH units, had only five carbon atoms derived from the parent sugar and its contiguous spin system contained also four protons. Therefore, this compound was a bis-DPH derivative (9, Figure 2) of a product of 2 oxidative decarboxylation.

#### P2O Catalysis with D-Galactose and Related P2O Activities

Based on the structures of free di- and tricarbonyl sugars deduced from the above identified hydrazones and HPLC monitoring of the time course of P2O catalysis, we



**Scheme 1.** Reaction scheme for double oxidation of D-galactose by pyranose oxidase from *Trametes versicolor* and *Oudemansiella mucida*. D-Galactose is successively oxidized at C-2 and C-3 to diketoaldose 2 (D-*erythro*-hexos-2,3-diulose), which is apparently susceptible to non-enzymatic oxidative decarboxylation giving 3 (D-*threo*-pentos-2-ulose) *via* a keto/enol intermediate (not shown). 1, intermediate D-*lyxo*-hexos-2-ulose; for simplicity, only one of the possible equilibrium cyclic forms is shown for 1 and 2, carbonyl hydration also takes place.

conclude that D-galactose is transformed by P2O and molecular oxygen to ketoaldose **1**, which is subsequently oxidized by the same enzyme at C-3 to give the diketoaldose **2** (Scheme 1). It is assumed that prior to the second P2O-catalyzed step, **1** is hydrated at C-2 carbonyl or the 1,5-pyranose isoform of **1** is spontaneously recycled into 2,6-pyranose<sup>[10]</sup> hydrated at the C-1 carbonyl.

The ability of fungal sugar oxidoreductases to catalyse C-2,3 double oxidation of aldoses was first demonstrated for P2O from *O. mucida*<sup>[6]</sup> and pyranose dehydrogenase of *Agaricus bisporus*<sup>[12]</sup> with D-glucose giving D-*erythro*-hexos-2,3-diulose epimeric to **2**. Pyranose dehydrogenase of *A. meleagris* was shown to catalyse C-2,3 oxidation of D-xylose to another tricarbonyl derivative, D-*erythro*-pentos-2,3-diulose.<sup>[14]</sup> To our knowledge, none of the aldos-2,3-diuloses exhibiting three adjacent carbonyl groups have been prepared to date by means of carbohydrate chemistry and this is the first report describing enzymatic formation of **2**. The search of Chemical Abstracts database for double oxidation products of D-galactose revealed one item, D-*lyxo*-hex-2-ulosonic acid. However, its formation involves an oxidation at C-1 and C-2. Similar C-2,3 dioxidation by P2O (*Peniophora gigantea*) was also observed with 1,5-anhydro-D-glucitol and 1,5-anhydro-D-galactitol yielding the corresponding 1,5-anhydro-hex-2,3diuloses.<sup>[5]</sup>

Oxidative decarboxylation of **2** to D-*threo*-pentos-2-ulose (**3**, Scheme 1, 2-dehydro-D-xylose) was inferred from the structure of the isolated hydrazone **9**. Formation of this by-product in yields of 5-10% was dependent on intensity of aeration of the reaction mixture. Analogous degradative reaction was earlier shown for C-3 carbonyl derivatives of 2-deoxy-D-glucose<sup>[5]</sup> and D-*glycero*-pentos-2,3-diulose.<sup>[14]</sup>

In the semipreparative transformations described, D-galactose was converted by *T. versicolor* or *O. mucida* P2O to **2** in yields approaching 80% (HPLC analysis). In contrast with D-*erythro*-hexos-2,3-diulose previously isolated also in a crystalline form,<sup>[15]</sup> we were unsuccessful in crystallizing **2** from the ultrafiltrate of a completed reaction mixture. The present study extends our earlier findings<sup>[6,12,16]</sup> demonstrating high potential of pyranose oxidoreductases for conversions of various sugars to their reactive di- and tricarbonyl derivatives, possible building blocks in synthetic carbo-hydrate chemistry. In view of the earlier designed enzymatic processes for isomerization of D-galactose to D-tagatose,<sup>[10,11]</sup> our present findings point to the importance of the fungal source of P2O, i.e., the enzyme of choice should have no or minimum activity for the C-3 oxidation.

#### EXPERIMENTAL

#### **Enzyme Source, Purification, and Assay**

Fungi *Trametes versicolor*, CCBAS 614 and *Oudemansiella mucida*, CCBAS 428 were obtained from the Culture Collection of Basidiomycetes at the Institute of Microbiology, Prague. The fungi were grown under submerged conditions on a complex medium<sup>[17]</sup> for three days (*T. versicolor*) or a synthetic medium<sup>[6]</sup> for four days (*O. mucida*). *T. versicolor* P2O was purified from mycelial extracts to apparent homogeneity (39 U/mg protein) following substantially the three-step procedure described previously.<sup>[17]</sup> P2O from *O. mucida* was partially purified by applying the first step, hydrophobic interaction chromatography.

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The enzyme activities were assayed spectrophotometrically by measuring at 590 nm the production of  $H_2O_2$  in a coupled peroxidase/chromogen (3-methyl-2-benzo-thiazolinone hydrazone-3-dimethylaminobenzoic acid) system.<sup>[17]</sup> One unit (U) of the enzyme activity oxidizes D-glucose at 1  $\mu$ mol min<sup>-1</sup> under the assay conditions.

# P2O Catalyzed Transformation of D-Galactose

The analytical transformations of D-galactose were performed for 20 h at 30°C under stirring with 13.5 mg (50 mM) D-galactose, 6.2 U purified P2O (T. versicolor or O. mucida) and 350 Sigma Units of bovine liver catalase (Reanal, Budapest, Hungary) in 10 mM bisTris HCl buffer, pH 7.2 (1.5 mL). Excess catalase was added in order to prevent P2O deactivation and product decomposition by co-produced  $H_2O_2$ . Time course of the reaction was followed by HPLC analysis of the samples (60  $\mu$ L) withdrawn at time intervals, passed through Ultrafree-MC 30,000 NMWL filter units (Millipore, Bedford, MA) and diluted 10 times with water. HPLC monitoring was performed on an SP 8800 liquid chromatograph (Spectra Physics, San Jose, CA, USA) fitted with a refractive index detector, using Ostion LG KS 0800 Ca<sup>2+</sup> column 250 x 8 mm (Watrex, Prague, Czech Republic), eluted at 80°C with deionized water (0.5 mL  $\min^{-1}$ ). The reaction mixture (25 mL) for semipreparative transformations (for 28 h at 25°C under stirring) contained 225 mg D-galactose, 60 U P2O (T. versicolor), 2000 Sigma Units catalase added in three aliquots at 0, 6, and 14 h, and 0.03% ProClin 300 (Supelco, Bellefonte, PA) antimicrobial preservative in 50 mM 2(N-morpholino)ethanesulfonic acid (MES), pH 7.0.

# **Derivatization of D-Galactose Oxidation Products**

The semipreparative reaction mixture was vacuum concentrated to 15 mL, passed through a YM 30 membrane (Amicon, Danvers, MA), and the filtrate was supplemented with the same volume of ethanol and 366  $\mu$ L of freshly distilled *N*,*N*-diphenylhydrazine (Koch-Light Lab., Colnbrook, UK). After acidification (0.3 mL 99 % acetic acid) and vigorous stirring for 2 h, the mixture was left standing overnight at 22°C and then the solvent removed by vacuum evaporation. The solid obtained was extracted into 2 mL of methanol, most of crystalline MES being separated. The hydrazones contained in the methanol fraction were isolated using TLC on Silica Gel 60 F<sub>254</sub> aluminium sheets (Merck, Darmstadt, Germany) in 50:3 CHCl<sub>3</sub>–MeOH and UV/VIS detection. Pale-yellow to orange streaks of six major hydrazones were excised, combined separately, and extracted into methanol. The individual hydrazones were further purified by rechromatography and spectroscopically identified.

#### **Spectral Analysis**

<sup>1</sup>H and <sup>13</sup>C NMR spectra of the isolated *N*,*N*-diphenylhydrazones were measured on a Varian INOVA-400 spectrometer in CD<sub>3</sub>OD at 30°C. The residual solvent signals were used as an internal standard ( $\delta_{\rm H}$  3.33,  $\delta_{\rm C}$  49.3). Carbon signal multiplicity was determined by APT (Attached Proton Test). 2D NMR experiments (gCOSY, TOCSY, HMQC, and HMBC) were performed using manufacturer's software. 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  $(1 \times 10^{-5} \text{ bar})$  was used as a bombarding gas.

**D**-*lyxo*-**Hexos-2-ulose 1-**(*N*,*N*-**diphenylhydrazone**) (4). Compound of  $R_f 0.11$ , rechromatographed using 50:1 CHCl<sub>3</sub>–MeOH, and crystallized from EtOH, pale yellow needles (27 mg), mp 148–149°C, UV/VIS:  $\lambda_{max}$  (MeOH) 229, 247, 412 nm. FAB-MS, *m/z* (% rel. int.): 367 [M+Na]<sup>+</sup> (72), 345 [M+H]<sup>+</sup> (33), 327 (58), 254 (12), 223 (18), 195 (5), 183 (9), 168 (100), 121 (3).

**D**-*lyxo*-**Hexos-2-ulose 1,2-bis**(*N*,*N*-**diphenylhydrazone**) (5). R<sub>f</sub> 0.28, rechromatographed in 50:1 CHCl<sub>3</sub>-MeOH, dark yellow syrup (45 mg). UV/VIS:  $\lambda_{max}$  (MeOH) 212, 293, 300, 344, 389 nm. FAB-MS, *m/z* (% rel. int.): 533 [M+Na]<sup>+</sup> (29), 511 [M+H]<sup>+</sup> (26), 493 (1), 449 (2), 419 (5), 389 (1), 342 (4), 327 (6), 252 (7), 222 (14) 195 (6), 183 (7), 168 (100).

**D**-*threo*-**Hexos-2,3-diulose 1,2-bis**(*N*,*N*-**diphenylhydrazone**) (6). R<sub>f</sub> 0.45, rechromatographed in 100:1 CHCl<sub>3</sub>-MeOH, silica gel 60 foils, orange syrup (36 mg). UV/ VIS:  $\lambda_{max}$  (MeOH) 210, 240, 288, 394 nm. FAB-MS, *m/z* (% rel. int.): 531 [M+Na]<sup>+</sup> (48), 509 [M+H]<sup>+</sup> (10), 508 (24), 491 (1), 389 (2), 340 (3), 325 (4), 222 (32), 195 (9), 183 (8), 168 (100).

**D-threo-Hexos-2,3-diulose 1,3-bis**(*N*,*N*-**diphenylhydrazone**) (7). R<sub>f</sub> 0.39, rechromatographed using 100:1 CHCl<sub>3</sub>-MeOH and aluminium foils with concentration zone, deep yellow syrup (63 mg). UV/VIS:  $\lambda_{max}$  (MeOH) 212, 239, 278, 357 nm. FAB-MS, *m/z* (% rel. int.): 531 [M+Na]<sup>+</sup> (62), 509 [M+H]<sup>+</sup> (26), 508 (18), 491 (6), 447 (2), 340 (2), 325 (3), 285 (1), 250 (3), 223 (47), 195 (6), 183 (7), 168 (100).

**D-***threo***-Hexos-2,3-diulose 1,2,3-tris**(*N*,*N***-diphenylhydrazone) (8).** R<sub>f</sub> 0.74, rechromatographed in CHCl<sub>3</sub>, orange solid (99 mg). UV/VIS:  $\lambda_{max}$  (MeOH) 215, 242, 289, 390 nm. FAB-MS, *m/z* (% rel. int.): 697 [M+Na]<sup>+</sup> (12), 675 [M+H]<sup>+</sup> (3), 613 (2), 506 (3), 491 (4), 446 (1), 415 (2), 323 (1), 222 (4), 195 (4), 168 (100).

**D**-*threo*-**Pentos-2-ulose 1,2-bis**(*N*,*N*-**diphenylhydrazone**) (9). R<sub>f</sub> 0.54, rechromatographed in 100:1 CHCl<sub>3</sub>-MeOH, yellow syrup (15 mg). UV/VIS:  $\lambda_{max}$  (MeOH) 239, 294, 344, 388 nm. FAB-MS, *m/z* (% rel. int.): 503 [M+Na]<sup>+</sup> (12), 481 [M+H]<sup>+</sup> (10), 480 (15), 463 (2), 419 (3), 389 (2), 312 (5), 297 (7), 252 (5), 222 (11), 195 (7), 183 (6), 168 (100).

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