Cellulase-catalysed, Stereoselective Synthesis of Oligosaccharides

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Oligosaccharides are synthesised enzymatically using β-lactosyl fluoride as glycosyl donor and a variety of mono-, di- and tri-saccharides as acceptors; the nature of the active site of the catalyst cellulase is discussed on the basis of these reactions.

Enzymes have gained increasing interest in organic synthesis in recent years.[1] Owing to the high regio- and stereoselectivities, enzymes can be used for the synthesis of multifunctional compounds with only little or no need for protecting groups. Recently, the concept of enzymatic reactions in polymer chemistry has been proposed and proved successfully, for example, using hydrolases and oxidases.[2–6] In the case of saccharides, β-cellobiosyl fluoride can be polymerized using the hydrolytic enzyme cellulase as catalyst to yield the polysaccharide cellulose, a natural substrate for cellulase.[7] Based on these results cellulase was used as a catalyst for the enzymatic synthesis of cellooligosaccharides.[8,9] In the present study, β-lactosyl fluoride **1**, which is accepted by cellulase but cannot be polymerized owing to the axial OH-group at the 4′-position, has been used as the glycosyl donor. As glycosyl acceptors, a variety of natural mono-, di- and tri-saccharides **2a–l** as well as non-natural glucosides **2n–q** in the form of their methyl glycosides have been examined. The enzyme-catalysed condensation reaction hence leads to tri-, tetra- and penta-saccharides, respectively (see Scheme 1).

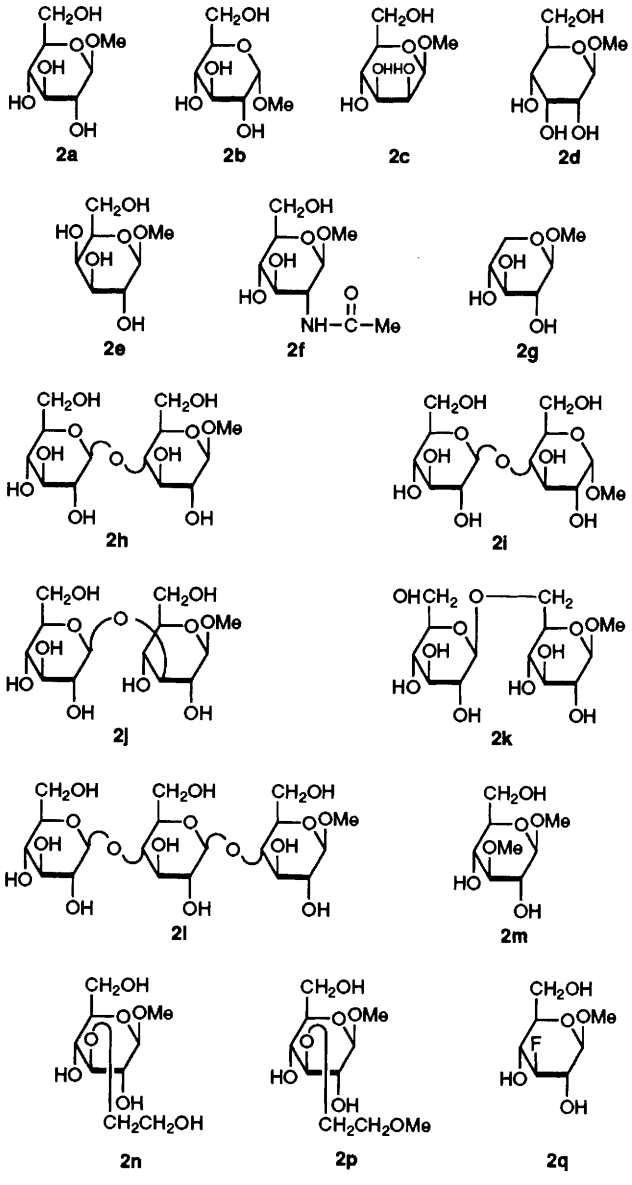
The reactions were carried out in a 0.05 mol dm^{-3} acetate buffer (pH 5) or in a mixture of buffer/organic solvent.† The organic solvent decreases the enzyme activity especially for the backward reaction, the hydrolysis of glycosidic bonds. The yields are very much dependent on the nature of the glycosyl acceptor and are in the range of 0 to 60% (see Table 1). The lactosylation takes place at the 4-position of the non-reducing end of the glycosyl acceptor forming the (1–4)-β-glycosidic bond, exclusively.‡ It is shown for the first time that cellulase also can accept non-glucose substrates, like mannose **2c**, xylose **2g** and functionalized glucose derivatives **2m** and **2q**. This paves the way for the enzymatic synthesis of sequential oligosaccharides, employing only one kind of enzyme. The advantages of this method are the complete regio- and stereo-selectivity and the unnecessity of protecting groups during the enzyme reaction.

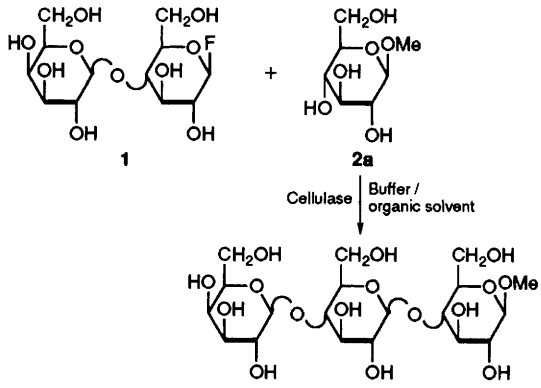
High yields of the lactosylation were obtained using methyl β-glucoside **2a** (51%), methyl β-mannoside **2c** (52%), methyl

† Typical reaction procedure: 0.1 mmol of **2q** and 0.1 mmol of **1** are dissolved in 700 μl of buffer and kept at 30 °C. 5 wt% for **1** of the cellulase (Onozuka R-10, *Trichoderma viride*, Yakult Co., Japan, activity 6.5 units/mg, 1 unit produces reducing sugar equivalent to 1 μmol glucose per min from carboxymethylcellulose at pH 4.5 and 40 °C) in 300 μl buffer was added. For workup, the reaction mixture was diluted with acetonitrile in order to reduce enzyme activity and kept at 100 °C for 10 min prior to HPLC analysis.

‡ ^1H and ^{13}C NMR data are given for a typical example, the lactosylated product of methyl β-mannoside **2c**. ^1H NMR (250 MHz, solvent D_2O, standard 1,4-dioxane): δ 4.58 (1 H, s, H-1), 4.51 (1 H, d H-1′ $J_{1',2'}$ 7.9 Hz), 4.44 (1 H, d, H-1″ $J_{1'',2''}$ 7.6 Hz), 3.6–4.1 (18 H, sugar-H), 3.53 (3 H, s, OMe). Coupling constants in the range of 7–8 Hz for the anomeric protons H-1′ and H-1″ indicate β-glycosidic bonds.

^{13}C NMR (62.8 MHz, solvent D_2O, standard 1,4-dioxane): δ 103.7 (C-1″), 103.3 (C-1′), 101.7 (C-1), 78.9 (C-4′), 77.5 (C-4), 76.2 (C-5″), 75.8 (C-5), 75.7 (C-5′), 74.9 (C-3′), 73.7 (C-2′), 73.3 (C-3″), 72.5 (C-3), 71.7 (C-2″), 70.6 (C-2), 69.4 (C-4″), 61.9 (C-6″), 61.2 (C-6), 60.8 (C-6′), 57.6 (OMe). The resonances of the anomeric carbons are located *ca.* δ 100, a value typical for β-glycosidic bonds. The resonance of the C-4 at δ 77.5 clearly shows the 1–4-glycosidic bond.





Scheme 1 Enzymatic synthesis of oligosaccharides using lactosyl fluoride **1** as glycosyl donor and various acceptors, for example **2a**

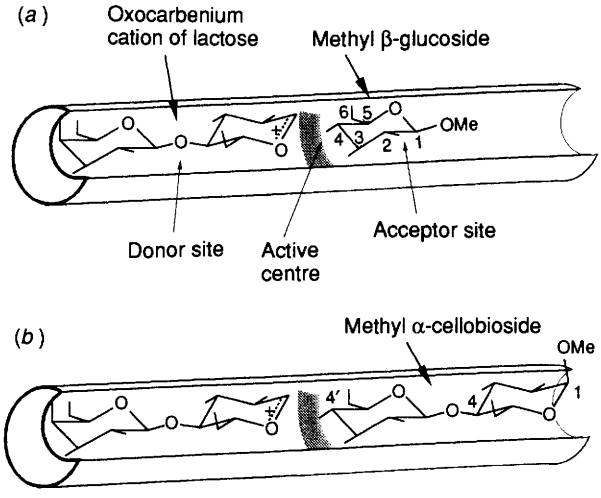


Fig. 1 Proposed model of the active site of cellulase and the orientation of glycosyl acceptor and glycosyl donor in the transition state. (*a*) Methyl β-glucoside **2a** as glycosyl acceptor, (*b*) methyl α-cellobioside **2i** as glycosyl acceptor

Table 1 Glycosylation reaction using lactosyl fluoride **1** as glycosyl donor and **2a–q** as glycosyl acceptors

Acceptor	Solvent ratio (v/v)[a]	Conc. (M)[b]	*t*/h	Yield (%)[c]
2a	MeCN/buffer 1 : 2	0.15	0.5	51
2b	MeCN/buffer 1 : 2	0.15	0.5	0
2c	MeCN/buffer 1 : 2	0.15	0.5	52
2d	MeCN/buffer 1 : 2	0.15	0.5	0
2e	MeCN/buffer 1 : 2	0.15	0.5	0
2f	MeCN/buffer 1 : 2	0.15	0.5	Trace
2g	MeCN/buffer 1 : 2	0.15	1	25
2h	MeOH/buffer 4 : 1	0.025	6	36[d]
2i	Buffer	0.15	0.5	60
2j	MeCN/buffer 1 : 3	0.05	0.5	23
2k	MeCN/buffer 1 : 3	0.05	0.5	26
2l	MeOH/buffer 4 : 1	0.025	8	27
2m	MeCN/buffer 1 : 1	0.1	2	8
2n	Buffer	0.1	6	0[e]
2p	Buffer	0.1	6	0
2q	Buffer	0.1	2	18

[a] 0.05 mol dm^{-3} acetate buffer, pH 5. [b] Equimolar amounts of glycosyl acceptors **2a–q** and **1**. [c] Determined by HPLC. [d] Isolated yield. [e] Inhibition of enzyme, no formation of lactose, the hydrolysis product of **1**.

β-cellobioside **2h** (36%) and methyl α-cellobioside **2i** (60%). Other derivatives, like allose **2d** and glucose derivatives carrying bulky groups (**2f, 2n** or **2p**) seem not to be accepted by cellulase and only little or no lactosylation of these substrates occurs. Furthermore, the orientation of the methoxy group at the anomeric centre plays an important role in the reaction. The equatorial methoxy group in **2a** leads to high yield, but the methyl α-glucoside **2b** does not react at all. The disaccharide having a free OH-group at the 4-position **2j** as acceptor was lactosylated exclusively at the 4′-position, which is not surprising when the negative results of the lactosylation of **2n** and **2p** are taken into account. The disaccharide **2k** also gave the 4′-product. These results indicate that the glucosides with a substituent at the 3- or 6-position are too bulky to fit into the active site and the 4-position is sterically too hindered for lactosylation. In general, bulky equatorial groups at the 2-, 3- or 6- position, *e.g.* **2f, 2j, 2k, 2n, 2p**, weaken binding to the enzyme and no lactosylation product at the 4-position is produced.

These results can be used for the estimation of the size and shape of the active site of cellulase and the orientation of the glycosyl acceptor and donor. In spite of the detailed information of the peptide sequence of a variety of cellulases,[10,11] there is no information about the tertiary structure of the enzymes and therefore none about the shape of the active site. The present results can be summarized as the following statements for the nature of the active site (Fig. 1).

(*i*) The lactosylation reaction yields exclusively a (1–4)-β-glycosidic bond and therefore the glycosyl donor and acceptor must be oriented in a linear way. The retention of the configuration of the anomeric carbon in **1** can be explained by a 'double displacement mechanism'[12] involving the formation of a reactive intermediate, *e.g.* an oxocarbenium ion stabilized by the enzyme, and the nucleophilic attack of the 4-OH-group of the glycosyl acceptor.[7]

(*ii*) The monosaccharide-acceptors having an axial group at the 1- **2b** or 3-position **2d** cannot be lactosylated, therefore this group is directed towards the enzyme, inhibiting the binding owing to steric repulsion between the group and the enzyme.

(*iii*) An axial group at the 2-position of the monosaccharide **2c** or at the 1-position of the disaccharide **2i** does not inhibit binding, therefore this group is directed away from the enzyme.

(*iv*) A bulky equatorial group at the 2-, 3- or 6-position (**2f, 2j, 2k, 2n** and **2p**) inhibits binding to the enzyme, implying a channel-like structure with narrow edges.

(*v*) The missing OH-group at the 6- **2g** or 3-position **2q** leads to drastically lowered yields. This indicates that the OH-group in these positions is responsible for hydrogen bonding, but the orientation of the OH-group at the 2-position plays little role in it (high yield in the case of **2c**).

This model will be helpful for the future design of promising substrates for the facile syntheis of new oligosaccharides using cellulase as a catalyst.

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