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ARABINOSYL:HYDROXYPROLINE TRANSFERASE ACTIVITY IN *CHLAMYDOMONAS REINHARDTII*

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Key Word Index—*Chlamydomonas reinhardtii*; glycoproteins; arabinosyl transferase; lipid intermediate.

Abstract—A crude membrane preparation of the unicellular green alga *Chlamydomonas reinhardtii* was found to catalyse the transfer of [^{3}H]arabinose from UDP-arabinose to protein. Highest incorporation rates were found at 25° and pH 6.5. Hydrolytic studies on the labelled product revealed the presence of arabinose and an arabinose disaccharide in the acid hydrolysate. The transfer of arabinose to lipid was also monitored. The addition of dolichol-phosphate as an intermediate had no effect on the label incorporation into lipid. However, it had a marked inhibitory effect on the label incorporated into protein. This inhibitory effect was examined kinetically and indicated mixed-type inhibition.

INTRODUCTION

The cell wall of the unicellular alga *Chlamydomonas reinhardtii* does not contain cellulose but is composed of concentric layers of hydroxyproline-rich glycoproteins whose overall sugar composition resembles the insoluble hydroxyproline-rich glycoproteins of higher plants [1, 2]. They are structurally distinct from the arabinogalactan proteins secreted by plant cells in suspension culture [3–6]. In the cell wall preparations from *Chlamydomonas reinhardtii* hydroxyproline was found to be *O*-glycosidically linked either to galactose or a mixture of hetero-oligosaccharides composed of arabinose and galactose [7]. This is in contrast to the structure of cell wall hydroxyproline-rich glycoproteins of higher plants. Monosaccharides such as glucose, galactose and mannose have also been detected in total hydrolysates of wall preparations of *Chlamydomonas* [1, 8].

Lipid–sugar intermediates such as polyprenyl phosphate derivatives are often involved in sugar transfer reactions. They have been shown to take part in the biosynthesis of glycoproteins and polysaccharides in eukaryotic cells [9–11] as well as in the biosynthesis of cell wall material of bacteria [12].

It is already known however that there is no evidence for a lipid intermediate in galactosylation of the cell wall glycoprotein of *Chlamydomonas* [13]. Since arabinose is the most abundant sugar in the cell wall of *Chlamydomonas reinhardtii* we have investigated the arabinosyl transferase enzyme in this alga and also the possibility of a lipid intermediate in the transfer of arabinose from UDP-arabinose to protein.

RESULTS

Incorporation of arabinose from UDP-β-L-[1-^{3}H]arabinose

The crude particulate fraction prepared from *Chlamydomonas reinhardtii* was incubated with UDP-β-L-[1-^{3}H]arabinose. Figure 1 shows the optimum conditions for arabinose incorporation into products insoluble in TCA. Incubations were carried out at 25° for 30 min. Longer incubation times did not increase the amounts of incorporation (Fig. 1a). The greatest stimulation of incorporation was obtained with Mg^{2+} and Mn^{2+} (Fig. 1b). The membranes were prepared in the absence of Mg^{2+} when the effect of the divalent cations was measured. The pH optimum was found to be 6.5; this is in agreement with the value found by Karr [14]. The kinetic data obtained from the TCA precipitable products gave a value of 0.247 ± 0.024 mM for the K_m of the transferase.

Properties of labelled products

The TCA precipitated material that was collected on the GF/C filters was dissolved in 10 mM Tris–HCl (pH 8.0) concentrated and passed through a column of Sephadex G-75 to show the presence of high M_r compounds. Both the radioactivity and absorbance at 280 nm of the fractions eluted from the column were monitored. The result (Fig. 2a) showed that 96% of the total radioactivity added was present in high M_r compounds which were proteins. However when these compounds were subjected to SDS polyacrylamide electrophoresis on a 15% separating gel [15] they did not enter the gel. This property is similar to that of the high M_r compounds released into the media of asynchronously grown *Chlamydomonas reinhardtii* [16]. The high M_r compounds were hydrolysed either by $Ba(OH)_2$ or strong acid, the hydrolysates were passed through a G-75 Sephadex column (Figs 2b–c). The measurements of radioactivity and the absorbance at 280 nm indicated that predominantly low M_r compounds were present in both hydrolysates, although a small amount of non-radioactive polypeptide was present as well. The $Ba(OH)_2$ hydrolysate electrophoresed at pH 2.0 gave rise to spots with $R_{hydroxyproline}$ 0.62, 0.76 and 0.89. The spot with $R_{hydroxyproline}$ 0.62 corresponds to *cis*-hydroxyproline *O*-linked to three arabinosyl units and constituted 48% of the total radioactivity present, while the other two spots correspond to hydroxyproline linked to two or a single arabinosyl unit and constituted 20 and

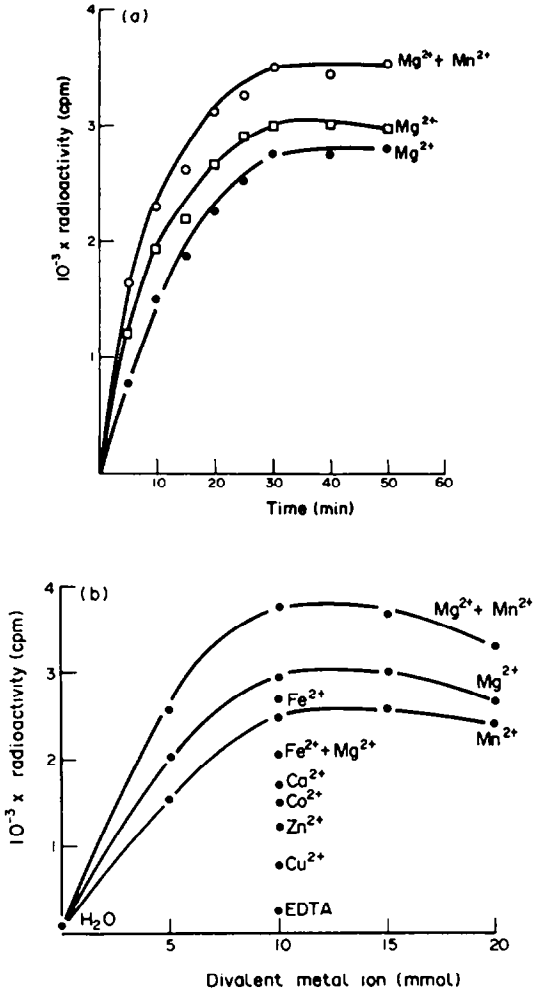

Fig. 1. The optimum conditions for the arabinosyl transferase assay: (a) time-course with divalent cation concentration constant at 10 mM; (b) effect of divalent cations. The standard membrane preparation was used in a total volume of 50 μl, except during (b) where the membranes were prepared in the absence of Mg^{2+}.

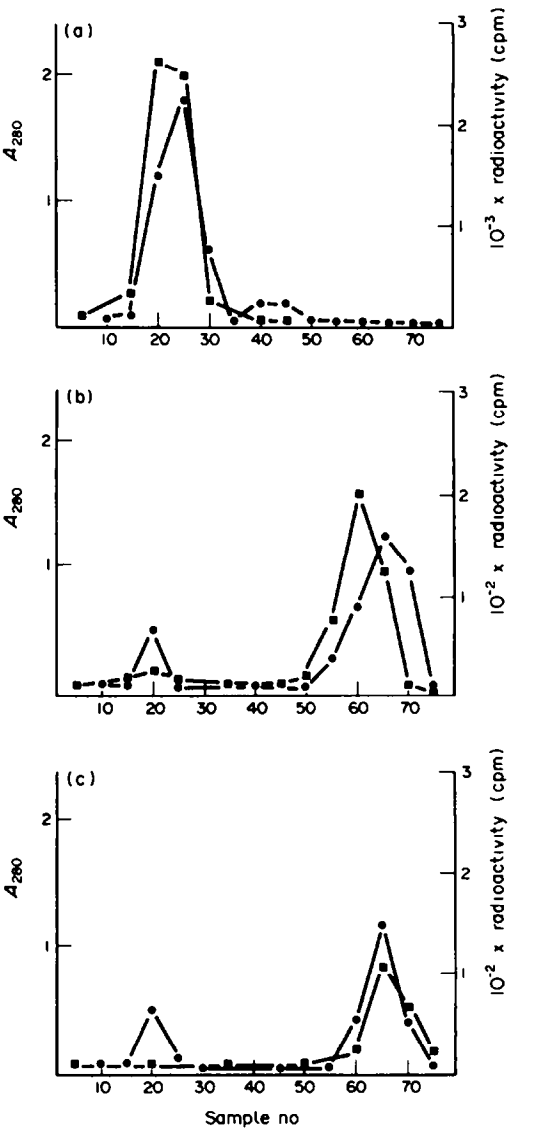

Fig. 2. Sephadex G-75 gel chromatography of the radioactive material after incorporation of [^{3}H]arabinose from UDP-β-[^{3}H]arabinose (a) before hydrolytic treatment, (b) after hydrolysis by $Ba(OH)_2$, (c) after hydrolysis by strong acid. The column was equilibrated and eluted with 10 mM Tris–HCl pH 8.0, 1 ml fractions were collected after elution of the void volume (fraction 20). (●—●) A_{280} nm; (■—■) radioactivity.

29% of the total radioactivity, respectively [17]. The acid hydrolysate was chromatographed in solvents A and B when the only radioactive product was identified as arabinose. The TCA precipitable products were hydrolysed in 0.01 N HCl in 50% (v/v) propanol at 100° for various times ranging from 100 min to 1 hr. These mild acid hydrolysates gave two radioactive products on a paper chromatogram (solvent B) which were identified as monomeric arabinose, which accounted for 63% of the radioactivity added, and an arabinose disaccharide (R_{ara}, 0.5; the R_{ara} for cellobiose was 0.38) which accounted for 24% of the added radioactivity. The disaccharide was present even when the hydrolysis with mild acid was continued for up to 60 min.

Effect of exogenous lipid on arabinosyl transferase activity

For measurement of incorporation of arabinose into total lipid as well as into TCA precipitable products, the amount of material in the assay system was doubled. The radioactive lipid present in the chloroform solution was chromatographed on a DEAE-cellulose column to demonstrate the possible presence of monophosphate and diphosphate groups. Two types of experiment were carried out, one with and one without the addition of dolichol-phosphate. The lipid that was labelled in the absence of dolichol-phosphate was eluted from the column with 99% methanol, suggesting that it was all neutral lipid (Table 1). The products labelled in the presence of dolichol-phosphate contained, in addition to the neutral lipid, a small amount of material that was only eluted from the column with 0.2 M ammonium acetate in 99% methanol. Analysis of this material was not possible due to the small amount of radioactivity present.

Table 1. Fractionation on DEAE-cellulose of arabinolipid fractions synthesized in the presence or absence of added dolichol-monophosphate

Assay conditions	cpm incorporated		
	Neutral lipid	Lipid monophosphate	Lipid diphosphate
No additions	1845	0	0
Dolichol-phosphate added	1930	75	0

Each sample was applied to the column in 99% (v/v) methanol followed by stepwise elution with (a) 99% methanol (neutral lipid); (b) 0.2 M ammonium acetate in 99% methanol (monophosphate); (c) 0.4 M ammonium acetate in 99% methanol (diphosphate).

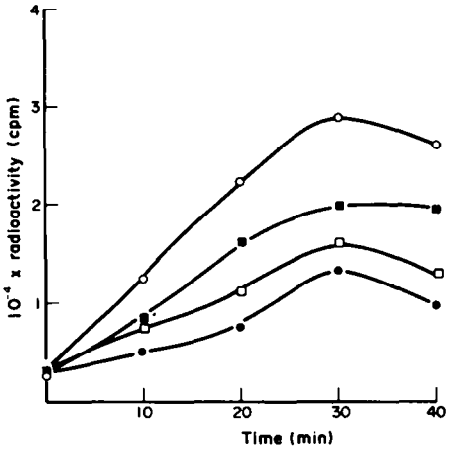

Fig. 3. Time-course of the transfer reactions. Incorporation of radioactivity from UDP-L-[^{3}H]arabinose into protein (○) and lipid (□) in the absence and presence (filled symbols) of 10 mM dolichol-phosphate. The volume of the standard incubation mixture was 100 μl. At various times half the incubation mixture was removed and incorporation into TCA precipitable products measured. Chloroform–methanol (2:1, v/v, 2 ml) was added to the remainder and the radioactivity in these lipid fractions determined after Folch-washing.

As can be seen from Fig. 3 the increase in the amount of radioactive incorporation into total lipid was very small even after the dolichol-phosphate was added. However the addition of dolichol-phosphate had a marked inhibitory effect on the production of radioactive TCA precipitable products. This inhibition was examined kinetically (Fig. 4) and the plots of slopes (Fig. 4b) and intercepts (Fig. 4c) against dolichol-phosphate concentration indicated a mixed inhibition with a value of 11.8 $\pm$ 1.18 μM for the K_{iE} and 74 $\pm$ 7.4 μM for the K_{iES}.

DISCUSSION

The cell wall of *Chlamydomonas reinhardtii* is composed of hydroxyproline-rich glycoproteins in which the hydroxyproline residues are glycosylated with either galactose or arabinose [7]. In this work an arabinosyl:hydroxyproline transferase has been identified in a membrane preparation of the organism and this enzyme system may be responsible for the glycosylation of the cytoplasmic precursor of the cell wall glycoprotein and/or the matrix glycoproteins.

The particulate enzyme preparation from *Chlamydomonas reinhardtii* catalysed the transfer of arabinose from UDP-arabinose to high M_r polypeptides. These polypeptides were unresolved in SDS-polyacrylamide gels which probably indicated that they were similar to the high M_r glycoproteins released into the media by asynchronously growing *C. reinhardtii*. On alkaline degradation in 0.2 M $Ba(OH)_2$ the radioactivity was found exclusively in low M_r material, probably oligosaccharide. Strong acid hydrolysis showed that the oligosaccharides were completely hydrolysed to give radioactive arabinose. However, mild acid hydrolysis gave products containing radioactive arabinose which were of higher M_r than the arabinose which was also produced. These products appear to be disaccharides containing arabinose. The disaccharide in the mild acid hydrolysate was found even after hydrolysis for 1 hr, suggesting that some of the arabinose was probably in the pyranose form, which may be the case for the soluble matrix glycoproteins. This was in contrast with a methylation analysis [14] of *C. reinhardtii* cell walls which indicated that the arabinose present in cell wall glycoproteins was in the furanose form.

The optimum conditions for the transarabinosylation were in agreement with those found for other enzyme systems studied in higher plants [14, 18], although in the system studied here the effect of Mg^{2+} and Mn^{2+} gave higher incorporation rates than Mg^{2+} alone. The presence of a polyprenyl-type intermediate involved in the transfer of arabinose from UDP-arabinose to endogenous protein has not been shown. The addition of dolichol-phosphate as an exogenous intermediate did not enhance the incorporation of label into total lipid but it had an inhibitory effect on the incorporation into TCA precipitable products which contained both polypeptides and arabinose and were therefore glycoproteins. This unexpected inhibitory effect was examined kinetically over a wide range of dolichol-phosphate concentrations and showed that the dolichol-phosphate gave a mixed-type inhibition, increasing the K_m value and lowering the apparent affinity of the enzyme for the substrate. It is difficult therefore to see how it could act as an intermediate during the glycosylation of the glycoproteins. This conclusion supports and extends the work of Lang [13] on galactosyl transferase.

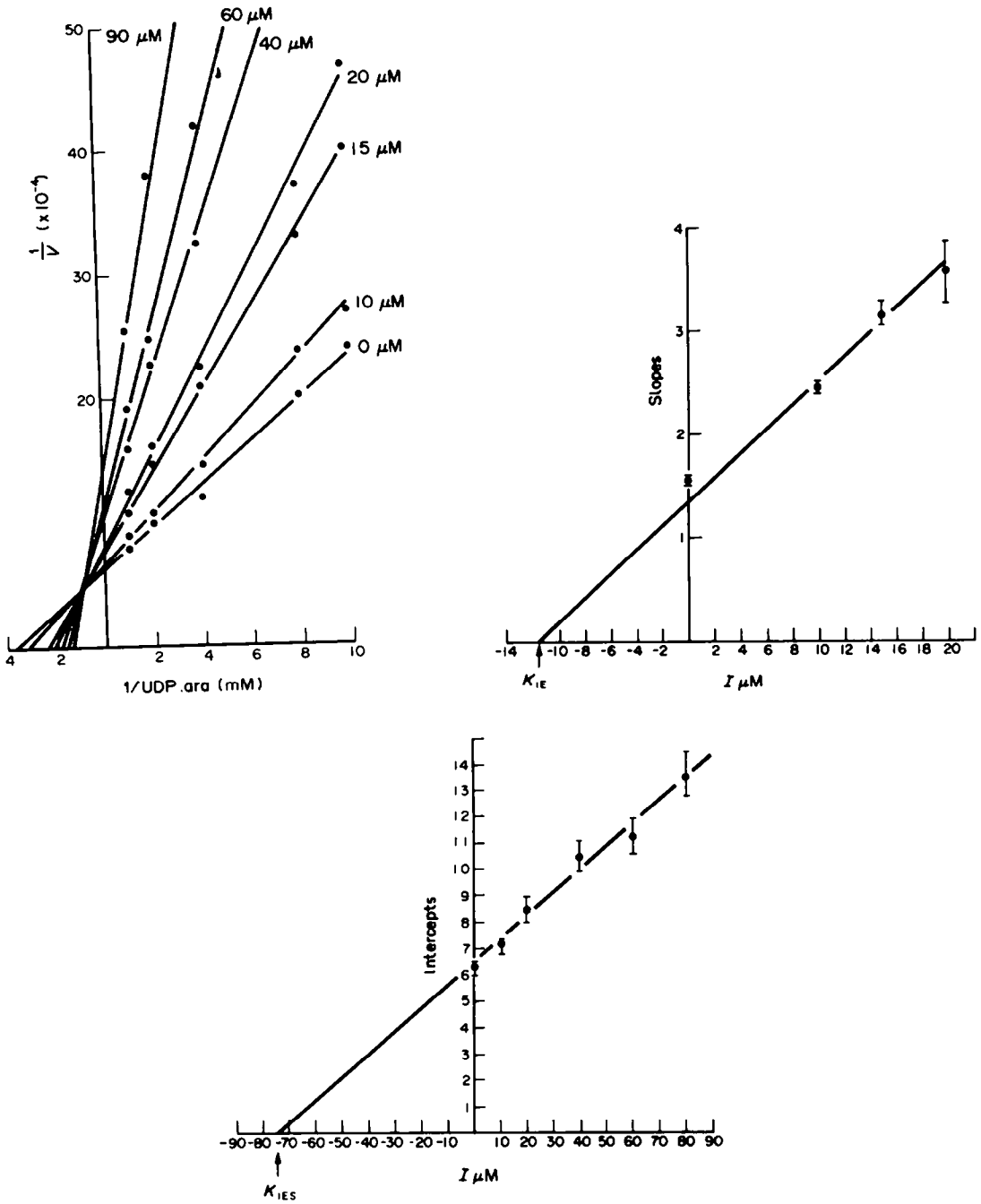

Fig. 4. Kinetic plots showing (a) the influence of increasing concentrations of dolichol-phosphate (0–90 μM) on the K_m and V_{max} values, (b) the slopes obtained from Lineweaver–Burk plots with various dolichol-phosphate concentrations, (c) the intercepts obtained from Lineweaver–Burk plots with various dolichol-phosphate concentrations, plotted against inhibitor (dolichol-phosphate) concentration. The plots show a mixed type inhibition with different inhibition constants for enzyme and enzyme-substrate complex (K_{iE} and K_{iES}).

EXPERIMENTAL

Suspension cultures of Chlamydomonas reinhardtii 11/32mt(+) (Culture Centre for Algae and Protozoa, Cambridge, U.K.) were grown under the conditions described in ref. [2] modified to include a 16 hr light, 8 hr dark cycle.

L-[1-^{3}H)*Arabinose* was prepared at Amersham International U.K., by catalytic exchange of pure arabinose with tritiated H_2.

L-*Arabinokinase* was extracted from mung bean seedlings and phosphorylations were carried out with ATP and $MgCl_2$ in the presence of 40 mM KF [19]. The *β-L-arabinopyranose-1-phosphate* was isolated by paper electrophoresis (pH 3.5). A crude mixture of UDP-sugar *pyrophosphorylases* was also extracted from mung bean seedlings [20] and the reaction of the sugar-1-phosphates with excess UTP in the presence of $MgCl_2$ and 40 mM KF was carried out as described in ref. [20]. The *UDP-β-*

L-*arabinopyranose* was isolated by paper electrophoresis (pH 3.5) [21].

Preparation of crude membrane pellet and arabinosyl transferase assay. The crude membrane preparation and the assay were based on a modification of the method of ref. [22]. The packed cell vol. of 36 ml harvested from 9 l. of culture was ground with acid washed sand and suspended in 50 mM Tris–HCl buffer (pH 7.4), containing 250 mM sucrose, 5 mM β-mercaptoethanol and 10 mM $MgCl_2$ before being centrifuged at 1000 *g* for 10 min. The supernatant was centrifuged at 48 000 *g* for 30 min, then the pellet was resuspended in 5 ml of 50 mM Tris–maleate buffer (pH 6.5), containing 10 mM β-mercaptoethanol, 40% glycerol and 5 mM $MgCl_2$. This crude enzyme preparation contained 5–10 mg protein per ml. Arabinosyl transferase was measured in a total vol. of 50 μl. Except for optimizing experiments the reaction mixture comprised 5 μl of a soln containing 0.1 M $MgCl_2$ and 0.1 M $MnCl_2$, 5 μl UDP-L-[^{3}H]arabinose and 40 μl of the crude particulate enzyme preparation. The reaction mixture was incubated at 25° for 30 min. When dolichol-phosphate was added, it was dried under a stream of N_2 and dissolved in 0.45% (w/w) Triton X100, the assay vol. being adjusted accordingly.

Transfer of arabinose from [^{3}H]*arabinose to labelled products.* Incorporation of radioactivity into a TCA precipitable product was measured by collecting the products on glass-fibre (GF/C) discs and washing in chilled 5% TCA, MeOH and $CHCl_3$–MeOH (2:1). These discs were then counted in a toluene-based scintillant. Controls when the enzyme was inactivated by heat and when UDP-β-L-[^{3}H]arabinose was added after the TCA precipitation were found to be free of radioactivity. Incorporation of radioactivity into the lipid fraction was measured by adding 2 ml of $CHCl_3$–MeOH (2:1) to the assay mixture, followed by a brief centrifugation to separate insoluble material. After addition of 400 μl of $MgCl_2$ the lower phase was washed according to ref. [23]. A sample was then dried and counted in a toluene-based scintillant.

Analysis of products. The TCA precipitable products were hydrolysed in 0.2 M $Ba(OH)_2$ for 6 hr at 100° or in 3% H_2SO_4 (strong acid hydrolysis) at 120° [24]. The unhydrolysed and hydrolysed materials were chromatographed on a (0.9 × 75 cm) Sephadex G-75 column, eluted with 10 mM Tris–HCl pH 8.0.

The 'Folch-washed' lipids were run on a small column (0.5 × 2 cm) of DEAE-cellulose (acetate) and eluted sequentially with 99% MeOH, 0.2 M ammonium acetate in 99% MeOH and 0.4 M ammonium acetate in 99% MeOH. The radioactivity in each eluate was determined.

High voltage paper electrophoresis was performed at pH 2.0 ($HOAc$–HCO_2H–H_2O, 4:1:45) and 5 kV for 45 min. Hydroxyproline-arabinosides were stained with ninhydrin-isatin reagent [25].

Paper chromatography using Whatman no. 1 paper was performed in the following solvents (A) EtOAc–Pyr–H_2O (8:2:1); (B) *n*-BuOH–Pyr–H_2O (10:3:3). Chromatograms were stained with silver [26] in order to locate the sugar standards.

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