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One- and two-step enzymatic synthesis of polymerizable vinyladipoyl mono- and diesters of non-reducing trisaccharides

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Abstract

The enzymatic transesterification of non-reducing trisaccharides raffinose and melezitose with divinyl adipate using two lipases (Novozym 435, Lipozyme TL IM) and the protease subtilisin Carlsberg is described. Polymerizable vinyladipoyl sugar esters were obtained with good to excellent selectivity and high yields. Monoesters prepared with lipase catalysis have also been used as substrates for a second acylation step catalyzed by the protease. Diesters were obtained with high selectivity although the isolated yields were slightly lower than those obtained for the corresponding monoester derivatives. It is important to note that the preferential acylation positions for subtilisin are the same in the free sugar and in the corresponding used monoesters for both trisaccharides. This two-step enzymatic approach allows regioselective control in the incorporation of the sugar inside the polymer structure.

Keywords: Oligosaccharide; Vinyl ester monomer; Enzymatic transesterification; Lipase; Protease

1. Introduction

Synthetic polymers containing sugar branches have attracted considerable interest due to their role as biomimetic analogues, their potential for commercial applications and their biodegradability [1–3]. These polymers normally consist of a main chain, a spacer arm and a sugar moiety. Although the carbohydrate is clearly one of the tools to modulate polymer structure diversity and therefore polymer properties, most of the polymers described in the literature use mono- and disaccharides polymerizable monomers. In fact, these monomers are easier to prepare than those for larger oligosaccharides since chemical synthesis usually involves long protection–deprotection strategies [4] and enzymatic synthesis is limited due to solubility of oligosaccharides in organic solvents where enzymes, specially lipases, are still active [5]. Nevertheless, a few interesting examples of glycopolymers that incorporate oligosaccharides have been described in order to study carbohydrate–protein [6,7] or carbohydrate–carbohydrate interactions [8] or to explore their physicochemical and structural properties [9–12].

Vinyl sugar esters are typical water-soluble monomers which can be polymerized by radical reactions to yield such glycoconjugate polymers [11,13]. Carbohydrate bearing vinyl acyl esters can be obtained by selective enzyme-catalyzed transesterifications. An interesting approach reported by Kitagawa and Tokiwa [14] employs divinyl dicarboxylates as the spacer where one vinyl ester group is used to promote hydrolase transesterification and the other is the polymerizable group. Most of the research performed in this area has been devoted to the synthesis of mono- and disaccharide monomers using both lipases and proteases as biocatalysts [15–17]. Nevertheless, larger carbohydrate polymerizable monomers have only been prepared to date with protease catalysis taking advantage that these enzymes are active in polar organic solvents like pyridine or DMF capable of solubilizing big sugars, such as raffinose or cyclodextrin, to a large extent [18–20]. Recently, we have described a highly efficient enzymatic transesterification of non-reducing tri- and tetrasaccharides with vinyl laurate using different hydrolases,

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including lipases, which could be used to prepare polymerizable sugar units [21].

In this work, we have extended our enzymatic methodology to synthesize new polymerizable vinyladipoyl monoesters of raffinose (1) and melezitose (2) for the future preparation of novel carbohydrate-containing polymers. At the same time, we describe the sequential two-step enzymatic synthesis of vinyladipoyl diesters of the same trisaccharides where the monoesters prepared with lipase catalysis are substrates for a second acylation step catalyzed by the protease subtilisin Carlsberg. Analogous diesters derived from sucrose have been enzymatically polymerized in the presence of diols like 1,8-octanediol to yield linear glycopolyesters [22]. This two-step enzymatic strategy allows regioselective control in the incorporation of the sugar inside the polymer structure. The regioselectivity of all acylation reactions has been studied by LC/MS [23] and ^{13}C NMR experiments showing the formation of a main regioisomer in all cases. The second acylation carried out over the trisaccharide monoesters clearly proves that the protease shows the same preferential acylation position in both the free and monoacylated trisaccharides.

2. Experimental

2.1. Materials and methods

Anhydrous pyridine were supplied by Fluka; anhydrous *tert*-butanol, molecular sieves (3 Å, 8–12 mesh) and methyl-β-cyclodextrin from Aldrich; raffinose and melezitose from Sigma and divinyl adipate from TCI Chemicals. The carbohydrates were used in their amorphous form prepared by lyophilization of the corresponding aqueous solutions.

The following enzymes were used: granulated lipase from *T. lanuginosus* (Lipozyme TL IM), immobilized lipase from *C. antarctica* B (Novozym 435), and subtilisin Carlsberg (purified powder). All the enzymes were kindly donated by Novozymes A/S. The protease was employed as a solid powder obtained by colyophilization with methyl-β-cyclodextrin (MβCD-subtilisin Carlsberg preparation), as previously described [24]. The catalytic activity of each enzyme, according to the manufacturer, is the following: Lipozyme TL IM: Catalytic activity = 250 IUN/g. One Interesterification Unit Novo is defined as 0.01% (w/w) converted tristearin/minute (initial rate) under the following batch interesterification conditions: substrate (fully hydrogenated soybean oil/ soybean oil; 27/73% (w/w)); temperature: 70 °C. No co-solvents. Novozym 435: Catalytic activity = 10,000 PLU/g. The ester synthesis activity of Novozym 435 is expressed in Propyl Laurate Units per gram (PLU/g). Subtilisin Carlsberg: Catalytic activity = 25 AU/g (AU = Anson Units). One Anson unit is defined as the amount of enzyme which, under specified conditions, digests urea-denatured hemoglobin at an initial rate such that there is liberated an amount of TCA-soluble product per minute which gives the same color with Folin–Ciocalteu Phenol reagent as one milliequivalent of tyrosine. For this enzyme, the "specified conditions" were 25 °C, pH 7.50 and reaction time of 10 min.

Molecular sieves were preactivated at ca. 350 °C for 12 h. 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 (2:1) for the synthesis of monoesters and (2.5:1) for the synthesis of diesters. Products were purified by flash chromatography with Aldrich Silica gel 60 (200–400 mesh) using a gradient of chloroform:methanol 5:1–2:1 (v/v) for the monoesters and 6:1–3.5:1 (v/v) for the trisaccharide diesters.

NMR spectra were recorded on a Bruker AVANCE 300 at room temperature for solutions in DMSO-d_6 (monoesters) or CD_3OD (diesters). Chemical shifts are referred to the residual solvent signal. (DMSO: δ_C 39.6; CD_3OD: δ_C 49.3). Optical rotations (sodium D line) were measured at 20 °C with a Perkin-Elmer 241 for pure regioisomers (compounds obtained with >95% regioselectivity). High resolution FAB (+) mass spectral analyses were obtained on a Micromass AutoSpec-Q spectrometer.

Analysis of the regioisomeric distribution of the isolated mono- and diesters were carried out by HPLC/MS [23]. A Waters Alliance 2695 separation module equipped was employed with a Waters Spherisorb 3 μm ODS2 column (4.6 mm × 250 mm) and a Waters Micromass ZQ mass spectrometry detector. The temperature of the column was set to 40 °C. Flow rate was 0.3 mL/min. Mobile phases were acetonitrile:water mixtures in isocratic conditions. The ratio changed depending on the application as follows: for the analysis of the regioisomeric distribution of trisaccharide monoesters, acetonitrile:water 17:83 (v/v) and for the less polar trisaccharide diesters, 35:65 (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 50 V. 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 (Figs. 1–3) as follows: [(area peak mean regioisomer)/(∑area all peaks)] × 100%.

2.2. Synthesis of monoesters catalyzed by Novozym 435

Raffinose or melezitose (3.03 g, 6.0 mmol) were dissolved in anhydrous pyridine (66 mL) at 60 °C before careful addition of preheated (60 °C) anhydrous *tert*-butanol (84 mL). Novozym 435 (1.5 g) and 3 Å molecular sieves (1.5 g) were then added and the suspension maintained 30 min at 60 °C with orbital shaking (250 rpm). Finally, divinyl adipate (5950 mg, 30.0 mmol) was added. When conversion stopped as estimated by TLC, the mixture was cooled and filtered. The solvent was evaporated under vacuum at 45 °C eliminating last traces of pyridine by co-evaporation with toluene. The remaining residue was subjected to flash chromatography. Concentration of pure fractions in vacuo afforded the monoesters as amorphous white solids.

2.2.1. 6-O-Vinyladipoylraffinose (1a)

The general procedure outlined above was followed. After 3 days the reaction was stopped and the monoester isolated

(1.665 g, 42%). Only regioisomer **1a** was obtained (HPLC/MS). $R_f = 0.20$; $[\alpha]_D = +89.7$ (c 7 in methanol); HRMS (FAB): calcd for $C_{26}H_{42}O_{19}Na$ ($M + Na^+$) 681.2218, found 681.2210; ^{13}C NMR: see assignment in Table 2.

2.2.2. Monovinyladipoylmelezitose

The general procedure outlined above was followed. After 3 days the reaction was stopped and the monoester fraction isolated (1.504 g, 38%). $R_f = 0.25$; HRMS (FAB): calcd for $C_{26}H_{42}O_{19}Na$ ($M + Na^+$) 681.2218, found 681.2224. Regioisomeric proportion **2a/2c/2b** = 62/24/14 (HPLC/MS). ^{13}C NMR: see assignment in Table 2.

2.3. Synthesis of monoesters catalyzed by Lipozyme TL IM

The same method as the Novozym 435 catalyzed reactions was used only changing the biocatalyst to Lipozyme TL IM.

2.3.1. 6-O-Vinyladipoylraffinose (1a)

The general procedure outlined above was followed. After 24 h the reaction was stopped and the monoester isolated (2.98 g, 75%). Only one regioisomer **1a** was obtained (HPLC/MS) which is the same obtained with Novozym 435 catalysis.

2.3.2. Monovinyladipoylmelezitose

The general procedure outlined above was followed. After 3 days the reaction was stopped and the monoester fraction isolated (1.98 g, 50%). $R_f = 0.25$; HRMS (FAB): calcd for $C_{26}H_{42}O_{19}Na$ ($M + Na^+$) 681.2218, found 681.2226. Regioisomeric proportion **2b/2a/2c** = 57/37/4 + 2% other regioisomers (HPLC/MS). ^{13}C NMR: see assignment in Table 2.

2.4. Synthesis of monoesters catalyzed by MβCD-subtilisin carslberg

A solution of raffinose or melezitose (1.01 g, 2.0 mmol) in anhydrous pyridine (10 mL) and divinyl adipate (1.2 g, 6.0 mmol) were shaken with orbitalic stirring (250 rpm) at 40 °C in the presence of MβCD-subtilisin Carlsberg (360 mg). When conversion stopped as estimated by TLC, the mixture was cooled and filtered. The solution was concentrated under vacuum at 45 °C eliminating last traces of pyridine by co-evaporation with toluene. The remaining residue was subjected to flash chromatography. Concentration of pure fractions in vacuo afforded the monoesters as amorphous white solids.

2.4.1. 1″-O-Vinyladipoylraffinose (1b)

The general procedure outlined above was followed. After 24 h the reaction was stopped and the monoester isolated (857 mg, 65%). Regioisomer **1b** was obtained with 96% regioselectivity (HPLC/MS). $R_f = 0.19$; $[\alpha]_D = +90.6$ (c 7 in methanol); HRMS (FAB): calcd for $C_{26}H_{42}O_{19}Na$ ($M + Na^+$) 681.2218, found 681.2224. ^{13}C NMR: see assignment in Table 2.

2.4.2. Monovinyladipoylmelezitose

The general procedure outlined above was followed. After 48 h the reaction was stopped and the monoester isolated (725 mg, 55%). $R_f = 0.22$; HRMS (FAB): calcd for $C_{26}H_{42}O_{19}Na$ ($M + Na^+$) 681.2218, found 681.2209. Regioisomeric proportion **2c/2a/2b** = 77/13/5 + 4% other regioisomers (HPLC/MS). ^{13}C NMR: see assignment in Table 2.

2.5. Synthesis of diesters catalyzed by MβD-subtilisin Carlsberg

For the synthesis of the diesters the following monoesters were used as substrates: monoester prepared from raffinose with Lipozyme TL IM (containing only regioisomer **1a**); monoester prepared from melezitose with Lipozyme TL IM (containing this regioisomeric distribution, **2b/2a/2c** = 57/37/4 + 2% of other regioisomers); monoester prepared from melezitose with Novozym 435 (containing this regioisomeric distribution, **2a/2c/2b** = 62/24/14)

In a typical experiment a solution of monoester (660 mg, 1.0 mmol) in anhydrous pyridine (5 mL) and divinyl adipate (595 mg, 3.0 mmol) were shaken with orbitalic stirring (250 rpm) at 40 °C in the presence of MβCD-subtilisin Carlsberg (180 mg). After 4 days the mixture was cooled and filtered, although the majority of starting material was not converted as seen by TLC. The solution was concentrated under vacuum at 45 °C eliminating last traces of pyridine by co-evaporation with toluene. The remaining residue was subjected to flash chromatography. Concentration of pure fractions in vacuo afforded the diesters as amorphous white solids. The unreacted starting material was also recovered.

2.5.1. 6,1″-Di-O-Vinyladipoylraffinose (1c)

The general procedure outlined above was followed, using as substrate the monoester prepared from raffinose with Lipozyme TL IM. After 96 h the reaction was stopped and the diester isolated (358mg, 44%). $R_f = 0.55$; $[\alpha]_D = +77.9$ (c 7 in methanol); Regioisomer **1c** was obtained with 95% regioselectivity (HPLC/MS). HRMS (FAB): calcd for $C_{34}H_{52}O_{22}Na$ ($M + Na^+$) 835.2848, found 835.2843. ^{13}C NMR: see assignments in Table 3.

2.5.2. 6′,6″-Di-O-vinyladipoylmelezitose (2d)

The general procedure outlined above was followed, using as substrate the monoester prepared from melezitose with Novozym 435. After 96 h the reaction was stopped and the diester isolated (212 mg, 26%). $R_f = 0.60$; regioisomer **2d** was obtained with 87% regioselectivity (HPLC/MS). HRMS (FAB): calcd for $C_{34}H_{52}O_{22}Na$ ($M + Na^+$) 835.2848, found 835.2844. ^{13}C NMR: see assignments in Table 3.

2.5.3. Divinyladipoylmelezitose

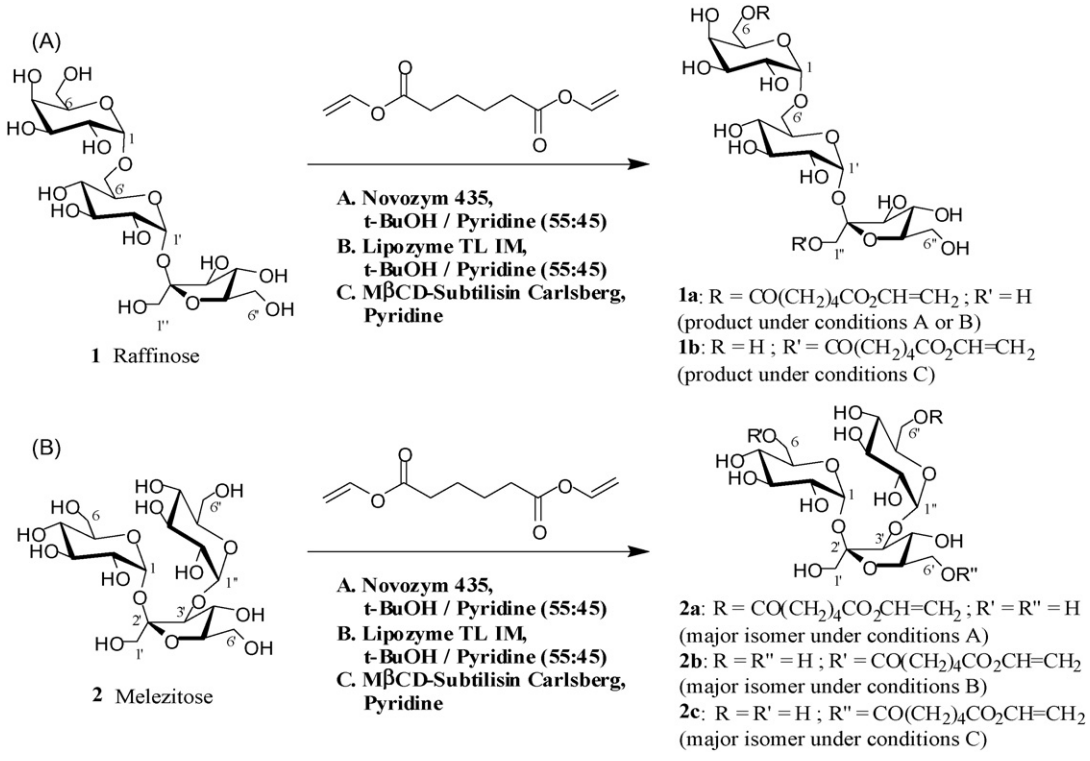
The general procedure outlined above was followed, using as substrate the monoester prepared from melezitose with Lipozyme TL IM. After 96 h the reaction was stopped and the diester isolated (81 mg, 10%). Regioisomer **2d** was obtained with 67% regioselectivity (HPLC/MS).

3. Results and discussion

One of the main problems related to the enzymatic acylation of free mono-, and especially, di- and trisaccharides is the low solubility of these compounds in the non-polar organic solvents that are more suitable to preserve the catalytic activity of lipases. The main approach to overcome this problem consists in the use of organic solvents mixtures such as *t*-amyl alcohol–DMSO [25], *t*-butanol–pyridine [21,26] or acetonitrile–DMSO [27] capable of maintaining a satisfactory enzymatic activity and to solubilize the carbohydrates to a large extent. In the case of some proteases like subtilisin Carlsberg, the enzymatic acylation can be carried out in DMF or pyridine [28] and consequently the solubility of the sugar in the reaction media is not a limitation.

Our initial goal was the acylation of raffinose (**1**) and melezitose (**2**) (see Scheme 1) with divinyl adipate using as biocatalysts the immobilized lipases from *Thermomyces lanuginosus* (Lipozyme TL IM) and *Candida antarctica* B (Novozym 435), and the protease subtilisin Carlsberg prepared by lyophilization with methyl-β-cyclodextrin (MβCD) as excipient [24]. The reaction conditions employed were essentially identical to the conditions we have previously described for the same substrates using vinyl laurate as acylating agent [21]. Briefly, the trisaccharides are employed in their amorphous form obtained by lyophilization and a mixture of *t*-BuOH-pyridine at 60 °C is used as the reaction media for the reactions catalyzed by lipases and pure pyridine at 40 °C for the reactions catalyzed by the protease. Table 1 summarizes the isolated yield of monoester obtained in each case. The yields obtained are similar for lipases and slightly lower for protease subtilisin than those obtained using vinyl laurate as the acylating agent [21].

The regioisomeric distribution in the isolated vinyl fatty acid sugar esters was determined by HPLC/MS [23] and the position of acylation in the main regioisomer obtained in each case was verified by ^{13}C NMR experiments according to the general strategy described by Yoshimoto et al. [29]. When compared with the parent carbohydrate, the ^{13}C NMR spectra of all new derivatives (Tables 2 and 4) showed the expected downfield shift of the peak corresponding to the *O*-acylated carbon and an upfield shift of the peak corresponding to the neighbouring carbon. On the other hand, routine ^{13}C NMR acquisition in these regioisomeric mixtures easily allowed the identification of the signals corresponding to the main regioisomer in the mixture due to their larger intensity [23]. The ^{13}C NMR chemical shifts assignments (Tables 2 and 4) are also based in the comparison with the corresponding monolaurates [21]. Regioisomeric distribution, i.e. chromatographic profiles (Fig. 1) is in excellent agreement with our previously reported results where vinyl laurate was used as the acylating agent [21]. This is not surprising, since the differences in overall length and size among vinyl laurate and divinyl adipate seem to be small. Moreover, the reported effect [30] that states that the chain length of the acylating agent may influence the regioselectivity of the transesterifications catalyzed by subtilisin is not observed. The vinyladipoyl monoesters prepared from these trisaccharides may be polymerized by radical reactions [11,19] to yield novel glycopolymers.



Scheme 1.

Table 1
Acylation of trisaccharides with divinyl adipate as acylating agent and different enzymes as biocatalyst

Carbohydrate	Enzyme[b]	Reaction time (h)	Yield (%)[a]	Main regioisomer	Selectivity (%)
Raffinose	A	72	42	6-*O*-Vinyladipoylraffinose	>99
Melezitose	A	72	38	6″-*O*-Vinyladipoylmelezitose	62
Raffinose	B	24	75	6-*O*-Vinyladipoylraffinose	>99
Melezitose	B	72	50	6-*O*-Vinyladipoylmelezitose	57
Raffinose	C	24	65	1″-*O*-Vinyladipoylraffinose	96
Melezitose	C	24	55	6′-*O*-Vinyladipoylmelezitose	77

[a] Yields are referred to isolated monoester fraction and selectivity corresponds to percentage of main regioisomer as found by HPLC/MS.
[b] Enzymes: A (Novozym 435 in *t*-BuOH-pyridine, 55:45 (v/v), at 60 °C), B (Lipozyme TL IM in *t*-BuOH-pyridine, 55:45 (v/v), at 60 °C), C (MβCD-subtilisin Carlsberg in pyridine at 40 °C).

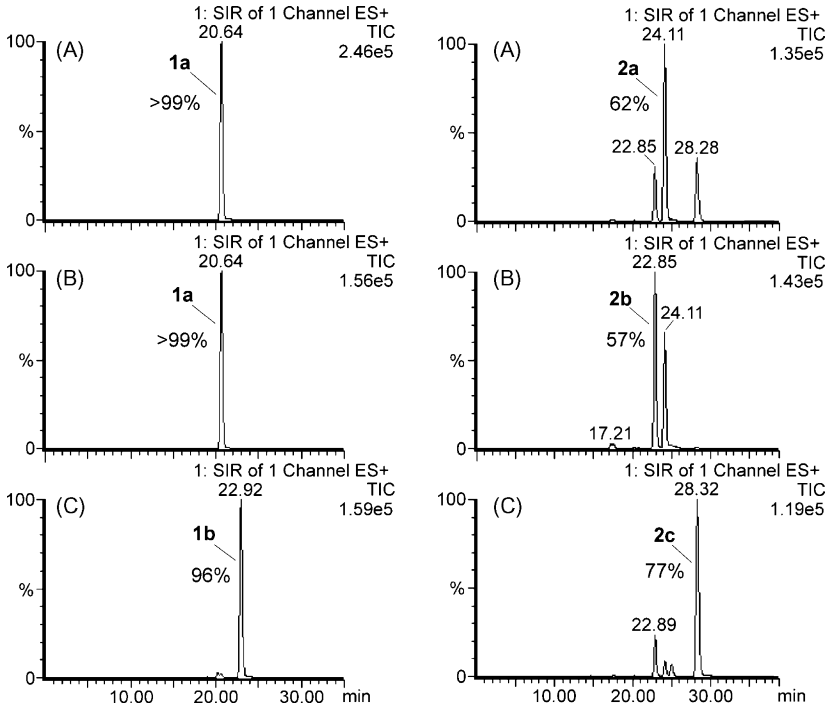


Fig. 1. HPLC/MS chromatograms showing the regioisomeric distribution in the isolated monoesters of each biocatalyzed reaction (raffinose derivatives: left chromatograms, melezitose derivatives: right chromatograms). Biocatalysts: (A) Novozym 435, (B) Lipozyme TL IM, (C) MβCD-subtilisin Carlsberg. Percentage of the main regioisomer obtained in each case is also indicated.

Next, we prepared vinyladipoyl diesters of raffinose and melezitose that could be polymerized enzymatically in the presence of diols to yield linear polyesters as it has been previously described for analogous diesters of sucrose [22]. We used the monoesters prepared with lipase catalysis as starting material and transesterified them with divinyl adipate using MβCD-subtilisin Carlsberg as biocatalyst to obtain the corresponding diesters (see Scheme 2). A similar approach to obtain sucrose fatty acid diesters has been previously described using sucrose monoesters prepared by protease catalysis as starting material and lipases as biocatalyst for the second acylation step [30,31]. In our case, the reaction conditions employed were essentially identical to the conditions used for the acylation of the free trisaccharides (see Section 2). It should be noted that the starting material contains only one regioisomer in the case of raffinose and more than one in the case of melezitose derivatives (Fig. 1). In both cases less conversion of the starting monoester than in the case of the free sugar was observed, despite the longer reaction time (96 h), which resulted in low to moderate yields of diester product (Table 3). Nevertheless, a main regioisomer is formed for both trisaccharides (Fig. 2), and ^{13}C NMR experiments (Table 4) confirm that the preferential position of acylation is the expected based on the results obtained with the free sugar (1″-OH in raffinose and 6′-OH in melezitose), producing diesters **1c** and **2d**. The raffinose monoester used as starting material contains only regioisomer **1a** (Fig. 1), and the preferential acylation position for this protease in the case of the trisaccharide raffinose, 1″-OH, is available for the second acylation step. As a result, the obtained diester is the expected 6,1″-di-*O*-vinyladipoylraffinose (**1c**).

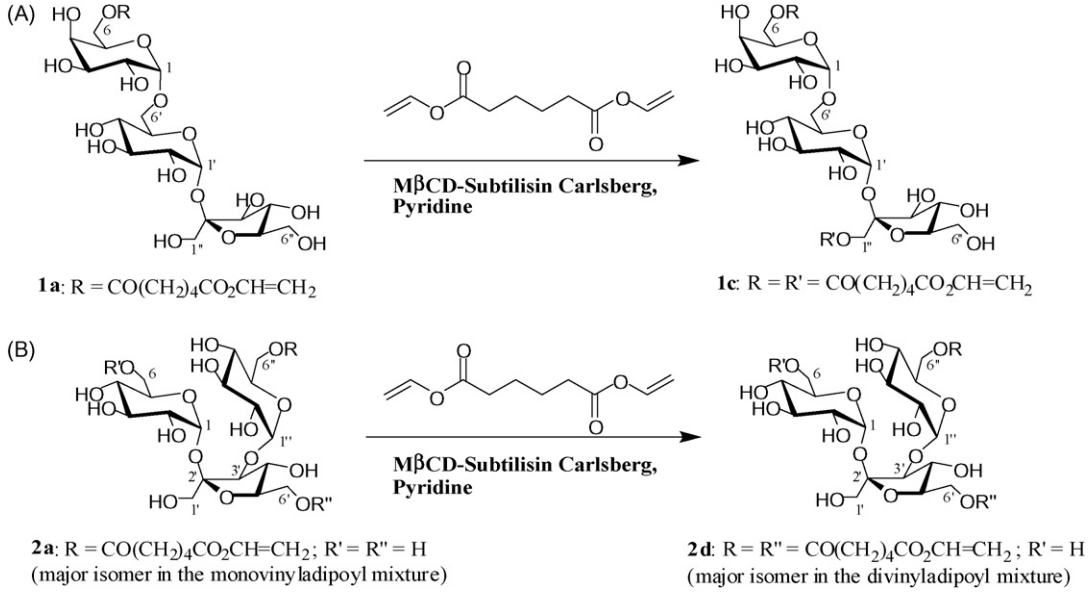
In the case of reactions with melezitose monoesters as starting material, the HPLC/MS analysis of the recovered unreacted

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

Compound	C-1	C-2	C-3	C-4	C-5	C-6	C=O	$-CH=CH_2$	$-CH_2-$
1 (Raffinose)									
α-Galactopyranosyl-(1 → 6)	99.2	68.8	69.7	69.1	71.1	**60.8**			
α-Glucopyranosyl-(1 → 2)	91.8	71.7	73.1	70.5	71.5	66.9			
β-Fructofuranose	**62.4**	**104.2**	77.2	74.4	82.5	62.4			
1a (6-*O*-Vinyladipoylraffinose)	62.0	104.1	77.0	74.3	82.4	62.3			23.4
α-Galactopyranosyl-(1 → 6)									33.0
α-Glucopyranosyl-(1 → 2)	98.9	68.2	69.0	68.2	**69.0**	**63.7**	172.5	141.2	32.6
β-Fructofuranose	91.7	71.4	72.7	70.3	71.1	66.7	170.2	98.1	23.6
1b (1″-*O*-Vinyladipoylraffinose)	**62.4**[a]	**102.1**	76.5	73.3	82.6	62.0			23.4
α-Galactopyranosyl-(1 → 6)									33.0
α-Glucopyranosyl-(1 → 2)	99.1	68.4	69.4	68.8	71.1	60.6	172.1	141.2	32.6
β-Fructofuranose	91.9	71.3	72.6	70.2	71.2	66.7	170.2	98.1	23.6
2 (Melezitose)									
α-Glucopyranosyl-(1 → 2)	91.9	71.8	73.3	70.1	**72.7**	**60.8**			
β-Fructofuranosyl-(3 → 1)	62.6	104.4	81.5	73.4	**82.8**	**62.0**			
α-Glucopyranose	98.2	72.1	73.7	70.1	**72.5**	**60.8**			
2a (6″-*O*-Vinyladipoylmelezitose)	98.2	71.8	73.7	69.8	**71.7**	**63.1**			23.5
α-Glucopyranosyl-(1 → 2)									32.9
β-Fructofuranosyl-(3 → 1)	91.8	71.7	73.1	70.0	72.4	60.7	172.7	141.2	32.7
α-Glucopyranose	62.5	104.3	81.5	73.5	82.9	62.3	170.2	98.0	23.7
2b (6-*O*-Vinyladipoylmelezitose)	98.3	72.0	73.5	70.0	72.6	60.7			23.5
α-Glucopyranosyl-(1 → 2)									33.0
β-Fructofuranosyl-(3 → 1)	91.7	71.7	73.0	69.8	**71.6**	**63.7**	172.7	141.2	32.7
α-Glucopyranose	62.8	104.3	81.4	73.4	82.8	62.7	172.0	98.0	23.7
2c (6′-*O*-Vinyladipoylmelezitose)	98.1	72.0	73.9	70.2	72.5	60.9			23.4
α-Glucopyranosyl-(1 → 2)							172.6	141.2	32.9
β-Fructofuranosyl-(3 → 1)	91.9	72.0	73.1	70.0	72.6	60.7	170.2	98.0	32.6
α-Glucopyranose	61.6	104.7	80.7	73.5	**79.3**	**65.8**			23.7

All spectra were acquired in DMSO-d_6. Data for **1** and **2** are taken from Ref. [32]. The carbons were the induced shift effect due to acylation is observed are indicated in bold.

[a] Downfield shift is not observed in this case, but clear upfield is observed for the vecinal C-2.



Scheme 2.

Table 3
Acylation of monoesters with divinyl adipate as acylating agent and MβCD-subtilisin Carlsberg as biocatalyst

Substrate[b]	Reaction time (h)	Yield (%)[a]	Main regioisomer	Selectivity (%)
A	96	44	6,1″-Di-*O*-vinyladipoylraffinose	95
B[c]	96	10	6′,6″-Di-*O*-vinyladipoylmelezitose	67
C[c]	96	26	6′,6″-Di-*O*-vinyladipoylmelezitose	87

[a] Yields are referred to isolated diester fraction and selectivity corresponds to percentage of main regioisomer as found by HPLC/MS.
[b] Substrates: A (monoester prepared from raffinose with Lipozyme TL IM); B (monoester prepared from melezitose with Lipozyme TL IM); C (monoester prepared from melezitose with Novozym 435). Reactions were carried out in pyridine at 40 °C.
[c] Mixture of regioisomers.

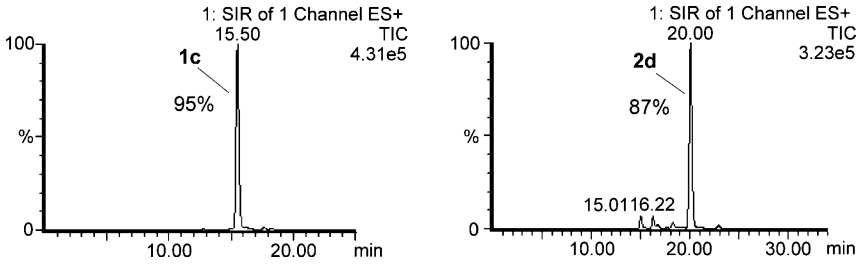


Fig. 2. HPLC/MS chromatograms showing the regioisomeric distribution in the isolated diesters. Starting materials were raffinose monoester prepared with catalysis by Lipozyme TL IM (left chromatogram) and melezitose monoester prepared with catalysis by Novozym 435 (right chromatogram). Percentage of the main regioisomer obtained in each case is also indicated.

material compared with the starting material (Fig. 3) clearly shows that the peak corresponding to 6″-*O*-acylmelezitose (**2a**) has relatively decreased more rapidly than the others, which indicates that monoester **2a** is a better substrate for subtilisin than the other regioisomers. This observation may partially explain the yields of diester obtained in each case for the melezitose diesters (Table 3) since the starting material prepared with Novozym 435, where the 6″-*O*-derivate is the main regioisomer (62%), shows a better conversion than the monoester prepared with Lipozyme TL IM, where the same regioisomer is present as a 37% of the regioisomeric mixture. In summary, the preferential acylation positions for subtilisin are the same in the free sugar and the corresponding used monoesters for both trisaccharides, indicating that the acyl chain does not affect the carbohydrate–enzyme interaction.

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

Compound	C-1	C-2	C-3	C-4	C-5	C-6	C=O	−CH=CH$_2$	−CH$_2$−
1 (Raffinose)									
α-Galactopyranosyl-(1 → 6)	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			
1c (6,1″-di-*O*-vinyladipoylraffinose)									25.1, 25.0
α-Galactopyranosyl-(1 → 6)	100.4	70.4	71.2	70.9	**69.9**	**65.0**	174.9	142.4	34.6, 34.5
α-Glucopyranosyl-(1 → 2)	94.0	72.9	74.5	72.2	73.2	68.5	174.3	98.0	34.2
β-Fructofuranose	**64.2**[a]	**104.2**	78.9	75.0	83.7	63.2	172.0		25.4, 25.3
2 (Melezitose)									
α-Glucopyranosyl-(1 → 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**			
2d (6′,6″-di-*O*-vinyladipoylmelezitose)									25.1, 25.0
α-Glucopyranosyl-(1 → 2)	93.0	73.2	75.2	71.7	**71.8**	**64.7**	175.2	142.4	34.5
β-Fructofuranosyl-(3 → 1)	64.7	105.5	85.4	75.7	**80.5**	**66.4**	175.0	98.0	34.2
α-Glucopyranose	101.7	73.6	74.8	71.9	74.0	62.8	172.0		25.4, 25.3

All spectra were acquired in CD_3OD. Data for **1** and **2** are taken from reference [33]. The carbons were the induced shift effect due to acylation is observed are indicated in bold.
[a] Downfield shift is not observed in this case, but clear upfield is observed for the vecinal C-2.

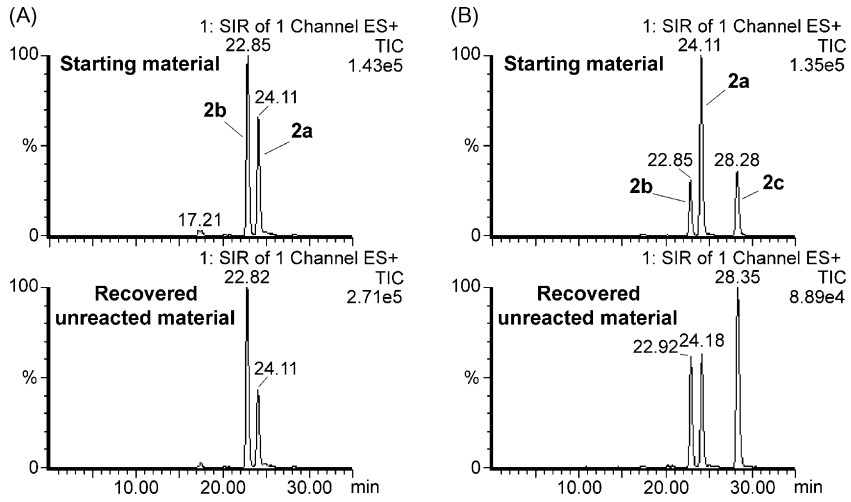


Fig. 3. HPLC/MS chromatograms showing a comparison between the regioisomeric distribution in the starting material and the recovered unreacted monoester fraction after processing the reaction of acylation catalyzed by MβCD-subtilisin Carlsberg. Melezitose monoesters used as substrates were prepared by catalysis with Lipozyme TL IM (left) or Novozym 435 (right).

4. Conclusions

In this work, novel polymerizable vinyladipoyl monoesters derived from the non-reducing trisaccharides raffinose and melezitose have been prepared with good selectivity and medium to high yields using different hydrolases as biocatalysts. The use of the monoesters prepared with lipase catalysis as substrates for a second acylation step catalyzed by subtilisin Carlsberg have lead to new vinyladipoyl diesters which could also be enzymatically polymerized in the presence of diols to yield linear polyesters. Unsurprisingly, the protease shows the same preferential acylation position in both the free and monoacylated trisaccharides. It is important to note that the isolated yields of diester are lower for both substrates than the isolated yields of monoester, indicating that the vinyl adipoyl chain of the monoester seems to affect the kinetics of transesterification with respect to the free sugar as substrate for the acylation.

References

[1] S.L. Flitsch, Curr. Opin. Chem. Biol. 4 (2000) 619.
[2] Q. Wang, J.S. Dordick, R.J. Lindhardt, Chem. Mater. 14 (2002) 3232.
[3] V. Ladmiral, E. Melia, D.M. Haddleton, Eur. Polym. J. 40 (2004) 431.
[4] A.H. Haines, Adv. Carbohydr. Chem. Biochem. 39 (1981) 13.
[5] F.J. Plou, M.A. Cruces, M. Ferrer, G. Fuentes, E. Pastor, M. Bernabé, M. Christensen, F. Comelles, J.L. Parra, A. Ballesteros, J. Biotechnol. 96 (2002) 55.
[6] R. Roy, W.K.C. Park, O.P. Srivastava, C. Foxall, Bioorg. Med. Chem. Lett. 6 (1996) 1399.
[7] W.J. Sanders, E.J. Gordon, O. Dwir, P.J. Beck, R. Alon, L. Kiessling, J. Biol. Chem. 274 (1999) 5271.
[8] K. Matsuura, H. Kitakouji, N. Sawada, H. Ishida, M. Kiso, K. Kitajima, K. Kobayashi, J. Am. Chem. Soc. 122 (2000) 7406.
[9] Y. Qiu, T. Zhang, M. Ruegsegger, R.E. Marchant, Macromolecules 31 (1998) 165.
[10] D.M. Haddleton, K. Ohno, Biomacromolecules 1 (2000) 152.
[11] Y. Miura, T. Ikeda, K. Kobayashi, Biomacromolecules 4 (2003) 410.
[12] L. Ferreira, M.H. Gil, R. Carvalho, C.F.G.C. Geraldes, D.-Y. Kim, J.S. Dordick, Chem. Mater. 14 (2002) 4009.
[13] R.A. Gross, A. Kumar, B. Kalra, Chem. Rev. 101 (2001) 2097.
[14] M. Kitagawa, Y. Tokiwa, Biotechnol. Lett. 20 (1998) 627.
[15] Q. Wu, D. Lu, Y. Cai, X. Xue, Z. Chen, X. Lin, Biotechnol. Lett. 23 (2001) 1981.
[16] Q. Wu, N. Wang, Y.-M. Xiao, D.-S. Lu, X.-F. Lin, Carbohydr. Res. 339 (2004) 2059.
[17] Y. Miura, N. Wada, Y. Nishida, H. Mori, K. Kobayashi, J. Polym. Sci. 42 (2004) 4598.
[18] Q. Wu, D.-S. Lu, Z.-C. Chen, G.-H. Yu, X.-F. Lin, Chem. J. Chin. Univ. Chin. 24 (2003) 1806.
[19] Q. Wu, Z.-C. Chen, D.-S. Lu, X.-F. Lin, Macromol. Biosci. 6 (2006) 78.
[20] Y.-M. Xiao, Q. Wu, N. Wang, X.-F. Lin, Carbohydr. Res. 339 (2004) 1279.
[21] I. Pérez-Victoria, J.C. Morales, Tetrahedron 62 (2006) 878.
[22] O.-J. Park, D.-Y. Kim, J.S. Dordick, Biotechnol. Bioeng. 70 (2000) 208.
[23] I. Pérez-Victoria, A. Zafra, J.C. Morales, Carbohydr. Res. 342 (2007) 236.
[24] I. Montanez-Clemente, E. Alvira, M. Macias, A. Ferrer, M. Fonseca, J. Rodríguez, A. Gonzalez, G. Barletta, Biotechnol. Bioeng. 78 (2002) 53.
[25] M. Ferrer, M.A. Cruces, F.J. Plou, M. Bernabé, A. Ballesteros, Tetrahedron 56 (2000) 4053.
[26] P. Degn, W. Zimmermann, Biotechnol. Bioeng. 74 (2001) 485.
[27] P. Simerská, A. Pisvejcová, M. Kuzma, P. Sedmera, V. Kren, S. Nicotra, S. Riva, J. Mol. Catal. B: Enzyme 29 (2004) 219.
[28] S. Riva, J. Chopineau, A.P.G. Kieboom, A.M. Klibanov, J. Am. Chem. Soc. 110 (1988) 584.
[29] K. Yoshimoto, Y. Itatani, T. Tsuda, Chem. Pharm. Bull. 28 (1980) 2065.
[30] G. Carrea, S. Riva, F. Secundo, B. Danieli, J. Chem. Soc., Perkin Trans. I (1989) 1057.
[31] P. Potier, A. Bouchu, G. Descotes, Y. Queneau, Synthesis 3 (2001) 458.
[32] J.C. Christofides, B.D. Davies, J. Chem. Soc., Perkin Trans. 2 (1984) 481.
[33] I. Pérez-Victoria, J.C. Morales, Tetrahedron 62 (2006) 2361.