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Subtilisin-catalyzed esterification of di- and oligosaccharides containing a D-fructose moiety

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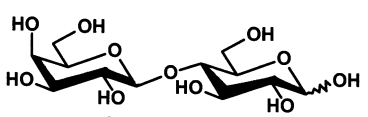
Abstract

Several di- and oligosaccharides containing a D-fructose moiety have been acylated by protease subtilisin in anhydrous dimethylformamide in the presence of the activated ester trifluoroethyl butanoate. Under the reaction conditions used, all the substrates were converted into the corresponding monobutanoates in ca. 50% isolated yields. Structural determination of the products by ^{13}C NMR indicated a strong preference of subtilisin towards the regioselective esterification of the primary hydroxyls of the fructose moiety and, specifically, of the C-1 OH, as already observed with sucrose.

Keywords: Subtilisin; Regioselectivity; Transesterification; Fructose; Lactulose; Disaccharide acylation

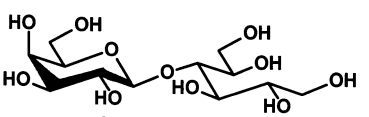
1. Introduction

The upgrading of agriculture by-products is one of the key issues of the food industry. In some instances, raw materials are enriched in specific molecules that can be potentially exploited as chemical commodities [1].



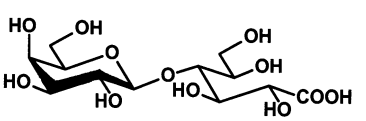
lactose (1)

Lactose (**1**) is one of these molecules since, throughout the processing of milk, it accumulates in very large amounts (estimated as 1.2 million tons worldwide annually) from the dairy by-product cheese whey. Research is in progress to develop biocatalytic processes of industrial relevance that might convert lactose into more valuable products;



lactitol (2)

that is, either into its 'first generation' derivatives such as lactitol (**2**), lactobionic acid (**3**) and lactulose (**4**) or into novel lactose-based materials, i.e., surfactants and polymers [2].



lactobionic acid (3)

In general, sugar-based surfactants can be obtained by selective esterification catalyzed by lipases or proteases in organic solvents [3], while esterification with acrylate derivatives can allow the isolation of sugar-based monomers suitable for chemical polymerization [4]. As recently reviewed, most of the research performed in this area has been devoted to the modification of monosaccharides or of the non-reducing disaccharide su-

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crose (**5**), and to the investigation of the performances of different lipases and proteases with these compounds [5,6]. Specifically, sucrose-based polymers and surfactants have been synthesized and characterized [7,8], while to the knowledge of the authors, lactose has never been considered as an additional freely available disaccharidic starting material for the production of such materials.

This paper presents the preliminary results obtained in the acylation of lactose and of its derivatives catalyzed by the protease subtilisin (EC 3.4.21.62), as well as an application of this methodology to several di- and oligosaccharides containing a D-fructose moiety.

2. Results and discussion

Our investigation on the enzymatic esterification of lactose **1** and of its derivatives **2**–**4** started by using the activated ester trifluoroethyl butanoate (TFEB) and the protease subtilisin suspended in anhydrous dimethylformamide, according to the protocol reported in the first paper published on the biocatalytic regioselective acylation of di- and oligosaccharides [9]. As previously reported [9], subtilisin-catalyzed acylation of lactose was mainly directed to the C-6 primary hydroxyl group of the galactosyl moiety. Under the same conditions, lactitol (**2**) and lactobionic acid (**3**) gave a mixture of monoesters, as deduced by ^{13}C NMR spectroscopy. On the other hand, the acylation of lactulose (**4**) was quite regioselective, affording a single monoester in 48% isolated yield (Fig. 1). The structure of 1-*O*-butanoyl-lactulose (**4a**) for this derivative was suggested by the analysis of its ^{13}C NMR spectrum, which indicated a small downfield shift of the fructosyl C-1 signal and a concomitant upfield shift of the neighboring fructosyl C-2 signal [10]. Moreover, the product structure was confirmed by submitting **4a** to the catalytic action of a β-galactosidase, which split it into two monosaccharides, identified as D-galactose and 1-*O*-butanoyl-D-fructose by comparison with authentic samples [11].

This result was quite peculiar and matched the previously reported [9] subtilisin-catalyzed selective acylation of sucrose to give 1′-*O*-butanoyl sucrose (**5a**). Compounds **4** and **5** have several structural differences: the other monosaccharidic moiety is galactose in **4**, whereas it is glucose in **5**; the glycosidic bond is β in **4** and α in **5**; the fructose hydroxyl implicated in the glycosydic bond is the C-4 hydroxyl in **4** and the C-2 hydroxyl in **5**. Therefore, we decided to extend our investigation on subtilisin regioselectivity to other di- and oligosaccharides containing a D-fructose moiety. To our knowledge, not only is this information not already available in the literature, but more generally, very few reports have been published on the selective esterification of commercially available di- and oligosaccharides catalyzed by lipases and/or proteases [12–14], despite the fact that these compounds can be useful building blocks for the preparation of more complex oligosaccharides.

The commercially available compounds that have been considered in this investigation are shown in Fig. 2. Specifically, compounds **6**–**8** are a homogeneous set of fructose-containing disaccharides in which a D-glucosyl moiety is α-linked to the fructose C-4 hydroxyl in maltulose (**6**), C-6 hydroxyl in palatinose (**7**) and C-3 hydroxyl in turanose (**8**). Additionally, we have considered the disaccharide leucrose (**9**),

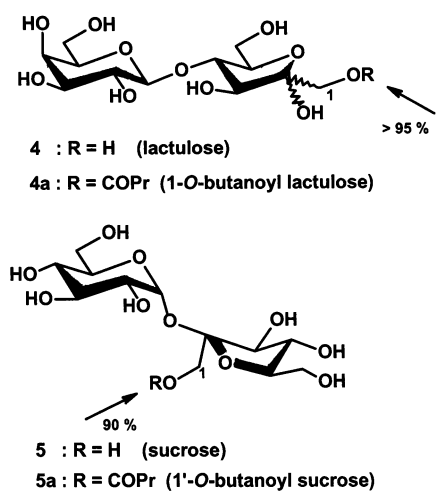

Fig. 1. Acylation of lactulose (**4**) and sucrose (**5**) catalyzed by subtilisin in anhydrous dimethylformamide. Arrows indicate the acylation site. Numbers indicate the percentage of regioselectivity.

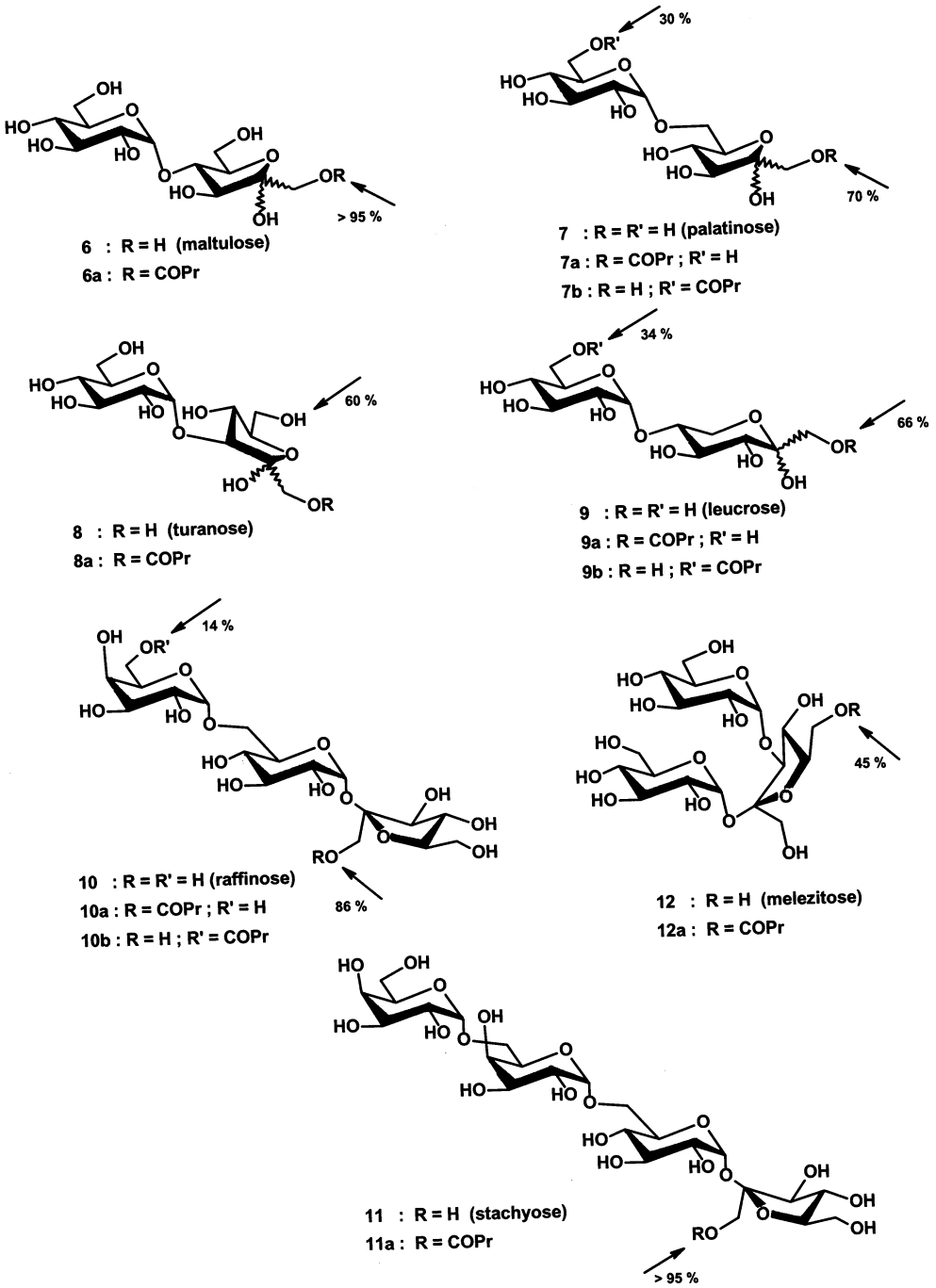

Fig. 2. Di- and oligosaccharides acylated by subtilisin in anhydrous dimethylformamide. Arrows indicate the acylation site. Numbers indicate the percentage of regioselectivity.

in which the fructosyl moiety is forced into a chair conformation due to the fact that D-glucose is α-linked to the fructose C-5 hydroxyl. Moreover, the tri- and tetrasaccharides raffinose (**10**) and stachyose (**11**), which can be considered as sucrose molecules substituted at C-6 hydroxyl groups, were investigated. Finally, the trisaccharide melezitose (**12**), which can be considered either as a C-3′ hydroxyl-glucosylated sucrose or as a C-2 hydroxyl-glucosylated turanose, was checked.

All these compounds were acylated by subtilisin following the usual protocol, and the corresponding monoesters were isolated by flash chromatography and characterized by ^{13}C NMR spectroscopy using the well-estab-

lished acylation-induced shift methodology [10] following the attribution of the signals of the carbons of the starting sugars as reported in refs. [15–19]. The results (Tables 1 and 2), clearly indicate that the fructose moiety is indeed highly preferred by subtilisin in the esterification reaction. Some of the sugars considered (i.e., maltulose **6** and the tetrasaccharide stachyose **11**, besides the already mentioned lactulose, **4**, and sucrose, **5**) were regioselectively acylated at the fructose C-1 hydroxyl, giving the corresponding monoester in ca. 50% isolated yield. Compounds **7**, **9** and **10** gave more complex mixtures of monoesters, in which, however, the corresponding 1-*O*-acyl derivatives were the most abundant products. At variance with turanose (**8**) and melezitose (**12**), the preferred acylation site was still the fructose moiety, but the C-6 hydroxyl was esterified.

The similar selectivity observed with palatinose (**7**) and leucrose (**9**) ($\approx$ 65–70% acylation at fructose C-1 OH and $\approx$ 30–35% acylation at glucose C-6 OH) rules out the possibility that a lower regioselectivity is determined by the absence of a fructopyranosyl ring structure (as in **7**) or of a fructofuranosyl structure (as in **9**). On the other hand, the excellent results obtained with the non-reducing sugars sucrose (**5**), stachyose (**11**) and to a lesser extent, raffinose (**10**), exclude the need for an open fructosyl chain to get better enzyme selectivity.

As it is obvious that the spatial orientation of the hydroxyl groups plays an essential role in the regioselective acylation of sugars by hydrolases, molecular modeling has been used recently to investigate a plausible rationalization of the observed selectivity [20–22]. Specifically, concerning sucrose, it has been shown [20] that the glucosyl moiety is exposed to a favorable interaction with a polar solvent (such as pyridine or dimethylformamide) when the fructosyl C-1 hydroxyl is acylated by subtilisin BPN′. On the other hand, acylation at the glucosyl C-6 hydroxyl would require that the entire sucrose molecule must be buried within the enzyme binding pocket, giving a sterically less favorable spatial orientation of the complex enzyme–substrate. The same approach also explains the results obtained with raffinose (**10**) and stachyose (**11**), as these compounds can be considered as sucrose derivatives modified at their glucosyl C-6 hydroxyl. The acylation of melezitose (**12**) and turanose (**8**) at their fructosyl C-6 hydroxyl, could also be explained by docking these two molecules into subtilisin active site according to the procedure suggested by ref. [20].

Recently, Sheldon and co-workers have reported the results obtained in the acylation of disaccharides by action of *Candida antarctica* lipase [12]. As it is shown in Fig. 3, the

Table 1
Subtilisin-catalyzed acylation of fructose-containing di- and oligosaccharides[a]

Substrate (mg)	Temperature (°C)	Reaction time (h)	Monoester(s) isolated yield [mg (%)]	Acylated OH
4 (684)	45	15	390 (48)	Fructosyl 1-OH
6 (342)	45	15	182 (44)	Fructosyl 1-OH
7 (342)	45	9	217 (53)[b]	Fructosyl 1-OH ($\approx$ 70%) and Glucosyl 6-OH ($\approx$ 30%)
8 (342)	45	24	227 (55)	Fructosyl 6-OH ($\approx$ 60%)[c]
9 (342)	45	15	243 (59)[b]	Fructosyl 1-OH ($\approx$ 66%) and Glucosyl 6-OH ($\approx$ 34%)
10 (504)	30	24	247 (43) 34 (6)	Fructosyl 1-OH Galactosyl 6-OH
11 (333)	30	24	181 (49)	Fructosyl 1-OH
12 (504)	30	24	287 (50)	Fructosyl 6-OH ($\approx$ 45%)[c]

[a] See Section 3.
[b] As a mixture.
[c] Main product in mixture with other unidentified monoesters.

Table 2
Physical data for acylated sugars[a]

Substrate	Product structure	Eluent[a]	R_f	$[\alpha]_D$ (H_2O, c 0.5)	Elemental analysis	
					Calcd for	Found
4	**4a**	A	0.24	−23.2	$C_{16}H_{28}O_{12}$ C, 46.60; H, 6.80	C, 46.48; H, 6.75
6	**6a**	A	0.34	47.2	$C_{16}H_{28}O_{12}$ C, 46.60; H, 6.80	C, 46.58; H, 6.84
7	Mixture of monoesters	A	0.29	n.d.[b]		
8	Mixture of monoesters	A	0.32	n.d.		
9	Mixture of monoesters	A	0.25	n.d.		
10	**10a**	B	0.23	102.2	$C_{22}H_{38}O_{17}$ C, 45.99; H, 6.62	C, 45.87; H, 6.75
	10b	B	0.39	87.4	$C_{22}H_{38}O_{17}$ C, 45.99; H, 6.62	C, 46.12; H, 6.71
11	**11a**	C	0.31	114.4	$C_{28}H_{48}O_{22}$ C, 45,65; H, 6.52	C, 45.46; H, 6.48
12	Mixture of monoesters	B	0.35	n.d.		

[a] A: 16:4:1 EtOAc:MeOH:H_2O; B: 85:30:10 EtOAc:MeOH:H_2O; C: 5:5:1 EtOAc:MeOH:H_2O.
[b] n.d., not determined.

esterification of the fructose-containing disaccharides **5**, **6** and **7** catalyzed by this lipase was mainly directed to the C-6 hydroxyl of the non-reducing glucose unit, even though the selectivity was not very high. Compared with the data reported by Sheldon's group, our results offer an additional example of complementary regioselective acylation by action of different hydrolases on the same molecule [23,24].

According to the initial goal of this investigation, we will exploit the information obtained in the regioselective modification of **1** and **4** to get new sugar-based surfactants and polymers.

3. Experimental

General methods.—Subtilisin Carlsberg (protease from *Bacillus licheniformis*) and β-galactosidase from *Aspergillus oryzae* were from Sigma. The sugars lactose (**1**), lactitol (**2**), lactobionic acid (**3**), maltulose (**6**), palatinose (**7**), turanose (**8**), raffinose (**10**), stachyose (**11**) and melezitose (**12**) were from Aldrich, while lactulose (**4**) and leucrose (**9**) were from Fluka. NMR spectra (see Tables 3–5) were recorded with a Bruker AC 300 spectrometer for solutions in D_2O or Me_2SO-d_6. Optical rotations were measured using a Perkin–Elmer 141 polarimeter. TLC were performed on E. Merck Silica Gel 60 F_{254} plates, and

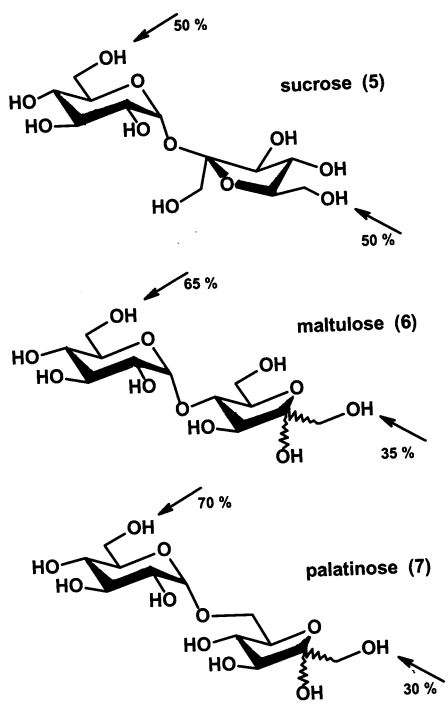

Fig. 3. Disaccharides acylated by Novozym 435 (lipase from *Candida antarctica*) in *tert*-butanol. Arrows indicate the acylation site. Numbers indicate the percentage of regioselectivity (see ref. [12]).

Table 3
^{13}C NMR data for compounds **4a**, **6a**, **7a** and **7b**

1-*O*-Butanoyl-lactulose (**4a**)[a]				1-*O*-Butanoyl-maltulose (**6a**)[a]			1-*O*-Butanoyl-palatinose (**7a**)[b]		6′-*O*-butanoyl-palatinose (**7b**)[b]	
	β-*P*	β-*F*	α-*F*		β-*P*	β-*F*	α-*F*		α-*F*	β-*F*
Galactosyl				Glucosyl			Glucosyl		Glucosyl	
C-1	101.1	103.1	103.7		103.3	101.2	100.4	99.1	99.1	
C-2	71.0	71.0	71.0		74.6	74.0	74.0	72.1	72.1	
C-3	72.8	72.8	72.8		75.6	75.6	75.6	73.6	73.6	
C-4	68.9	68.9	68.9		72.5	72.3	72.3	70.4	70.3	
C-5	75.9	75.9	75.9		75.1	75.1	75.1	72.6	69.7	
C-6	61.4	61.4	61.4		63.4	63.3	63.3	61.2	61.0	62.7	
Fructosyl				Fructosyl			Fructosyl		Fructosyl	
C-1	65.6	65.6	64.6		68.1	67.1	67.1	64.7	64.2	63.8	63.2
C-2	97.3	101.2	104.8		100.0	103.2	106.8	103.4	100.5	104.2	102.4
C-3	66.7[c]	75.6	81.4		70.1	78.9	83.7	82.4	75.3	83.0	75.7[c]
C-4	77.3	84.0	85.2		80.6	83.7	84.4	77.5	76.7	77.1	75.8[c]
C-5	66.8[c]	80.4	81.2		71.7	83.0	84.1	79.6	80.0	79.5	79.9
C-6	63.5	62.6	62.6		66.5	65.3	64.2	68.4	68.4	68.4	68.4
Butanoyl				Butanoyl			Butanoyl		Butanoyl	
C=O	176.6				176.8			172.4		172.4	
C-2	35.9				35.8			35.6		35.6	
C-3	18.2				18.1			17.9		17.9	
C-4	13.2				13.2			13.2		13.2	

[a] Solvent D_2O.
[b] Solvent: Me_2SO-d_6.
[c] Assignments may be reversed.

Table 4
^{13}C NMR data for compounds **8a**, **9a** and **9b**

6-*O*-Butanoyl-turanose (**8a**)[a]			1-*O*-Butanoyl-leucrose (**9a**)[b]	6′-*O*-Butanoyl-leucrose (**9b**)[b]
	α-*F*	β-*F*	β-*P*	β-*P*
Glucosyl			Glucosyl	Glucosyl
C-1	97.8	99.2	101.4	101.3
C-2	72.0	72.2	72.6	72.4
C-3	73.5	73.5	73.7	73.2
C-4	70.2	70.2	69.8	70.0
C-5	75.6	75.3	72.6	70.1
C-6	61.2	61.2	60.9	63.6
Fructosyl			Fructosyl	Fructosyl
C-1	61.9	63.6	64.5	64.4
C-2	105.0	102.5	96.8	97.9
C-3	85.4	80.8	68.4	68.8
C-4	73.2	73.2	70.1	70.3
C-5	80.8	79.0	79.9	79.9
C-6	65.2	65.2	62.1	62.0
Butanoyl			Butanoyl	Butanoyl
C=O	177.5		172.7	172.7
C-2	36.5		35.3	35.4
C-3	18.8		17.9	17.9
C-4	13.7		13.5	13.5

[a] Solvent D_2O.
[b] Solvent Me_2SO-d_6.

Table 5
^{13}C NMR data for compounds **10a**, **10b**, **11a** and **12a**

Compound		C-1	C-2	C-3	C-4	C-5	C-6
α-Galactopyranosyl-(1→6)-	**10a**[a]	99.3	69.3	70.3	70.1	71.8	62.0
α-glucopyranosyl-(1→2)-		93.4	71.7	73.5	70.3	72.3	66.8
β-fructofuranose		63.4	103.3	77.5	74.3	82.3	63.0
butanoate		176.9	36.5	18.8	13.7		
α-Galactopyranosyl-(1→6)-	**10b**[a]	99.1	69.1	70.1	70.1	69.6	64.9
α-glucopyranosyl-(1→2)-		93.4	71.7	73.5	70.3	72.3	66.8
β-fructofuranose		62.4	104.6	77.2	74.9	82.3	63.0
butanoate		177.3	36.5	18.8	13.7		
α-Galactopyranosyl-(1→6)-	**11a**[b]	99.0	68.6[c]	69.4	69.0[c]	71.2	60.6
α-galactopyranosyl-(1→6)-		98.9	68.6[c]	69.8	69.0[c]	68.8[c]	66.6
α-glucopyranosyl-(1→2)-		92.1	71.4	72.8	70.2	71.2	66.6
β-fructofuranoside		62.4	102.3	76.7	73.6	82.7	62.2
butanoate		172.2	35.5	18.0	13.4		
α-Glucopyranosyl-(1→3)-	**12a**[b]	98.2	72.1	73.6	70.2	72.6	60.8
β-fructofuranosyl-(2→1)-		61.7	104.8	80.9	73.4	79.5	65.8
α-glucopyranoside		92.0	71.8	73.3	70.2	72.7	60.8
butanoate		172.8	35.3	17.9	13.5		

[a] Solvent D_2O.
[b] Solvent Me_2SO-d_6.
[c] Assignments may be reversed.

visualized by spraying with a molybdate reagent prepared by dissolving $CeSO_4$ (1 g) and $(NH_4)_6Mo_7O_{24}{\cdot}4\ H_2O$ (21 g) in water (500 mL) and adding concd H_2SO_4 (31 mL) followed by heating. Preparative chromatography was performed by flash chromatography using Silica Gel 60A (230–400 mesh) with the appropriate eluent.

*General procedure for the acylation of the sugars **1**–**4**, **6**–**12**.*—In a typical procedure a 0.1 M solution of the sugars **1**–**3**, **6**–**10** and **12** in anhyd DMF, or 0.2 M of **4** or 0.05 M of **11** (10 mL) and trifluoroethyl butanoate (2 mL) were shaken in the presence of pH-adjusted subtilisin (150 mg) [9] at the temperature and for the reaction time reported in Table 1. Filtration of the enzyme and evaporation of the solvent gave a crude mixture, which was purified by flash chromatography using the eluent reported in Table 2.

*Hydrolysis of 1-*O*-butanoyl-lactulose* (**4a**) *catalyzed by β-galactosidase.*—The ester **4a** (167 mg) was dissolved in 50 mM acetate buffer pH 4.5 (10 mL). β-Galactosidase from *Aspergillus oryzae* (10 mg, ≈900 units) was added and the solution was left at rt for 6 h (TLC, 80:20:5 AcOEt:MeOH:H_2O). Water was lyophilized and the residue was purified by flash chromatography to give 80 mg (79% isolated yield) of 1-*O*-butanoyl-D-fructose (identified by comparison with an authentic sample [11]).

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