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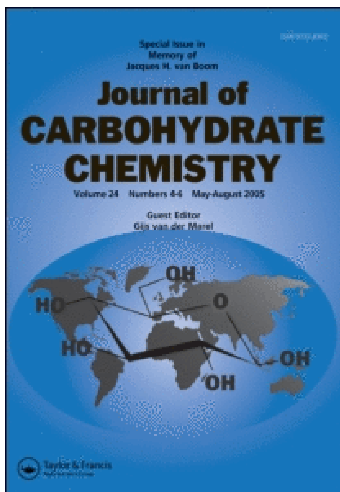

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ENZYMATIC FORMATION OF DICARBONYL SUGARS: C-2 OXIDATION OF 1→6 DISACCHARIDES GENTIOBIOSE, ISOMALTOSE AND MELIBIOSE BY PYRANOSE 2-OXIDASE FROM *TRAMETES MULTICOLOR*

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ABSTRACT

Pyranose 2-oxidase, a homotetrameric FAD-flavoprotein from the fungus *Trametes multicolor*, catalyzes the oxidation of 1→6 disaccharides gentiobiose (β-D-Glc*p*-(1→6)-D-Glc), melibiose (α-D-Gal*p*-(1→6)-D-Glc), and isomaltose (α-D-Glc*p*-(1→6)-D-Glc) at C-2 of their reducing moiety. The products were identified as their hydrazone derivatives.

INTRODUCTION

Pyranose 2-oxidases (P2O, EC 1.1.3.10, glucose 2-oxidase), a growing family of sugar oxidoreductases that are expressed in many wood-degrading basidiomycete fungi, are gaining increased interest in carbohydrate chemistry as valuable biocatalysts for synthesis of di- and tricarbonyl sugar derivatives. These enzymes are intracellular FAD flavoproteins of different size and subunit composition (mostly ca. 300 kDa homotetramers) catalyzing the C-2 oxidation of several aldoses, viz. D-Glc (the preferred substrate), D-Xyl, D-All and D-Gal, and D-glucono-1,5-lactone with variable relative

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activities depending on the enzyme source.[1] In contrast to chemical syntheses of dicarbonyl sugars, which often give low yields and a number of by-products, the enzymatic oxidations of aldoses and ketoses to dicarbonyl derivatives typically show high regioselectivity while using advantageously unprotected sugar substrates as well as nearly complete conversions of these substrates to the corresponding products. The enzymatic introduction of a carbonyl group into the sugar structure generates a site for selective chemical syntheses.[2] The oxidation of a variety of monosaccharides on a laboratory scale using both free and immobilized P2O preparations has been reported.[3,4,5] P2O catalyzed transformation of D-Glc to D-*arabino*-hexos-2-ulose (2-dehydro-D-glucose, 2-keto-D-glucose) has a potential application in food technology as the key step in the process for production of D-Fru, mannitol or D-glucitol from D-Glc.[6,7,8]

Our research has been focused on production and characterization of P2O from the wood-rotting fungus *Trametes multicolor*,[9] and its utilization in sugar transformations.[8,10] In general, P2Os prefer monosaccharide substrates. Out of the various oligo-saccharides we tested, only gentiobiose (β-D-Glc*p*-(1→6)-D-Glc), melibiose (α-D-Gal*p*-(1→6)-D-Glc), and isomaltose (α-D-Glc*p*-(1→6)-D-Glc) served as *T. multicolor* P2O substrates. In the present paper we report the use of P2O (*T. multicolor*) for semipreparative production of carbonyl derivatives of these 1→6 disaccharides and their identification. To our knowledge, the recent report of P2O from another basidiomycete *Peniophora gigantea* is the only one describing P2O activity towards an oligosaccharide (gentiobiose), supported by structural analysis of the respective oxidation product.[11]

RESULTS AND DISCUSSION

The time course of transformation of gentiobiose in batch procedure as followed by HPLC demonstrated that the sugar was converted almost quantitatively to its carbonyl derivative (Fig. 1). Isomaltose and melibiose gave also a single oxidation product (not shown). Kinetic measurements demonstrated that gentiobiose was the best substrate [K_m=62.4 mM, v_{max}=22.7% (Glc v_{max}=100%)] followed by melibiose (K_m=120.3 mM, v_{max}=8.6%), while kinetic constants for isomaltose with the lowest P2O affinity could not be determined because of the contaminating Glc in the commercial preparation. On TLC, the oxidation products of the three disaccharides afforded blue spots upon detection with diphenylamine–aniline reagent, which is characteristic of aldos-2-uloses.[4]

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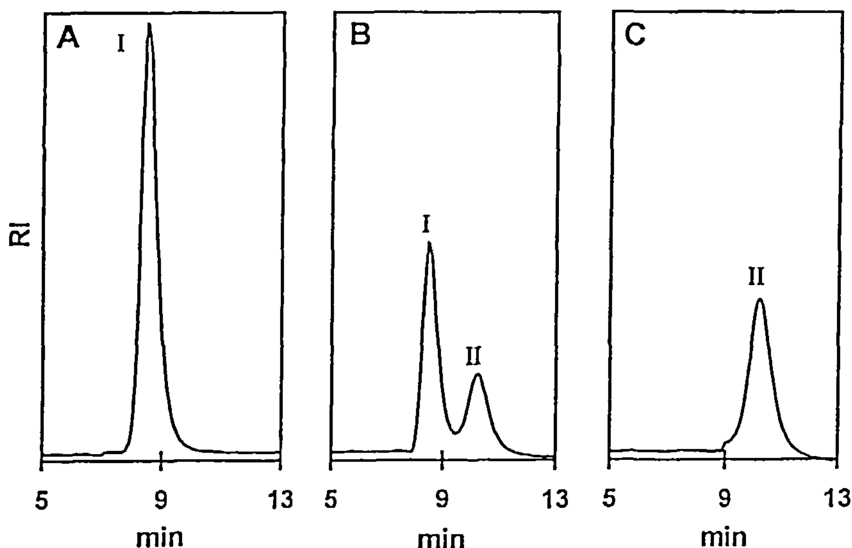

Figure 1. HPLC monitoring of gentiobiose oxidation to the corresponding C-2 carbonyl derivative by pyranose 2-oxidase at incubation times of (A) immediately after adding the enzyme, (B) 5 h, and (C) 24 h, respectively. I, gentiobiose; II, β-D-Glc*p*-D-*arabino*-hexos-2-ulose (2-ketogentiobiose); for composition of the reaction mixture, see Experimental.

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Since the P2O reaction products were expected to occur in an equilibrium of several tautomeric forms, complicating direct analysis, they were converted to their *N,N*-diphenylhydrazone (DPH) derivatives. The isolated hydrazones of oxidation products from gentiobiose (Fig.2, **1**), melibiose (**2a**) and isomaltose (**2b**, **3**) were subjected to analysis by mass spectrometry and NMR spectroscopy. According to pseudomolecular $[M+H]^+$ and $[M+Na]^+$ ions observed in FAB mass spectra and NMR data (Tables 1–3), compounds **1**, **2a**, and **2b** are mono-DPH derivatives; **3** is a bis-DPH derivative. Spin systems picked-up by COSY and TOCSY belong to an unchanged glycosyl residue (α- or β-Glc*p*, or α-Gal*p*) and to a system of the oxidized glucose involving atoms C-3 to C-6 in which C-1 resonates as a singlet. That fact indicates an absence of protons at C-2. The nature of this carbon is evident from ^{13}C NMR spectra (Table 3): a ketone group in **1**, **2a**, and **2b**, a C=N– moiety in the bis-DPH derivative **3**. The downfield shift of C-6 of the hydrazone residue confirms an attachment of the glycosyl moiety at this site.

The transformation product of gentiobiose was also examined in free form without derivatization. In water solution, the chemical shifts of reducing end anomeric protons and

Table 2. Proton-proton Coupling Constants [Hz]

[i,j]	1	2a	2b	3
3,4	1.7	1.8	1.9	1.5
4,5	9.2	8.6	9.0	8.9
5,6u	6.6	n.d.[a]	2.0	2.4
5,6d	2.5	n.d.	4.9	4.8
6u,6d	10.5	n.d.	10.0	10.2
1',2'	7.8	3.8	3.7	3.7
2',3'	9.1	9.8	9.7	9.7
3',4'	9.3	3.3	9.3	9.4
4',5'	9.3	1.2	9.3	9.1
5',6'u	5.2	5.5	1.5	n.d.
5',6'd	2.1	6.6	4.9	n.d.
6'u,6'd	11.9	11.2	13.9	n.d.

a. n.d., not determined

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Table 3. ^{13}C NMR Chemical Shifts for Compounds **1**, **2a**, **2b** and **3** [ppm]

Carbon	1	2a	2b	3
1	131.23	131.51	131.54	128.66
2	**199.86**	**199.99**	**200.06**	**161.43**
3	74.44	74.67	74.40	70.44
4	75.07	74.62	74.72	74.75
5	71.80	71.38	71.29	71.60
6	**73.77**	**70.91**	**70.83**	**71.05**
1'	105.15	100.71	100.39	100.42
2'	75.42	70.84	73.83	74.15
3'	71.85	71.88	75.62	75.70
4'	78.24	71.23	71.98	72.01
5'	78.27	72.69	74.06	73.87
6'	62.95	63.04	62.88	62.92
ipso-	143.60	143.82	144.20	150.40
ortho-	123.82[a]	123.69[a]	123.85[a]	122.96[a]
meta-	131.54[a]	131.60[a]	131.54[a]	130.59[a]
para-	127.87	128.11	127.85	124.84
ipso-				143.95
ortho-				123.85[a]
meta-				131.41[a]
para-				126.98

a. 2 C
Chemical shifts diagnostic for C-2 and C-6 substitution are given in bold

carbons agreed (upon calculation to the same reference) with those reported earlier for β-D-Glc*p*-(1→6)-D-*arabino*-hexos-2-ulose,[11] but one more species was observed (^{1}H, 4.677 ppm; ^{13}C, 93.73 ppm). The data indicate four tautomeric species present, all having a hydrated carbonyl at C-2 of the reducing glucose moiety. On the contrary, fresh DMSO solution contains eight forms, four keto sugars and four C-2 hydrates that gradually dehydrate leaving the keto forms only (93.63 d, 93.94 d, 94.43 d, 97.27 d; 202.09 s, 201.92 s, 205.42 s, 205.20 s). H-1 resonating as a doublet (coupling to an OH) and HMBC crosspeaks between C-2 and H-1 and H-3 also place the keto group at C-2.

In summary, the three 1→6 disaccharides, gentiobiose, melibiose and isomaltose, were stereospecifically oxidized by P2O from *T. multicolor* to β-D-Glc*p*- (Fig.2, 4), α-D-Gal*p*- and α-D-Glc*p*-(1→6)-D-*arabino*-hexos-2-ulose, respectively, all selectively at the disaccharide reducing end (Fig. 2). β-D-Glc*p*-(1→6)-D-*arabino*-hexos-2-ulose has only recently been characterized as product of gentiobiose oxidation (80% conversion) by P2O from another fungus, *Peniophora gigantea* by direct ^{1}H/^{13}C NMR spectroscopy and analysis of its peracetylated hydrogenation derivatives.[11] Apparently, P2Os from different sources can be used for the above sugar transformations to 2-ketodisaccharides. The activities found earlier with maltose and cellobiose[12,13] might have been due to D-Glc contamination in the commercial sugar preparations, residual glycosidase activity in the P2O preparations used, or due to hydrolytic activity of P2O itself.[11] In our assays, *T. multicolor* P2O did not exert any activity towards these two 1→4 disaccharides of chromatography grade quality. Conversion of several oligosaccharides to their C-3 carbonyl derivatives is well documented for the bacterial D-glucoside 3-dehydrogenase EC 1.1.99.13.[14] Out of them ß-D-Fru*f*-(2↔1)-α-D-*ribo*-hexopyranosid-3-ulose (3-keto-sucrose) received attention as a chiral synthon in organic chemistry.[15]

EXPERIMENTAL

Enzyme source, purification and assay. The cultivation in a laboratory fermentor of the basidiomycete *Trametes multicolor* (= *T. zonata*) MB 49, and the purification to homogeneity of P2O from the fungal biomass were carried out as described previously.[9] P2O activity was measured spectrophotometrically using a coupled peroxidase assay with 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) as a chromogene.[1]

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Sugar conversions by P2O and derivatization of the reaction products. The transformations of the 1→6 disaccharides into corresponding glycosyl D-*arabino*-hexos-2-uloses were performed batchwise for 24 h in 300-mL shaken (120 rpm) baffled flasks containing 1 g (55 mM) gentiobiose or melibiose (both Sigma, St. Louis, MO), or 450 mg isomaltose (ICN Pharmaceuticals, Costa Mesa, CA), 100 U P2O and 50,000 Sigma units catalase (bovine liver, Sigma) in 50 mM potassium phosphate, pH 6.5 (50 mL) at 30 °C.

The mixtures of sugar transformation products were each supplemented successively with the same volume of ethanol and 1 mL freshly distilled *N,N*-diphenylhydrazine (Koch-Light Lab., Colnbrook, UK) without acidification, vigorously stirred for 1 h and left overnight at room temperature. The residue obtained after vacuum evaporation of the solvents was dissolved in 2 mL of methanol and subjected to TLC as below. Yellow streaks of major hydrazones were cut out and extracted into methanol. The compounds were further purified by rechromatography to substantial homogeneity.

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The P2O oxidation product of gentiobiose was also recovered in nonderivatized free form as a white solid. The enzyme reaction mixture was the same as above except that ammonium acetate buffer was used. After incubation, the enzyme proteins were separated by ultrafiltration (YM 10 membrane, Amicon, Denwers, CO), the ultrafiltrate was concentrated in a Büchi rotation evaporator at 40 °C, lyophilized and product, recovered in ca. 95% yield, was stored at -20 °C.

Analytical methods. HPLC was performed on an S 1000 SYKAM liquid chromatograph (Gilching, Germany) fitted with an RI detector using ligand exchange chromatography on Ostion LG KS 0800 Ca^{2+} column (250x8 mm, Watrex, Prague, Czech Republic) eluted at 85 °C with deionized water at a flow rate of 0.85 mL min^{-1}. TLC analyses of free sugar oxidation products were performed on Silica Gel 60 F_{254} aluminium sheets (Merck, Darmstadt, Germany) developed with 5:2:1:1 EtOAc–EtOH–AcOH–boric acid (saturated in water). Sugars were visualized using a diphenylamine–aniline reagent [16]. *N,N*-Diphenylhydrazone derivatives were purified on the same foils using 5:1 MeOH–$CHCl_3$ (twice developed) and UV/VIS detection. ^1H and ^{13}C NMR spectra were measured on a Varian INOVA-400 spectrometer at 30 °C; the compounds **1**, **2a**, **2b** and **3** in CD_3OD, **4** in D_2O and d_6-DMSO. TSP was used as an internal standard in D_2O, the residual solvent signals were employed otherwise (δ_H 3.33, δ_C 49.3 with CD_3OD, δ_H 2.50,

δ_C 39.6 with d_6-DMSO). Carbon signal multiplicity was determined by APT (Attached Proton Test). 2D NMR experiments (HOM2DJ, COSY, delay-COSY, 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 ($1x10^{-5}$ bar) was used as a bombarding gas.

β-D-Glucopyranosyl-(1→6)-D-*arabino*-hexos-2-ulose 1-(*N,N*-Diphenylhydrazone) (1). Compound of R_f 0.07, crystallized from $CHCl_3$ (yellow needles, 152 mg), mp 178–180 °C, decomp. UV/VIS: λ_{max} (MeOH) 232, 276, 347 nm. ^1H NMR and ^{13}C NMR data see Tables 1–3. FAB MS: *m/z* (% rel. int.): 507 $[M+H]^+$ (11), 506 (12), 489 (24), 345 (3), 327 (27), 254 (4), 223 (24), 195 (7), 183 (8), 169 (56), 168 (100).

α-D-Galactopyranosyl-(1→6)-D-*arabino*-hexos-2-ulose 1-(*N,N*-Diphenylhydrazone) (2a). Compound of R_f 0.07, pale-yellow rectangular crystals (MeOH), 104 mg, mp 107–110 °C. UV/VIS: λ_{max} (MeOH) 232, 280, 345 nm. NMR see Tables 1–3. FAB MS: *m/z* (% rel. int.): 529 $[M+Na]^+$ (45), 507 $[M+H]^+$ (5), 489 (8), 345 (3), 327 (15), 254 (2), 223 (18), 195 (5), 183 (7), 169 (45), 168 (100).

α-D-Glucopyranosyl-(1→6)-D-*arabino*-hexos-2-ulose 1-(*N,N*-Diphenylhydrazone) (2b). Compound of R_f 0.07, obtained as a pale-yellow syrup, 89 mg. UV/VIS: λ_{max} (MeOH) 232, 277, 346 nm. NMR see Tables 1–3. FAB MS: *m/z* (% rel. int.): 529 $[M+Na]^+$ (60), 507 $[M+H]^+$ (3), 489 (9), 345 (1), 327 (15), 254 (2), 223 (20), 195 (6), 183 (6), 169 (48), 168 (100).

α-D-Glucopyranosyl-(1→6)-D-*arabino*-hexos-2-ulose 1,2-Bis(*N,N*-diphenylhydrazone) (3). Compound of R_f 0.14, obtained as an orange syrup, 27 mg. UV/VIS: λ_{max} (MeOH) 241, 298, 342, 391 nm. NMR see Tables 1–3. FAB MS: *m/z* (% rel. int.): 695 $[M+Na]^+$ (23), 673 $[M+H]^+$ (19), 419 (5), 252 (7), 222 (11), 195 (6), 183 (8), 169 (45), 168 (100).

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