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Enzymatic synthesis of 3-*O*-methyl-4-*O*-β-D-galactopyranosyl-D-glucose (3-*O*-methyl-lactose); a potential agent for the assessment of intestinal lactase activity

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Abstract

Enzymatic synthesis of 3-*O*-methyl-4-*O*-β-D-galactopyranosyl-D-glucose (3-*O*-methyl-lactose) has been attempted using both galactosyltransferase and galactosidase activities. The transferase-catalysed reaction produces exclusively the desired product in β-1,4-glycosidic linkage whereas the galactosidase-catalysed reactions predominantly form a 1,6-linked disaccharide. With galactosidase, in order to change the regioselectivity, blocking of the 6-position of 3-*O*-methyl-D-glucose and anomeric modification of the acceptor structure were investigated. Although acetylation of the 6-position of 3-*O*-methyl glucose catalysed by lipase was successful, the synthesis of the desired disaccharide did not occur.

Keywords: Lactasia; Enzymatic synthesis; Galactosyltransferase; β-Galactosidase; 3-*O*-methyl-lactose

1. Introduction

Internal disaccharidases in higher animals are located in the luminal membrane of the intestinal mucosa, where they hydrolyse disaccharides prior to the absorption of sugars as monosacchrides [1]. Internal disaccharidase deficiency is a frequent cause of gastrointestinal symptoms and it is reported that as many as 50–90% of adult non-white subjects may be lactase deficient [2–4]; hence, its diagnostic evaluation is important in pediatrics and gastroenterology.

There are conventional non-invasive assessments to evaluate intestinal lactase activity such as the oral lactose tolerance test [5], the appearance of hydrogen in the breath after ingestion of disaccharide [6–9] or assay of disaccharidase activity in tissue obtained by jejunal biopsy specimen [10,11]. These methods, however, are subject to limitations. An efficient method to evaluate intestinal lactase which avoids errors due to variations in monosaccharide transport and metabolism and may thus permit a more specific

Abbreviations: PNP-Gal, 4-nitrophenyl-β-D-galactopyraniside; 3-*O*-methyl-lactose, 3-*O*-methyl-4-*O*-β-D-galactopyranosyl-D-glucose; 3-*O*-methyl-allolactose, 3-*O*-methyl-6-*O*-β-D-galactopyranosyl-D-glucose; UDP-Glc, Uridine-5′-diphospho[1]-α-D-glucopyranoside; UDP, uridine-5′-diphosphate; TEP, triethylphosphate

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and quantitative assessment of intestinal disaccharidase activity is therefore desirable.

A possible non-invasive procedure could be based on the oral administration of '3-*O*-methyl-lactose' which is known to be an acceptable substrate for intestinal lactase for the diagnosis of hyperlactasia by oral administration, followed by estimation of 3-*O*-methyl-glucose in the urine [12–14]. The 3-*O*-methyl-D-glucose is efficiently absorbed in the small intestine but slowly metabolised [15] and there is no evidence that it is converted to another sugar in the intestine [16].

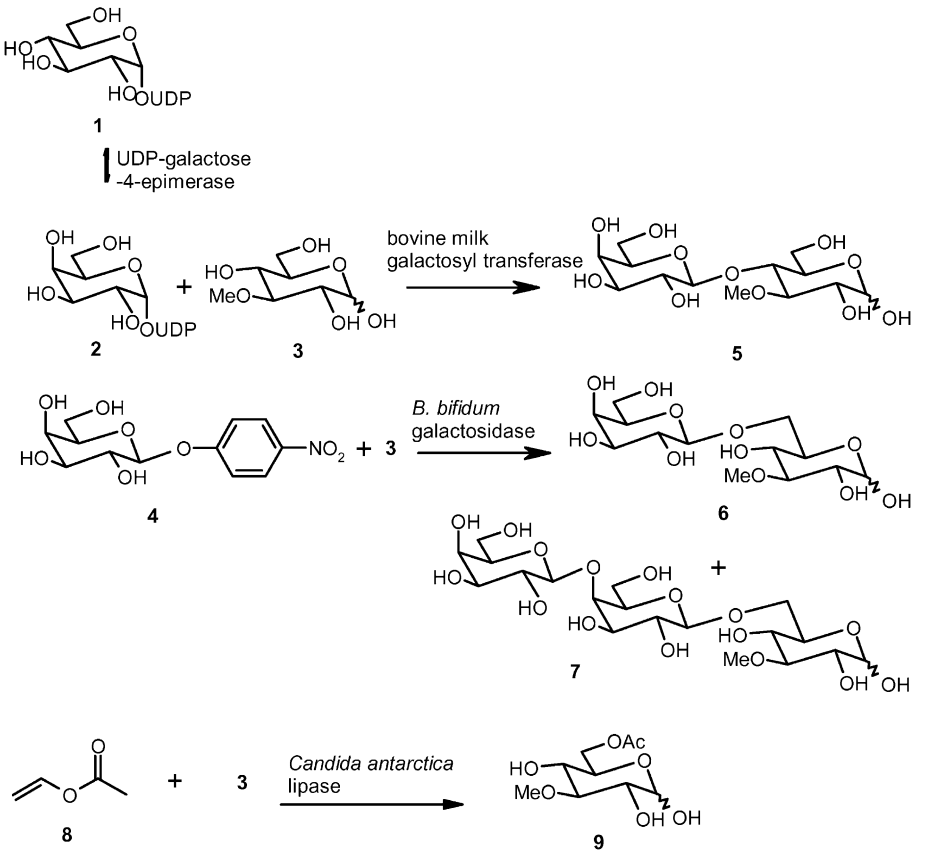
Chemical syntheses of this disaccharide have been achieved by Martin-Lomas and co-workers [13,14]; the most efficient gave a yield of 6% in six steps, including three column separations, starting from lactose [14].

We are, however, interested in introducing enzymes for the synthesis of the disaccharide because of the potential simplicity of the procedures. In this work, the synthesis with β-1,4-galactosyltransferase and various β-galactosidases has been investigated. In the presence of lactalbumin β-1,4-galactosyltransferase transfers galactose efficiently to glucose [17], however, with 3 substituted derivatives a reduction in rate of 90% was reported [18]. The β-galactosidases offered the prospect of lower cost and absence of cofactor requirement, but possibly low yield and undesired regioselectivity. To this end, several different strategies were attempted (see Scheme 1).

2. Experimental

2.1. General methods

Chemicals were purchased from Sigma and Aldrich (Dorset, UK) and were used without further



Scheme 1.

purification, unless otherwise stated. Lipase, Novozyme 435 (Candida antarctica lipase) was kindly donated by Novo Industries. The β-1,4-galactosyltransferase from bovine milk (E.C 2.4.1.22) and UDP-galactose 4-epimerase (UDP-glucose 4-epimerase EC 5.1.3.2) were purchased from Sigma. Pectinase G "Amano", Biodiasmin B-100, Transglucosidase L "Amano", Gluzyme NL4.2, Hemicellulase "Amano" 90, Biozyme S Conc., Cellulase A "Amano" 3 and Enzyme RP-1 were donated by Amano (Japan). The β-galactosidase from *B. bifidum* was cultured as previously described [18]. The α,β-methyl- and αβ-ethyl-3-*O*-Me-glucopyransides were synthesised by Fischer glycosidation.

^{1}H and ^{13}C NMR spectra were recorded in D_2O and CD_3OD on a Bruker AMX 600 spectrometer (^{1}H, 600.14 MHz; 13 C 150.92 MHz) with TMS or TSP (Trimethylsilyl-1-Propanesulfonic acid) as reference. Peak assignments were based on COSY and DEPT experiments. The NMR data of 3-*O*-Me-Glc-containing products are listed in Table 2.

The HPTLC was performed on pre-coated glass-backed plates (Silicagel 60 without fluorescent indicator, 10 cm × 20 cm, Merck) with *n*-propanol:AcOH:H_2O (5:4:1 v/v) as eluent and detection by charring with sulfuric acid. Column chromatography was performed on Kieselgel 60 (Merck, 230–440 mesh) using the same eluent (4:5:1). The product was extracted with methanol, filtered and the solvent was removed by rotary evaporation. The FABMS was performed on a Jeol DX-303 HF spectrometer. An HPLC system equipped with an evaporative light-scattering dedector (ELSD) was used with an Asahipak NH2P-50 column (4.6 mm × 250 mm) eluted with water–acetonitrile, 20:80% v/v,1 ml/min).

2.2. 3-*O*-methyl-4-*O*-β-D-galactopyranosyl-D-glucose (**5**)

The UDP-glucose (**1**) or UDP-galactose (**2**) (275 mg, 0.12 mmol) as the donor, 3-*O*-methyl glucose as the acceptor (**3**, 96 mg, 0.48 mmol), UDP-galactose epimerase (10 units), β1-4 galactosyltransferase (4 units), α-lactalbumin (0.5 mg), $MnCl_2$ (5 mM) and NaN_3 (0.02%) were dissolved in Tris-HCl buffer (100 mM, 5 ml, pH 7.0) (Scheme 1). The reaction was carried out at 37°C for 7 days. Samples (20 μl) were taken at various time intervals. Each sample was heated at 100°C for 2 min. The 5 μl aliquots were analysed by HPTLC and separated by preparative HPTLC. The yield of product was 6.6 mg (15%, based on the donor added). MS: *m/z* 357 $[M + H]^+$: NMR data are listed in Table 2.

2.3. 3-*O*-methyl-6-*O*-β-D-galactopyranosyl-D-glucose (**6**)

The β-galactosidase from *B. bifidum* culture broth (0.1 ml, 10 units) was incubated with PNPGal (**4**, 194 mg, 0.65 mmol) as the donor and 3-*O*-Me-Glc (**3**, 500 mg, 2.5 mmol) as the acceptor in buffer (50 mM CH_3COONa, pH 5.1, 0.4 ml) containing 50% v/v triethyl phosphate. The system was kept at 37°C for 48 h. Samples (20 μl) were taken at various time intervals. Each sample was heated at 100°C for 2 min and made up to 100 μl with deionised water. After centrifugation, 5 μl aliquots were applied to HPLC. Compound **6** was obtained in a yield of 26.1% based on donor.

The β-galactosidases from *Bacillus circulans*, *Streptococcus* 6646 K, Bovine testes, Pectinase G "Amano". Biodiasmin B-100, Transglucosidase L "Amano", Gluczyme NL4.2, Hemicellulase "Amano" 90, Biozyme S Conc., Cellulase A "Amano" 3 and Enzyme RP-1 were used with the same donor and acceptor in a buffer (50 mM CH_3COONa, pH 5.1). Reaction conditions and procedures were as described above.

2.4. 3-*O*-methyl-4-*O*-β-D-galactopyranosyl-D-galactopyranosyl-6-*O*-β-D-glucose (**7**)

Under the conditions described above one other product was found in reactions catalysed by *B. bifidum*. It was separated by liquid chromatography and identified as **7** (6%). MS: *m/z* 519 $[M + H]^+$.

2.5. 6-*O*-acetyl 3-*O*-methyl-D-glucose (**8**)

The reaction mixture contained 3-*O*-Me-Glc (7 g, 36 mmol), vinyl acetate (3.1 g, 36 mmol), lipase (Novozyme, 9 g), silica gel (14 g), molecular sieve 3 Å (36 g) and diisopropyl ether (200 ml). The reaction was carried out at room temperature with vigorous stirring for 24 h. The mixture was then diluted with

200 ml of methanol and filtered. After the solvent was removed, the concentrate was transferred to a silica gel chromatography column and eluted with chloroform-methanol (10/1v/v). The 6-*O*-acetyl-3-*O*-methyl-D-glucose (**8**, 4.82 g, 57% yield) was obtained. MS: *m/z* 237 $[M + H]^+$.

2.6. Synthesis using galactosidases with galactosides as acceptors

To solutions of PNP-Gal (635 mg, 2.1 mmol) and α,β-methyl 3-*O*-methyl-D-glucopyranoside (500 mg, 2.1 mmol) or αβ-ethyl 3-*O*-methyl-D-glucopyranoside (470 mg, 2.1 mmol) in sodium phosphate buffer (1.5 ml, pH 4.2, 50 mM), containing triethyl phosphate (1.5 ml, 50%) was added β-galactosidase (*B. bifidum*, 70 units). The other procedures were the same as described above.

3. Results and discussion

The 3-*O*-methyl-lactose was synthesised from UDP-Glc and 3-*O*-methyl-D-glucose (donor: acceptor ratio 1:4) using UDP-Gal epimerase and β-galactosyltransferase in the presence of lactalbumin. The components of the reaction mixture were identified as UDP-Glc (**2**, R_f 0.1), UDP (0.22), 3-*O*-methyl-lactose (**5**, 0.38) and 3-*O*-methyl-glucose (**3**, 0.58) by HPTLC. The 3-*O*-methyl-lactose was isolated by preparative HPTLC in a yield of 15% based on the amount of nucleotide donor.

Although galactosyltransferase exclusively produces 3-*O*-methyl-lactose, the cost of enzyme and donor would be impracticably high for large scale synthesis. We therefore screened β-galactosidases from a number of microbes for their ability to effect the same synthesis.

The enzymes from 12 different sources, viz. *Bacillus circulans*, *B. bifidum*, *Streptococcus* 6646 K, Bovine testes, Pectinase G "Amano", Biodiasmin B-100, Transglucosidase L "Amano", Glucozyme NL 4.2, Hemicellulase "Amano" 90, Biozyme S Conc., Cellulase A "Amano" 3 and Enzyme RP-1 were examined. However, with PNP-Gal as the donor and 3-*O*-methyl-glucose as the acceptor in 50% TEP which we have shown to be a useful solvent in galactosidase-catalysed synthesis [19,20], the 1,6-linkage was formed preferentially. Although not optimised, the yields of disaccharides in the glycosidase-catalysed syntheses were in the range 4–26% (see Table 1).

Fig. 1 shows the HPLC chromatogram of a reaction mixture containing PNP-Gal and 3-*O*-methyl glucose (donor:acceptor ratio 1:4), with *B. bifidum* β-galactosidase. The major peaks: PNP-Gal (**4**, 4.0 min), 3-*O*-methyl-glucose (**3**, 5.8 min), 3-*O*-methyl-allolactose (**6**, 9.65 min) and β-Gal-(1,4)- β-Gal-(1,6)-3-*O*-methyl-glucose (**7**, 13.6 min) were identified by isolation, NMR and mass spectrometry (see Section 2 and Table 2).

The yield of 3-*O*-methyl-allolactose reached a maximum after 34 h and declined thereafter under the conditions used. The trisaccharide product presumably arose from galactosyl transfer to 3-*O*-methylallolac-

Table 1
Yields of 3-*O*-methyl-allolactose using β-galactosidases from different sources (50 mM sodium acetate buffer, pH 5.1, 37°)

Source of β-galactosidase	Enzyme amount	PNP-Gal (donor, g)	3-*O*-Me-Glc (acceptor, g)	TEP (%v/v)	Reaction (time, h)	Yield (%)
Bifibobacterium bifidum	10 unit	0.194	0.5	50	34	26.1
Bacillus circulans	54 units	0.02	0.05	50	11	19.8
Streptococcus 6646 K	0.1 unit	0.01	0.026	20	22	17.2
Bovine testes	0.5 unit	0.05	0.129	50	28	3.6
Pectinase G "Amano"	12 mg	0.02	0.05	50	11	13.7
Biodiasmin B-100	23 mg	0.02	0.05	50	11	4.0
Transglucosidase L "Amano"	20 μl	0.02	0.05	50	11	7.6
Gluczyme NL4.2	20 μl	0.02	0.05	50	11	13.9
Hemicellulase "Amano" 90	11 mg	0.02	0.05	50	11	11.5
Biozyme S Conc.	15 mg	0.02	0.05	50	11	8.1
Cellulose A "Amano" 3	22 mg	0.02	0.05	50	11	0
Enzyme RP-1	12 mg	0.02	0.05	50	11	17.7

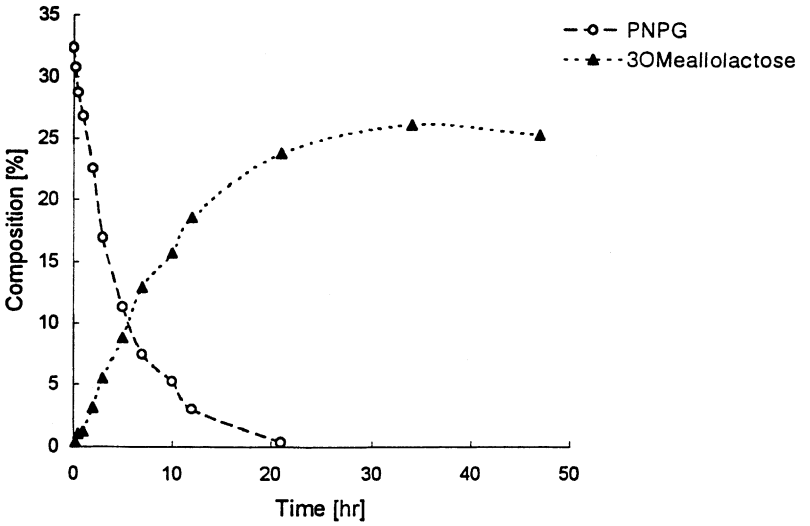


Fig. 1. HPLC chromatogram of mixture from reaction of 3-*O*-methyl-glucose (**3**) and PNP-Gal (**4**) catalysed by *B. bifidum* β-galactosidase.

tose, a feature which may used for the synthesis of trisaccharide. It is noteworthy that the second galactosyl transfer to the terminal non-reducing β-galactosyl residue occurs preferentially to the 4-position as distinct from the 6-position acceptor in 3-*O*-methyl-glucose.

The β-galactosidase from *B. circulans* and *B. bifidum* have been generally used to produce β-(1,4)-linked disaccharides [21,22]; in the present study, however, these enzymes gave exclusively 1,6-linked products. The change in regioselectivity with the introduction of the 3-*O*-methyl-substituent is attributed to steric hindrance of the reaction of the 4-hydroxyl group and/or formation of an unfavourable enzyme-acceptor conformation.

With β-galactosidase from *B. bifidum* an effect of acceptor structure was observed in our previous investigation [20]. Previously, Dumortier et al. [23] had used this enzyme with lactose to transfer galactose preferentially to the 3-position to form galactosyl-oligosaccharide derivatives. When we applied the same enzyme with GlcNAc and GalNAc as acceptors it preferentially transferred galactose to the 4-position in good yield (33%). We concluded that the

Table 2
^{13}C NMR data of 3-*O*-methyl-glucose-containing products

Compound	Residue	C-1	C-2	C-3	C-4	C-5	C-6	CH_3 (3-*O*-Me)	CH_3 (acetyl)	C=O
5	Gal (1–4)	106.3	73.8	75.6	71.8	78.1	64.0			
	3-*O*-Me-Glc-α	95.1	73.5	85.5	79.5	76.3	63.7	63.0		
	3-*O*-Me-Glc-β	98.0	73.7	88.2	80.1	77.7	63.2	62.7		
6	Gal (1–6)									
	3-*O*-Me-Glc-α	95.1	73.5	85.5	71.6	76.3	71.4	63.0		
	3-*O*-Me-Glc-β	98.0	73.7	88.2	71.6	77.7	71.6	62.7		
7	Gal (1–4)	106.9	74.1	75.8	71.5	77.8	63.7			
	Gal (1–6)	105.9	73.9	75.4	79.7	77.5	63.2			
	3-*O*-Me-Glc-α	94.8	73.3	85.3	71.3	76	71.3	62.7		
	3-*O*-Me-Glc-β	98.6	73.6	87.9	71.3	77	71.3	62.4		
8	6-*O*-Ac-3-*O*-Me-Glc-α	95.4	75.0	86.3	72.1	77.4	66.4	63.0	22.7	174.5
	6-*O*-Ac-3-*O*-Me-Glc-β	99.6	76.5	89.0	72.1	81.0	66.5	62.8	22.7	174.5

substitution of the 2 –OH group by an *N*-acetylamino group in the acceptor led to a different orientation in the enzyme acceptor site and altered the regioselectivity from 1,3 to 1,4. In a different study, Martin-Lomas and co-workers [24,25] investigated recognition and hydrolysis by intestinal lactase of lactose derivatives and found that 3-*O*-methyl derivatives were substrates; the V_{max} and K_m values for 3-*O*-methyl-lactose were estimated to be ca. 14 times lower and 24 times higher than those of the natural substrate, respectively. They concluded from these results that methylation introduces a significant restriction on the enzyme galactosidation step; either steric hindrance for the initial recognition or the lack of hydrogen donor capacity accounted for the lower affinity. The enzymes interacts with the 3 –OH group in the periphery of the binding site, where large substituents are not easily accommodated. This restriction clearly applies also to the reaction in the synthetic direction.

Modification of the acceptor by protection of the hydroxyl group of the 6-position to exclude it from the reaction was attempted. The 6-position of 3-*O*-methyl-glucose was successfully acetylated with vinyl acetate using lipase Novozym 435 in a yield of 57%. Confirmation was achieved by ^{13}C NMR analysis which showed a distinct downfield shift in the C-6 carbon (60.8–65.5 ppm). Unfortunately with 6-*O*-acetyl-3-*O*-methyl glucose as the acceptor, disaccharide synthesis did not occur. It is assumed that the methyl in the 3-position and acetyl in the 6-position hindered the formation of any disaccharides.

Martin-Lomas and co-workers [24,25] also evaluated the importance of the 6 –OH group on the glucose moiety for recognition by intestinal lactase using various methyl lactosides. The V_{max} values were not greatly altered (0.4–1.7 fold), but the K_m values were >10 times higher than for lactose. These results again show that the 6 –OH group is important for efficient binding with the enzyme and that it may participate as a hydrogen bond donor in the binding site.

A number of reports have shown that the regioselectivity of glycosidase catalysed transglycosylation can be changed by using glycosides of different anomeric configuration, size and hydrophobicity as acceptors [26–30]. We therefore attempted the galactosidase-catalysed synthesis with methyl and ethyl 3-*O*-methyl glucosides as acceptors. The 1,6-linked disaccharides were again the main products.

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