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Regioselectivity in acylation of oligosaccharides catalyzed by the metalloprotease thermolysin

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Abstract—Investigation of the acylation scope of carbohydrates by metalloprotease thermolysin immobilized on Celite as biocatalyst has been carried out. The reactions were performed in DMSO, a good solvent for carbohydrates, where the enzyme has previously shown its activity in transesterifications of sucrose, maltose and maltose-containing oligosaccharides. Surprisingly, no reaction was observed for glucose or the glucose-containing disaccharides, trehalose and lactose. In contrast, laurate monoesters of several sucrose-containing tri- and tetrasaccharides were synthetized through a one step transesterification using vinyl laurate as the acylating agent. Enzyme regioselectivity was accurately determined by HPLC/MS and the structure of the main regioisomers was established by a combination of NMR experiments. The preferred position of acylation in all cases was the 2-OH of the α-D-glucopyranose moiety linked 1→2 to the β-D-fructofuranose unit. These results correlate with the regioselectivity observed in the case of the disaccharide sucrose. A general carbohydrate binding motif for catalysis by thermolysin is proposed.

1. Introduction

Carbohydrate fatty acid esters are an important class of biodegradable and non-toxic surfactants with broad applications in food, cosmetic and pharmaceutical industries.[1] Their emulsifying and surfactant properties[2] may be modulated by the type of fatty acid and the sugar moiety. Actually, the degree of substitution and the position of attachment to the fatty acid is also important as shown by the different CMC values found for regioisomeric sucrose monoesters.[3] Moreover, this type of compounds also present interesting biological properties like the recently reported antibacterial[4] and antitumoral[5] activities of maltotriose fatty acid esters.

Regioselective chemical monoacylation of carbohydrates is not easy due to their multifunctionality[6] and frequently, protection/deprotection sequences are needed.[7] On the other hand, enzymatically catalyzed sugar fatty acid esterification reactions are, in general, reasonably specific and the regioselective acylation of several carbohydrates with both lipases[8] and proteases[9] has been reported.[10] The hydrolases used in these processes share in common a similar mechanism, which involves the formation of an acyl enzyme intermediate and the acylation takes place on the less hindered primary hydroxyls.

We have recently reported on the enzymatic preparation of oligosaccharide fatty acid esters showing how different regioisomers substituted on primary hydroxyls can be prepared by selecting the appropriate hydrolase preparation.[11] We were also interested in investigating enzymes that could provide regioselective esterification on secondary hydroxyl groups. Lipases are able to acylate secondary hydroxyls groups in sugars solely when the primary hydroxyls are blocked (with a protecting group)[12] and there are only two examples of serine proteases, which have shown regioselectivity towards secondary hydroxyls in unprotected carbohydrates.[13–15] A very promising enzyme employed to date for secondary hydroxyl acylation in carbohydrates is thermolysin, the thermostable neutral metalloprotease from *Bacillus thermoproteolyticus*. This enzyme was first used by Pedersen et al.[16] to catalyze the transesterification of sucrose with vinyl laurate in DMSO with selectivity towards the 2-OH of the glucose moiety. Recently, the same authors have extended the use of this enzyme to prepare cyclodextrin esters.[17]

In this work, we study the transesterification of carbohydrates catalyzed by thermolysin. Acylation of the sucrose-containing oligosaccharides raffinose, melezitose, 1-kestose and stachyose with vinyl laurate in DMSO is described (Scheme 1).

Keywords: Oligosaccharide acylation; Thermolysin; Carbohydrate fatty acid esters; Transesterification; Regioselectivity.
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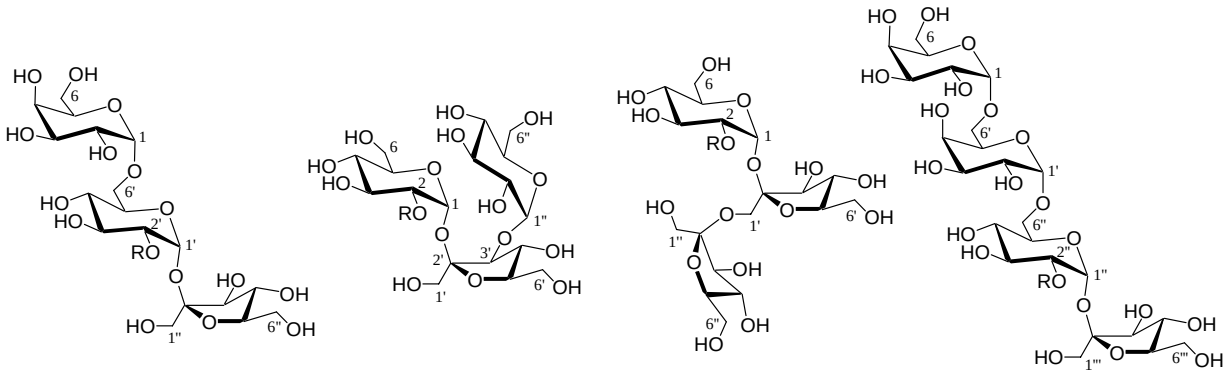


1 :R = H (raffinose) **2** : R = H (melezitose) **3** : R = H (1-kestose) **4** : R = H (stachyose)
1a: R = $CO(CH_2)_{10}CH_3$ **2a**: R = $CO(CH_2)_{10}CH_3$ **3a**: R = $CO(CH_2)_{10}CH_3$ **4a**: R = $CO(CH_2)_{10}CH_3$

Scheme 1. Oligosaccharides used in this study and their corresponding main acylated regioisomers found after thermolysin catalysis.

The results obtained allow us to suggest a possible reaction-mechanism for carbohydrate acylation by thermolysin.

2. Results

Our investigation on the acylation capacity of thermolysin over a range of carbohydrates used the reaction conditions previously described for sucrose.[16] We decided to employ the amorphous form of each oligosaccharide obtained by lyophilization in order to remove most of the crystallization water[18] and thus avoid the formation of fatty acid (by thermolysin-catalyzed hydrolysis of the vinyl ester). The amorphous form also has the additional advantage of being dissolved much more rapidly in DMSO than the crystalline carbohydrate. The enzyme was immobilized onto Celite as previously described[16] but the preparation was dried by lyophilization instead of overnight vacuum drying.

First, we tried transesterification of monosaccharide glucose since sucrose is acylated at the 2-OH of the glucose unit by thermolysin. Suprisingly, no reaction was observed by TLC after 24 h (Fig. 1). Furthermore, neither trehalose or lactose, two disaccharides that contain glucose in their structures, showed acylation by the metalloprotease. Although maltose and sucrose, both containing glucose in their structures, are acylated at secondary hydroxyl groups, it seems that other structural or electrostatic features are needed in the carbohydrate to interact with thermolysin and allow catalysis to take place.

Next, we investigated the acylation of raffinose (**1**) that can be considered a sucrose molecule substituted at the C-6 hydroxyl group with a galactose unit. After 24 h, a mono-acylated product was observed by TLC (Fig. 1). The reaction was stopped after 72 h, when traces of diester were observed although some starting material remained unreacted. No reaction was observed with the Celite control prepared in the same manner as the immobilized enzyme. The reaction mixture of the enzyme-catalyzed transesterification was processed and finally subjected to flash chromatography. The monoester fraction was separated from remaining starting material and minor amounts of

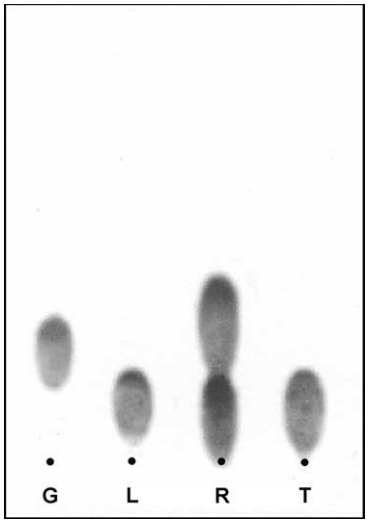


Figure 1. TLC of reaction after 24 h with the saccharides G = glucose; L = lactose; R = raffinose and T = trehalose. Only product formation is observed in the raffinose reaction. Alliquots were extracted with *n*-hexane to remove excess of vinyl laurate and diluted with methanol prior to analysis. Eluent: chloroform–methanol (2.5/1, v/v)

Table 1. HRMS [FAB (+)] of the oligosaccharide monoesters. R_f and yields are also indicated

Carbohydrate	Monolaurate[a] Isolated yield (%)[c]	R_f[b]	Formula	M_W (M+Na) Calculated	Found
Raffinose	27	0.31	$C_{30}H_{54}O_{17}Na$	709.3259	709.3246
Melezitose	22	0.32	$C_{30}H_{54}O_{17}Na$	709.3259	709.3263
1-Kestose	32	0.32	$C_{30}H_{54}O_{17}Na$	709.3259	709.3248
Stachyose	12	0.63	$C_{36}H_{64}O_{22}Na$	871.3787	871.3785

[a] As a mixture of regioisomers.
[b] See TLC solvent in Section 4.
[c] See Section 4 for yield calculation.

diacylated compounds. The FTIR analysis of the isolated monoester (as a mixture of regioisomers) showed the presence of the ester carbonyl group signal at 1730 cm^{-1} and HRMS analysis gave the expected molecular weight (Table 1). The isolated yield obtained is slightly lower than for sucrose.[16]

Then, we extended the reaction to other sucrose-containing oligosaccharides to check the possible influence of other sugar substituents in the structure of sucrose on acylation with themolysin. Two trisaccharides and a tetrasaccharide were examined: melezitose (**2**) and 1-kestose (**3**), that can be considered as a C-3′ hydroxyl-glucosylated sucrose and a C-1′ hydroxyl-fructosylated sucrose, respectively, and stachyose (**4**), that can be considered a raffinose extended on C-6 with galactose. All reactions showed some diester formation and remaining starting sugar by TLC after 72 h, similarly to the raffinose case. After processing and purification, monoacylated compounds were isolated (as a mixture of regioisomers) with the yields shown in Table 1.

In order to study the regioselectivity of the acylation by thermolysin, the purified monoester fraction for each reaction was analyzed by HPLC/MS (see Section 4).[11] The regioisomeric distribution obtained by this method is much more accurate than the typically reported based on ^{13}C NMR. The high chromatographic sensitivity achieved by acquisition in SIR mode (selected-ion recording mode) allows the detection of regioisomers present in the sample in very small quantities; on the other hand, the intensities observed in the routine ^{13}C NMR spectrum are highly dependent on the number of acquisition scans (minor regioisomers may not even appear in the spectrum) and a particular carbon in different regioisomers may present different relaxation times. HPLC/MS chromatograms in Figure 2 show the substitution pattern obtained for each oligosaccharide. It is clear the presence of a major regioisomer in all cases, although the selectivity is not the same, decreasing in the order: raffinose ≈ stachyose > melezitose > 1-kestose.

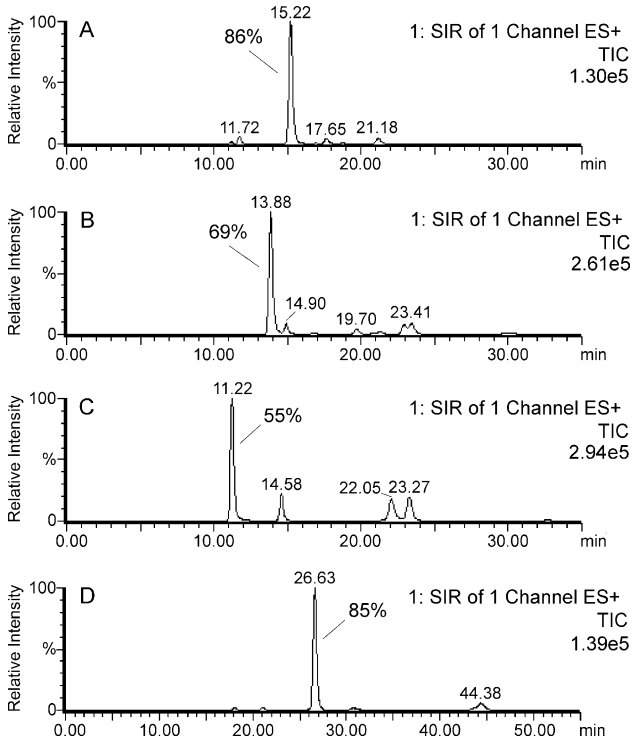


Figure 2. HPLC/MS chromatograms showing the regioisomeric distribution in the isolated monoesters. (A) Raffinose monolaurate regioisomers. (B) Melezitose monolaurate regioisomers. (C) 1-Kestose monolaurate regioisomers. (D) Stachyose monolaurate regioisomers. The percentage of the main regioisomer in each mixture is also indicated.

A combination of one and two-dimensional NMR experiments was then used to establish the position of acylation in the main regioisomer. Using raffinose monolaurate as example, the HMBC spectrum shows the correlation between the carbon peak of the carbonyl of the newly created ester linkage with the proton of the carbon bearing the oxygen atom of the ester function (Fig. 3A). The edited-HSQC spectrum corroborates that this carbon is a methine and thus acylation has taken

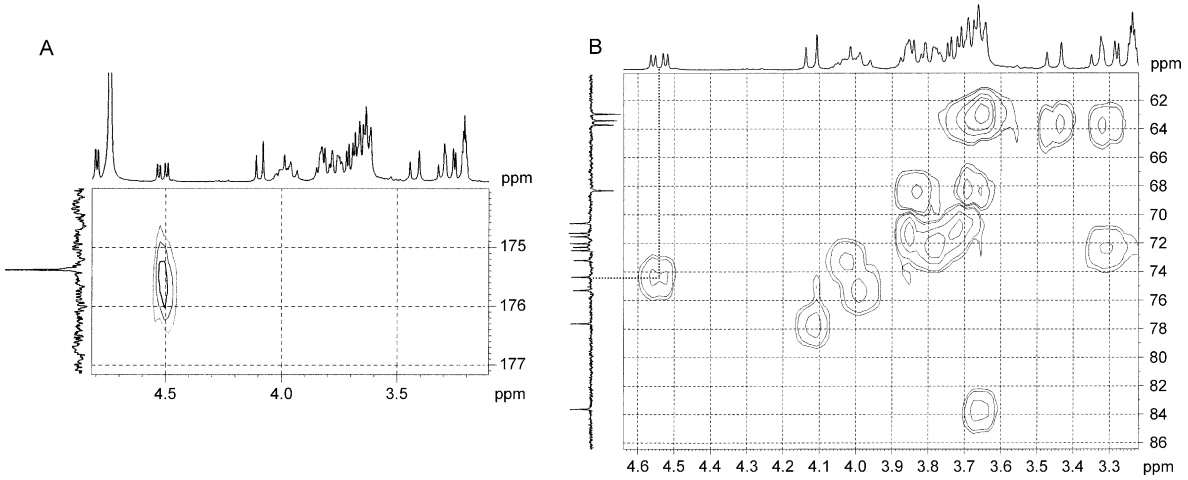


Figure 3. (A) Amplification of the HMBC spectrum of isolated 2′-*O*-lauroylraffinose (**1a**) (86% regioisomeric purity) showing the key cross peak involving the carbonyl ester signal. (B) Part of the edited-HSQC spectrum of the same product. The correlation between the acylated carbon of raffinose (a methine) and its corresponding proton is indicated.

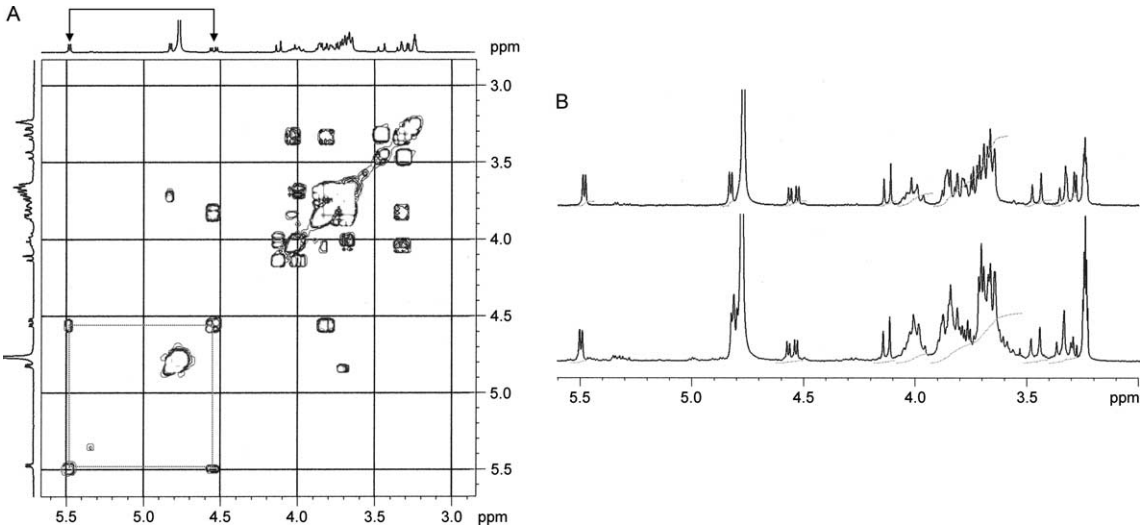


Figure 4. (A) Part of the COSY spectrum (carbohydrate region) of 2′-*O*-lauroylraffinose (**1a**) (86% regioisomeric purity) with indication of the vicinal coupling between the anomeric glucose proton and the proton of the acylated position. (B) Comparison between the proton spectra (carbohydrate region) of 2′-*O*-lauroylraffinose (**1a**) (86% regioisomeric purity) (upper spectrum) and 2″-*O*-lauroylstachyose (**4a**) (85% regioisomeric purity) (lower spectrum).

place in a secondary hydroxyl (Fig. 3B). Finally, a COSY experiment reveals vicinal scalar coupling between the proton of this methine and the anomeric proton of the glucose moiety (Fig. 4A) providing the final evidence that acylation has taken place in the 2-OH of the glucose moiety in raffinose. A similar approach was used for the other oligosaccharide monolaurates. In the case of the stachyose derivative a simple comparison between its 1D proton spectrum and that of the acylated raffinose (Fig. 4B) proves at a glance that acylation has also taken place in the 2-OH of the glucose moiety in stachyose. In the cases of melezitose and 1-kestose, where a more complex regioisomeric mixture was observed, selective 1D-TOCSY experiments were very helpful. Comparison between the spectrum obtained by selective excitation of the anomeric glucose proton in both 1-kestose and its derivative clearly shows that the 2-OH of this (spin system) residue was acylated (Fig. 5A). In the case of melezitose the same approach shows that acylation has taken place in the 2-OH of the α-D-glucopyranose moiety linked 1→2 to the β-D-fructofuranose unit (Fig. 5B). When compared with the parent carbohydrate, the ^{13}C NMR spectra of all new derivatives (Table 2) showed the expected downfield shift of the peak corresponding to the acylated glucosyl C-2 and a similar upfield shift of the peaks corresponding to the neighboring glucosyl C-1 and C-3, in agreement with the general trend observed by Yoshimoto et al.[19] In all these sucrose-containing oligosaccharides acylation occurred preferentially at the 2-OH of the α-D-glucopyranose moiety linked 1→2 to β-D-fructofuranose, which is in agreement with the regioselectivity reported in the case of sucrose acylation catalyzed by the same enzyme[16] (see structures **1a**–**4a** in Scheme 1).

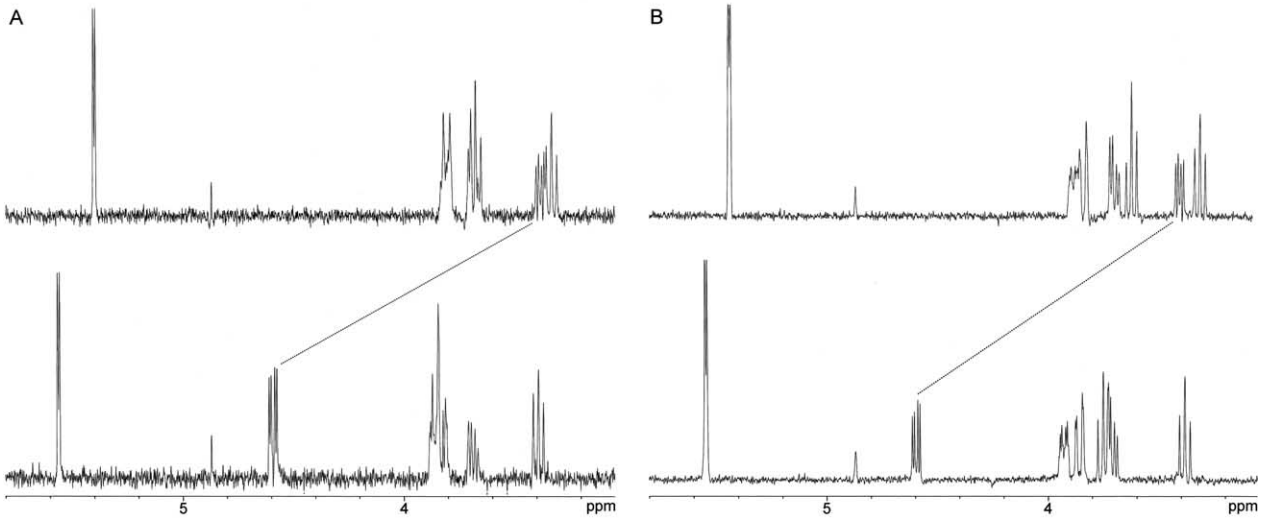


Figure 5. Comparison of 1D TOCSY spectra obtained after selective excitation of the anomeric proton of α-D-glucopyranose moiety linked 1→2 to β-D-fructofuranose in: (A) 1-kestose (upper) and its monolauroyl ester (lower); (B) melezitose (upper) and its monolauroyl ester (lower). Deshielding of the 2-H of the glucose residue consequence of esterification is evident as shown in the figure.

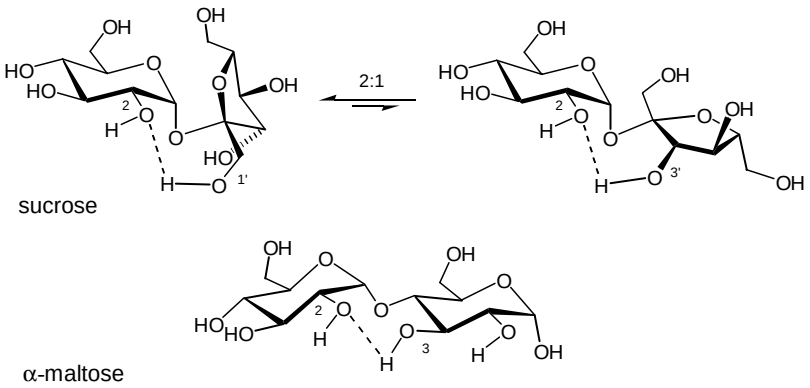
Table 2. ^{13}C NMR chemical shifts (ppm) of compounds **1a**, **2a**, **3a**, **4a** and their parent carbohydrates **1**, **2**, **3** and **4**

Compound		C-1	C-2	C-3	C-4	C-5	C-6
α-Galactopyranosyl-(1→6)-	**1**	100.5	70.5	71.4	71.0	72.4	62.8
α-Glucopyranosyl-(1→2)-		**93.4**	**73.0**	74.4	72.0	73.3	68.3
β-Fructofuranose		64.2	105.3	79.2	75.3	83.4	63.2
α-Galactopyranosyl-(1→6)-	**1a**	100.6	70.5	71.4	71.2	72.3	62.8
α-Glucopyranosyl-(1→2)-		**90.7**	**74.2**	**72.1**	71.9	73.0	68.2
β-Fructofuranose		63.6	105.8	77.5	75.2	83.4	63.2
Laurate		175.4 (C=O)	35.1 (–*C*H$_2$–CO–)	33.0–23.7 (–*C*H$_2$– chain)	14.4 (*C*H$_3$–)		
α-Glucopyranosyl-(1→2)-	**2**	**93.3**	**73.2**	**75.1**	71.7	74.1	62.4
β-Fructofuranosyl-(3→1)-		64.2	105.3	85.8	74.4	83.4	63.3
α-Glucopyranose		101.9	73.8	74.9	72.2	74.0	62.9
α-Glucopyranosyl-(1→2)-	**2a**	**90.3**	**74.3**	**72.4**	71.4	74.1	62.3
β-Fructofuranosyl-(3→1)-		63.9	105.0	85.2	74.3	83.2	63.3
α-Glucopyranose		102.7	73.7	74.8	71.8	74.0	62.4
Laurate		175.2 (C=O)	35.1 (–*C*H$_2$–CO–)	33.0–23.7 (–*C*H$_2$– chain)	14.4 (*C*H$_3$–)		
α-Glucopyranosyl-(1→2)-	**3**	**94.1**	**73.4**	**74.7**	71.5	74.4	62.4
β-Fructofuranosyl-(1→2)-		63.0	105.3	79.9	75.6	83.6	63.2
β-Fructofuranose		62.4	105.0	78.9	76.4	83.5	63.8
α-Glucopyranosyl-(1→2)-	**3a**	**91.1**	**74.3**	**72.0**	71.6	74.3	62.2
β-Fructofuranosyl-(1→2)-		63.2	105.5	77.8	75.0	83.6	63.2
β-Fructofuranose		63.0	105.2	78.9	76.4	83.4	63.5
Laurate		175.5 (C=O)	35.1 (–*C*H$_2$–CO–)	33.1–23.7 (–*C*H$_2$– chain)	14.5 (*C*H$_3$–)		
α-Galactopyranosyl-(1→6)-	**4**[a]	99.8	70.2[b]	71.3[b]	70.8	72.3	62.5
α-Galactopyranosyl-(1→6)-		100.1	70.1[b]	71.1[b]	70.8	70.3[b]	67.7
α-Glucopyranosyl-(1→2)-		**93.4**	**72.8**	**74.4**	71.5	72.9	67.7
β-Fructofuranose		63.8	105.2	78.7	75.3	83.3	63.4
α-Galactopyranosyl-(1→6)-	**4a**	100.1	70.3[b]	71.5[b]	71.1[b]	72.4	62.7
α-Galactopyranosyl-(1→6)-		100.4	70.2[b]	71.3[b]	71.0[b]	70.3[b]	67.9
α-Glucopyranosyl-(1→2)-		**90.7**	**74.2**	**72.1**	71.9	72.9	68.1
β-Fructofuranose		63.6	105.9	77.5	75.2	83.5	63.3
Laurate		175.4 (C=O)	35.1 (–*C*H$_2$–CO–)	33.0–23.7 (–*C*H$_2$– chain)	14.4 (*C*H$_3$–)		

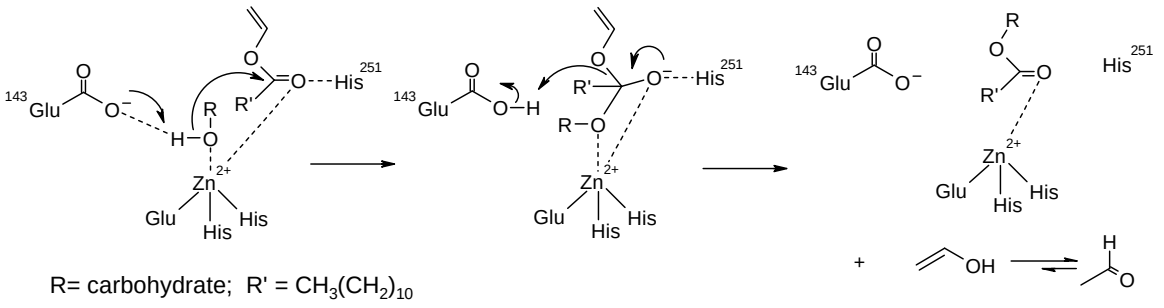
All spectra were acquired in CD_3OD. The carbons where the induced shift effect due to acylation is observed are indicated in bold.
[a] Solvent CD_3OD–D_2O (7.5/1).
[b] Assignments marked with the same letter may be reversed.

3. Discussion

Acylation of a number of carbohydrates using metalloprotease thermolysin has been investigated. In this work, sucrose-containing oligosaccharides have been esterified with vinyl laurate. At the same time, transesterification of sucrose, maltose, maltotetraose, maltoheptaose and cyclodextrins has been reported by Pedersen et al.[16,17] The major regioisomer obtained for sucrose and cyclodextrins has shown acylation at the secondary hydroxyl group in position 2 of glucose whereas no regioselectivity has been reported for maltose and maltose-containing oligosaccharides. It is important to note that monosaccharide glucose and disaccharides trehalose and lactose, both containing glucose in their structure, gave no acylation on the same reaction conditions. It seems that a common structural motif is necessary for a proper binding of the carbohydrate with the enzyme so that catalysis occurs. In fact, an inter-residue H-bond is present in sucrose,[20] (1′-OH···O-2 or 3′-OH···O-2) maltose and cyclodextrins[21] (3-OH···O-2′), where glucose OH-2 participates as hydrogen bond acceptor (Scheme 2).



Scheme 2. Inter-residual hydrogen bond found in DMSO-d_6 for sucrose (equilibrium of two conformations) and α-maltose.



Scheme 3. Proposed reaction mechanism for transesterification of carbohydrates with vinyl laurate by metalloprotease thermolysin.

It is important to note that this hydrogen bond organizes both involved hydroxyl groups in a very similar spatial position for both disaccharides. This motif is not possible in glucose, trehalose or lactose, and may arrange the sucrose- or maltose-containing saccharides in a specific way that allows appropriate interaction with the metalloprotease for catalysis to take place. In fact, sucrose is able to act as a bidentade ligand towards heavy atoms yielding isolable complexes like Δ-$[Co(III)(phen)_2(sucrose)]^{3+}$ in which coordination takes place at the 2-OH of the glucose unit and the 1′-OH of the fructose unit, but no complex is formed with the monosaccharide glucose in such a case, probably because the specific spatial placement of the OHs achieved by sucrose is needed.[22] On the other hand, the hydroxamate moiety of some inhibitors of thermolysin form a bidentade complex with the active site's zinc.[23] Thus, it would be reasonable to think that the carbohydrate binding to the zinc cation in the active site follows a similar bidentade approach, that would be possible for sucrose- or maltose-containing saccharides, but not for glucose, trehalose or lactose.

When we compared the regioselectivity found in the transesterification of the sucrose-containing oligosaccharides with the obtained for sucrose, a decrease is observed in the order sucrose ≈ rafinnose ≈ stachyose > melezitose > 1-kestose. Actually, from the hydrolytic mechanism accepted for thermolysin,[24] a putative mechanism for the carbohydrate transesterification may be proposed to help understand the regioselectivity obtained. In this mechanism (Scheme 3) the nucleophilicity of the attacking hydroxyl is enhanced by aminoacid Glu143, which functions as a general-basic catalyst (GBC) in the reaction. A possible explanation for the regioselectivity observed could be related with the relative acidity of this hydroxyl in the different carbohydrates, bearing in mind the GBC role of Glu143. ^{1}H NMR experiments have shown the presence of two intramolecular hydrogen-bond conformations for sucrose in DMSO-d_6, in which the glucosyl 2-OH acts as acceptor for 1′-OH or 3′-OH of the fructofuranosyl residue. The two hydrogen-bond conformations exist in a competitive equilibrium with the 1′-OH···O-2 hydrogen bond favoured over the 3′-OH···O-2 hydrogen bond in a ratio ~2:1, assuming that the magnitudes of the isotope effects reflect the relative 'strengths' of the hydrogen bonds (Scheme 2).[20] Semiempirical calculations based on these hydrogen bond conformations clearly establish that the glucosyl 2-OH is the most acidic position of the sucrose molecule.[25] This is also corroborated experimentally in the base-catalyzed acylation of sucrose with vinyl laurate in DMSO, which produces 2-O-lauroylsucrose as the main regioisomer.[26] Similar NMR experiments have shown that the same competitive hydrogen-bond equilibrium found in sucrose also occurs in raffinose and stachyose[27] and thus a similar acidity of the corresponding glucosyl 2-OH should be expected. Both melezitose and 1-kestose have one of the possible interresidue H-bonds excluded in their sucrose moiety due to substitution with a sugar unit. The other possible inter-residual H-bond, the 1′-OH···O-2 hydrogen bond for melezitose and the 3′-OH···O-2 hydrogen bond for 1-kestose may take place, since O-3′ sucrose derivatives and 1′-sucrose derivatives with similar structures have showed its presence in DMSO-d_6.[28,29] It is important to note that missing one of the two-possible hydrogen-bonds may reduce the actual acidity of the OH-2, and also that the 3′-OH···O-2 hydrogen bond is weaker than the 1′-OH···O-2.[20] This takes us to the following order of relative acidity of the glucosyl 2-OH for each carbohydrate: sucrose ≈ rafinnose ≈ stachyose > melezitose > 1-kestose, which correlates quite well with the substitution pattern observed.

In summary, we have extended the study of acylation with the metalloprotease thermolysin to several mono-, di-, tri- and tetrasaccharides. It seems that a common structural motif in the carbohydrate is needed for proper binding so that catalysis may take place. This motif may locate the two hydroxyl groups involved in an inter-residual H-bond in a position where they are able to bind the zinc ion via a bidentade mode. Accurate HPLC/MS analysis along with NMR spectroscopic studies show that the acylation occurs preferentially in the 2-OH of the glucose moiety involved in an inter-residue H-bond with the vicinal sugar moiety. Moreover, it is the first time that sucrose containing tri- and tetrasaccharides are acylated enzymatically in a secondary hydroxyl group. Studies of the biological and physical properties of these new non-ionic surfactants are in progress.

4. Experimental

4.1. Chemicals

Anhydrous dimethyl sulfoxide (DMSO) and vinyl laurate were supplied by Fluka; raffinose and melezitose from Sigma and stachyose and 1-kestose from TCI Chemicals. All the carbohydrates were used in their amorphous form prepared by lyophilization of the corresponding aqueous solutions. Thermolysin from *Bacillus thermoproteolyticus*

rokko (Type X, lyophilized powder, 50–100 units/mg protein) and acid-washed Celite were from Sigma.

4.2. Enzyme immobilization

Enzyme adsorption onto Celite was done essentially as previously described.[16] Basically, thermolysin (100 mg) was dissolved in 3.33 ml of 50 mM 3-morpholinopropanesulfonic acid (Na-MOPS) buffer at pH 7.5. The enzyme solution was mixed thoroughly with 3.33 g of acid-washed Celite and subsequently lyophilized to ensure complete drying of the preparation. Celite control was prepared as the immobilized enzyme.

4.3. General procedure for ester synthesis and product isolation

In a typical experiment a solution of raffinose, melezitose, 1-kestose (150 mg, 0.30 mmol) or stachyose (200 mg, 0.30 mmol) in anhydrous DMSO (8.5 ml) and vinyl laurate (228 mg, 1 mmol) were shaken with orbitalic stirring (325 rpm) at 45 °C in the presence of immobilized enzyme (1500 mg). When traces of diester were observed by TLC, the mixture was cooled and filtered. Then, *n*-hexane was added (5 ml) and the mixture vigorously stirred and then cooled to −20 °C. The *n*-hexane, which took up residual vinyl laurate, was decanted. The DMSO reaction solution was allowed to warm at room temperature and then mixed with 10 ml of water. The mixture was extracted with *n*-butanol–cylohexane (3/1 v/v for trisaccharide reactions and 4:1 v/v for the stachyose reaction) (3×40 ml). The organic phases were pooled and washed with 15 ml of brine to remove residual DMSO, and solvents were evaporated off. The remaining residue was subjected to flash chromatography. Concentration of pure fractions in vacuo afforded the monolaurates (mixture of regioisomers) as amorphous white solids. Yields of oligosaccharide monolaurates (Table 1) are based on weight of the isolated fraction containing the monoesters.

All reactions were monitored by TLC on precoated Silica-Gel 60 plates (Alugram Sil G/UV_{254} supplied by Macherey-Nagel), and detected by heating with Mostain (500 ml of 10% H_2SO_4, 25 g of $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$, 1 g $Ce(SO_4)_2\cdot 4H_2O$). The elution system was $CHCl_3$–MeOH (2.5/1) for the reactions involving trisaccharides and EtOAc–MeOH–H_2O (7/5/1) for the reactions with stachyose. Flash chromatography was performed with Aldrich Silica gel 60 (200–400 mesh) using a gradient of chloroform/methanol 5:1–2:1 (v/v) for the trisaccharides monolaurates and 5:1 to 1:1 v/v for stachyose monolaurate.

4.4. HPLC/MS analysis

Analysis of the regioisomeric distribution of the isolated monoesters was carried out by HPLC/MS.[11] A Waters Alliance 2695 separation module was employed with a Waters Spherisorb 3 μm ODS2 column (4.6×250 mm) and a Waters Micromass ZQ mass spectrometer detector. The temperature of the column was set to 40 °C. Flow rate was 1.1 ml/min with splitting before the detection module (so that 0.20 ml/min enters the detector). Mobile phases were acetonitrile:water mixtures in isocratic conditions. The ratio changed depending on the compounds as follows: for the analysis of the regioisomeric distribution of trisaccharide monolaurates, acetonitrile/water 35:65 (v:v) and for the more polar stachyose monolaurate, 30:70 (v:v). Detection was done with positive ESI ionization in both Scan and SIR (selecting the mass of the Na^+ adduct, i.e., M+Na) modes. Cone voltage was set to 40 V to have the maximum possible intensity for the molecular ion and no fragmentation. Samples were prepared as water solutions (ca. 1 ppm) and immediately analyzed. Percentage of main regioisomer in each mixture was calculated by integration (using MassLynx version 3.5 software) of the corresponding SIR chromatogram (Fig. 2) as follows: [(Area peak mean regioisomer)/(ΣArea all peaks)]×100%. This calculation assumes that all regioisomers in the sample have the same response in the detector, that is, all of them ionize equally in the ESI source. The chromatographic conditions we have employed (constant flow rate, isocratic eluent, absence of additives in the mobile phase, sample diluted enough to avoid signal saturation, etc.) and the obvious structural similarity between all regioisomers (all of them have the same hydrophilic–lipophilic balance and are polar enough to form stable adducts with Na^+ in the source) ensure that the equal response requirement is met.

4.5. Spectroscopic analysis

NMR spectra of the parent oligosaccharides and their corresponding isolated monolaurates were recorded on either a Bruker AVANCE 300 or ARX 400 [300 or 400 MHz (^{1}H) and 75 or 100 (^{13}C)] at room temperature for solutions in CD_3OD. Chemical shifts are referred to the methanol multiplet, centered at 3.31 ppm for ^{1}H NMR and 49.0 ppm for ^{13}C NMR. All spectra were acquired using standard pulse sequences, instrument settings and procedures (selective excitation in 1D selective TOCSY spectra was achieved by excitation sculpting with a PFGSE sequence). High resolution FAB (+) mass spectral analyses were obtained on a Micromass AutoSpec-Q spectrometer. Infrared spectra (KBr disks) were recorded using a Nicolet 20SXB FTIR spectrophotometer.

4.5.1. 2′-*O*-Lauroylraffinose (1a). The general procedure outlined above was followed. After 72 h the reaction was stopped and the monoester fraction isolated (56 mg, 27%). R_f=0.31, ν_{max} (cm^{-1}) (KBr disks): 3410 br (O–H), 1730 (C=O); HRMS (FAB+): calcd for $C_{30}H_{54}O_{17}Na$ (M+Na) 709.3259, found 709.3246. Compound **1a** was obtained with 86% regioisomeric purity (HPLC/MS). NMR assignments of main regioisomer **1a**: ^{1}H NMR (CD_3OD, 300 MHz): δ 5.55 (d, 1H, $J_{1'-2'}$=3.7 Hz, H-1′), 4.90 (d, 1H, J_{1-2}=3.4 Hz, H-1), 4.61 (dd, 1H, $J_{2'-3'}$=9.7 Hz, $J_{1'-2'}$=3.7 Hz, H-2′), 4.19 (d, 1H, $J_{3''-4''}$=8.8 Hz, H-3″), 4.10 (m, 1H, H-5′), 4.06 (t, 1H, $J_{3''-4''}=J_{4''-5''}$=8.8 Hz, H-4″), 3.93 (m, 1H, H-3), 3.93 (dd, 1H, $J_{6'a-b}$=11.2 Hz, $J_{6'a-5'}$=5.7 Hz, H-6′a), 3.88 (t, 1H, $J_{2'-3'}=J_{3'-4'}$=9.7 Hz, H-3′), 3.85 (m, 1H, H-5), 3.80 (m, 1H, H-4), ca. 3.77 (m, 1H, H-2), 3.75 (m, 1H, H-6′b), ca. 3.73 (m, 3H, H-5″, H-6″a, H-6″b), ca. 3.71 (m, 2H, H-6a, H-6b), 3.53 (d, 1H, $J_{1''a-b}$=11.9 Hz, H-1″a), 3.39 (t, 1H, $J_{3'-4'}=J_{4'-5'}$=9.7 Hz, H-4′), 3.38 (d, 1H, $J_{1''a-b}$=11.9 Hz, H-1″b), 2.38 (t, 2H, J=7.4 Hz, –CH_2–CO–), 1.63 (m, 2H, CH_2–CH_2–CO–),

1.29 (m, 16H, –CH_2– chain), 0.90 (t, 3H, J=6.7 Hz, CH_3–); ^{13}C NMR (CD_3OD, 75 MHz): see assignment in Table 2.

4.5.2. 2-*O*-Lauroylmelezitose (2a). The general procedure outlined above was followed. After 72 h the reaction was stopped and the monoester fraction isolated (45 mg, 22%). R_f=0.32, ν_{max} (cm^{-1}) (KBr disks): 3400 br (O–H), 1725 (C=O); HRMS (FAB+): calcd for $C_{30}H_{54}O_{17}Na$ (M+Na) 709.3259, found 709.3263. Compound **2a** was obtained with 69% regioisomeric purity (HPLC/MS). NMR assignments of main regioisomer **2a**: ^{1}H NMR (CD_3OD, 300 MHz): δ 5.55 (d, 1H, J_{1-2}=3.7 Hz, H-1), 5.07 (d, 1H, $J_{1''-2''}$=3.7 Hz, H-1″), 4.61 (dd, 1H, J_{2-3}=9.7 Hz, J_{1-2}= 3.7 Hz, H-2), ca. 4.26 (m, 2H, H-3′, H-4′), 3.94 (m, 1H, H-5), 3.88 (m, 1H, H-5″), 3.87 (dd, 1H, J_{6a-b}=12.1 Hz, J_{6a-5}=2.1 Hz, H-6a), ca. 3.82 (m, 2H, H-6″a, H-6″b), ca. 3.78 (m, 3H, H-5′, H-6′a, H-6′b), 3.76 (t, 1H, J_{2-3}=J_{3-4}= 9.7 Hz, H-3), 3.72 (dd, 1H, J_{6a-b}=12.1 Hz, J_{6b-5}=6.1 Hz, H-6b), 3.70 (t, 1H, $J_{2''-3''}$=$J_{3''-4''}$=9.6 Hz, H-3″), 3.67 (d, 1H, $J_{1'a-b}$=11.9 Hz, H-1′a), 3.44 ((dd, 1H, $J_{2''-3''}$=9.6 Hz, $J_{1''-2''}$=3.7 Hz, H-2″), 3.43 (t, 1H, $J_{3''-4''}$=$J_{4''-5''}$=9.6 Hz, H-4″), 3.39 (t, 1H, J_{3-4}=J_{4-5}=9.7 Hz, H-4), 3.28 (d, 1H, $J_{1'a-b}$=11.9 Hz, H-1′b), 2.38 (t, 2H, J=7.5 Hz, –CH_2–CO–), 1.63 (m, 2H, CH_2–CH_2–CO–), 1.29 (m, 16H, –CH_2– chain), 0.89 (t, 3H, J=6.8 Hz, CH_3–); ^{13}C NMR (CD_3OD, 75 MHz): see assignment in Table 2.

4.5.3. 2-*O*-Lauroyl(1-kestose) (3a). The general procedure outlined above was followed. After 72 h the reaction was stopped and the monoester fraction isolated (66 mg, 32%). R_f=0.32, ν_{max} (cm^{-1}) (KBr disks): 3400 br (O–H), 1730 (C=O); HRMS (FAB+): calcd for $C_{30}H_{54}O_{17}Na$ (M+Na) 709.3259, found 709.3248. Compound **3a** was obtained with 55% regioisomeric purity (HPLC/MS). NMR assignments of main regioisomer **3a**: ^{1}H NMR (CD_3OD, 400 MHz): δ 5.57 (d, 1H, J_{1-2}=3.7 Hz, H-1), 4.61 (dd, 1H, J_{2-3}=9.7 Hz, J_{1-2}=3.7 Hz, H-2), 4.27 (d, 1H, $J_{3'-4'}$= 8.4 Hz, H-3′), 4.15 (d, 1H, $J_{3''-4''}$=8.3 Hz, H-3″), ca. 4.04 (m, 2H, H-4′, H-4″), 3.87 (m, 1H, H-5), 3.86 (m, 1H, H-3), ca. 3.84 (m, 1H, H-6a), ca. 3.84-3.77 (m, 2H, H-1′a, H-1′b), ca. 3.77–3.72 (6H, H-5′, H-5″, H-6′a, H-6′b, H-6″a, H-6″b), 3.70 (dd, 1H, J_{6a-b}=12.0 Hz, J_{6b-5}=5.2 Hz, H-6b), ca. 3.64–3.60 (2H, H-1″a, H-1″b), 3.41 (t, 1H, J_{3-4}=J_{4-5}= 9.7 Hz, H-4), 2.38 (t, 2H, J=7.5 Hz, –CH_2–CO–), 1.63 (m, 2H, CH_2–CH_2–CO–), 1.29 (m, 16H, –CH_2– chain), 0.90 (t, 3H, J=6.8 Hz, CH_3–); ^{13}C NMR (CD_3OD, 100 MHz): see assignment in Table 2.

4.5.4. 2″-*O*-Lauroylstachyose (4a). The general procedure outlined above was followed. After 72 h the reaction was stopped and the monoester fraction isolated (31 mg, 12%). R_f=0.63, ν_{max} (cm^{-1}) (KBr disks): 3410 br (O–H), 1730 (C=O); HRMS (FAB+): calcd for $C_{36}H_{64}O_{22}Na$ (M+Na) 871.3787, found 871.3785. Compound **4a** was obtained with 85% regioisomeric purity (HPLC/MS). NMR assignments of main regioisomer **4a**: ^{1}H NMR (CD_3OD, 300 MHz): δ 5.55 (d, 1H, $J_{1''-2''}$=3.7 Hz, H-1″), 4.89 (d, 1H, H-1), 4.88 (d, 1H, $J_{1'-2'}$=3.6 Hz, H-1′), 4.61 (dd, 1H, $J_{2''-3''}$=9.7 Hz, $J_{1''-2''}$=3.7 Hz, H-2″), 4.20 (d, 1H, $J_{3'''-4'''}$= 8.6 Hz, H-3‴), 4.08 (m, 1H, H-5″), 4.07 (m, 1H, H-5′), 4.05 (t, 1H, $J_{3'''-4'''}$=$J_{4'''-5'''}$=8.6 Hz, H-4‴), 3.93 (m, 1H, H-3′), 3.93 (dd, 1H, $J_{6''a-b}$=11.1 Hz, $J_{6''a-5''}$=5.6 Hz, H-6″a), ca. 3.89 (m, 1H, H-3), 3.87 (t, 1H, $J_{2''-3''}$=$J_{3''-4''}$=9.7 Hz, H-3″), ca. 3.85 (m, 1H, H-6′a), ca. 3.83 (m, 1H, H-5), ca. 3.80 (m, 1H, H-4), ca. 3.76 (m, 2H, H-2, H-2′), ca. 3.73 (m, 4H, H-4′, H-5‴, H-6‴a, H-6‴b), ca. 3.71 (m, 2H, H-6a, H-6b), 3.70 (m, 1H, H-6″b), ca. 3.65 (m, 1H, H-6′b), 3.53 (d, 1H, $J_{1'''a-b}$=11.9 Hz, H-1‴a), 3.39 (t, 1H, $J_{3''-4''}$= $J_{4''-5''}$=9.7 Hz, H-4″), 3.38 (d, 1H, $J_{1'''a-b}$=11.9 Hz, H-1‴b), 2.38 (t, 2H, J=7.4 Hz, –CH_2–CO–), 1.63 (m, 2H, CH_2–CH_2–CO–), 1.29 (m, 16H, –CH_2– chain), 0.90 (t, 3H, J=6.7 Hz, CH_3–); ^{13}C NMR (CD_3OD, 75 MHz): see assignment in Table 2.

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