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Phosphorolytic synthesis of cellodextrins

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Abstract

Transglucosylation reactions catalyzed by cellodextrin phosphorylase from *Clostridium thermocellum* were reinvestigated by using a series of cellobiosyl residues as glycosyl acceptors and α-D-glucopyranosyl phosphate (Glc-1-P) as glycosyl donor. When cellobiose was used as an acceptor, various unsubstituted cellodextrins, ranging from water-soluble products to crystalline precipitates were obtained, depending on the concentration in acceptor. 4-Thiocellobiose, methyl β-cellobioside, and methyl 4-thio-α-cellobioside could also be used as acceptors with the same efficiency as cellobiose. The enzyme showed higher activity when β-cellobiosides bearing strong hydrophobic aglycons were used. A practical preparation of methyl β-cellotrioside, cellotetraoside and cellopentaoside was achieved, using a 1:5 molar ratio of methyl β-cellobioside to the glucosyl donor.

Keywords: Enzymatic synthesis; Phosphorylase; Methyl β-cellodextrins; X-ray diffraction of cellodextrins; Electron microscopy of cellodextrins

1. Introduction

Cello-oligosaccharides (cellodextrins) form an important homologous family that has proven very useful to model the physical [1–3] and chemical [4,5] properties of cellulose together with its susceptibility towards cellulases [6,7]. The preparation of cellodextrins by acetolysis or acid hydrolysis has been established for a long time [8–10]. This method can be monitored to yield mixtures of degree of polymerization (DP) 3–6 that have to be fractionated to give the individual cellooligomers. In a different approach,

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several laboratories have attempted to prepare cellodextrins through a stepwise chemical synthesis. This method is somewhat more difficult since it is not easy to obtain suitable D-glucopyranosyl derivatives having only the C-4 position unprotected. Further, the low reactivity of the hydroxyl group at C-4 leads to the selection of strong acceptors such as derivatives of 1,6-anhydro-β-D-glucopyranose [11,12] or 2,3,6-tri-*O*-benzyl-glucosides [13]. These compounds are not easily prepared in quantities. For this reason, the stepwise methods have not been used so far for large scale synthesis of cello-oligosaccharides.

The chemo-enzymatic or the purely enzymatic routes appear to be promising alternatives for the synthesis of tailor-made cellodextrins. Along this line and following the work of Hehre et al. [14] who showed that the transglycosylation of α-maltosyl fluoride could be catalyzed by alpha-amylases, Kobayashi et al. [15,16] have recently reported the transglucosylation of β-cellobiosyl fluoride using catalysis by crude cellulases in an acetonitrile–acetate buffer solution. Initially, this method gave crystalline cellodextrins having a DP ~ 22 and the structure of cellulose II. More recently, the technique was further refined by using purified cellulase fractions and optimized acetonitrile–acetate buffer mixtures. Under such conditions, the system was even able to lead to the first in vitro synthesis of crystalline microfibrils of cellulose I [17].

The present work deals with another approach to the enzymatic synthesis of cellodextrins, by using an enzyme system that is different from that used by Kobayashi and his group. Our synthesis takes advantage of one enzyme, namely a cellodextrin phosphorylase that is produced by *Clostridium thermocellum* when the bacteria are grown on cellulose-based media [18,19]. In addition to its phosphorolytic action (Eq. 1, pathway *a*), the most interesting feature of cellodextrin phosphorylase is its additional ability to synthesize cellodextrins when the enzymes are incubated with a primer, $G_{n\ (n \geq 2)}$ and D-glucopyranosyl phosphate (Glc-1-P) (Eq. 1, pathway *b*) [19].

$$[G_{n+1} + Pi \underset{b}{\overset{a}{\rightleftharpoons}} G_n + \text{Glc-1-P} \qquad (1)$$

The present report describes the phosphorolytic synthesis and the characterization of cellodextrins, together with those of methyl β-cellotrioside, -cellotetraoside and -cellopentaoside. In addition, we also present a study of acceptor specificity toward cellodextrin phosphorylase.

2. Results and discussion

When *Clostridium thermocellum* was grown on cellulose, cellobiose phosphorylase, cellodextrin phosphorylase and traces of cellulases were found intracellularly. The use of cellodextrin phosphorylase for the in vitro synthesis of cellodextrins requires a purification step to eliminate the cellulase activity. This purification, achieved on the bacterial cell extracts, is summarized in Table 1. It involved at first a precipitation by protamine sulfate, followed by a fractionation with ammonium sulfate. This treatment gave a high yield of cellodextrin phosphorylase as well as cellobiose phosphorylase that was nearly

Table 1
Partial purification of cellodextrin phosphorylase from *Clostridium thermocellum*

Fractions	Volume (mL)	Enzymatic activity (U mL^{-1})			Yield (%)	
		Cellulases	Cellobiose phosphorylase	Cellodextrin phosphorylase	Cellulases	Cellodextrin phosphorylase
Crude extract	170	0.125	0.63	0.19	100	100
Supernatant after protamine sulfate precipitation	170	0.025	0.53	0.18	20	95
After fractionation with ammonium sulfate 60–80%	6	0.06	13.2	4.65	1.6	86

devoid of any cellulase activity. Further purification was not attempted since the pure phosphorylases were found to be quite unstable [19].

Synthesis of unsubstituted cellodextrins. — The phosphorolytic synthesis of unsubstituted cellodextrins was assayed with three different concentrations of cellobiose (Fig. 1). At low concentration (2.5 mM), a white crystalline precipitate was formed after the liberation of 20 mM of inorganic (Pi) phosphate. Its structure was investigated by electron microscopy imaging, electron and X-ray diffraction analysis, together with ^{1}H and ^{13}C NMR. When observed by electron microscopy, it was found that the precipitate consisted of more or less regular elongated platelet crystals with average lateral size of 0.1 μm $\times$ 1 μm and a thickness of around 10 nm (Fig. 2). By low dose imaging and electron-diffraction analysis on a bundle of crystals (Fig. 3), a sharp electron-diffraction diagram could be recorded (insert in Fig. 3). This pattern presents three main diffraction arcs: one of them, calibrated at 0.72 nm is positioned perpendicular to the long axis of the crystals whereas two arcs at 0.44 and 0.40 nm are roughly aligned with their long axis. These diffraction results are corroborated with X-ray data recorded on powder of the crystals which show again very clearly the three strong rings at 0.72, 0.44 and 0.40 nm (Fig. 4) These values are exactly those expected for crystalline cellodextrins [20]. In addition, the orientation of the crystals with respect to their diffraction diagram indicates that within the individual platelets, the molecular axis of the cellodextrin are perpendicular to the plane of the platelet and that the crystalline planes giving the 0.72 nm spacing corresponds to the growth planes of the crystals. Quite interestingly, these crystals, together with their electron diffraction patterns resemble strongly the crystals and the diffraction diagrams of low molecular weight cellulose II [21] or those of cellotetraose [22].

The crystalline precipitate was soluble in dimethyl sulfoxide at 353 K and 0.2 M sodium hydroxide at room temp. Its ^{13}C NMR spectrum (Me_2SO) had resonance bands whose chemical shift matched exactly those of a spectrum of cellulose oligomers [23]. In addition, in the ^{1}H NMR spectrum (NaOD), a quantitative measurement obtained by comparing the integral of the anomeric protons to the sugar ring protons, indicated that the precipitate corresponded to a cellodextrin having an average DP of about 8. At such DP, cellodextrins are almost completely insoluble in water [8]. Thus, in the present

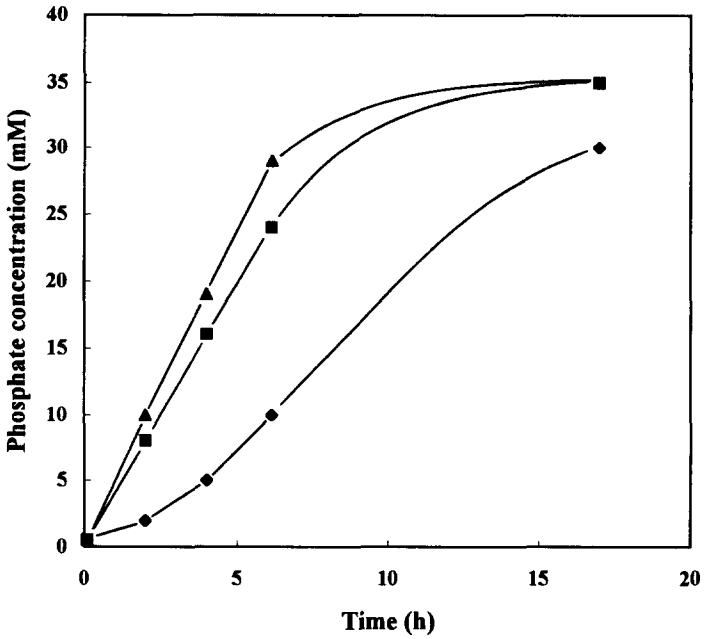

Fig. 1. Effect of cellobiose concentration on the phosphate liberation by interacting cellodextrin phosphorylase (0.15 U mL^{-1}) with G-1-P. (◆) 0 mM; (■) 2.5 mM; (▲) 10 mM. The initial concentration in Glc-1-P was 100 mM.

Fig. 2. Electron micrograph after shadowing with W/Ta of a typical preparation of crystalline cellodextrin after incubation of cellodextrin phosphorylase with 2.5 mM of cellobiose.

Fig. 3. Low dose electron micrograph of a bundle of crystals of cellodextrin obtained as in Fig. 2. In insert is shown a typical electron diffraction pattern of the circled area.

synthesis, we believe that as soon as celloheptaose or cellooctaose was produced, it crystallized readily and therefore was not accessible to further chain elongation. It was indeed shown in the synthesis of amylose by potato phosphorylase [24], that there was a

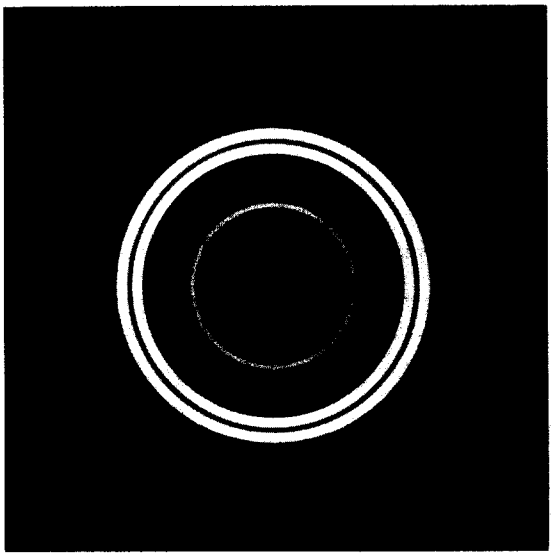

Fig. 4. Powder X-Ray diagram recorded on a preparation of crystalline cellodextrin as in Fig. 2 and 3.

complete dissociation between the enzyme and the product after each addition of a glucose moiety. Thus, for subsequent chain elongation, it was essential to have soluble products [25]. Such behavior, analogous to that reported for starch phosphorylase, is assumed for the present cellodextrin phosphorylase, even though the reactions of the two enzymes differ in their stereochemistry. This must explain why the polymerization will terminate as soon as the product reaches a DP where it becomes insoluble. In the case reported by Kobayashi et al. [15,16], the synthesis of higher DP cellodextrins may be explained by a different enzymatic mechanism, in particular where the enzyme would remain bound to its substrate throughout the synthesis, even after the precipitation of the product. Thus, even after reaching a cellodextrin DP of 8, the enzymatic system should still be active provided that it would not be buried within the solid substrate.

No precipitate was observed when the initial cellobiose concentration was 10 mM or higher, probably because an increase of primer to Glc-1-P ratio contributed to a decrease in DP, therefore to soluble products.

In the absence of cellobiose, a lag phase was first observed, but with time, the reaction began, its rate increased and a precipitate occurred. After 17 h, the same amount of inorganic phosphate was liberated as in the case where 2.5 mM of cellobiose had been used as primer (Fig. 1). This behavior may be explained by a slow side enzymatic reaction where Glc-1-P is hydrolyzed into glucose. This liberated glucose could act as a primer for the cellobiose phosphorylase that is still present and which converts glucose into cellobiose. This cellobiose in small amount could then become acceptor for the cellodextrin phosphorylase and, after a few steps, generate insoluble cellodextrins.

Synthesis of cellodextrins substituted at their reducing end. — The cellodextrin phosphorylase from *Clostridium thermocellum* proved also very useful for the preparation of various cellodextrins substituted at their reducing end. To be specific however, this synthesis required the removal of all glucose produced as side product in the hydrolysis of Glc-1-P. This removal was achieved by oxidation, with glucose oxidase in the presence of catalase [26].

The rate of phosphorolytic synthesis of cellodextrin phosphorylase in the presence of several primers is presented in Table 2. This table shows that methyl α-4-thiocellobioside and methyl β-cellobioside are as good primers as cellobiose or 4-thiocellobiose. On

Table 2
Relative initial rate of phosphorolytic synthesis by purified cellodextrin phosphorylase in presence of various primers

Primers	Relative rate (%) [a]
None	8
Cellobiose	1004
4-Thiocellobiose	100
Methyl 4-thio-α-cellobioside	100
Methyl β-cellobioside	100
Phenyl β-cellobioside	150
Benzophenone β-cellobioside	160

[a] Calculated from the amount of phosphate liberated after 2 h incubation with 0.075 U mL^{-1} of cellodextrin phosphorylase, 5 mM of primer and 20 mM of Glc-1-P.

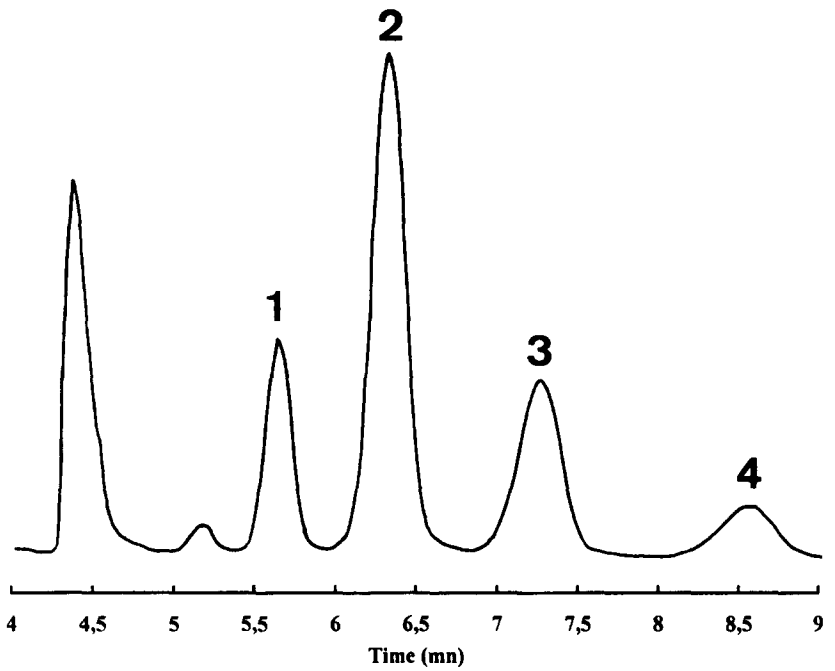

Fig. 5. LC profile recorded during the phosphorolytic synthesis of cellodextrins by cellodextrin phosphorylase using methyl β-cellobioside as acceptor in the presence of glucose oxidase and catalase (peak 1: methyl β-cellobioside; 2: methyl β-cellotrioside; 3: methyl β-cellotetraoside; 4: methyl β-cellopentaoside).

the other hand, cellobiosides bearing a strongly hydrophobic aglycon were even better acceptors than cellobiose itself. In the present work, mixtures of methyl β-cellotrioside, -cellotetraoside and -cellopentaoside were obtained when methyl β-cellobioside was incubated for 72 h with Glc-1-P in the presence of cellodextrin phosphorylase (Fig. 5). These compounds were isolated by LC with yields of 36, 15 and 6% (respectively from methyl β-cellobioside).

The phosphorolytic synthesis of cellodextrins substituted at their reducing end is not limited to the methyl β-cellodextrin series. It can also be applied to several other cellodextrins that are important for the study of cellulases, including reduced cello-oligosaccharides or oligomers with 2-chloro-4-nitrophenyl and umbelliferyl aglycons [27,28]. The present synthesis opens an easy route to quantities of these compounds. Their synthesis, starting from 2-chloro-4-nitrophenyl or umbelliferyl cellobiosides, is in progress and will be reported shortly.

3. Experimental

Production and purification of cellodextrin phosphorylase.— Clostridium thermocellum (DSM 1237) was obtained from the German collection of microorganisms. Cells

were grown in 10-L carboys containing 8 L of the following medium : NH_4Cl, 1 g L^{-1} ; yeast extract, 4 g L^{-1} ; $MgSO_4 \cdot 7H_2O$, 0.15 g L^{-1} ; $CaCl_2 \cdot 2H_2O$, 0.012 g L^{-1}; KH_2PO_4, 7 g L^{-1}; microgranular Whatman cellulose CC31, 5 g L^{-1}; cysteine · HCl, 0.5 g L^{-1}. After autoclaving, the carboy was flushed with N_2 and cooled to 55° C. A $NaHCO_3$ soln (1 M, 400 mL) was then added and the resulting medium was inoculated (5% v/v) and incubated for 3–4 days at 55° C with constant stirring. When a decrease of the gas production was observed, the cells were harvested by centrifugation (45 min at 5000 g). They were then resuspended in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.5 mM DTT (10 mL g^{-1} of wet cell) and were broken by sonication. After centrifugation (30 min, 30.000 g), the supernatant (crude extract) was treated with protamine sulfate (1.5 mg mL^{-1}) and centrifuged (30 min, 30.000 g). An ammonium sulfate fractionation was then performed following the method of Alexander [19]

Enzyme assays; cellobiose and cellodextrin phosphorylases. — These activities were determined and distinguished by measuring the release of Pi from α-D-glucopyranosyl phosphate (Glc-1-P) in the presence of glucose (for cellobiose phosphorylase) or cellobiose (for cellodextrin phosphorylase). The enzymes were incubated (30 min) in MOPS buffer (20 mM, pH 7.0) containing 1 mM EDTA, 0.5 mM DTT, 10 mM glucose or cellobiose. The synthesis was initiated by the addition of Glc-1-P (20 mM). Throughout the reaction, one reacting unit was defined as the amount of enzymes which catalyzed the liberation of 1 mmol min^{-1} of phosphate at 30° C.

Cellulase activities. — These activities were determined spectrophotometrically by following the increase in absorbency at 405 nm due to the liberation of 2-chloro-4-nitrophenol ($\epsilon = 16$ m M^{-1}) from 2-chloro-4-nitrophenyl lactoside [29]. The lactoside (1 mM) was dissolved in MOPS buffer (20 mM, pH 7.0) and the reaction was initiated by the addition of the enzyme complex. One unit of cellulase was defined as the amount which catalyzed the liberation of 1 mmol min^{-1} of 2-chloro-4-nitrophenol at 25° C.

Analytical techniques. — (A) Phosphate concentration was quantified colorimetrically by the method of Tashima [30] for enzyme assay and by the method of Fiske-Subbarow [31] for phosphorolytic synthesis. (B) ^1H and ^{13}C NMR spectra were recorded with a Bruker AC-300 spectrometer at 296 K and 353 K respectively. (C) Electron microscopy was achieved on the undried cellodextrin precipitate that was washed several times by centrifugation in 10% EtOH. Drops of the suspended precipitate were deposited on coated grids. After drying, some of these grids were shadow casted with W/Ta. Others were observed directly under low dose conditions. All electron microscopy was done with a Philips EM400 T operated at 80 kV for shadowed samples and 120 kV for unshadowed specimen. Electron diffraction diagrams were obtained on unshadowed specimens by choosing never observed areas. (D) X-Ray analysis was performed after inserting the dried cellodextrin precipitates into thin walled X-ray capillaries. The powder diffraction diagrams were recorded with a Warhus flat film camera mounted on a Philips X-ray generator operated with Cu-$K\alpha$ radiation. (E) The course of the reactions and the homogeneity of the products were monitored by LC on an analytic μ-Bondapak NH_2 column (Waters, Milford, MA, USA) with MeCN–water as the eluent. The separation of methyl β-cellotrioside, -cellotetraoside and -cellopentaoside was achieved by using a preparative μ-Bondapak NH_2 column (125 Å, 10 μm, 19 × 150 mm) with 70:30 MeCN–water as eluent. (F) Low resolution mass

spectra were recorded on a Nermag R-1010C spectrometer (FAB mode) using acidic (0.1 N HCl) glycerol matrix (1:4 v/v).

Preparation of insoluble (1 → 4)-β-D-glucans. — Cellodextrin phosphorylase (7.5 U) was added to a MOPS buffer solution (25 mM, pH 7.0, 180 mL) containing cellobiose (2.5 mM), DTT (0.5 mM), EDTA (1 mM) and Glc-1-P (100 mM). After an incubation time of 40 h at 35° C, a suspension resulted that was recovered by centrifugation and purified by washing with 10% EtOH (3 ×). The wet precipitate was used for electron microscopy and X-Ray analyses. When dried under reduced pressure at 60° C, the resulting product was obtained as a powder (600 mg). ^{13}C NMR: δ 102.4 (C-1); 79.6 (C-4); 74.7 (C-5); 74.5 (C-3); 73.0 (C-2); 60.2 (C-6).

Methyl β-cellotrioside, -cellotetraoside and -cellopentaoside. — To a solution of methyl β-cellobioside [32] (710 mg, 2 mmol) in MOPS buffer (25 mL, 0.1 M, pH 7) containing EDTA (4 mM) and DTT (2 mM) was added cellodextrin phosphorylase (1 mL, 4.6 U), glucose oxidase (500 U), catalase (200 μL, 18600 U mg^{-1}) and then Glc-1-P disodium salt hydrate (Sigma, 3 g, 10 mmol) in water (75 mL). The resulting solution was stirred at 25° C until the LC tracing indicated the formation of methyl β-cellohexaoside (~ 72 h). The solution was then heated at 100° C for 2 min to inactivate the enzyme, and some MB-3 Amberlite resin was added. After removal of the resin, the product was freeze-dried. It was redissolved into a minimum of water and purified by LC, resulting in the isolation of residual methyl β-cellobioside (170 mg, 23%). Methyl β-cellotrioside (375 mg, 36%): [α]D −11.8° (*c* 0.86, water), (lit. [9], [α]D −13.7°); FAB$^+$MS: *m/z* 519, ([M + H]$^+$); 541 ([M + Na]$^+$); ^{13}C NMR (D_2O): δ 105.2 (C-1); 104.5, 104.3 (C-1′, C-1″); 81.0, 80.8 (C-4′, C-4″); 78.1–75.1 (C-2, C-2′, C-2″, C-3, C-3′, C-3″, C-5, C-5″); 71.8 (C-4); 62.9, 62.5, 62.4 (C-6, C-6′, C-6″, C-6‴); 59.1 (CH_3); Anal. Calcd for $C_{19}H_{34}O_{16} \cdot H_2O$: C, 42.53; H, 6.76; Found: C, 42.07; H, 6.83. Methyl β-cellotetraoside (186 mg, 14%): [α]D −7.6° (*c* 0.54 water), (lit. [9], [α]D −10.0°); FAB$^+$MS: *m/z* 681, ([M + H]$^+$); FAB$^-$MS: *m/z* 679, ([M − H]$^-$); ^{13}C NMR (D_2O): δ 105.2 (C-1); 104.5–104.3 (C-1′, C-1″, C-1‴); 80.9, 80.8, 80.6 (C-4′, C-4″, C-4‴); 78.1–75.1 (C-2, C-2″, C-2‴, C-3, C-3′, C-3″, C-3‴, C-5, C-5′, C-5″, C-5‴); 71.8 (C-4); 62.9, 62.5, 62.4 (C-6, C-6′, C-6″, C-6‴); 59.1 (CH_3); Anal. Calcd for $C_{25}H_{44}O_{21} \cdot 2H_2O$: C, 41.90; H, 6.75; Found: C, 42.06; H, 6.68. Methyl β-cellopentaoside (101 mg, 6%): [α]D −5.5° (*c* 0.51, water), (lit. [9], [α]D −8.1°); FAB$^+$MS *m/z* 843, ([M + H]$^+$); ^{13}C NMR (D_2O): δ 105.2 (C-1); 104.5–104.3 (C-1′, C-1″, C-1‴, C-1⁗); 80.9–80.7 (C-4′, C-4″, C-4‴, C-4⁗); 78.1–75.1 (C-2, C-2′, C-2″, C-2‴, C-2⁗, C-3, C-3′, C-3″, C-3⁗, C-5, C-5′, C-5″, C-5‴, C-5⁗); 71.8 (C-4); 62.9-62.4 (C-6, C-6′, C-6″, C-6‴, C-6⁗); 59.1 (CH_3); Anal. Calcd for $C_{31}H_{54}O_{26} \cdot 4H_2O$: C, 40.70; H, 6.83; Found: C, 40.3, H, 6.53

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