Chemo-Enzymatic Synthesis of Disaccharide Fatty Acid Esters

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A novel enzymatic method for the synthesis of disaccharide fatty acid esters was developed with immobilized *Mucor miehei* lipase (Lipozyme IM-60; Novo Nordisk, Bagsvaerd, Denmark) as a catalyst. A range of lactose and maltose monoesters was prepared in overall yields of 48–77% from the corresponding sugar acetals and fatty acids.

KEY WORDS: Enzymatic esterification, lactose-esters, lipase, maltose-esters, sugar fatty acid esters.

Enzymatic approaches to the production of sugar-based surfactants have been actively explored over the last few years (1,2). The main advantages associated with the use of biocatalysts are mild reaction conditions and the high regioselectivity typically displayed by enzymes. Although numerous recent reports have dealt with the preparation of monosaccharide (3–11) and alkyl glycoside esters (12–14), the synthesis of disaccharide esters still remains a challenging problem. This is due to the low solubility of disaccharides in those organic solvents that are generally considered suitable for biocatalysis, whereas only a few enzymes are known to retain their catalytic activity in pyridine or DMSO, which can solubilize these sugars at high concentrations (3).

To circumvent this problem, we have adopted an alternative approach previously developed for the preparation of monosaccharide fatty acid esters (9), namely the acetalization of disaccharides was attempted to improve their solubility in or miscibility with fatty acids, thus avoiding the use of highly polar solvents in the reaction medium. This communication describes the synthesis of lactose and maltose monoesters enzymatically prepared from the corresponding disaccharide acetals. The final products were obtained in good yields after acid-catalyzed cleavage of the acetal groups.

EXPERIMENTAL PROCEDURES

Chemicals. Lipozyme IM-60 (EC 3.1.1.3 lipase from *Mucor miehei*) was supplied by Novo Nordisk A/S (Bagsvaerd, Denmark). Fatty acids, disaccharides, silica gel and all organic solvents used in this study were obtained from Aldrich Chemical Co. (Gillingham, United Kingdom) and were of the highest purity available. Tetrafluoroboric acid (50% aqueous solution) and silica gel C60 TLC plates were supplied by Merck (Eastleigh, United Kingdom).

Analytical. High-performance liquid chromatography (HPLC) was carried out with a light-scattering mass detector, as previously described (9). Gas chromatography analysis was performed on a Hewlett-Packard (Winnersh, United Kingdom) series 5890A gas chromatograph equipped with flame-ionization detection (FID). One μL of trimethylsilyl derivatives, prepared according to Sweeley *et al.* (15), was applied to a Hewlett-Packard Ultra 2, 25 m × 0.2 mm fused-silica capillary column as previously described (10). Thin-layer chromatography was used for the qualitative analysis of disaccharide acetal derivatives (eluent = ethyl acetate) and disaccharide esters (ethyl acetate/2-propanol/water, 6:2:1) on silica gel C60 plates. Nuclear magnetic resonance (NMR) spectra were recorded on a JEOL (Tokyo, Japan) EX 270 Fourier transform spectrometer at 67.8 MHz with d_6-dimethylsulfoxide (DMSO) as the solvent. All the chemical shifts (δ) are reported in parts per million (ppm) relative to tetramethylsilane. Fast atom bombardment mass spectrometry (FAB-MS) spectra were obtained on a Kratos (Manchester, United Kingdom) MS9/50TC spectrometer with Xenon at 5-8 KeV. Accurate mass measurements were recorded at 1.0 milliamu resolution with PEG 600 ions as reference.

Synthesis of disaccharide acetals. 4-*O*-(4′,6′- *O*-isopropylidene-β-D-galactopyranosyl)-D-glucopyranose[4′,6′-*O*-isopropylidene- lactose] **1** and 4-*O*-(3′,4′- *O*-isopropylidene-β-D-galactopyranosyl)-D-glucopyranose [3′,4′-*O*-isopropylidene- lactose] **2** were prepared in 70 and 40% yields, respectively, by using anhydrous lactose, *p*-toluenesulfonic acid (*p*-TSA) and 2,2-dimethoxypropane in dimethylformamide (DMF) at room temperature (**1**) or at 85°C (**2**) according to Baer and Abbas (16). 4-*O*-(3′,4′-*O*-isopropylidene-β-D-galactopyranosyl)-2,3:5,6-di-*O*-isopropylidene-1,1-di-*O*-methyl-D-glucose [lactose tetra-acetal (LTA)] **3** was prepared with 50% yield by refluxing a suspension of α-lactose monohydrate and *p*-TSA in 2,2- dimethoxypropane and purified as described by Thelwall *et al.* (17). The crude preparation of **3** was obtained by neutralization of *p*-TSA with ion exchange resin (Amberlite IRA-400), filtration and evaporation of 2,2-dimethoxypropane. The resulting syrup consisted of 80% LTA, partial lactose acetals and traces of lactose. 4-*O*-(α-D-glucopyranosyl)-2,3:5,6-di-*O*-isopropylidene-1,1-di-*O*-methyl-D-glucose [maltose tri-acetal] was prepared in 20% yields by refluxing a suspension of maltose and *p*-TSA in 2,2- dimethoxypropane, and the product was subsequently purified on a silica gel column eluted with ethyl acetate. The structures of disaccharide acetals and ester products derived therefrom are shown in Figure 1.

Preparation of disaccharide acetal esters. Enzymatic esterification reactions were performed either on a 2-g scale in open-top glass vials in an incubator-shaker at 75°C (180 rpm), or on a 15-g scale in 100-mL, round-bottom flasks stirred at 180 rpm at 75°C. The reactions were conducted with an initial addition of toluene or *t*-butyl acetate (50% vol/wt) unless otherwise specified, to aid miscibility of the substrates. The solvent was then allowed to evaporate after 6 or 10 h (refer to Fig. 2 legend) after initiation of the reaction by removing the caps from the vials or by applying a vacuum (20 mbar) with the larger-scale synthesis.

*General preparation of LTA monoesters **3a–g** and lactose monoesters **4a–g**.* This approach is exemplified by the enzymatic esterification of 4-*O*-(3′,4′-*O*-isopropylidene- β-D-galactopyranosyl)-2,3:5,6-di-*O*-isopropylidene-1,1-di-*O*-methyl-D-glucose[LTA] **3** with octadecanoic acid (stearic acid) to form 6′-*O*- octadecanoyl-4-*O*-(3′,4′-*O*-isopropylidene-β-D-galactopyranosyl)-2,3:5,6-di-*O*-isopropylidene-1,1-di-*O*-methyl-D-glucose [LTA monostearate] **3f** followed by cleavage of acetal groups to yield 6′-*O*-octadecanoyl-4-*O*-(β-D- galactopyranosyl)-α,β-D-glucopyranose [lactose monostearate] **4f**.

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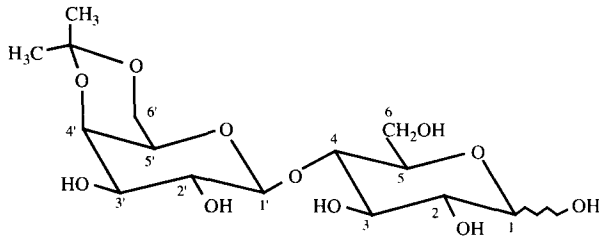
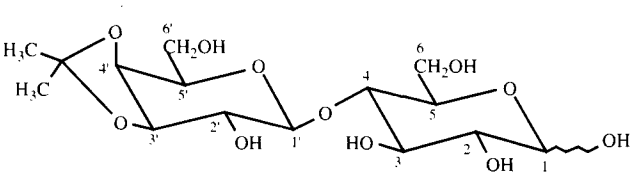
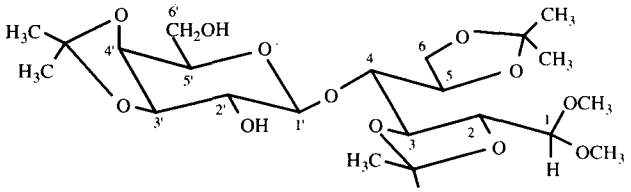


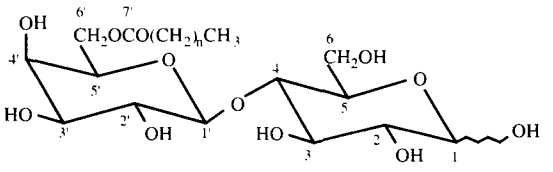
4',6'-*O*-Isopropylidene lactose **1**



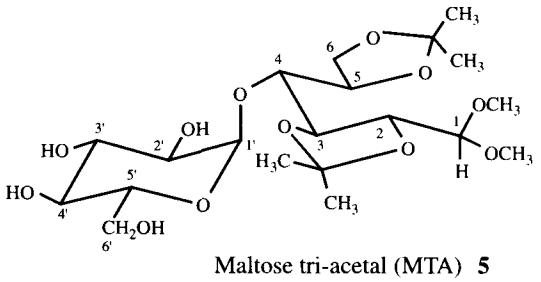
3',4'-*O*-Isopropylidene lactose **2**



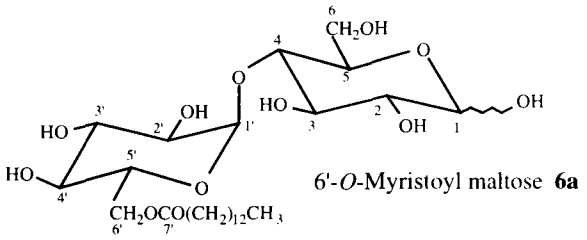
Lactose tetra-acetal (LTA) **3**



Lactose 6'-*O*-monoester **4a-g**



Maltose tri-acetal (MTA) **5**



6'-*O*-Myristoyl maltose **6a**

FIG. 1. Structures of disaccharide acetals and products obtained therefrom.

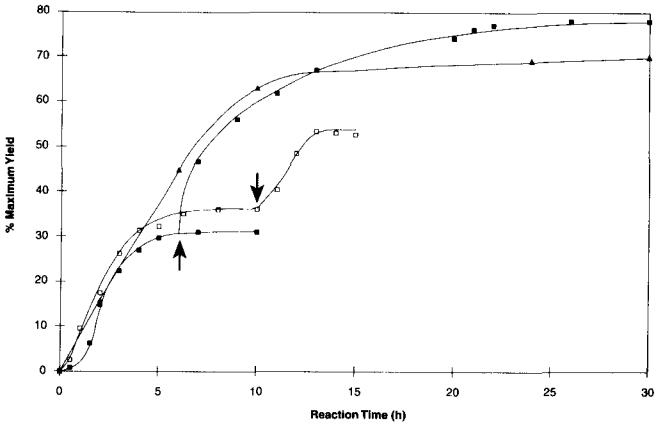


FIG. 2. Kinetics of Lipozyme-catalyzed esterification of lactose tetra-acetal 3 and maltose tri-acetal 4. ■, Pure lactose tetra-acetal, cap off after 6 h; □, pure maltose tri-acetal, cap off after 10 h; ▲, crude lactose tetra-acetal, solvent-free. Arrows indicate the times at which caps were removed from the vials to allow evaporation of the solvent.

An equimolar mixture of LTA **3** (1.00 g, 1.95 mmol) and octadecanoic acid (0.56 g, 1.95 mmol) were mixed together in a 12-mL glass vial with 1.0 mL toluene (water-saturated, 25°C) and heated to 75°C to give a homogenous liquid phase. Lipozyme (10% w/w, 156 mg) was added, and the mixture was shaken at 75°C (180 rpm) for 6 h, after which the vial was opened to allow evaporation of the solvent. After 80% conversion had been reached the reaction mixture was diluted with acetone (5 mL) and filtered, and the filtrate was evaporated under vacuum at room temperature. The syrup obtained was dissolved in petroleum ether (16 mL) and washed twice with distilled water to recover unreacted **3**. Minor amounts of **3f** were back-extracted from the combined water phases into petroleum ether (8 mL). Unreacted fatty acids were removed from the combined ether phases by washing three times with 0.2 M K_2CO_3/0.3 M $NaCl_{(aq)}$ (10 mL). The organic phase was dried with Na_2SO_4 and evaporated under vacuum at room temperature to yield a viscous, colorless syrup **3f** (1.18 g, 78%, purity >99%).

Compound **3f** (1.18 g) was dissolved in tetrafluoroboric acid/water/acetonitrile (1:5:500) (11.8 mL) in a stoppered flask and shaken for 2 h at 30°C. The final product, lactose monostearate, was insoluble in this mixture and hence precipitated out. This white solid (>95% pure) was filtered and washed with acetonitrile to remove traces of residual acid and subsequently dried before recrystallization from

TABLE 1

Yields and Analytical Data for Disaccharide Fatty Acid Esters

Enzymatic yield (%)[a]	Final product	Overall yield (%)[b]	FAB-MS[c] [M − H]
78 (**3a**)	**4a** (lactose C8:0)	61	467.2143 (467.2128)
77 (**3b**)	**4b** (lactose C10:0)	65	495.2522 (495.2441)
77 (**3c**)	**4c** (lactose C12:0)	69	523.2834 (523.2754)
81 (**3d**)	**4d** (lactose C14:0)	77	551.2932 (551.3067)
83 (**3e**)	**4e** (lactose C16:0)	76	579.3407 (579.3380)
82 (**3f**)	**4f** (lactose C18:0)	75	607.3762 (607.3693)
80 (**3g**)	**4g** (lactose C18:1)	74	605.3659 (605.3606)
52 (**5a**)	**6a** (maltose C14:0)	48	551.3093 (551.3067)

[a]Yields of disaccharide acetal esters after enzymatic reaction.
[b]Yields of monoesters after cleavage of isopropylidene groups calculated on the basis disaccharide acetals.
[c]Accurate mass measurements, calculated values are reported in brackets. FAB-MS, fast atom bombardment-mass spectrometry.

TABLE 2

^{13}C Nuclear Magnetic Resonance Chemical Shifts of Disaccharide Fatty Acid Esters[a]

	4a	**4b**	**4c**	**4d**	**4e**	**4f**	**4g**	**6a**
C-1α	91.9	91.8	91.8	92.1	91.8	92.1	91.9	92.0
C-1β	96.6	96.6	96.6	96.8	96.6	96.8	96.7	96.8
C-2α	69.6	69.6	69.6	69.8	69.6	69.9	69.7	71.9
C-2β	74.4	74.4	74.4	74.7	74.4	74.6	74.4	74.4
C-3α	71.2	71.2	71.2	71.4	71.2	71.4	71.2	72.6
C-3β	74.5	74.5	74.5	74.8	74.5	74.7	74.5	76.5
C-4α	80.8	80.9	80.9	81.0	80.9	81.1	80.9	80.8
C-4β	80.4	80.4	80.4	80.6	80.4	80.7	80.5	80.4
C-5α	72.1	72.0	72.1	72.3	72.1	72.2	72.1	70.4
C-5β	74.7	74.7	74.7	74.9	74.7	74.9	74.7	75.2
C-6α	60.6	60.6	60.6	60.8	60.6	60.3	60.3	60.5
C-6β	60.6	60.6	60.7	60.8	60.6	60.7	60.6	60.7
C-1′	103.3	103.3	103.3	103.5	103.5	103.6	103.4	100.9
C-2′	70.0	70.2	70.0	70.4	70.2	70.2	70.1	72.4
C-3′	72.8	72.8	72.8	72.9	72.8	73.0	72.9	73.2
C-4′	68.1	68.1	68.1	68.3	68.1	68.4	68.2	70.2
C-5′	72.3	72.3	72.3	72.5	72.3	72.5	72.4	70.6
C-6′	63.0	63.0	63.0	63.2	63.0	63.4	63.0	63.5
C-7′	172.6	172.6	172.6	172.8	172.6	173.2	172.7	172.7
CH_2 chain	33.2	33.2	33.1	33.3	33.2	33.5	33.2	33.4
	30.8	31.0	31.0	31.2	31.0	31.4	31.0	31.2
	28.2	28.6	28.7	28.9	28.7	29.1	28.9	29.2
	28.0	28.4	28.6	28.8	28.6	29.0	28.6	28.9
	24.1	28.3	28.4	28.6	28.4	28.8	28.4	28.8
	21.7	28.2	28.2	28.4	28.3	28.6	28.3	28.5
		24.1	24.1	24.3	24.1	24.4	26.4	28.4
		21.8	21.7	22.0	21.8	22.2	24.1	24.3
							21.8	21.9
CH_3	13.6	13.6	13.6	13.8	13.6	14.0	13.6	13.7

[a]At 67.80 MHz in d_6-dimethylsulfoxide (DMSO), in ppm from tetramethylsilane.

methanol to yield white crystals **4f** (>99% pure). Overall synthetic yields and FAB-MS of **4a–g** are summarized in Table 1, and ^{13}C NMR data are detailed in Table 2.

6′-*O*-(α-D-glucopyranosyl)-2,3;5,6-di-*O*-methyl-D-glucose [maltose tri-acetal monomyristate] **5a** and 6′-*O*-tetradecanoyl-4-*O*-(α-D-glucopyranosyl)-α,β-D-glucopyranose [maltose monomyristate] **6a** were synthesized from **5** by applying the procedures described in the previous paragraph. This involved lipase-catalyzed esterification of maltose tri-acetal **5** with tetradecanoic acid (myristic acid) to form maltose tri-acetal monomyristate **5a**, followed by HBF_4-catalyzed cleavage of the acetal groups (18) to yield maltose monomyristate **6a**. Yields and analytical data are detailed in Tables 1 and 2.

RESULTS AND DISCUSSION

Initially, we investigated Lipozyme-catalyzed esterification of lactose acetals **1–3** (Fig. 1) with myristic acid in a range of organic solvents (toluene, *t*-butyl acetate, 2-hexanone, *t*-amyl alcohol, hexane). Only traces of product were detected when 4′,6′-*O*-isopropylidene-lactose **1** was employed as substrate after five days of incubation at 60°C. Although the reaction occurred more readily with 3′,4′-*O*-

isopropylidene-lactose **2** under the same conditions, the yields of 15–20% were unattractive for preparative synthesis. On the contrary, LTA **3** was found to be a much better substrate despite potential steric hindrances, and up to 80% conversion was obtained in 24 h with this substrate (Fig. 2). The comparison of results obtained with **1**, **2** and **3** suggested that the *M. miehei* lipase exhibited the highest reactivity toward the primary hydroxyl group of the glucose ring.

LTA **3** was sufficiently soluble in the molten fatty acid for the reaction to proceed, even in the absence of added solvents. Thus, the esterification kinetics of a crude preparation of **3** (syrup, obtained as described in the Experimental Procedures section) were virtually identical with and without addition of solvents. However, when highly purified crystalline **3** was used, the enzyme kinetics were much improved after the initial addition of *t*-butyl acetate or toluene (50% vol/wt). This was probably due to the facilitation of mass transfer in an otherwise semi-solid reaction mixture. The solvent was allowed to evaporate after 6 h (Fig. 2), leaving a viscous liquid in which the reaction was driven to completion by removal of the water produced. This indicated that the formation of a liquid phase was of primary importance and that the nature of the solvents used did not influence the reaction. The addition of solvents appeared to be even more crucial in the esterification of maltose tri-acetal **5**. This substrate is less soluble in molten fatty acids, and poor conversion was observed in the absence of added solvents. The high regioselectivity of *M. miehei* lipase led to the exclusive formation of 6′ monoesters within the reaction time used. However, some diesters of **5** were detected after 24 h and, hence, this reaction was routinely carried out for 15 h.

Having established optimal conditions for the esterification of the disaccharide acetals, the reaction was scaled up and monoesters of lactose and maltose were prepared from a range of saturated fatty acids and oleic acid. The results are summarized in Table 1. The final products, 6′-*O*-acyl lactoses **4a–g** and 6′-*O*-myristoyl maltose **6a** were then obtained after acid-catalyzed cleavage of the acetal groups. HBF_4 was the most efficient catalyst and was used on a routine basis. Similar results were obtained with acetic acid (1:1:4 $CH_3COOH/H_2O/CH_3CN$, 75°C), but the cleavage was significantly slower (30 h), and a range of partially deprotected products was observed by HPLC and thin-layer chromatographic analysis at intermediate stages of the reactions. Final products precipitated directly from the reaction mixtures to yield both α and β anomers of the disaccharide esters (Table 2), where the ratio was dependent on the deprotection conditions, as shown by NMR spectroscopy. Pure α anomers of lactose esters **4a–g** could be obtained, however, by subsequent recrystallization from methanol. Overall yields quoted in Table 1 are based on disaccharide acetals because highly purified, crystalline starting materials were used in these syntheses. It should be noted that only slightly lower yields were obtained, based on lactose, when these reactions were carried out with the crude syrup.

We conclude that the developed methodology provides an attractive synthetic route to the regioselective synthesis of some disaccharide fatty acid monoesters. LTA **3** proved to be the best substrate for the preparation of 6′-*O*-acyl-lactoses. The initial addition of a small amount of solvent (50% vol/wt) proved to be beneficial for esterification of pure, crystalline **3** and maltose tri-acetal **5**. However, no purification of LTA was required prior to enzymatic esterification, and the reaction was carried out under solvent-free conditions when using crude **3**, which was obtained as an oil containing a range of partial acetals. Owing to the high regioselectivity of the lipase, partial acetals present in the oil either remained unreactive (e.g., 6′-protected species) or were esterified to yield the same final product after hydrolysis of the acetal groups.

All products were obtained in good yields, and their emulsifying properties are currently being investigated in comparison with commercially available sucrose esters. It should be added that esters of reducing sugars cannot be prepared in satisfactory yields through conventional high-temperature (trans)esterification.

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