Novel dialkoxytriazine-type glycosyl donors for cellulase-catalysed lactosylation†

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Novel glycosidic compounds, 4,6-dialkoxy-1,3,5-triazin-2-yl β-lactosides (DAT-β-Lac), have been prepared directly in water from lactose. The reaction was carried out on a laboratory scale without protecting the hydroxy groups of lactose. The resulting triazine derivatives were found to be recognized by *endo*-β1,4-glucanase III from *Trichoderma reesei* (EGIII). The EGIII-catalysed transglycosylation of 4,6-dimethoxy-1,3,5-triazine derivative (DMT-β-Lac) with various glycosyl acceptors has successfully been demonstrated, affording the corresponding lactosylated products.

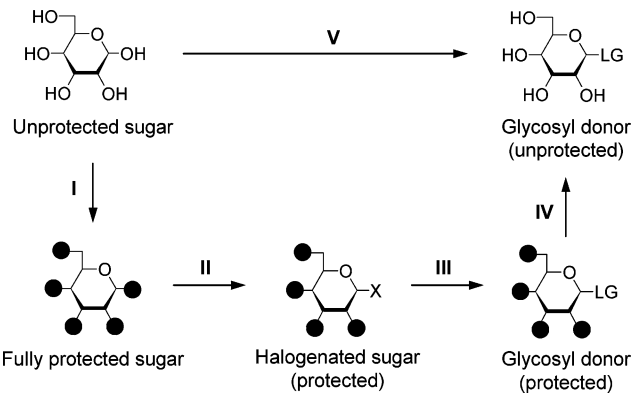
Introduction

The role of enzyme catalysts in stereo-selective synthesis of oligosaccharides has become increasingly important in recent years.[1] In particular, much attention has been paid to glycosidase-catalysed glycosylations[2] because glycosidases satisfy strong requirements as catalysts for industrial applications. Most glycosidases are considerably stable and easily available. In addition, the substrates for glycosidases are normally inexpensive.

In principle, glycosidase-catalysed transglycosylations utilise activated glycosyl donors such as *p*-nitrophenyl glycosides,[3] glycosyl fluorides,[4] or sugar oxazolines,[5] the use of which greatly contribute to the increase of transglycosylation yields. However, the preparation of these glycosyl donors necessitates laborious tasks including the protection of all of the hydroxy groups, the regio-selective introduction of a bromine or chlorine into the anomeric center under acidic conditions, the nucleophilic substitution of these halogen atoms by, for example, *p*-nitrophenol or fluorine atom, and the removal of the protecting groups (Scheme 1, Route I→II→III→IV).

Furthermore, in the case of employing oligosaccharides with higher molecular weights or acid-labile functional groups as starting materials, the cleavage of the inner glycosidic bonds by an acid are often observed in the course of the introduction of a leaving group at the anomeric center. Therefore, development of a new class of glycosyl donors that can be prepared directly from the corresponding unprotected sugars under mild reaction conditions has been necessary in order to establish a practical chemo-enzymatic glycosylating process (Scheme 1, Route V).

Recently, several attempts for direct anomeric activation of unprotected sugars have been made in polar organic solvents such as *N*,*N*-dimethylformamide.[6] For example, *p*-nitrophenyl glycosides have successfully been synthesized by using Mitsunobu coupling between unprotected monosaccharides and *p*-nitrophenol without using any protecting groups.[6c] However, it is extremely difficult to apply this method to oligosaccharides with higher molecular weights due to their poorer solubilities in organic solvents.



Scheme 1 Synthetic routes of glycosyl donors for enzymatic glycosylation reactions. Lower: Conventional route that consists of (I) protection of hydroxy group (● = protecting group), (II) halogenation at the anomeric position (X = Cl or Br), (III) introduction of a leaving group to the anomeric position (LG = leaving group), and (IV) deprotection. Upper: One-step route without using protecting groups (V).

In this paper, we wish to report that 4,6-dialkoxy-1,3,5-triazin-2-yl β-lactosides (DAT-β-Lac, **2a–d**) can be directly prepared starting from lactose in water without protecting the hydroxy groups, and can be recognized by a cellulase catalyst (*endo*-β1,4-glucanase).[7] Based on these findings, a facile chemo-enzymatic route for lactosylation *via* the one-step preparable DAT-type glycosyl donor has successfully been demonstrated.

Results and discussion

Design and formation of 4,6-dialkoxy-1,3,5-triazine-type β-lactosides

In general, a monosaccharide unit possesses three kinds of hydroxy groups, the primary hydroxy group, the secondary hydroxy groups, and the hemiacetal. The hemiacetal is more acidic than other hydroxy groups, and can be easily deprotonated to give the anomeric oxyanion. These facts make it possible for the hemiacetal to increase its nucleophilicity towards an electrophile.

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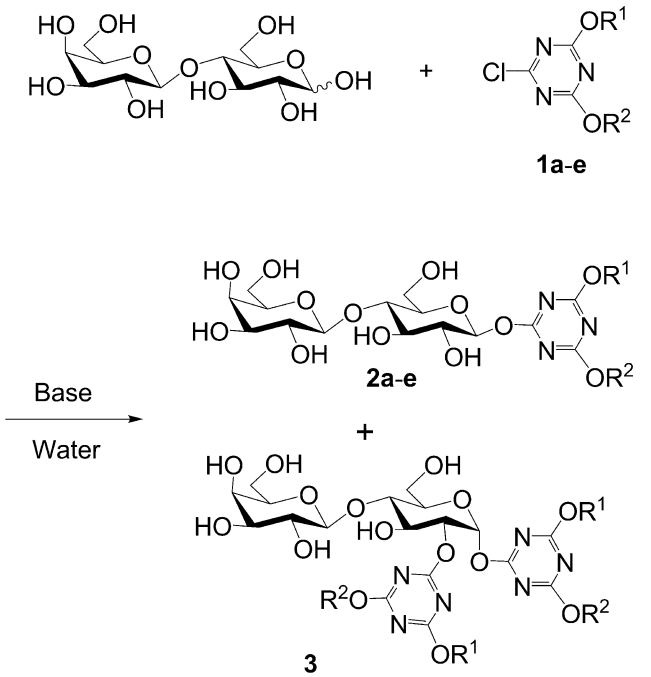
Consequently, a preferential attack of the hemiacetal over other hydroxy groups would be achieved. On the other hand, the use of water as reaction media would make the nucleophilic attack of the primary and secondary hydroxy groups disadvantageous as a result of competitive nucleophilic attack of hydroxy anions to an electrophile. It is, therefore, extremely important to carry out the reaction in an aqueous solution in order to restrict the nucleophilic attack of the primary and secondary hydroxy groups in the saccharide moiety. These hypotheses are partly supported by the fact that the pK_a value of water (15.7) is located between those of the hemiacetal (12.2)[8] and the primary and secondary hydroxy groups (*ca*. 16). It was, therefore, postulated that a chemo-selective nucleophilic attack with the hemiacetal towards an electrophilic reagent would be possible in water without any protection of the primary and secondary hydroxy groups, giving rise to a glycosyl adduct that can be used as a glycosyl donor for glycosidase-catalysed transglycosylation.[9]

We chose 2-chloro-4,6-dialkoxy-1,3,5-triazine derivatives as appropriate candidates for the electrophiles due to the following reasons: 1) 4,6-Dialkoxy triazine derivatives are considerably soluble in water and facilitate the nucleophilic substitution reaction. 2) The 4,6-dialkoxy triazine moiety can be constructed by the reaction of cyanuric chloride and the corresponding alcohols both of which are easily available and inexpensive, and can be modulated by changing alcohols.[10] 3) The resulting glycosyl adducts, 4,6-dialkoxy-1,3,5-triazin-2-yl glycosides (DAT-glycosides), possess a partial structure of O–C═N that could be activated by protonation by an acidic amino acid in the catalytic site of glycosidases, resulting in the formation of a glycosyl-enzyme intermediate.

In the presence of a base, lactose was treated with five kinds of 2-chloro-4,6-dialkoxy-1,3,5-triazines (DAT-chlorides) (**1a–e**) which had been prepared *via* the nucleophilic substitution reaction of cyanuric chloride with the corresponding alcohols (R^1OH and/or R^2OH) (Scheme 2). In case of using 2-chloro-4,6-dimethoxy derivative (**1a**: $R^1 = R^2 = CH_3$), the substitution reaction by the hemiacetal of lactose took place smoothly in the presence of *N*-methylmorpholine (NMM),[11] giving rise to the corresponding 4,6-dimethoxy-1,3,5-triazin-2-yl lactoside **2a** in 82% yield (Table 1, entry 1). When other 2-chlorotriazine derivatives, **1b** ($R^1 = CH_3$, $R^2 = CH_2CH_3$), **1c** ($R^1 = R^2 = CH_2CH_3$), and **1d** ($R^1 = CH_3$, $R^2 = CH_2CF_3$) were employed as an electrophile, lactose could be converted to the corresponding DAT-lactosides in moderate



Scheme 2 Preparation of DAT-β-Lac **2** in water by the reaction of lactose and DAT-chlorides **1** (**a**: $R^1 = R^2 = CH_3$, **b**: $R^1 = CH_3$, $R^2 = CH_2CH_3$, **c**: $R^1 = R^2 = CH_2CH_3$, **d**: $R^1 = CH_3$, $R^2 = CH_2CF_3$, **e**: $R^1 = R^2 = CH_2CF_3$) accompanied by the formation of disubstituted compounds **3a–e**.

yields (Table 1, entries 2, 3, and 4). The 2-chlorotriazine derivative having two trifluoroethoxy groups **1e** gave no glycosyl adduct due to its poorer solubility in water (Table 1, entry 5).

It was assumed that the reactions proceeded by the initial attack of *N*-methylmorpholine to the 2-position of a DAT-chloride, affording a 4-(4,6-dialkoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DAT-MM) *in situ*. Then, the hemiacetal of the lactose attacked the 2-position of the resulting DAT-MM. These explanations were partially supported by the fact that 4,6-dimethoxy-1,3,5-triazin-2-yl lactoside (DMT-Lac) could be obtained when commercially available 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM)[12] was used as electrophile instead of DMT-chloride (Table 2). It has also been found that the reaction with DMT-MM requires a general base to enhance the nucleophilicity of the hemiacetal. Various

Table 1 Synthesis of DAT-β-Lac **2** by the reaction of lactose and DAT-chlorides **1**[a]

Entry	DAT-chloride	DAT-β-Lac	Yield (%)
1	**1a**	**2a**	82[b] (40)[c]
2	**1b**	**2b**	42[c]
3	**1c**	**2c**	45[c]
4	**1d**	**2d**	53[d]
5	**1e**	**2e**	ND[e]

[a] The reactions were carried out in water starting from β-enriched lactose (β/α = 74/26) for 24 h at room temperature in the presence of 2 equivalent of DAT-chloride and 2 equivalent of *N*-methylmorpholine (NMM). [b] Determined by ^{1}H NMR by comparing the integrals of the anomeric proton of the product and that of lactose in D_2O. [c] Isolated yield after crystallization from methanol. [d] Isolated yield after silica gel column chromatography. [e] No product was detected by ^{1}H NMR in D_2O.

Table 2 Synthesis of DMT-β-Lac **2a** by using DMT-MM in the presence of base[a]

Entry	Lactose (β/α)	DMT-MM (equiv.)	Base (equiv.)	Yield (%)
1	74/26	2	2,6-lutidine (0.5)	71[b]
2	74/26	2	2,6-lutidine (1.0)	73[b]
3	74/26	2	2,6-lutidine (2.0)	70[b]
4	10/90	2	2,6-lutidine (2.0)	40[c]
5	74/26	2	NMM (1.0)	72[c]
6	74/26	2	$NaHCO_3$ (1.0)	67[c]
7	74/26	2	$(i\text{-}Pr)_2NEt$ (1.0)	66[c]
8	74/26	2	Et_3N (1.0)	63[c]
9	74/26	2	pyridine (1.0)	62[c]

[a] The reactions were carried out in water for 18-24 h at r.t. [b] Isolated yield. [c] Determined by ^{1}H NMR by comparing the integrals of the anomeric proton of the product and that of lactose in D_2O.

organic bases as well as sodium bicarbonate as an inorganic base can be used to give to 4,6-dimethoxy-1,3,5-triazin-2-yl β-lactoside (DMT-β-Lac) **2a** by the reaction of lactose and DMT-MM.

In this reaction, the yield of the resulting DAT-β-glycosides **2** was strongly influenced by the anomeric ratio (β/α) of starting lactose. When an α-anomerically-enriched lactose (β/α = 10/90) was reacted with DMT-MM in the presence of 2,6-lutidine, the yield decreased to 40% (Table 2, entry 4). In addition to the formation of DAT-β-Lac **2**, a disubstituted product **3** having triazine rings at the 1 and 2 positions of lactose was produced as by-product in 55% yield. The ^{1}H NMR of the product showed a doublet signal at 6.7 ppm with a coupling constant of 3.5 Hz assigned to the anomeric proton. These results clearly indicate that the stereochemistry of the anomeric center of the by-product is α-type. Although at the early stage of this reaction, the formation of a monosubstituted α-type product was observed by ^{1}H NMR spectroscopy, this compound could not be isolated because it was converted to the disubstituted product immediately. The nucleophilicity of the hydroxy group at the 2 position of the monosubstituted product may be intramolecularly enhanced by the action of the nitrogen atom in the triazine ring, resulting in the formation of the disubstituted product.

It is, therefore, necessary to utilize a β-anomerically-enriched lactose (β/α = 74/26) as a starting material in order to increase the yield. These results clearly indicate that both of the reaction rates of the aromatic nucleophilic attack of the α-anomer and β-anomer are considerably large and presumably comparable with that of the anomeric interconversion between two anomers in water.

The ^{1}H NMR spectrum of DMT-β-Lac **2a** showed a singlet peak at 3.91 ppm (6H) due to the 4,6-dimethoxy groups on the triazine ring. A doublet peak at 5.81 ppm with the coupling constant of 8.1 Hz is ascribable to the anomeric proton of the reducing end of the lactoside. These results clearly indicate that the 4,6-dimethoxy triazine moiety is connected to the anomeric carbon of lactose with a β-configuration.

EGIII-catalysed hydrolysis and transglycosylation using DAT-β-lactosides as glycosyl substrates

All of the DAT-β-lactosides (DAT-β-Lac) **2a–d** were found to be smoothly hydrolyzed by the action of *endo*-β1,4-glucanase III from *Trichoderma reesei* (EGIII), affording lactose and 2-hydroxy-4,6-dialkoxy-1,3,5-triazine (4,6-dialkoxy-1,3,5-triazine-2-one) in acetate buffer (pH 5.5). The enzymatic hydrolysis was monitored by quantitating the amount of DAT-β-Lac by HPLC (UV at 214 nm). The Michaelis constants (K_m) and the first-order rate constants (k_{cat}) for each substrate have been determined (Table 3).

The detailed mechanism for EGIII-catalysed hydrolysis of DAT-β-Lac has not been made clear. It is assumed that the $^4C_1$ conformation of the pyranose ring, located in the -1 subsite changes to a half boat conformation in order that one of the lone pairs on the ring oxygen could be in an antiperiplanar position to the glycosidic bond connecting the anomeric carbon and the DAT moiety. Then, the nitrogen atom (1 or 3 position) in the triazine ring or the glycosidic oxygen are protonated by an acidic amino acid in the catalytic site, and the DAT moiety was liberated from the anomeric center, affording an oxocarbenium ion intermediate or a covalent α-glycosyl-enzyme intermediate. The resulting intermediate is then attacked by water at the anomeric position to give the hydrolysate, lactose.

Table 3 Michaelis–Menten kinetics for hydrolysis DAT-β-Lac **2** catalysed by EGIII

Entry	Substrate	K_m/mM	k_{cat}/s^{-1}
1	**2a**	12 ± 1	5.4 ± 0.2
2	**2b**	13 ± 3	5.6 ± 0.2
3	**2c**	15 ± 3	4.2 ± 0.4
4	**2d**	13 ± 1	12 ± 1.6

Based on these results obtained in the hydrolysis experiments, we tried EGIII-catalysed transglycosylation reactions using DAT-β-Lac **2** as glycosyl donors. Firstly, we investigated the influence of pH on the transglycosylation yields by using DMT-β-Lac **2a** as a glycosyl donor and phenyl l-thio-α-cellobioside **4** as a glycosyl acceptor (Fig. 1).[13] The yield of lactosylated product reached a maximum when the reaction was performed in pH 5.5. These results were in agreement with the previous report that the optimum pH for the hydrolysis of nitrophenyl-β-cellobioside catalysed by EGIII is 5.5.[14]

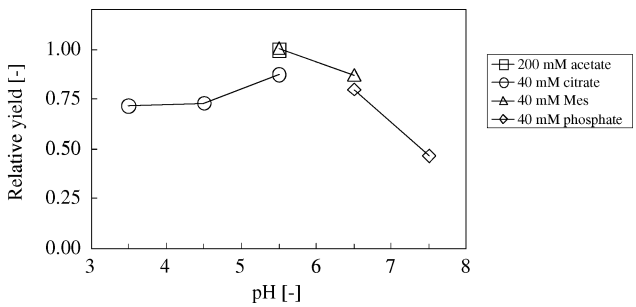
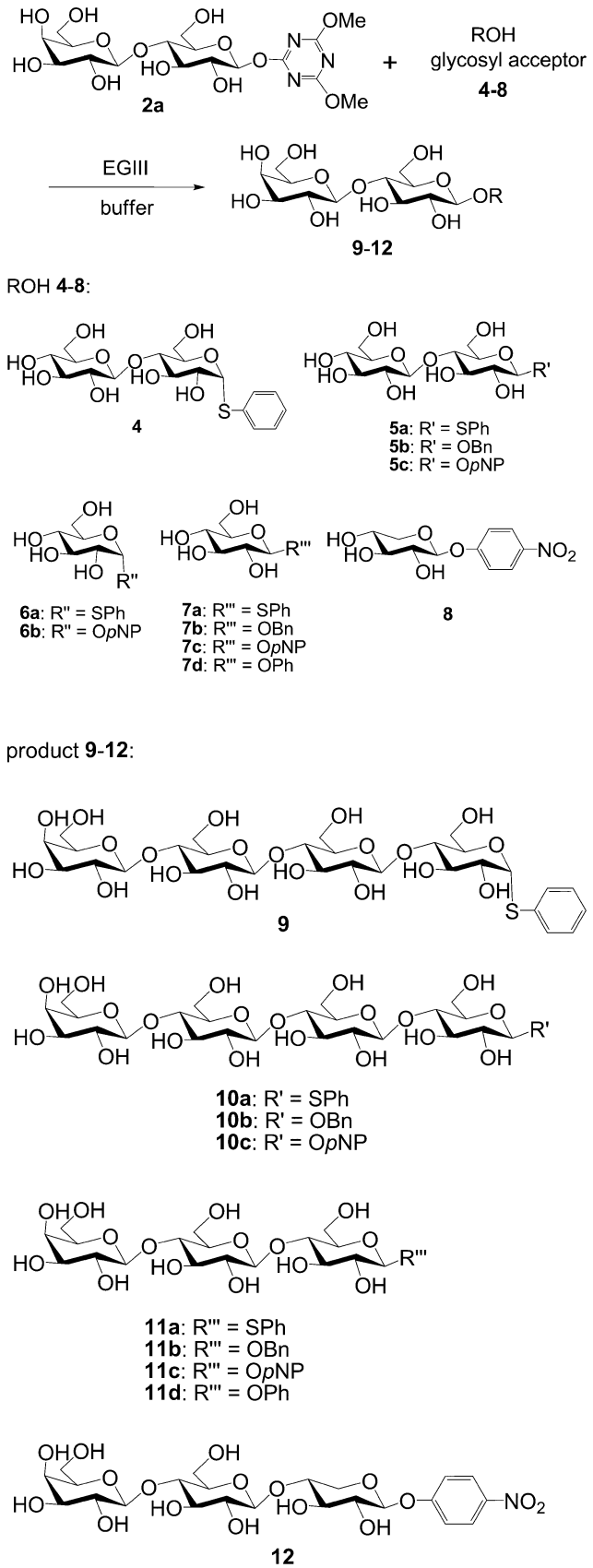


Fig. 1 pH-Dependence of EGIII-catalysed lactosylation reaction for 3 h at 30 °C using DMT-β-Lac **2a** as a glycosyl donor and **4** as a glycosy acceptor. (□: 200 mM acetate, ○: 40 mM citrate, △: 40 mM Mes, ◇: 40 mM phosphate).

Various glycosyl acceptors **4–8** have been enzymatically transglycosylated, affording the corresponding tetrasaccharides or trisaccharides (Scheme 3 and Table 4). In the absence of the enzyme catalyst, no transglycosylated products could be observed by HPLC analysis, showing that the lactosylated products are enzymatically transglycosylated.

The ^{13}C NMR spectra of the resulting oligosaccharides show signals at around 102 and 78 ppm due to the anomeric and C4 carbon atoms of the newly formed glycosidic bonds, clearly indicating that β1,4 glycosidic linkages were stereoselectively constructed between the glycosyl donor and glycosyl acceptors. No signals derived from a β1,3 or β1,6 glycosidic bond were detected.

When the enzymatic reaction was carried out using DMT-β-Lac **2a** as a glycosyl donor under the condition of donor : acceptor ratio 1 : 1, the yield of tetrasaccharide **9** was 66% (Table 4, entry 1). The other sugar triazine derivatives **2b–d** having an electron-releasing ethoxy group or electron-withdrawing trifluoroethoxy group on the triazine ring were also able to be used as glycosyl donors (Table 4, entries 2–4). In the condition of donor : acceptor ratio 1 : 0.4, the yield of **9** significantly increased to 95% on the basis of acceptor (Table 4, entry 6). Interestingly, the lactosylating yield of phenyl l-thio-α-cellobioside **4** was much higher than that of phenyl 1-thio-β-cellobioside **5a** (Table 4, entries 6 and 7).



Scheme 3 EGIII-catalysed lactosylation of glycosyl acceptors **4–8** by using DMT-β-Lac **2a** as a glycosyl donor to give lactosylated products **9–12**.

Table 4 EGIII-catalysed lactosylation using DAT-β-Lac **2** as glycosyl donors[a]

Entry	Donor/Acceptor (mol/mol)	Time/h	Product	Yield (%)[b]
1	**2a/4** (1/1)	3	**9**	66
2	**2b/4** (1/1)	2	**9**	48
3	**2c/4** (1/1)	2	**9**	51
4	**2d/4** (1/1)	2	**9**	53
5	**2a/4** (1/0.8)	3	**9**	76
6	**2a/4** (1/0.4)	1	**9**	95
7	**2a/5a** (1/0.4)	10	**10a**	33
8	**2a/5b** (1/0.4)	0.25	**10b**	3
9	**2a/5c** (1/0.4)	0.25	**10c**	trace
10	**2a/6a** (1/0.4)	—	—	ND[c]
11	**2a/6b** (1/0.4)	—	—	ND[c]
12	**2a/7a** (1/0.4)	3	**11a**	21
13	**2a/7b** (1/0.4)	3	**11b**	47
14	**2a/7c** (1/0.4)	1	**11c**	45
15	**2a/7d** (1/0.4)	1	**11d**	39
16	**2a/8** (1/0.4)	3	**12**	16

[a] The reactions were carried out in 200 mM acetate buffer (pH 5.5) at 30 °C. [b] Determined by HPLC based on acceptor. [c] No product was detected.

We have observed that the rate of disappearance for the glycosyl donor **2a** in the presence of **5a** as glycosyl acceptor is much slower than the case of using **4** as glycosyl acceptor. These results may be explained by assuming a competitive inhibition between **5a** and **2a** towards the donor site of the EGIII enzyme, because the structure of these two compounds having a β-glycosidic linkage at their reducing ends is very similar. In the case of the transglycosylation by using **4** as glycosyl acceptor, the enzyme may not be inhibited because the affinity of **4** towards the donor site of EGIII is considered to be much lower due to the steric hindrance of the axial SPh group of **4**.

The present EGIII-catalysed lactosylation reaction was found to be applied to monosaccharide acceptors having a β-glycosidic bond including phenyl β-glucoside derivatives as well as a xyloside (Table 4, entries 12–16). No product was obtained when α-glucosyl acceptors **6a** and **6b** were used, because of the steric hindrance of the axial group in the +1 subsite of EGIII enzyme (Table 4, entries 10 and 11).

Conclusions

Novel glycosidic compounds, 4,6-dialkoxy-1,3,5-triazin-2-yl β-lactosides (DAT-β-Lac), can be directly prepared from lactose in water by using 4,6-dialkoxy-1,3,5-triazine-type agents in aqueous media. The resulting one-step preparable triazine-type β-lactosides could be used as efficient glycosyl donors for EGIII-catalysed transglycosylation reactions, affording the corresponding lactosylated products. Compared with the conventional chemo-enzymatic process, the present methodology requires fewer tasks for preparation of glycosyl donors. In addition, the fact that the DAT-derivatives can be prepared in aqueous media without using any protecting groups enables us to develop a one-pot procedure without isolating the DAT intermediate.[7] The present chemo-enzymatic process would pave a way which leads to an efficient and practical synthesis of oligo- and poly-saccharides in the synthetic field of glycochemistry.

Experimental

General

All chemical agents and solvents were commercially available and purchased. EGIII was expressed and purified by the previous report.[15] The specific activity of the EGIII was 13 U/mg. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of 2-hydroxy-4,6-dialkoxy-1,3,5-triazine per min. ^{1}H and ^{13}C, NMR spectra were recorded on Bruker DRX-500 at room temperature. Where applicable, assignments were based on the combination of several 2D NMR techniques (H–H COSY, HMQC, HMBC). ESI MS spectra were recorded on Bruker Daltonics APEXIII. MALDI-TOF MS spectra were recorded on Shimadzu AXIMA-CFR *plus* using 2,5-dihydroxybenzonic acid as a matrix. Specific rotations were measured on Jasco DIP-1000 polarimeter using a 10 cm cell.

Chemicals

2,4-Dichloro-6-methoxy-1,3,5-triazine. Cyanuric chloride (13.0 g, 70 mmol) was added to a solution of sodium hydrogen carbonate (6.5 g, 77 mmol) in methanol (480 ml) at 0 °C and the resulting mixture was stirred for 2 h at room temperature. After a solid was removed by filtration, the filtrate was concentrated *in vacuo* to give 2,4-dichloro-6-methoxy-1,3,5-triazine (10.0 g, 63 mmol, 82%).

δ_H (500 MHz, CD_3OD) 4.10 (3H, s, OCH_3); δ_C (126 MHz, CD_3OD) 172.0 (2C, N–C–Cl), 171.0 (1C, N–C–O), 56.2 (1C, OCH_3).

2-Chloro-4,6-dimethoxy-1,3,5-triazine (1a). Cyanuric chloride (228.7 g, 1.24 mol) was added to a solution of sodium hydrogen carbonate (260.0 g, 3.0 mol) in methanol (2000 ml) at 0 °C and the resulting mixture was stirred for 2.5 h at room temperature and subsequently 2 h at 55 °C. After removal of a solid by filtration, the filtrate was concentrated *in vacuo*. The residue was crystallized from methanol to give 2-chloro-4,6-dimethoxy-1,3,5-triazine (123.6 g, 0.70 mol, 57%).

δ_H (500 MHz, CD_3OD) 4.08 (6H, s, OCH_3); δ_C (126 MHz, CD_3OD) 172.6 (2C, N–C–O), 172.1 (1C, N–C–Cl), 55.4 (2C, OCH_3).

2-Chloro-4-ethoxy-6-methoxy-1,3,5-triazine (1b). 2,4-Dichloro-6-methoxy-1,3,5-triazine (0.95 g, 5.3 mmol) was added to a solution of sodium carbonate (0.56 g, 5.3 mmol) in ethanol (8 ml) at 0 °C and the resulting mixture was stirred for 10 h at 60 °C. After a solid was removed by filtration, the filtrate was concentrated *in vacuo*. The residue was diluted with AcOEt, washed with water, dried over Na_2SO_4, and concentrated *in vacuo* to give 2-chloro-4-ethoxy-6-methoxy-1,3,5-triazine (0.75 g, 4.0 mmol, 75%).

δ_H (500 MHz, CD_3OD) 4.49 (2H, q, OCH_2), 4.05 (3H, s, OCH_3), 1.40 (3H, t, CH_3); δ_C (126 MHz, CD_3OD) 172.6 and 172.1 (2C, N–C–O), 172.0 (1C, N–C–Cl), 65.2 (1C, OCH_2), 55.3 (1C, OCH_3), 13.0 (1C, CH_3).

2-Chloro-4,6-diethoxy-1,3,5-triazine (1c). Cyanuric chloride (1.9 g, 10 mmol) was added to a solution of sodium hydrogen carbonate (1.9 g, 22 mmol) in ethanol (10 ml) at 0 °C and the resulting mixture was stirred for 1 h at room temperature, and subsequently for 10 h at 60 °C. After a solid was removed by filtration, the filtrate was concentrated *in vacuo*. The residue was diluted with AcOEt, washed with water, dried over Na_2SO_4, and concentrated *in vacuo* to give 2-chloro-4,6-diethoxy-1,3,5-triazine (1.7 g, 8.3 mmol, 83%).

δ_H (500 MHz, CD_3OD) 4.52 (4H, q, OCH_2), 1.45 (6H, t, CH_3); δ_C (126 MHz, CD_3OD) 172.6 (1C, N–C–Cl), 172.0 (2C, N–C–O), 65.4 (2C, OCH_2), 14.2 (2C, CH_3).

2-Chloro-4-(2′,2′,2′-trifluoroethoxy)-6-methoxy-1,3,5-triazine (1d). To a solution of 2,4-dichloro-6-methoxy-1,3,5-triazine (2.2 g, 12 mmol) in acetone (60 ml) was added potassium carbonate (2.2 g, 16 mmol) and 2,2,2-trifluoroethanol (1.1 ml, 16 mmol), and the resulting mixture was stirred for 5 h at room temperature. After a solid was removed by filtration, the filtrate was concentrated *in vacuo*. The residue was purified by silicagel column chromatography (hexane–AcOEt = 19/1) to give 2-chloro-4-(2′,2′,2′-trifluoroethoxy)-6-methoxy-1,3,5-triazine (2.1 g, 8.6 mmol, 72%).

δ_H (500 MHz, CD_3OD) 4.85 (2H, q, OCH_2), 4.11 (3H, s, OCH_3); δ_C (126 MHz, CD_3OD) 173.0 (1C, N–C–O), 172.0 (1C, N–C–Cl), 171.0 (1C, N–C–O), 122.0 (1C, q, CF_3), 64.2 (1C, OCH_2), 56.0 (1C, OCH_3).

2-Chloro-4,6-bis(2′,2′,2′-trifluoroethoxy)-1,3,5-triazine (1e). To a solution of cyanuric chloride (0.46 g, 2.5 mmol) in acetone (20 ml) was added potassium carbonate (1.4 g, 10 mmol) and 2,2,2-trifluoroethanol (0.54 ml, 7.5 mmol), the mixture was stirred for 4 h at room temperature. After a solid was removed by filtration, the filtrate was concentrated *in vacuo* to give 2-chloro-4,6-bis(2′,2′,2′-trifluoroethoxy)-1,3,5-triazine (0.68 g, 2.2 mmol, 88%).

δ_H (500 MHz, CD_3OD) 4.95 (4H, q, OCH_2); δ_C (126 MHz, CD_3OD) 173.0 (2C, N–C–O), 171.0 (1C, N–C–Cl), 122.0 (2C, q, CF_3), 64.0 (2C, OCH_2).

4,6-Dimethoxy-1,3,5-triazin-2-yl β-D-lactoside (DMT-β-Lac, 2a). [Method A] (Table 1, entry 1) 2-Chloro-4,6-dimethoxy-1,3,5-triazine (**1a**, 527 mg, 3.0 mmol) was added to a solution of β-D-lactose (513 mg, 1.5 mmol, containing 26% α-lactose) and *N*-methylmorpholine (0.33 ml, 3.0 mmol) in water (15 ml), and the resulting mixture was stirred for 24 h at room temperature. After concentration *in vacuo*, the residue was crystallized from methanol to give 4,6-dimethoxy-1,3,5-triazin-2-yl β-D-lactoside (289 mg, 1.2 mmol, 40%).

[Method B] (Table 2, entry 2) 2,6-Lutidine (23 μl, 0.20 mmol) was added to a solution of β-D-lactose (68.5 mg, 0.20 mmol, containing 26% α-lactose) and 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (110.7 mg, 0.40 mmol) in water (1.0 ml), and the resulting mixture was stirred for 18 h at room temperature. After concentrating the solution *in vacuo*, the residue was crystallized from ethanol to give 4,6-dimethoxy-1,3,5-triazin-2-yl β-D-lactoside (70.0 mg, 0.145 mmol, 73%).

δ_H (500 MHz, D_2O) 5.81 (1H, d, H-1, $J_{1,2}$ = 8.1 Hz), 4.36 (1H, d, H-1′, $J_{1',2'}$ = 8.6 Hz), 3.91 (6H, s, OCH_3), 3.86–3.81 (2H, m, H-6^a, H-4′), 3.74–3.61 (7H, m, H-3, H-4, H-5, H-6^b, H-5′, H-6′^{a}, H-6′^{b}), 3.60–3.50 (2H, m, H-2, H-3′), 3.45 (1H, t, H-2′, $J_{1',2'}$, $J_{2',3'}$ = 8.4 Hz); δ_C (126 MHz, D_2O) 173.3 and 171.9 (3C, triazine), 102.9 (1C, C-1′), 96.8 (1C, C-1), 77.5 (1C, C-4), 75.5 (1C, C-5), 75.3 (1C, C-5′), 74.0 (1C, C-3), 72.5 (1C, C-3′), 71.8 (1C, C-2), 70.9 (1C, C-2′), 68.5

(1C, C-4′), 61.0 (1C, C-6′), 59.6 (1C, C-6), 55.9 (2C, OCH_3); *m/z* (ESI) 504.1434 (M+Na^+. $C_{17}H_{27}N_3O_{13}$ requires 504.1436); $[\alpha]_D^{23}$ –20.1 (*c* 1.0 in H_2O); Decomposed at 163 °C without melting.

4,6-Dimethoxy-1,3,5-triazin-2-yl 2-*O*-(4,6-dimethoxy-1,3,5-triazin-2-yl)-α-D-lactoside (3a). 2,6-Lutidine (46 μl, 0.40 mmol) was added to a solution of D-lactose monohydrate (72.1 mg, 0.20 mmol, β/α = 10/90) and 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (110.7 mg, 0.40 mmol) in water (1.0 ml), and the resulting mixture was stirred for 13 h at room temperature. After concentrating the solution, the residue was purified by silicagel column chromatography ($CHCl_3$–MeOH = 5/1) to give 4,6-dimethoxy-1,3,5-triazin-2-yl 2-*O*-(4,6-dimethoxy-1,3,5-triazin-2-yl)-α-D-lactoside (67.7 mg, 0.109 mmol, 55%).

δ_H (500 MHz, D_2O) 6.73 (1H, d, H-1, $J_{1,2}$ = 3.5 Hz), 5.25 (1H, dd, H-2, $J_{1,2}$ = 3.6 Hz, $J_{2,3}$ = 10.1 Hz), 4.42 (1H, d, H-1′, $J_{1',2'}$ = 7.8 Hz), 4.24 (1H, t, H-3, $J_{2,3}$, $J_{3,4}$ = 9.2 Hz), 3.99 (1H, m, H-5), 3.89 (1H, m, H-4), 3.87 (6H, s, OCH_3), 3.82 (3H, m, H-6^a, H-6^b, H-4′), 3.80 (6H, s, OCH_3), 3.69–3.63 (3H, m, H-5′, H-6′^{a}, H-6′^{b}), 3.58 (1H, dd, H-3′, $J_{2',3'}$ = 10.0 Hz, $J_{3',4'}$ = 3.4 Hz), 3.49 (1H, m, H-2′, $J_{1',2'}$, $J_{2',3'}$ = 8.9 Hz); δ_C (126 MHz, D_2O) 173.1, 173.0, 172.2, and 171.3 (6C, triazine), 102.8 (1C, C-1′), 92.4 (1C, C-1), 77.0 (1C, C-4), 75.4 (1C, C-5′), 75.1 (1C, C-2), 73.3 (1C, C-5), 72.5 (1C, C-3′), 70.1 (1C, C-2′), 69.2 (1C, C-3), 68.5 (1C, C-4′), 61.0 (1C, C-6′), 59.4(1C, C-6), 55.8 and 55.7 (4C, OCH_3); *m/z* (ESI) 643.1816 (M+Na^+. $C_{22}H_{32}N_6O_{15}$ requires 643.1818).

4-Ethoxy-6-methoxy-1,3,5-triazin-2-yl β-D-lactoside (2b). 2-Chloro-4-ethoxy-6-methoxy-1,3,5-triazine (**1b**, 569 mg, 3.0 mmol) was added to a solution of β-D-lactose (513 mg, 1.5 mmol, containing 26% α-lactose) and *N*-methylmorpholine (0.33 ml, 3.0 mmol) in water (15 ml), and the resulting mixture was stirred for 24 h at room temperature. After concentrating the solution *in vacuo*, the residue was crystallized and washed from methanol and dried *in vacuo* to give 4-ethoxy-6-methoxy-1,3,5-triazin-2-yl β-D-lactoside (312 mg, 0.63 mmol, 42%).

δ_H (500 MHz, D_2O) 5.80 (1H, d, H-1, $J_{1,2}$ = 8.1 Hz), 4.36 (1H, d, H-1′, $J_{1',2'}$ = 7.9 Hz), 4.37 (2H, m, OCH_2), 3.91 (3H, s, OCH_3), 3.82–3.80 (2H, m, H-6^a, H-4′), 3.74–3.60 (7H, m, H-3, H-4, H-5, H-6^b, H-5′, H-6′^{a}, H-6′^{b}), 3.59–3.54 (2H, m, H-2, H-3′), 3.46 (1H, t, H-2′, $J_{1',2'}$, $J_{2',3'}$ = 8.4 Hz), 1.27 (3H, t, OCH_2CH_3); δ_C (126 MHz, D_2O) 173.3, 172.7, and 171.9 (3C, triazine), 102.9 (1C, C-1′), 96.8 (1C, C-1), 78.3 (1C, C-4), 75.5 (1C, C-5), 75.4 (1C, C-5′), 74.1 (1C, C-3), 72.5 (1C, C-3′), 71.8 (1C, C-2), 70.9 (1C, C-2′), 68.5 (1C, C-4′), 65.6 (1C, OCH_2), 61.0 (1C, C-6′), 59.7 (1C, C-6), 55.8 (1C, OCH_3), 13.3 (1C, OCH_2CH_3); *m/z* (ESI) 518.1594 (M+Na^+. $C_{18}H_{29}N_3O_{13}$ requires 518.1593); $[\alpha]_D^{23}$ –4.9 (*c* 0.5 in H_2O); Decomposed at 148 °C without melting.

4,6-Diethoxy-1,3,5-triazin-2-yl β-D-lactoside (2c). 2-Chloro-4,6-diethoxy-1,3,5-triazine (**1c**, 611 mg, 3.0 mmol) was added to a solution of β-D-lactose (513 mg, 1.5 mmol, containing 26% α-lactose) and *N*-methylmorpholine (0.33 ml, 3.0 mmol) in water (15 ml), and the resulting mixture was stirred for 24 h at room temperature. After concentrating the solution *in vacuo*, the residue was crystallized from methanol to give 4,6-diethoxy-1,3,5-triazin-2-yl β-D-lactoside (344 mg, 0.68 mmol, 45%).

δ_H (500 MHz, D_2O) 5.77 (1H, d, H-1, $J_{1,2}$ = 8.1 Hz), 4.36 (1H, d, H-1′, $J_{1',2'}$ = 7.9 Hz), 4.36 (4H, m, OCH_2), 3.83–3.78 (2H, m, H-6^a, H-4′), 3.74–3.60 (7H, m, H-3, H-4, H-5, H-6^b, H-5′, H-6′^{a}, H-6′^{b}), 3.59–3.53 (2H, m, H-2, H-3′), 3.46 (1H, t, H-2′, $J_{1',2'}$, $J_{2',3'}$ = 8.4 Hz), 1.26 (6H, t, CH_3); δ_C (126 MHz, D_2O) 172.7 and 171.8 (3C, triazine), 102.9 (1C, C-1′), 96.7 (1C, C-1), 77.5 (1C, C-4), 75.5 (1C, C-5), 75.3 (1C, C-5′), 74.0 (1C, C-3), 72.4 (1C, C-3′), 71.7 (1C, C-2), 70.9 (1C, C-2′), 68.5 (1C, C-4′), 65.5 (2C, OCH_2), 61.0 (1C, C-6′), 59.6 (1C, C-6), 13.3 (2C, CH_3); *m/z* (ESI) 532.1747 (M+Na^+. $C_{19}H_{31}N_3O_{13}$ requires 532.1749).

4-(2′,2′,2′-Trifluoroethoxy)-6-methoxy-1,3,5-triazin-2-yl β-D-lactoside (2d). 2-Chloro-4-(2′,2′,2′-trifluoroethoxy)-6-methoxy-1,3,5-triazine (**1d**, 240 mg, 1.0 mmol) was added to a solution of β-D-lactose (170 mg, 0.5 mmol, containing 26% α-lactose) and *N*-methylmorpholine (0.11 ml, 1.0 mmol) in water (5 ml), and the resulting mixture was stirred for 24 h at room temperature. After concentrating the solution, the residue was purified by silicagel column chromatography ($CHCl_3$–MeOH = 3/1) to give 4-(2′,2′,2′-trifluoroethoxy)-6-methoxy-1,3,5-triazin-2-yl β-D-lactoside (90 mg, 0.27 mmol, 53%).

δ_H (500 MHz, D_2O) 5.80 (1H, d, H-1, $J_{1,2}$ = 8.1 Hz), 4.86 (2H, m, OCH_2), 4.34 (1H, d, H-1′, $J_{1',2'}$ = 7.8 Hz), 3.91 (3H, s, OCH_3), 3.81–3.78 (2H, m, H-6^a, H-4′), 3.73–3.60 (7H, m, H-3, H-4, H-5, H-6^b, H-5′, H-6′^{a}, H-6′^{b}), 3.59–3.51 (2H, m, H-2, H-3′), 3.46 (1H, t, H-2′, $J_{1',2'}$, $J_{2',3'}$ = 8.4 Hz); δ_C (126 MHz, D_2O) 173.5, 172.1, and 172.0 (3C, triazine), 122.5 (1C, q, CF_3), 102.9 (1C, C-1′), 97.0 (1C, C-1), 77.5 (1C, C-4), 75.6 (1C, C-5), 75.3 (1C, C-5′), 74.0 (1C, C-3), 72.5 (1C, C-3′), 71.7 (1C, C-2), 70.9 (1C, C-2′), 68.5 (1C, C-4′), 63.8 (1C, q, OCH_2), 61.0 (1C, C-6′), 59.6 (1C, C-6), 56.1 (1C, OCH_3); *m/z* (ESI) 572.1310 (M+Na^+. $C_{18}H_{26}F_3N_3O_{13}$ requires 572.1308).

Enzymatic hydrolysis of triazine-type β-lactosides by EGIII

A mixture of triazine-type β-lactoside and EGIII in 50 mM acetate buffer was incubated at 30 °C. The reaction mixture was analyzed by HPLC (column; Inertsil ODS-3 (ϕ4.6 × 250 mm, GL-Sciences), eluent; MeCN–H_2O = 5/95, flow rate; 1.0 ml min^{-1}, column oven; 40 °C, detection; UV (214 nm)). The hydrolysis activity of EGIII for substrates **2a–d** was evaluated by measuring the amount of unreacted substrates. The kinetic parameters and their standard errors were calculated using the nonlinear regression analysis program "KaleidaGraph 4.0J".

EGIII-catalysed lactosylation reactions

A mixture of glycosyl donor (**2a–d**, 0.3 mmol, final conc.: 30 mM), glycosyl acceptor (**4–8**), and EGIII (1 μl, 0.4 μg μl^{-1}) in buffer was incubated at 30 °C. The reaction mixtures were analyzed by HPLC (column; TOSOH TSK-Gel Amide-80 (ϕ4.6 × 250 mm), eluent; 80 vol.% MeCN aq., flow rate; 1.0 ml min^{-1}, column oven; 40 °C, detection; UV (214 nm)). The lactosylated products were isolated by preparative HPLC (column; TOSOH TSK-Gel Amide-80 (ϕ21.5 × 300 mm), eluent; MeCN–H_2O = 7/3, flow rate; 7.0 ml min^{-1}, column oven; 40 °C, detection; UV (214 nm)).

Phenyl 1-thio-4-*O*-(((4-*O*-β-D-galactopyranosyl)-4-*O*-β-D-glucopyranosyl)-β-D-glucopyranosyl)-α-D-glucopyranoside (9). δ_H (500 MHz, D_2O) 7.47 and 7.29 (5H, Ph), 5.52 (1H, d, H-1, $J_{1,2}$ = 5.4 Hz), 4.44 and 4.43 (2H, d, H-1′, H-1″, $J_{1',2'}$, $J_{1'',2''}$ = 8.0 Hz), 4.33 (1H, d, H-1‴, $J_{1''',2'''}$ = 7.8 Hz), 4.22 (1H, H-5), 3.90–3.50 (20H, m, sugar-H), 3.43 (1H, t, H-2‴, $J_{1''',2'''}$, $J_{2''',3'''}$ = 9.0 Hz), 3.27–3.23 (2H, m, H-2′, H-2″); δ_C (126 MHz, D_2O)

132.8, 132.3, 129.4, and 128.3 (6C, Ph), 102.9 (1C, C-1‴), 102.3 (2C, C-1′, C-1″), 88.9 (1C, C-1), 78.6 (1C, C-4), 78.2 and 78.0 (2C, C-4′, C-4″), 75.3 (1C, C-5‴), 74.8 (2C, C-5′, C-5″), 74.1 and 74.0 (2C, C-3′, C-3″), 72.9 and 72.8 (2C, C-2′, C-2″), 72.5 (1C, C-3‴), 72.1 (1C, C-3), 71.4 (1C, C-5), 70.9 (2C, C-2, C-2‴), 68.5 (1C, C-4‴), 61.0 (1C, C-6‴), 59.9, 59.8, and 59.7 (3C, C-6, C-6′, C-6″); *m/z* (MALDI-TOF) 782.1 (M+Na$^+$. $C_{30}H_{46}O_{20}S$ requires 781.8).

Phenyl 1-thio-4-*O*-(((4-*O*-β-D-galactopyranosyl)-4-*O*-β-D-glucopyranosyl)-β-D-glucopyranosyl)-β-D-glucopyranoside (10a). δ_H (500 MHz, D_2O) 7.47 and 7.29 (5H, Ph), 4.7 (1H, H-1, included in DOH), 4.41 (2H, d, H-1′, H-1″, $J_{1',2'}$, $J_{1'',2''}$ = 7.8 Hz), 4.33 (1H, d, H-1‴, $J_{1''',2'''}$ = 7.4 Hz), 3.90–3.45 (20H, m, sugar-H), 3.42 (1H, t, H-2‴, $J_{1''',2'''}$, $J_{2''',3'''}$ = 8.5 Hz), 3.30–3.20 (3H, m, H-2, H-2′, H-2″); δ_C (126 MHz, D_2O) 132.8, 132.3, 129.4, and 128.3 (6C, Ph), 102.9 (1C, C-1‴), 102.2 (2C, C-1′, C-1″), 87.1 (1C, C-1), 78.7 (1C, C-4), 78.0 (2C, C-4′, C-4″), 75.6, 75.3, 74.8, 74.0, 73.9, 72.9, 72.8, 72.3, 71.4, and 70.9 (12C, sugar-C), 68.5 (1C, C-4‴), 61.0 (1C, C-6‴), 60.0 (3C, C-6, C-6′, C-6″); *m/z* (MALDI-TOF) 782.1 (M+Na$^+$. $C_{30}H_{46}O_{20}S$ requires 781.8).

Phenyl 1-thio-4-*O*-((4-*O*-β-D-galactopyranosyl)-β-D-glucopyranosyl)-β-D-glucopyranoside (11a). δ_H (500 MHz, D_2O) 7.47 and 7.29 (5H, Ph), 4.7 (1H, H-1, included in DOH), 4.41 (1H, d, H-1′, $J_{1',2'}$ = 8.0 Hz), 4.32 (1H, d, H-1″, $J_{1'',2''}$ = 7.8 Hz), 3.86–3.80 (3H, m, H-6^a, H-6′^{a}, H-4″), 3.71–3.47 (12H, m, sugar-H), 3.42 (1H, t, H-2″, $J_{1'',2''}$, $J_{2'',3''}$ = 9.1 Hz), 3.30–3.21 (2H, m, H-2, H-2′); δ_C (126 MHz, D_2O) 132.8, 132.3, 129.4, and 128.3 (6C, Ph), 102.9 (1C, C-1″), 102.3 (1C, C-1′), 87.1 (1C, C-1), 78.7 (1C, C-4), 78.0 (2C, C-4′, C-5″), 75.6 and 74.1 (2C, C-3′, C-3″), 75.3 and 74.8 (2C, C-5, C-5′), 72.8 (1C, C-2′), 72.5 (1C, C-3), 71.5 (1C, C-2), 70.9 (1C, C-2″), 68.5 (1C, C-4″), 61.0 (1C, C-6″), 60.0 and 59.9 (2C, C-6, C-6′); *m/z* (MALDI-TOF) 619.7 (M+Na$^+$. $C_{24}H_{36}O_{15}S$ requires 619.7).

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