Synthesis of novel oligomeric anionic alkyl glycosides using laccase/TEMPO oxidation and cyclodextrin glucanotransferase (CGTase)-catalyzed transglycosylation

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Abstract
Modification of alkyl glycosides, to alter their properties and widen the scope of potential applications, is of considerable interest. Here, we report the synthesis of new anionic alkyl glycosides with long carbohydrate chains, using two different approaches: laccase/2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) oxidation of a long-carbohydrate-chain alkyl glycoside and cyclodextrin glucanotransferase (CGTase)-catalyzed elongation of anionic alkyl glycosides. The laccase/TEMPO oxidation of dodecyl β-D-maltoctaoside proceeded efficiently with the formation of aldehyde and acid products. However, depolymerization occurred to a large extent, limiting the product yield and purity. On the other hand, CGTase-catalyzed coupling/disproportionation reactions with α-cyclodextrin and dodecyl β-D-maltoside diuronic acid (DDM-2COOH) or octyl β-D-glucuronic acid (OG-COOH) as substrates gave high conversions, especially when the CGTase Toruzyme was used. It was found that pH had a strong influence on both the enzyme activity and the acceptor specificity. With non-ionic substrates (dodecyl β-D-maltoctaoside and octyl β-D-glucoside), Toruzyme exhibited high catalytic activity at pH 5–6, but for the acidic substrates (DDM-2COOH and OG-COOH) the activity was highest at pH 4. This is most likely due to the enzyme favoring the protonated forms of DDM-2COOH and OG-COOH, which exist at lower pH (pKa about 3).

KEYWORDS
cyclodextrin glucanotransferase (CGTase), dodecyl β-D-maltoside, dodecyl β-D-maltoside diuronic acid, laccase, octyl β-D-glucuronic acid, oxidation, TEMPO, Toruzyme™ 3.0 L

1 | INTRODUCTION
Alkyl glycosides (AGs) are non-ionic surfactants, possessing several good properties. They display good wetting, foam production, cleaning ability, as well as good biodegradability and ocular safety. Thus, they are applied in many household and personal care products (including body wash products, facial cleaning lotions, shampoos, oral care products, etc.). Although AGs have desirable properties and a constantly growing market, the class of anionic surfactants still has a dominant position, with 60% of the world production of surfactants...
(Aloui et al., 2009). Some of these are not biodegradable and pollutes the environment (Cain, 1994). Therefore, it would be attractive to modify the structure of AGs so that they can be used as potential substitutes. An approach of considerable interest is to produce anionic derivatives of AGs. In 2004, Konya et al. investigated some anionic derivatives of alkyl polyglycosides (APGs) comprising APG citrate, tartrate, and sulphosuccinate in the stabilization of cosmetic and pharmaceutical preparations. These surfactants expressed a marked decrease in interfacial tension and better wetting properties compared to the reference Tween 60. Another important AG modification approach is to elongate the carbohydrate chain. Studies of other non-ionic surfactants—polyethylene oxide surfactants, have shown that a higher degree of ethoxylation lowers the toxicity (Ekelund et al., 2005; Jurado et al., 2012).

Elongation of the carbohydrate chain of AGs has not been possible to achieve with chemical synthesis, but can be carried out efficiently employing a cyclodextrin glucanotransferase (CGTase) as a catalyst. CGTases belong to the α-amylase family (glycosyl hydrolase family) (Henrissat, 1991), but are known as multifunctional enzymes, which catalyze four types of reactions: cyclization, coupling, disproportionation, and hydrolysis (Van der Veen et al., 2000). They can convert starch to cyclodextrin by intramolecular transglycosylation (cyclization). In the coupling reaction (reverse of cyclization) the glucose units (Svensson et al., 2009a, 2009b; Mathew & Adlercreutz, 2012) have recently reported the efficient and selective laccase/2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) oxidation of primary alcohols on the carbohydrate chain of AGs, with a reaction yield of 86% (Ngo et al., 2012; Zehentgruber et al., 2011).

Concerning the introduction of negative charges into AGs, we have recently reported the efficient and selective laccase/2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) oxidation of primary alcohols on the carbohydrate chain of AGs, with a reaction yield of 86% (Ngo et al., 2020a, 2020b). Laccases are copper-containing enzymes, known to oxidize a wide range of substrates, coupled to the reduction of oxygen to water. TEMPO works as an electron shuttle that is easily oxidized by the laccase forming free radicals that transfer the electrons to the primary alcohol substrate that forms the corresponding aldehyde or acid.

In the current report, an attempt was made to combine both of the abovementioned modifications, to synthesize novel surfactants, anionic AGs with long carbohydrate chains using two approaches: the elongation of anionic alkyl glycosides by employing CGTases and the laccase/TEMPO oxidation of a long carbohydrate alkyl glycoside (dodecyl β-D-maltooctaoiside, DDMO). In the elongation, α-cyclodextrin (α-CD) and the oxidized AGs octyl β-D-glucuronic acid (OG-COOH) and dodecyl β-D-maltoside diuronic acid (DDMO) were prepared as described in previous publications (Ngo et al., 2020a, 2020b; Svensson et al., 2009).

2 MATERIALS AND METHODS

2.1 Materials

*B. macerans* CGTase, here called Amano (EC.2.4.1.19), was kindly provided by Amano enzyme Europe Ltd. and *Thermoanaerobacter* sp. ATCC 53627 CGTase (Toruzyme® 3.0 L) from Novozymes. These enzymes have been characterized previously, and the specific activity in a coupling reaction was reported to be 99 U/mg for Amano and 95 U/mg for Toruzyme (Rather et al., 2015). The protein concentrations in the preparations used were 3.22 mg/ml for Amano and 5.96 mg/ml for Toruzyme. α-CD was purchased from Wacker Chemie AG, octyl β-D-glucoside (OG), and dodecyl β-D-maltoside (DDM) from Anatrace Inc. Laccase from *Trametes versicolor* as a powder (specific activity 0.2125 U/mg), TEMPO, citric acid, and sodium citrate were purchased by Sigma-Aldrich. Acetonitrile, 2-propanol, and dimethyl sulfoxide (DMSO) were obtained from VWR. All the chemicals were of analytical grade.

Octyl β-D-glucuronic acid (OG-COOH), dodecyl β-D-maltoside diuronic acid (DDM-COOH), and dodecyl β-D-maltooctaoiside (DDMO) were prepared as described in previous publications (Ngo et al., 2020a, 2020b; Svensson et al., 2009).

2.2 Methods

2.2.1 General laccase/TEMPO oxidation

Reactions were conducted in 4.5 ml open glass vials. DDMO (20 mM) and TEMPO (25.6 mM) were dissolved in citrate buffer (100 mM, pH 5). The vials were incubated in a thermoshaker at 24°C. Then, laccase (141.7 U/L) was added to initiate the oxidation. The reaction was shaken vigorously at 750 rpm to ensure aeration. Samples (8 µl) were taken at scheduled times during 24 h and diluted with 92 µl DMSO before they were analyzed on High-performance liquid chromatography with charged aerosol detector (HPLC-CAD) and liquid chromatography–mass spectrometry (LC-MS).

Laccase activity was determined spectrophotometrically at 530 nm using the oxidation of syringaldazine at room temperature in 0.1 M citrate buffer at pH 5 (Ride, 1980). The enzyme activity unit was defined as the amount of laccase converting 1 µmol of syringaldazine per minute.

2.2.2 Elongation of OG-COOH/DDM-2COOH/OG/DDM

A comparison of the catalytic ability of two CGTases, Toruzyme, and Amano was carried out. The concentration of the acceptor (OG-COOH/DDM-2COOH/OG/DDM) and the donor (α-CD) employed were 15 and 120 mM in 2 ml citrate buffer (50 mM, pH 5.6), respectively. All substrates were added to a glass 4.5 ml vial and incubated at 60°C in a thermoshaker. The reaction was started by...
adding CGTases (Toruzyme 20 µl/Amano 40 µl). During the reaction, 10 µl samples were diluted in 90 µl DMSO and analyzed by HPLC.

Likewise, the Toruzyme-catalyzed reactions were done with varying pH (3.0–6.0) with the aim of investigating the pH dependence. The initial reaction rates were quantified in terms of acceptor consumption. Every experiment for determining initial reaction rates was repeated three times.

2.2.3 | Analytical methods

The reaction products were analyzed using a UHPLC (Ultimate 2.2.3 | Analytical methods

10 µl samples were diluted in 90 µl DMSO and analyzed by HPLC. The injection volume was 2.5 µl, and the autosampler and column temperature were set at 40°C, while the post-column cooler was held at 30°C. The separation of products was performed as follows:

Elution of oxidation products involved a gradient with acetonitrile (solvent A), acetic acid 0.1% (v/v) in water (solvent B), and 2-propanol (solvent C) at a flow rate of 0.28 ml/min. The elution conditions were as follows: 0–10 min, 10% A, 62%–50% B, and 28%–40% C; 10–20 min, 10% A, 50% B, and 40% C; 20–22 min, 10% A, 50%–62% B, and 40%–28% C; 22–32 min, 10% A, 62% B, and 28% C.

Gradient elution of transglycosylation products from the CGTase-catalyzed reactions was performed at a constant flow rate of 0.4 ml/min, with acetonitrile (solvent A), and 0.1% (v/v) acetic acid in water (solvent B) in steps: 0–10 min, 10% A, 62%–50% B, and 28%–40% C; 10–20 min, 10% A, 50% B, and 40% C; 20–22 min, 10% A, 50%–62% B, and 40%–28% C; 22–35 min, 10% A, 62% B, and 28% C.

Standards of the acceptors (OG‐COOH/DDM‐2COOH/OG/DDM) were used for quantification. The products were quantified approximately using the standard curves of the acceptors. The CAD detector was assumed to give an equal response on a weight basis for the quantification of similar substances.

The product masses were determined by LC–MS/MS. Sample analysis was conducted with HPLC (Thermo Scientific Accela) coupled to a Velos Pro–Orbitrap Mass spectrometer (Thermo Fisher Scientific) composed of an electrospray ionization source (HESI-II) in negative mode with an ion source voltage of −2.5 kV. Parameters for measurement were as follows: source heater temperature 300°C, capillary temperature 380°C, sheath gas flow 35 AU, auxiliary gas flow 15 AU, and sweep gas flow 1 AU. The column and elution method were the same as in the HPLC-CAD analysis.

2.2.4 | Homology modeling

The molecular structure of Toruzyme was obtained by hybrid homology modeling. The target protein sequence was deduced by theoretical translation (web. ExPasy.org server, translation tool) of the CGTase gene (GenBank accession code: Z35484) from *Thermoaerobacterium thermosulfurigenes* sp. ATCC 53627 (Kelly et al., 2009). Signal peptide analysis was performed using SignalP 4.1 Server (Nielsen, 2017). The protein sequence excluding the signal peptide was used for building a model using the YASARA software (Krieger et al., 2002; Krieger & Vriend, 2014) under default settings (Table S1). Possible templates were identified by running three PSI-BLAST iterations to extract a position-specific scoring matrix (PSSM) from UniRef90 and then searching the PDB for a match. The best matching templates (Table 1) were selected based on the BLAST alignment score. Backbones were generated by transferring atomic coordinates from the templates to the model. The atomic coordinates of side chains were copied from the templates to the model when they were conserved between templates and the target protein, their coordinates were also copied to the model; otherwise, the rotamers were predicted. Loops were optimized after exploring different conformations. Thereafter, while the backbone atomic coordinates were kept fixed, side chains and loops were minimized combining simulated annealing and steepest descend methods. Finally, unrestrained refinement of the full model was performed by energy minimization (simulated annealing) with explicit solvent molecules of water. The final model was validated based on the z-score, which describes how many standard deviations the model quality is away from the average high-resolution X-ray structure, where negative values indicate low quality with respect to the high-resolution X-ray structure.

2.2.5 | Modeling of Toruzyme with ligands

A complex of Toruzyme with both donor and acceptor ligands was modeled, including an α-CD surrounding the hydrocarbon chain of the alkyl glucoside acceptor. First, the covalent intermediate was built using the atomic coordinates of the crystallographic covalent

| Template | PDB entry | Source | Coverage (%) | Identity (%) |
|----------|-----------|--------|--------------|--------------|
| Homology model | | | | |
| 1 | 3BMV | *Thermoaerobacterium thermosulfurigenes* EM1 AT mutant S77P | 96 | 90.03 |
| 2 | 1OT1 | *Bacillus circulans* strain 251 | 96 | 70.01 |
| Donor covalent intermediate | | | | |
| 1 | 1CXL | *Bacillus circulans* | 100 | 70.01 |

**TABLE 1** Templates used for building the models of Toruzyme
complex 1CXL as a template (Uitdehaag et al., 1999). The acceptor (DDM) was built up and optimized using Avogadro software (Hanwell et al., 2012), and placed into the active site of the covalent complex previously built. The cyclodextrin ring was obtained from the Chemical Components in the PDB, accession code ACX (representative model), and placed surrounding the hydrocarbon chain of DDM. The complex was subjected to energy minimization using AMBER03 forcefield implemented in YASARA (Wang et al., 2004). Thereafter, a second complex was generated with the acceptor DDM-2COOH, which was generated substituting the hydroxymethyl groups of the maltoside moiety with carboxylic groups. This complex was also formed was determined by LC−MS, by comparing the obtained data with the calculated monoisotopic m/z values. As shown in Figure 2, oxidized DDMO products co-eluted before DDMO. The MS analysis indicated a mixture of DDMO-(CHO) (1–8) and DDMO-(COOH) (1–7) (Table S3).

Interestingly, almost complete conversion of DDMO was achieved after 11 h of oxidation (Figure 2b), which is shorter than the time needed to completely convert OG and DDM under the same conditions (Ngo et al., 2020a, 2020b). Also, this trend was seen in the oxidation of a mixture of dodecyl glycosides, with a DP ranging from 2 to 8. After the 5-h reaction, less DDMO remained in the solution compared to DDM (Figure S2). This might be due to the influence of the length of the carbohydrate chain on the reactivity with TEMPO: the longer the carbohydrate chain the faster the oxidation to aldehyde groups.

Besides the presence of aldehyde and carboxyl groups, the chromatogram also showed peaks corresponding to depolymerization products (Figures 2a and S1). An intense peak at m/z 603.26 was observed, suggested to be an [M+114-H]− adduct that fragmented in MS/MS to m/z 489.27 [M-H]−, corresponding to DDM-CHO-1C=C (7). It is worth noting that the presence of the fragment [M = 114] was also detected in other major peaks. For example, m/z 765.32, 927.37, 1089.42, 1251.48, 1413.53 corresponding to their [M+114-H]− adducts, with fragment ions at m/z 651.32 [M-H]− of DDM-1CHO-1C=C (6), m/z 813.37 [M-H]− of DDM-2G-1CHO-1C=C (5), m/z 975.42 [M-H]− of DDM-3G-1CHO-1C=C (4), m/z 1137.48 [M-H]− of DDM-4G-1CHO-1C=C (3), m/z 1299.53 [M-H]− of DDM-5G-1CHO-1C=C (2), respectively. The accurate mass determination of the [M = 114] adduct (m/z 113.9923) indicated a

3 RESULTS AND DISCUSSION

Anionic AGs were prepared using the two different approaches: oxidation of AGs already having long carbohydrate chains and elongation of the carbohydrate chain of anionic AGs having short carbohydrate chains (Figure 1).

3.1 Oxidation of alkyl glycoside with long carbohydrate moiety—DDMO

To prepare anionic AGs using the first approach, DDMO (1) was employed as substrate and laccase/TEMPO as an oxidation catalyst, to introduce carboxyl groups in the carbohydrate chain. In previous studies, optimum reaction conditions for oxidation of AGs having 1–2 glucose residues were shown to be pH 5°C and 24°C (Aracri et al., 2012; Ngo et al., 2020a, 2020b). TEMPO is known to selectively oxidize primary alcohols to corresponding aldehydes and acids. However, in the oxidation of long carbohydrate chains, such as cellulose, depolymerization occurs. This is a side reaction, which is promoted by the presence of aldehyde groups (Aracri et al., 2012).

The oxidative modification of DDMO was investigated during a 24-h reaction. The oxidation was demonstrated by the presence of a variety of aldehyde and acid products. The identity of the products formed was determined by LC−MS, by comparing the obtained data with the calculated monoisotopic m/z values. As shown in Figure 2, oxidized DDMO products co-eluted before DDMO. The MS analysis indicated a mixture of DDMO-(CHO) (1–8) and DDMO-(COOH) (1–7) (Table S3).

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![Image](https://example.com/image1.png)

**FIGURE 1** Scheme of two different approaches to synthesize anionic derivatives of alkyl glycosides having long oligomeric carbohydrate chain
molecular composition of HOOC-C≡C-COOH (calcd 113.9953). A previous study demonstrated the formation of the compound (HOOC-CH(OH)-CH(OH)-COOH) in TEMPO/NaOCl oxidation of glucose. Thus, we hypothesize that HOOC-C≡C-COOH is formed from HOOC-CH(OH)-CH(OH)-COOH during the MS analysis.

A strong correlation has been reported between the loss in molecular weight of the carbohydrate substrate during TEMPO-mediated oxidation and the aldehyde content in the chain (Aracri et al., 2012); the higher the aldehyde content, the severer is the loss. In the oxidation of DDMO, the degradation products are mainly formed by depolymerization. However, no matter how long the reaction was prolonged, the conjugated aldehyde group in the degradation products was not further oxidized (Figure 2c). This is ascribed to the conjugation of the aldehyde group and C=C double bond that excludes the hydration of the aldehyde in water and thereby prevents further oxidation. The depolymerization in DDMO oxidation is in agreement with a study that reported about an 85% decrease in molecular weight when pulp was applied to the laccase/TEMPO oxidation system (Quintana et al., 2017).

Although the DDMO oxidation method did produce anionic alkyl glycosides with a long carbohydrate chain, the yield was comparatively low due to the preference for depolymerization. Also, the presence of many by-products will be a significant obstacle in the purification of the desired product.

### 3.2 | Elongation of anionic alkyl glycosides

#### 3.2.1 | The choice of enzymes

With the aim of synthesizing anionic AGs with long carbohydrate groups using the second approach, OG-COOH and DDM-2COOH were used as acceptor substrates using two different CGTases: Toruzyme and Amano. α-CD was used as glycosyl donor substrate. Both reactions were conducted at pH 5.6 at 60°C, which is the optimal condition for Amano (Svensson et al., 2009b). The highest conversion achieved was around 70% for Toruzyme, while Amano gave less than 40% (Figure 3). Besides, to reach high conversions, the amount of the enzymes was higher than needed for the conversion of DDM to DDMO. This is likely due to the presence of COOH groups in the acceptor substrates, which may impact the interaction between them and the enzyme. These COOH groups were almost completely deprotonated at pH 5.6 forming anionic surfactants, which are claimed to cause more deactivation of enzymes (Naidu & Prabhu, 2011). The acceptor specificity of CGTases is also dependent on the carbohydrate chain length of the surfactant. The Toruzyme catalyzed reaction was faster with DDM-2COOH than with OG-COOH, which is in agreement with the literature on elongation of non-ionic AGs (Svensson et al., 2009a). Due to the high conversions obtained, Toruzyme was chosen as an enzyme for the rest of the study.
3.2.2 | Distribution of products

Analysis of the reaction mixture by LC–MS showed a mixture of elongated DDM-2COOH/OG-COOH with 1–6 extra glucose units, which are listed in Table S4.

CGTases are known to catalyze different reactions: cyclization to produce cyclodextrins from linear oligo/polysaccharides, coupling reaction between cyclodextrins and acceptors, disproportionation reactions between linear substrates and hydrolysis reaction to a limited extent. In the present study, α-CD was used as glycosyl donor, so the initial dominating reaction was the coupling reaction in which the glucose residues of the α-CD were added to the AGs acceptors. However, when following the time course of the reactions (Figure 4), no clear accumulation of the primary coupling products OG-COOH-G6 and DDM-2COOH-G6 were observed. This shows that disproportionation must have dominated already from an early stage.
stage of the reactions, which agrees with previous observations that Toruzyme is very efficient in catalyzing disproportionation reactions (Svensson et al., 2009b). As a result, rather complex product mixtures were obtained with alkyl glycosides having between 1 and 6 additional glucose residues compared to the substrates (Figure 4). As in the initial experiments, the conversion of DDM-2COOH was faster than that of OG-COOH. In the case of DDM-2COOH, it was observed that the concentration of the acceptor substrate passed through a minimum and increased again toward the end of the reaction. This indicates that DDM-2COOH is a poor acceptor compared to other potential acceptors available (DDM-2COOH-G2, DDM-2COOH-G6, etc.). Therefore, when the reaction was prolonged, the formation of DDM-2COOH dominated over reactions involving it as an acceptor. Interestingly, no formation of DDG-COOH was observed, which indicates that the 1,4-glycosidic linkage between the two glucuronic acid residues is not attacked by the enzyme.

3.2.3 | The influence of pH

The experiments described so far were carried out at pH 5.6, known to be suitable for Toruzyme (Zhou et al., 2010). Although the reactions produced the desired products, the formation rates were quite low. Since the acceptor substrates used contained carboxyl groups, it was expected that pH could be of special importance. Therefore, the pH dependence of the elongation of OG-COOH and DDM-2COOH was studied and compared with the corresponding reactions with non-carboxylated substrates. For OG and DDM, the highest reaction rates were observed at pH 5.0–5.6, as expected (Figure 5a). However, for the carboxylated substrates OG-COOH and DDM-2COOH, the reaction rate increased instead of decreasing pH to pH 4. At pH 3, the enzyme had completely lost its activity.

The behavior was similar for the two pairs of substrates. At pH 6 the non-carboxylated substrates were converted 125–150 times faster than the carboxylated ones, while at pH 4 the difference was only about eight times (Figure 5b). Thus, the enzyme clearly favors the protonated form of the substrates, and a major part of the difference can actually be explained using the actual substrate concentration and assuming first-order kinetics. For instance, at pH 4 the concentration of protonated OG-COOH is 2.6 mM, using the determined pKa of 3.32 and a total concentration of 15 mM, which corresponds to a six-time lower substrate concentration when comparing with the OG experiment. The concentration of protonated OG-COOH decreases with an increase in pH values, and thus an observed significant reduction in initial rates. It would have been favorable to use even lower pH values, but that would require an enzyme being more resilient to acidic conditions.

The pH dependence of the time course of the reactions was studied as well. The maximal conversion of both OG-COOH and DDM-2COOH was considerably higher at pH 4 than at pH 6 (Figure 6).

3.2.4 | Molecular modeling

To understand the mechanism behind the pH dependence of the Toruzyme catalyzed elongation of OG-COOH and DDM-COOH,
molecular modeling of the enzyme and docking experiments with the substrates were carried out.

Homology model
The full-length protein contains 708 amino acids, from which, the first 27 were predicted as a signal peptide. The validation of the hybrid model gave a good z-quality score of −0.304 (Table S2). The theoretical molecular weight of the mature protein (without signal peptide) was calculated to be 75.2 kDa. The computational model of the mature protein showed the expected three-dimensional structure organized in five domains, named A to E. Domain A corresponds to the TIM-barrel containing the catalytic site and it begins in the Ala 28 and extends until Tyr 433. Domain B is an insertion into the domain A, corresponding to the sequence between Asn 167 and Gln 230. Domain C begins in Gly 434 and ends in Thr 523. Domain D includes residues between Asn 524 and Leu 606. Domain E extends from Thr 607 to Gln 708 (Figure 7).

Enzyme/ligands model
Two complexes were modeled: the first one Toruzyme/maltotriosyl (donor substrate) covalently bounded to the enzyme/DDM (acceptor substrate)/α-CD (Figure 8a), and the second one containing DDM-2COOH as acceptor substrate instead of DDM (Figure 8b). The donor substrate was placed in the glycan subsites of the Toruzyme model based on the co-crystallized covalent complex of the cycloextrin glycosyltransferase (1CXL, Table S1) from Bacillus circulans. Thus, Asp 257 of Toruzyme was predicted as the catalytic nucleophile involved in the covalent interaction; while, Asp 356, was predicted as the catalytic base. The amino acids involved in the interactions with the maltotrioside moiety are very well conserved, side chains involved in potential hydrogen bonds with the donor include: His 168 and Asp 356 in subsite −1; Trp 129, Asp 398, and Arg 402 in subsite −2; Lys 74 and Asp 398 in the subsite −3. In addition, a well-conserved Tyr that makes an important stacking interaction in subsite −1 was predicted as Tyr 128.

On the other hand, the aglycon subsites show a smaller number of hydrogen bond interactions and more hydrophobic interactions than the glycone subsites. Thus, the acceptor ligand was placed with the sugar moiety of DDM or DDM-2COOH in the subsites +1 and +2, while the dodecyl tail was surrounded by a ring of α-CD was placed on the...
Detailed kinetic studies have shown that when alkyl glycosides act as acceptors in CGTase catalyzed transglycosylation reactions involving cyclodextrins, it is the cyclodextrin alkyl glycoside complex that is the true acceptor (Börner et al., 2014). Since the acceptor is sticking out from the enzyme, there is enough space to accommodate the bulky cyclodextrin ring. Furthermore, the interaction with the surrounding aqueous medium is more favorable for the outer surface of the cyclodextrin than for the alkyl chain of the alkyl glycoside. Well-conserved amino acids were found in subsites +1 and +2 when they were compared with the crystallographic complex (PDB code: 3BMW); Phe 223 in subsite +1, Phe 211, and Tyr 287 (Phe 260 in 3BMW) in subsite +2. In addition, Lys 260 can make hydrogen bonds with the acceptor in subsite +2. Interestingly, the Glu 292 and Thr 290, both located in the subsite +3, could make hydrogen bond interactions with the hydroxyl groups of the α-CD ring that surrounds the hydrophobic tail of the acceptor.

The protonation state of DDM-2COOH showed a strong effect on the enzymatic reaction rate. Dissociation of protons from the carboxylic groups generates negative charges, which could decrease the capability of DDM-2COO- to bind to the active site due to several factors. For instance, the aglycone subsites have several nonpolar residues, as described above. Also, a negative charge in the sugar (+1) would produce electrostatic repulsion with the negatively charged catalytic base, Asp 356, disturbing the acceptor binding. However, the protonated form of the ligand (DDM-2COOH) could bind into the aglycone binding sites. Lys 260 could bind the COOH in sugar (+2) through a hydrogen bond in a way similar to that of the OH of carbon 6 of DDM (Figure 8a,b).

**4 | CONCLUSION**

Briefly, two different routes to synthesize anionic alkyl glycosides with long carbohydrate moiety were for the first time examined: the laccase/TEMPO oxidation of a long-carbohydrate-chain alkyl glycoside and the CGTase-catalyzed elongation of anionic alkyl glycosides. In the first route, the oxidative treatment caused depolymerization, resulting in a variety of degradation by-products, but negative charges in DDMO were successfully introduced. In the second and preferred route, the Toruzyme-catalyzed coupling/disproportionation reactions produced a mixture of OG-COOH/DDM-2COOH glycosides ranging mainly in DP from 1 to 6. Adjustment to the optimal pH value (pH 4) increased the yield of anionic AGs with long oligomeric head groups. Since commercial AGs are complex mixtures, the products prepared here with components mainly differing in carbohydrate chain length might be promising candidates for the surfactant market. Additionally, the degradation products with carboxyl groups from the DDMO oxidation could be potential intermediates for the condensation with amines, which can provide AGs with a diversity of head groups and interesting surface activities. Altogether, this study demonstrates possibilities to increase widely the range of AGs with long oligomeric head groups, which is attractive because these are expected to be mild to living cells.

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**CONFLICT OF INTERESTS**

The authors declare that there is no conflict of interests.

**AUTHOR CONTRIBUTIONS**

Ngoc T. N. Ngo, Javier A. Linares-Pastén, Carl Grey, and Patrick Adlercreutz designed the study. Ngoc T. N. Ngo performed the
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