Cellulase-catalysed Glycosylation Reactions: Simple Route towards a Highly Selective Synthesis of Oligosaccharides

Olaf Karthaus, Shin-ichiro Shoda, Hiroshi Takano, Kei Obata and Shiro Kobayashi*
Department of Molecular Chemistry and Engineering, Faculty of Engineering, Tohoku University, Aoba, Sendai 980, Japan

Cellulase (EC 3.2.1.4) can be used for the enzyme-catalysed synthesis of oligosaccharides in a mixed buffer/organic solvent system. Besides glucopyranosides and cellooligosaccharides, non-glucosidic saccharides and modified glucoses as glycosyl acceptors also react with β-lactosyl fluoride as glycosyl donor. The reaction proceeds in a completely stereo- and regio-specific manner, giving rise to coupling products having β (1→4) glycosidic bonds exclusively. Based on these results, a model for the active site of cellulase (*Trichoderma viride*) and the orientation of the donor and acceptor therein is proposed.

Despite the long history of carbohydrate chemistry (more than 100 years), reactions involving saccharides are far from being trivial. In particular, the glycosylation reaction, the formation of the linkage between an anomeric carbon atom and either an aglycone or a second saccharide, is still a topic of current research.[1] In the latter case, besides the stereoselectivity at the anomeric position of the glycosyl donor the regiospecificity at the glycosyl acceptor has to be optimized. This often leads to 'tailor-made' reaction conditions which are applicable only for a few selected donors and acceptors and necessitate the extensive use of protecting groups.

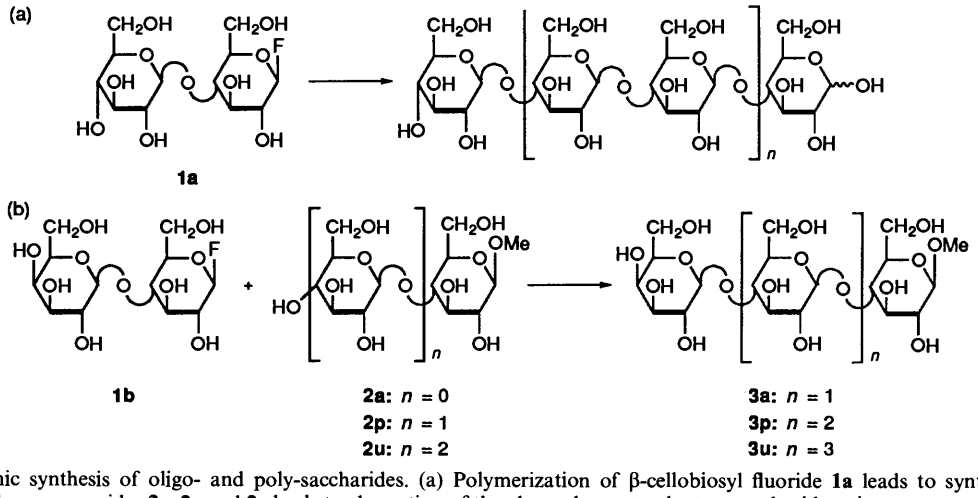
Oligo- and poly-saccharides are a very important class of natural compounds. Their enzyme-catalysed synthesis *in vivo* shows the perfect regio- and stereo-selectivity which is characteristic of biological systems. So far there have been several successful approaches to enzymic synthesis of oligosaccharides *in vitro*. Glycosyltransferases, enzymes which catalyse glycosylations in natural systems,[2] or glycosidases,[3] which are responsible for the cleavage of glycosidic bonds, have been used for the synthesis of oligosaccharides. Recently, it has been shown that a hydrolytic enzyme, cellulase, can be used for the synthesis of cellulose.[4] The substrate monomer used was β-cellobiosyl fluoride **1a**, which possesses both glycosyl-donor and glycosyl-acceptor properties. Nucleophilic attack of the 4'-OH-group of the glycosyl acceptor molecule at the anomeric centre of the glycosyl donor molecule leads to the formation of a β-glycosidic bond (see Scheme 1a). Consecutive reactions of this type, therefore, lead to the formation of a polymeric product, cellulose.

This is exactly the reverse of the natural reaction path for the cellulase catalysis, the hydrolysis of β(1→4) glucosidic bonds of cellulose. This concept was also used for the synthesis of new oligosaccharides[5] having a galactose moiety at the non-reducing end by treating β-lactosyl fluoride **1b** with cello-oligomers (see Scheme 1b). The axial OH group at the 4'-position of compound **1b** cannot attack the anomeric centre, thus polymerization is prevented. Still, lactose is accepted as a substrate by cellulase, therefore the fluoride **1b** can act as a glycosyl donor. According to this methodology, methyl β-D-glucopyranoside **2a**, methyl β-cellobioside **2p** or methyl β-cellotrioside **2u** as glycosyl acceptor can be stereoselectively lactosylated to afford compound **3a**, **3p** or **3u**, respectively, by elongation by two sugar residues.

The remarkable extension of this synthetic route is now due to the fact that cellulase, an enzyme specific for the hydrolysis of β(1→4) glucosidic bonds in cellulose, can also catalyse reactions with non-glucose saccharides as glycosyl acceptors and β-lactosyl fluoride **1b** as glycosyl donor.[6]

Results and Discussion

Table 1 shows the results of the lactosylation of a variety of glycosyl acceptors **2a**–**2u**. The yields range between 0 and 60% depending on the nature of the acceptor. In all cases the



Scheme 1 Enzymic synthesis of oligo- and poly-saccharides. (a) Polymerization of β-cellobiosyl fluoride **1a** leads to synthetic cellulose. (b) Lactosylation of glucopyranosides **2a**, **2p** and **2u** leads to elongation of the glycosyl acceptor by two saccharide units.

Table 1 Cellulase-catalysed lactosylation of various glycosyl acceptors **2a–2u**

Acceptor	Solvent ratio (v/v)[a]	Conc.[b] (mol dm^{-3})	Time (t/h)	Product	Yield (%)[c]
2a[g]	MeCN/buffer 1:2	0.15	0.5	**3a**	51
2b	MeCN/buffer 1:2	0.15	0.5		0
2c	MeCN/buffer 1:2	0.15	0.5	**3c**	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	**3g**	25
2h	EtOH/buffer 2:1	0.1	3	**3h**	30
2i	buffer	0.1	1	**3i**	36
2j	buffer	0.1	1.5	**3j**	43
2k	MeCN/buffer 1:2	0.1	8		0[d,e]
2l	buffer	0.1	2	**3l**	18
2m	MeCN/buffer 1:1	0.1	2	**3m**	8
2n	buffer	0.1	6		0[d]
2o	buffer	0.1	6		0
2p[g]	MeOH/buffer 4:1	0.025	6	**3p**	36[f]
2q	buffer	0.15	0.5	**3q**	60
2r	MeCN/buffer 1:1	0.1	4	**3r**	23
2s	MeCN/buffer 1:3	0.05	0.5	**3s**	23
2t	MeCN/buffer 1:3	0.05	0.5	**3t**	26
2u[g]	MeOH/buffer 4:1	0.025	8	**3u**	27

[a] 0.05 mol dm^{-3} acetate buffer, pH 5. [b] Equimolar amounts of glycosyl acceptors **2a–2t** and fluoride **1b**. [c] Determined by HPLC. [d] Inhibition of enzyme, no formation of lactose, the hydrolysis product of compound **1b**. [e] Diastereoselective hydrolysis of the sulfoxide. For details, see text footnote.* [f] Isolated yield. [g] See also ref. 5.

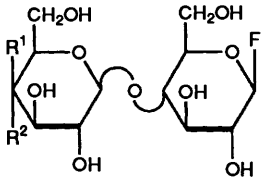
stereochemistry of the newly formed glycosidic bond was determined as β(1→4) by NMR spectroscopy. ^{1}H NMR analysis showed signals for the anomeric protons at δ ~4.5 with J-values of 6–8 Hz. The signals due to the anomeric carbon atoms in the ^{13}C NMR spectrum are at δ_C ~103. Both are characteristic values for β glycosidic bonds. The signals for the methyne carbon at the newly formed glycosidic bond are found at δ_C ~79, a value characteristic for the C-4 carbon of saccharides. No signals from the methylene carbon C-6 (δ_C 69) or from the methyne carbon C-3 (δ_C 85) adjacent to a β(1→6) or a β(1→3) glycosidic bond were observed in the products,[7] indicating that only products with β(1→4) linkages were formed.

Lactosylation of Glucopyranosides.—High yields are achieved with methyl β-D-glucopyranoside **2a**, both methyl α- and β-cellobioside **2q** and **2p** and methyl β-cellotrioside **2u**.[5] Interestingly, in the case of glucose, the orientation of the anomeric methoxy group is crucial for the reaction to proceed. Methyl β-D-glucopyranoside **2a** gives compound **3a** in high yields whereas the α-derivative **2b** does not react at all. For both methyl cellobiosides **2p** (β) and **2q** (α) lactosylation yields are in the same range. This fact gives a first hint of the orientation of substrates in the active site of cellulase (*vide infra*). The slightly reduced yield for (1→6) compound **2t** can be explained by a side reaction. Besides the condensation reaction between substrate **1b** and the acceptor, the cleavage of β(1→4) glucosidic bonds is also catalysed by the action of cellulase. In the case of compounds **3a** and **3q** only the formed glucosidic bond can be cleaved again. For compound **3p** two β(1→4) glucosidic bonds can be cleaved, and for compound **3u** there are three available glucosidic bonds for cellulase. Hence, cleavage is a significant side reaction in the latter case.

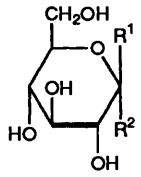
Thioglucopyranosides as Glycosyl Acceptors.—Thioglycopyranosides are reported to be inhibitors for various enzymes[8] and to exhibit antiinflammatory activity.[9] They are themselves also key compounds for chemical glycosylations.[10] Furthermore, they can be converted into the corresponding sulfoxide, which can be used as a leaving group in acid-catalysed glycosylations.[11] Therefore good yields in the lactosylation of β-D-thioglucopyranosides **2h–2j** pave the way for a combination of enzymic synthesis with conventional organic chemistry. In contrast to the sulfides, the glucose sulfoxide **2k** itself cannot be used directly for the enzymic lactosylation.* In analogy to *O*-cellobiosides **2p** and **2q**, methyl β-thiocellobioside **2r** can also be lactosylated in moderate yields. As in the case of the *O*-cellobioside **2p** the cleavage of glycosidic bonds take place as a side reaction.

Fig. 1 shows the time conversion curve of the lactosylation reaction of thiocellobioside **2r** with compound **1b** in a 0.1 mol dm^{-3} acetate buffer (pH 5). The reaction in buffer proceeds faster than in a mixed solvent system, but generally the yields are lower. The concentration of the saccharides was measured by high-performance liquid chromatography (HPLC) using a reversed-phase (RP) gel and water as eluent. In this system, the methyl β-glucopyranosides have characteristic retention times, depending on the length of the saccharide chain. Monosaccharides elute in the range 3–4 min, disaccharides have peaks at 5–7 min, tri- and tetra-saccharides at 8–10 and 15–20 min, respectively. During the reaction the concentration of both educts **1b** and **2r** is decreasing. In addition to the coupling reaction, the glycosidic bond in thiocellobioside **2r** is also cleaved by cellulase, leading to glucose and methyl β-thioglucopyranoside. After 1 h the maximum yield of compound **3r** is observed at 20%. In continuation of the reaction, the yield of

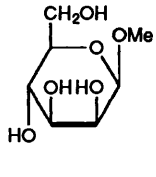
* The sulfoxide group provides an additional chiral centre adjacent to the anomeric position. In the synthesis, both diastereoisomers of the β-D-glucopyranoside are formed (d.e. 20%). Both isomers decrease enzyme activity, but, interestingly, diastereoselective hydrolysis of the glucosidic bond takes place. After 8 h one diastereoisomer of substrate **2k** is completely hydrolysed, whereas the other one remains unchanged. This is due to β-glucosidase, which is present in the crude cellulase mixture.[12] To the best of our knowledge, this is the first evidence of chiral discrimination using β-glucosidases. Current investigations are underway to elucidate the absolute configurations of the sulfoxides and their hydrolysis behaviour in detail, using β-glucosidases from different origins.



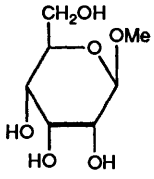
1a $R^1 = H$, $R^2 = OH$
1b $R^1 = OH$, $R^2 = H$



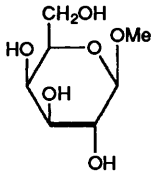
2a $R^1 = OMe$, $R^2 = H$
2b $R^1 = H$, $R^2 = OMe$



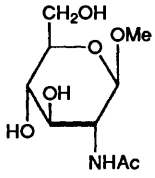
2c



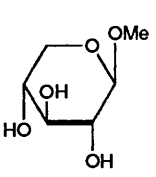
2d



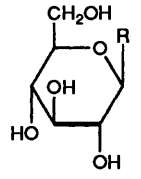
2e



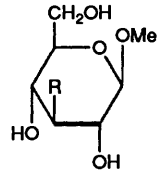
2f



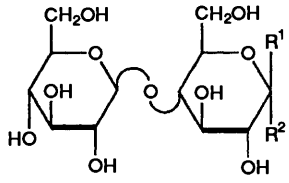
2g



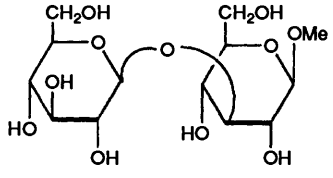
2h R = $SC_{12}H_{25}$
2i R = SPh
2j R = $SCH_2CONHPr$
2k R = S(O)Ph



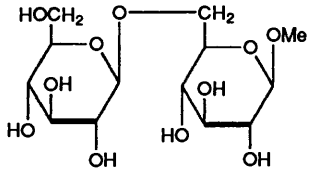
2l R = F
2m R = OMe
2n R = OCH_2CH_2OH
2o R = OCH_2CH_2OMe



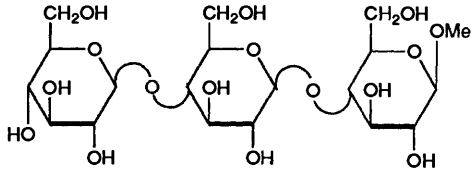
2p $R^1 = OMe$, $R^2 = H$
2q $R^1 = H$, $R^2 = OMe$
2r $R^1 = SMe$, $R^2 = H$



2s



2t



2u

compound **3r** decreases, accompanied by a further increase of glucose and methyl β-thioglucopyranoside. In addition, the glycosidic bond between galactose and glucose moieties in compound **3r** is slowly hydrolysed by cellulase, leading to methyl β-thiocellotrioside as side product.

Unnatural Substrates for Cellulase.—Interestingly, cellulase can also catalyse the lactosylation of glycosyl acceptors which differ in structure from the natural substrate for cellulase having β(1→4) glucosidic bonds exclusively. Among these are disaccharides having β(1→3) or β(1→6) glucosidic bonds, 3-modified glucopyranosides, and the epimers of D-glucose.

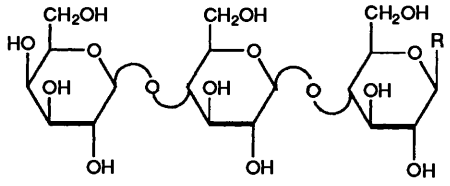
Methyl β-laminaribioside [Glc-β(1→3)-Glc-OMe] **2s** and methyl β-gentiobioside [Glc-β(1→6)-Glc-OMe] **2t** are lactosylated exclusively at the 4-position. No lactosylated product at the 4-position can be detected by ^{13}C NMR spectroscopy, indicating that the 4-OH group of the acceptors **2s** and **2t** is sterically hindered due to bulky groups in the 3- and 6-position, respectively.

Glucopyranosides, modified at the 3-position, can also be lactosylated, but only if the substituent is small, *e.g.* methyl 3-deoxy-3-fluoro-β-D-glucopyranoside **2l** or methyl 3-*O*-methyl-β-D-glucopyranoside **2m**. Longer alkyl chains at the 3-position of the acceptor do not lead to lactosylated products. Methyl 3-*O*-(2-hydroxyethyl)-β-D-glucopyranoside **2n** leads to inhibition of cellulase. The additional hydroxy group, spaced out from the glucose ring by 2 methylene units, may lead to a different orientation of the glucopyranoside in the active site of the enzyme. By capping this hydroxy group in methyl 3-*O*-(2-methoxyethyl)-β-D-glucopyranoside **2o** the inhibiting properties are lost, but still no lactosylation was observed, which can be explained by the steric hindrance of the bulky alkyl chain.†

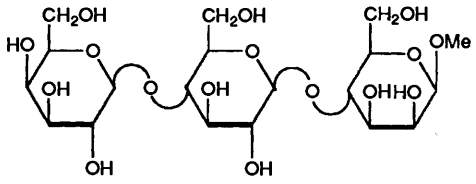
Another astonishing result is that cellulase can also be used for the lactosylation of certain non-glucose acceptors. High yields are achieved with methyl β-D-mannopyranoside **2c**, the 2-epimer of methyl glucopyranoside. Methyl β-D-xylopyranoside **2g** can also be lactosylated in moderate yields. The 3-epimer of methyl glucopyranoside, methyl β-D-allopyranoside **2d**, on the other hand, cannot be lactosylated. The same result is found for methyl *N*-acetyl-β-D-glucosamine **2f**, having a bulky group in the 2-position. These results will be discussed with regard to the elucidation of the shape of the active site of cellulase. As expected, the 4-epimer of methyl glucopyranoside, methyl β-D-galactopyranoside **2e**, cannot be lactosylated. In this case the axial OH group at the 4-position prevents nucleophilic attack at the anomeric carbon of compound **1b**.

In some reactions the order of addition of the glycosyl acceptor, donor and catalyst was altered. In the 'normal'

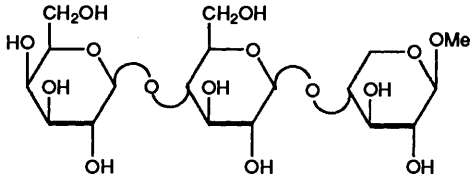
† The activity of cellulase is estimated by the hydrolysis rate of compound **1b**. A strongly decreased hydrolysis rate is interpreted as enzyme inhibition, whereas a rate similar to that of hydrolysis of compound **1b** in the absence of acceptor indicates no inhibition.



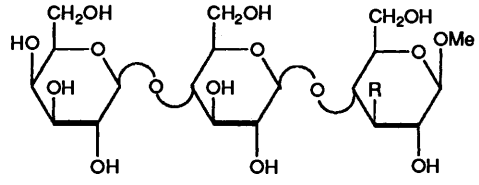
3a R = OMe
3h R = $SC_{12}H_{25}$
3i R = SPh
3j R = SCH_2CONH Pr



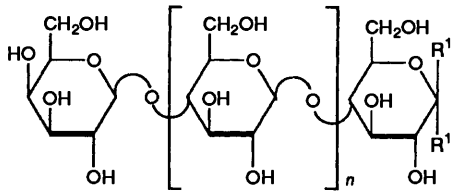
3c



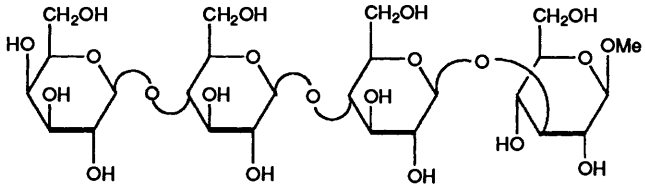
3g



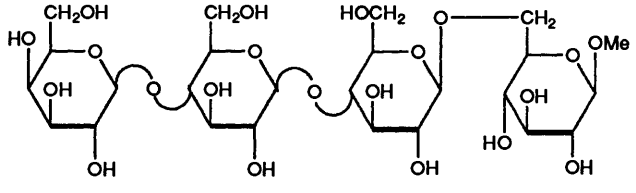
3l R = F
3m R = OMe



3p *n* = 2, R^1 = OMe, R^2 = H
3q *n* = 2, R^1 = H, R^2 = OMe
3r *n* = 2, R^1 = SMe R^2 = H
3u *n* = 3, R^1 = OMe, R^2 = H



3s



3t

reaction mode, the donor and acceptor were mixed prior to the addition of cellulase (see Experimental section). In the 'inverse' addition mode, the glycosyl acceptor was incubated with cellulase for 30 min followed by the addition of the donor **1b**. The 'inverse' reaction mode showed a slight, but significant, increase in the yields of lactosylated products (see Table 2). This can be explained as follows. In the solvent system only (*i.e.*, no cellulase), compound **1b** is fairly stable towards hydrolysis. In the presence of cellulase, fast hydrolysis occurs, when the donor subsite of the active site recognizes the substrate **1b**, to give a reactive intermediate. In the case of a vacant acceptor site, the attack of water on the activated cellulase/**1b** complex is likely, releasing lactose as the hydrolysis product. On the other hand, the incubation of cellulase with glycosyl acceptor first will lead to occupation of the acceptor site of cellulase. Addition of donor **1b** will therefore lead to a faster nucleophilic attack of the acceptor on the complex, increasing the yield of products **3a**, **3l** and **3m**, respectively.

Model of Active Site of Cellulase.—Besides the preparative aspect, the present results also allow some insight into the shape of the active site of cellulase to be obtained.[13] Large substituents in the equatorial position attached to the glycosyl donor (**2f**, **2n**, **2o** and the reducing-end glucose units of compounds **2s** and **2t**) prevent lactosylation. This points to a channel-like structure of the enzyme. A second supporting argument for a channel structure is the fact that hydrogen bonds between substrate and cellulase are necessary for substrate recognition (lower yields for **2l** and **2g**, respectively).

Furthermore, from the present data, the orientation of the

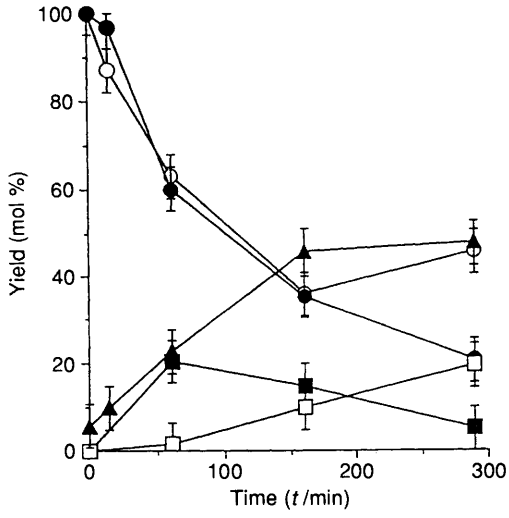


Fig. 1 Time–conversion curve for the lactosylation of compound **2r** with fluoride **1b**. (-○-) **1b**, (-●-) **2r**, (-■-) **3r**, (-□-) *S*-methyl 1-thio-β-cellotrioside, (-▲-) *S*-methyl 1-thio-β-D-glucopyranoside.

Table 2 Yield dependence of the lactosylation of glycosyl acceptors **2a**, **2l** and **2m** on the reaction mode

Acceptor	Reaction mode[a]	Time (*t*/min)	Yield[b] (%)
2a	normal	20	26
2a	inverse	30	45
2l	normal	10	4
2l	inverse	120	18
2m	normal	120	3
2m	inverse	60	8

Solvent: 0.05 mol dm^{-3} acetate buffer, pH 5, concentration of substrate: 0.1 mol dm^{-3}. [a] Order of addition is altered. Normal: **1b** + acceptor + cellulase. Inverse: acceptor + cellulase, 30 min incubation, then **1b**. [b] Estimated by HPLC.

donor and acceptor in the active site can be estimated (see Fig. 2). The results for compounds **2a**, **2b**, **2c** and **2d** indicate that the axial groups in the 1- (**2b**) and 3-position (**2d**) of the acceptor point towards the enzyme and thus prevent binding due to steric repulsion (Fig. 2). The axial group in the 2-position (**2c**) points away from the enzyme, not influencing the binding and leading to high yield of lactosylated product **3c**.

Having cellulose as the natural substrate, one can also assume that the orientation of the glucose residues is alternating along the polysaccharide chain, as in the crystal structure of natural cellulose. One characteristic feature of the crystal structure is the formation of intramolecular hydrogen bonds between C-3 and C-5 and between C-6 and C-2. From the energetics standpoint it is, therefore, favourable to retain these hydrogen bonds during the recognition process. Thus, the reducing end of a disaccharide acceptor has the opposite orientation to its non-reducing end. This explains how the α-cellobioside **2q** can be lactosylated in high yields, despite the fact that the α-glucoside **2b** does not react at all. Similar explanations can be given for the orientation of the donor moiety **1b**. The reducing end of the donor **1b** has to have the opposite orientation from that of the non-reducing end of the acceptor. Thus, the axial OH group at the non-reducing end in the 4'-position of the donor points away from the cellulase. Assuming the double displacement mechanism[14] for the lactosylation reaction a reactive intermediate, either an oxocarbenium ion of substrate **1b** or an activated ester with a carboxylic acid residue in the active site, has to be stabilized by cellulase. In this orientation, the OH group at the non-reducing end of the acceptor will attack the lactose moiety, leading to the observed β(1→4) glycosidic bond formation.

Based on these results, the screening of further substrates for cellulase is facilitated and a direct synthesis of tailor-made glycosyl donors and acceptors should be readily achieved.

Experimental

Materials and Measurements.—All organic solvents were distilled from the appropriate drying reagent before use. NMR data were obtained from a Bruker AT250T spectrometer. *J*-Values are given in Hz. For analytical and semipreparative HPLC a Hitachi 655 A-12 Liquid Chromatograph (1 cm^3 min^{-1}), equipped with a Hibar RT 250-4 column (25 × 0.4 cm), filled with Lichrosorb RP 18 (7 μm) gel (Merck), a Hitachi 655A-30 RI detector and a Hitachi D-2000 Chromato Integrator was used. Eluent was water. For compounds **2h** and **2j** a mixed solvent of water–methanol (5:1) was used as eluent.

D-Mannose (Nacalai), D-allose (Nacalai), *N*-acetyl-D-glucosamine (Nacalai), gentiobiose (Fluka), laminaribiose (KI Chemicals, Japan), pentaacetyl-D-glucose (Nacalai) and octaacetylcellobiose (Nacalai) were used without purification. The commercially available acceptors methyl β-D-glucopyranoside **2a**, methyl α-D-glucopyranoside **2b** and methyl β-D-xylopyranoside **2g** were used without further purification (Nacalai). All other methyl β-D-glycopyranosides (**2d–2f**, **2l–2p**, **2t**), except methyl β-D-mannopyranoside **2c**, were obtained by standard procedures from their peracetates *via* the α-D-glycosyl bromides.[15–17] Methyl β-D-mannopyranoside **2c**,[18] *S*-phenyl β-D-thioglucopyranoside **2i**,[19] 3-deoxy-3-fluoro-D-glucose,[20] 3-*O*-methyl-D-glucose[21] and the 3-*O*-alkyl ethers of D-glucose were synthesized according to literature.[22] Methyl α-cellobioside **2q** was synthesized in analogy to the literature.[23] Synthesis of methyl β-cellotrioside **2u** and β-lactosyl fluoride **1b** is described elsewhere.[5b] Cellulase Onozuka R-10 from *Trichoderma viride* (activity with carboxymethylcellulose, pH 4.5, 30 °C: 6.5 units mg^{-1}) was obtained from Yakult (Japan).

S-*Dodecyl* 1-*Thio*-β-D-*glucopyranoside* **2h**.—Synthesis according to ref. 8(*b*). Yield 97% (Found: C, 59.2; H, 9.7; S, 8.6. $C_{18}H_{34}O_5S$ requires C, 59.6; H, 9.5; S, 8.8%); δ_H(250 MHz; CD_3OD; 1,4-dioxane) 4.5 (1 H, d, *J* 7.2, 1-H), 3.7 (1 H, m, 5-H), 3.35–3.5 (3 H, m, 2-, 3- and 4-H), 3.3 (2 H, m, 6-H_2), 2.68 (2 H, m, SCH_2), 1.63 (2 H, m, SCH_2CH_2), 1.3 (16 H, m, CH_2) and 0.89 (3 H, t, Me).

S-(*Propylcarbamoylmethyl*)-1-*thio*-β-D-*glucopyranoside* **2j**.—(Found: C, 38.5; H, 7.6; N, 4.6; S, 8.6. $C_{11}H_{21}NO_6S{\cdot}3H_2O$ requires C, 37.8; H, 7.8; N, 4.0; S, 9.1%); δ_H(250 MHz; D_2O; 1,4-dioxane) 4.53 (1 H, d, *J* 7.2, 1-H), 3.92 (1 H, s, SCH_2), 3.87 (1 H, s, SCH_2), 3.7 (1 H, m, 5-H), 3.3–3.5 (5 H, m, 2-, 3- and 4-H and 6-H_2), 3.2 (2 H, t, *J* 6.9, NCH_2), 1.55 (2 H, m, CH_2Me) and 0.89 (3 H, t, Me); δ_C(62.8 MHz; D_2O; 1,4-dioxane) 170.8 (CO), 83.3 (C-1), 78.4 (C-5), 75.3 (C-2), 70.4 (C-4), 68.0 (C-3), 59.4 (C-6), 40.0 (NCH_2), 31.9 (SCH_2), 20.4 (CH_2Me) and 9.3 (Me).

Phenyl β-D-*Glucopyranoside Sulfoxide* **2k**.—*S*-Phenyl 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranoside (109 mg, 0.248 mmol) was dissolved in MeOH (20 cm^3) and the solution was cooled to 5 °C. A solution of magnesium monoperoxyphthalate[24] (61.1 mg, 0.124 mmol) in MeOH (10 cm^3) was added dropwise over a period of 2 h. The reaction was monitored by TLC [hexane–ethyl acetate (1:1)]. After evaporation of the solvent, the mixture was submitted to flash chromatography in the same eluent to give *peracetylated sulfoxide* **2k** (60%) (Found: C, 52.0; H, 5.1; S, 6.7. $C_{20}H_{24}O_{10}S$ requires C, 52.63; H, 5.30; S, 7.01%).

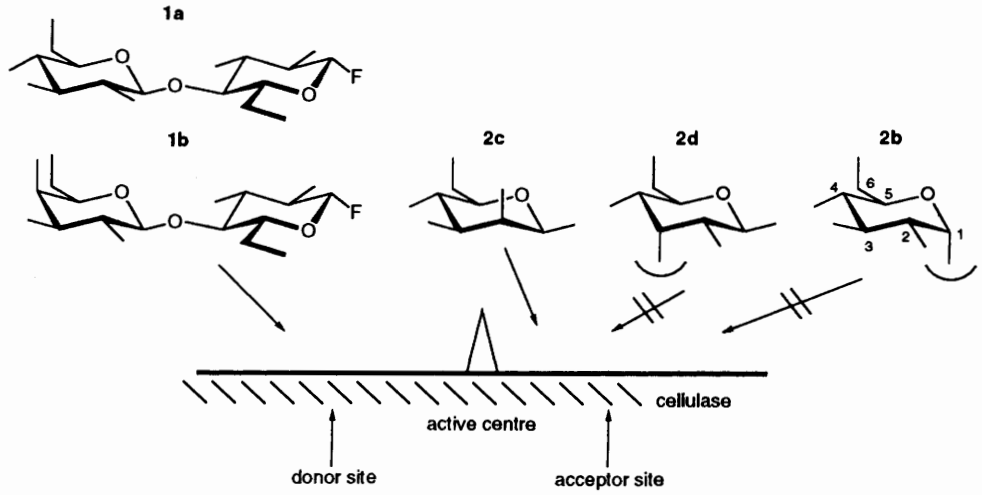


Fig. 2 Orientation of the glycosyl donors cellobiosyl fluoride **1a** and lactosyl fluoride **1b** and the glycosyl acceptors towards the enzyme surface. Compounds **2a** and **2c** can be lactosylated, whereas epimers **2b** and **2d** cannot be lactosylated due to steric hindrance of the axial groups in the 1- and 3-position

Subsequent methanolysis[17] led quantitatively to the deprotected glucopyranoside. ^1H as well as ^{13}C NMR analysis showed the presence of two diastereoisomeric oxidation products (d.e. 20%). This ratio could be estimated by integration of the C-1 proton or by HPLC; δ_H(250 MHz; CD_3OD; 1,4-dioxane) 4.50 (0.6 H, d, *J* 9.7, 1-H major diastereoisomer), 3.96 (0.4 H, d, *J* 9.7, 1-H minor diastereoisomer), 2.8–3.7 (6 H, m, 2-, 3-, 4- and 5-H and 6-H_2); δ_C(62.8 MHz; CD_3OD; MeOH) (major diastereoisomer) 139.0 (*ipso*), 133.2 (*o*), 130.0 (*p*), 127.8 (*m*), 94.3 (C-1), 83.5 (C-5), 71.0, 71.6 and 79.2 (C-2, -3 and -4), 63.1 (C-6); (minor diastereoisomer) 140.5 (*ipso*), 132.6 (*o*), 130.1 (*p*), 126.5 (*m*), 94.8 (C-1), 82.2 (C-5), 69.9, 70.0 and 79.1 (C-2, -3 and -4) and 61.8 (C-6).

General Lactosylation Procedure.—In a typical run, equimolar amounts (0.1 to 0.5 mmol) of compound **1b** and the glycosyl acceptor were dissolved in the organic solvent and $\frac{2}{3}$ of the buffer and the mixture was shaken at 30 °C. Then 5 wt% of cellulase for compound **1b**, dissolved in the last $\frac{1}{3}$ of buffer, was added. Yields were determined by taking aliquots of the sample and evaporation of the solvent prior to HPLC analysis. For final work-up the reaction mixture was heated to 100 °C for 10 min, prior to semipreparative HPLC to obtain samples for NMR analysis. All HPLC fractions containing oligosaccharide products were found to be chromographically homogeneous.

Inverse Lactosylation Procedure.—The glycosyl acceptors **2a**, **2l** and **2m** were each incubated with cellulase in half of the final amount of buffer under shaking. After 30 min at 30 °C compound **1b** was added, dissolved in the second half of the buffer. Yields were determined by taking aliquots of the sample and evaporation of the solvent prior to HPLC analysis. For final work-up the reaction mixture was heated to 100 °C for 10 min, prior to semipreparative HPLC to obtain samples for NMR analysis.

Methyl O-β-D-*Galactopyranosyl*-(1→4)-O-β-D-*glucopyranosyl*-(1→4)-β-D-*mannopyranoside* **3c**.—δ_H(250 MHz; D_2O; 1,4-dioxane) 4.6 (1 H, s, 1-H), 4.5 (1 H, d, *J* 6.6, 1′-H), 4.4 (1 H, d, *J* 6.7, 1″-H), 3.6 (3 H, s, OMe) and 3.2–4.1 [18 H, m, 2–6-, 2′–6′- and 2″–6″-$H_{(2)}$]; δ_C(62.8 MHz; D_2O; 1,4-dioxane) 103.7 (C-1′), 103.3 (C-1″), 101.7 (C-1), 79.0 (C-4′), 77.5 (C-4), 76.1 (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), 71.8 (C-2″), 70.6 (C-2), 69.4 (C-4″), 61.8 (C-6″), 61.2 (C-6), 60.8 (C-6′) and 57.6 (OMe).

Methyl O-β-D-*Galactopyranosyl*-(1→4)-O-β-D-*glucopyranosyl*-(1→4)-β-D-*xylopyranoside* **3g**.—δ_H(250 MHz; D_2O; 1,4-dioxane) 4.6 (1 H, d, *J* 7.8, 1-H), 4.4 (1 H, d, *J* 7.4, 1′-H), 4.3 (1 H, d, *J* 7.3, 1″-H), 3.6 (3 H, s, OMe) and 3.2–4.2 [16 H, m, 2′–6′-, 2″–6″- and 2–5-$H_{(2)}$]; δ_C(62.8 MHz; D_2O; 1,4-dioxane) 104.6 (C-1), 103.8 (C-1″), 101.8 (C-1′), 79.2 (C-4′), 76.2 (C-4), 75.6 (C-5″), 75.0 (C-5′), 74.7 (C-3′), 74.7 (C-3), 73.7 (C-2′), 73.42 (C-2), 73.36 (C-3″), 71.8 (C-2″), 69.4 (C-4″), 63.7 (C-5), 61.9 (C-6″), 60.9 (C-6′) and 58.0 (OMe).

S-*Dodecyl* O-β-D-*Galactopyranosyl*-(1→4)-O-β-D-*glucopyranosyl*-β-(1→4)-1-*thio*-β-D-*glucopyranoside* **3h**.—δ_H[250 MHz; $(CD_3)_2SO$; 1,4-dioxane] 4.5 (1 H, s, 1-H), 4.4 (1 H, d, *J* 6.6, 1′-H), 4.3 (1 H, d, *J* 6.7, 1″-H), 3.2–3.8 [18 H, m, 2–6-, 2′–6′- and 2″–6″-$H_{(2)}$], 2.68 (2 H, m, SCH_2), 1.53 (2 H, m, SCH_2CH_2), 1.24 (16 H, m, CH_2) and 0.86 (3 H, t, *J* 3.6, Me); δ_C(62.8 MHz; D_2O; 1,4-dioxane) 105.1 (C-1′), 102.9 (C-1″), 90.0 (C-1), 80.7 (C-4′), 80.4 (C-4), 79.0 (C-5″), 76.5 (C-5), 75.7 (C-5′), 75.0 (C-2′ and -3′), 73.4 (C-3″), 73.2 (C-3), 73.0 (C-2″), 70.7 (C-2), 68.4 (C-4″), 60.6 (C-6, -6′ and -6″), 36.5 (SCH_2), 34.5, 34.2, 34.1, 33.9, 33.8 and 33.6, (alkyl CH_2), 22.3 (CH_2Me) and 14.1 (Me).

S-*Phenyl* O-β-D-*Galactopyranosyl*-(1→4)-O-β-D-*glucopyranosyl*-(1→4)-1-*thio*-β-D-*glucopyranoside* **3i**.—δ_H(250 MHz; MeOD; 1,4-dioxane) 7.6 (5 H, m, Ph), 4.7 (1 H, overlapped with HDO, 1-H), 4.5 (1 H, d, *J* 6.3, 1′-H), 4.3 (1 H, d, *J* 6.7, 1″-H) and 3.2–3.8 [18 H, m, 2–6-, 2′–6′- and 2″–6″-$H_{(2)}$]; δ_C(62.8 MHz; MeOD; 1,4-dioxane) 135.5 (*ipso*), 133.1 (*p*), 128.7 (*m*), 128.2 (*o*), 102.9 (C-1′), 102.3 (C-1″), 87.2 (C-1), 80.4 (C-4), 78.6 (C-4′), 78.0 (C-5″), 75.7 (C-5), 75.2 (C-5′), 74.1 (C-3′), 72.6 (C-3″), 71.5 (C-2″ and C-3), 70.8 (C-2′), 69.4 (C-2), 68.5 (C-4″), 60.8 (C-6′ and -6″) and 59.7 (C-6).

S-(*Propylcarbamoylmethyl*) O-β-D-*Galactopyranosyl*-(1→4)-O-β-D-*glucopyranosyl*-(1→4)-1-*thio*-β-D-*glucopyranoside* **3j**.—δ_H(250 MHz; D_2O; 1,4-dioxane) 4.54 (2 H, d, *J* 7.2, 1- and 1′-H), 4.42 (1 H, d, *J* 6.8, 1″-H), 3.95 (1 H, s, SCH_2), 3.91 (1 H, s, SCH_2), 3.3–4.0 [18 H, m, 2–6-, 2′–6′- and 2″–6″-$H_{(2)}$], 3.18 (2 H, m, NCH_2), 1.53 (2 H, m, CH_2Me) and 0.89 (3 H, t, Me); δ_C(62.8 MHz; D_2O; 1,4-dioxane) 171.3 (CO), 102.0 (C-1′), 101.4 (C-1″), 84.0 (C-1), 77.8 (C-4′) 77.1 (C-5 and -5″), 74.5 (C-5′), 74.4 (C-2), 73.9 (C-3′), 73.2 (C-3″), 71.9 (C-2′), 71.5 (C-2″), 71.0 (C-4), 70.0 (C-4″), 67.6 (C-3), 60.1 (C-6″), 59.0 (C-6 and -6′), 40.7 (NCH_2), 32.4 (SCH_2), 20.8 (CH_2Me) and 9.7 (Me).

Methyl O-β-D-*Galactopyranosyl*-(1→4)-O-β-D-*glucopyranosyl*-(1→4)-O-3-*deoxy*-3-*fluoro*-β-D-*glucopyranoside* **3l**.[25]—δ_H(250 MHz; D_2O; 1,4-dioxane) 4.6 (1 H, d, *J* 6.6, 1-H), 4.5 (1 H, d, *J* 6.6, 1'-H), 4.4 (1 H, d, *J* 6.8, 1''-H), 3.6 (3 H, s, OMe) and 3.2–4.1 [18 H, m, 2–6-, 2'–6'- and 2''–6''-$H_{(2)}$]; δ_C(62.8 MHz; D_2O; 1,4-dioxane) 104.2 (C-1'), 103.6 (C-1), 103.5 (C-1''), 96.4 (d, $J_{C,F}$182, C-3), 79.5 (C-4'), 76.8 (d, $J_{C,F}$ 17.2, C-4), 76.6 (C-5''), 76.1 (C-5'), 75.4 (C-3'), 75.0 (d, $J_{C,F}$ 7.6, C-5), 74.2 (C-2'), 73.7 (C-3''), 72.9 (d, $J_{C,F}$ 18.1, C-2), 72.1 (C-2''), 69.8 (C-4''), 62.2 (C-6''), 61.2 (C-6), 60.9 (C-6') and 58.6 (OMe).

Methyl O-β-D-*Galactopyranosyl*-(1→4)-O-β-D-*glucopyranosyl*-(1→4)-O-3-O-*methyl*-β-D-*glucopyranoside* **3m**.[26]—δ_H(250 MHz; D_2O; 1,4-dioxane) 4.52 (1 H, d, *J* 7.8, 1-H), 4.44 (1 H, d, *J* 7.6, 1'-H), 4.37 (1 H, d, *J* 7.5, 1''-H), 3.6 (3 H, s, 1-OMe), 3.5 (3 H, s, 3-OMe) and 3.25–4.1 (18 H, m, 2–6-, 2'–6'- and 2''–6''-$H_{(2)}$]; δ_C(62.8 MHz; D_2O; 1,4-dioxane) 104.5 (C-1'), 104.4 (C-1), 103.7 (C-1''), 85.0 (C-3), 79.7 (C-4'), 77.1 (C-4), 76.8 (C-5), 76.5 (C-5''), 76.3 (C-5'), 75.8 (C-3'), 74.7 (C-2), 74.0 (C-2'), 73.6 (C-3''), 72.4 (C-2''), 70.0 (C-4''), 62.5 (3-OMe), 61.5 (C-6''), 61.4 (C-6), 60.6 (C-6') and 58.6 (1-OMe).

Methyl O-β-D-*Galactopyranosyl*-(1→4)-O-β-D-*glucopyranosyl*-(1→4)-O-β-D-*glucopyranosyl*-(1→4)-O-α-D-*glucopyranoside* **3q**.—δ_H(250 MHz; D_2O; 1,4-dioxane) 4.8 (1 H, overlapped with HDO, 1-H), 4.5 (2 H, d, *J* 8.4, 1'- and 1''-H), 4.4 (1 H, d, *J* 7.6, 1'''-H), 3.4 (3 H, s, OMe) and 3.3–4.1 [24 H, m, 2–6-, 2'–6'-, 2''–6''- and 2'''–6'''-$H_{(2)}$]; δ_C(62.8 MHz; D_2O; 1,4-dioxane) 103.8 (C-1'''), 103.2 (C-1' and -1''), 99.8 (C-1), 79.4 (C-4), 79.1 (C-4'), 79.0 (C-4''), 76.1 (C-5'''), 75.6 (C-5' and -5''), 74.9 (C-3''), 74.8 (C-3'), 73.8 (C-2'), 73.7 (C-5), 73.5 (C-3'''), 72.4 (C-3), 71.7 (C-2'' and -2'''), 71.1 (C-2), 69.4 (C-4'''), 61.5 (C-6''), 60.6 (C-6, -6' and -6''') and 55.9 (OMe).

S-*Methyl* O-β-D-*Galactopyranosyl*-(1→4)-O-β-D-*glucopyranosyl*-(1→4)-O-β-D-*glucopyranosyl*-(1→4)-1-*thio*-β-D-*glucopyranoside* **3r**.—δ_H(250 MHz; D_2O; 1,4-dioxane) 4.6 (1 H, d, *J* 7.8, 1''-H), 4.51 (1 H, d, *J* 7.8, 1'-H), 4.45 (1 H, d, *J* 7.7, 1'''-H), 4.2 (1 H, d, *J* 7.6, 1-H), 3.2–4.1 [24 H, m, 2–6-, 2'–6'-, 2''–6''- and 2'''–6'''-$H_{(2)}$] and 2.2 (SMe).

Methyl O-β-D-*Galactopyranosyl*-(1→4)-O-β-D-*glucopyranosyl*-(1→4)-O-β-D-*glucopyranosyl*-(1→3)-O-β-D-*glucopyranoside* **3s**.—δ_H(250 MHz; D_2O; 1,4-dioxane) 4.8 (1 H, d, *J* 7.8, 1'-H), 4.55 (1 H, d, *J* 7.8, 1''-H), 4.47 (1 H, d, *J* 9.5, 1'''-H), 4.44 (1 H, d, *J* 9.9, 1-H), 3.6 (3 H, s, OMe) and 3.3–4.1 [24 H, m, 2–6-, 2'–6'-, 2''–6''- and 2'''–6'''-$H_{(2)}$]; δ_C(62.8 MHz; D_2O; 1,4-dioxane) 103.8 (C-1 and -1'''), 103.5 (C-1'), 103.2 (C-1''), 85.4 (C-3), 79.4 (C-4'), 79.0 (C-4''), 76.4 (C-5), 76.2 (C-5'''), 75.7 (C-5' and -5''), 75.0 (C-3'), 74.9 (C-3''), 74.1 (C-2'), 73.69 (C-2''), 73.61 (C-2), 73.4 (C-3'''), 71.7 (C-2'''), 69.4 (C-4'''), 69.0 (C-4), 61.8 (C-6'''), 61.5 (C-6), 60.8 (C-6' and -6'') and 58.0 (OMe).

Methyl O-β-D-*Galactopyranosyl*-(1→4)-O-β-D-*glucopyranosyl*-(1→4)-O-β-D-*glucopyranosyl*-(1→6)-O-β-D-*glucopyranoside* **3t**.—δ_H(250 MHz; D_2O; 1,4-dioxane) 4.6 (2 H, d, *J* 7.8, 1'- and 1''-H), 4.5 (1 H, d, *J* 7.8, 1'''-H), 4.4 (1 H, d, *J* 7.6, 1-H) and 3.2–4.3 [27 H, m, 2–6-, 2'–6'-, 2''–6''- and 2'''–6'''-$H_{(2)}$ and OMe]; δ_C(62.8 MHz; D_2O; 1,4-dioxane) 104.2 (C-1), 103.8 (C-1'''), 103.5 (C-1'), 103.2 (C-1''), 79.3 (C-4'), 78.9 (C-4''), 76.5 (C-3), 76.2 (C-5'''), 75.7 (C-5), 75.66 (C-5''), 75.57 (C-5'), 75.0 (C-3', C-3''), 73.9 (C-2), 73.7 (C-2' and -2''), 73.4 (C-3'''), 71.8 (C-2'''), 70.2 (C-4), 69.4 (C-6 and -4'''), 61.8 (C-6'''), 60.7 (C-6' and -6'') and 58.2 (OMe).

Conclusions.—Cellulase, the enzyme responsible for the hydrolysis of β(1→4) glucosidic bonds in cellulose, proved to be useful for the synthesis of a large variety of oligosaccharides. The fact that non-glucose and non-natural saccharides can also be used as glycosyl acceptors broadens the applicability of this reaction tremendously. Information about the shape of the active site of cellulase and the orientation of the saccharides therein can be used for the tailoring of substrates suitable for further synthesis of oligosaccharides.

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References

1 H. Paulsen, *Angew. Chem., Int. Ed. Engl.*, 1982, **21**, 155; R. R. Schmidt, *Angew. Chem., Int. Ed. Engl.*, 1986, **25**, 121; H. Kunz, *Angew. Chem., Int. Ed. Engl.*, 1987, **26**, 294.
2 S. Sabesan and J. C. Paulson, *J. Am. Chem. Soc.*, 1986, **108**, 2068; M. M. Palcic, A. P. Venot, R. M. Ratcliffe and O. Hindsgaul, *Carbohydr. Res.*, 1989, **190**, 1; E. J. Toone, E. S. Simon, M. D. Bednarski and G. M. Whitesides, *Tetrahedron*, 1989, **45**, 5365; S. David, C. Aug and C. Gautheron, *Adv. Carbohydr. Chem. Biochem.*, 1991, **49**, 175.
3 K. G. I. Nilson, *Trends Biotechnol.*, 1988, **6**, 256; C. Bucke and R. A. Rastall, *Chem. Br.*, 1990, **26**, 675.
4 S. Kobayashi, K. Kashiwa, T. Kawasaki and S. Shoda, *J. Am. Chem. Soc.*, 1991, **113**, 3079; S. Kobayashi, K. Kashiwa, J. Shimada, T. Kawasaki and S. Shoda, *Makromol. Chem. Makromol. Symp.*, 1992, **54/55**, 509.
5 (*a*) S. Kobayashi, T. Kawasaki, K. Obata and S. Shoda, *Chem. Lett.*, 1993, 685; (*b*) S. Shoda, T. Kawasaki, K. Obata and S. Kobayashi, *Carbohydr. Res.*, 1993, **249**, 127.
6 S. Shoda, K. Obata, O. Karthaus and S. Kobayashi, *J. Chem. Soc., Chem. Commun.*, 1993, 1402.
7 P. A. J. Gorin, *Adv. Carbohydr. Chem. Biochem.*, 1981, **38**, 13.
8 (*a*) M. L. Chawla and O. P. Bahl, *Carbohydr. Res.*, 1974, **32**, 25; (*b*) P. L. Durette and T. Y. Shen, *Carbohydr. Res.*, 1978, **67**, 484.
9 L. Garuti, M. Roberti, G. Giovanninetti, R. Raggi, J. Defaye and H. Driquez, *Pharm. Acta Helv.*, 1988, **63**, 202.
10 P. Fugedi, P. J. Garegg, H. Lonn and T. Norberg, *Glycoconjugate J.*, 1987, **4**, 97.
11 S. Raghavan and D. Kahne, *J. Am. Chem. Soc.*, 1993, **115**, 1580.
12 B. Srey and C. Lambert, *FEMS Microbiol. Lett.*, 1984, **23**, 227.
13 P. J. Kraulis, G. M. Clore, M. Nilges, T. A. Jones, G. Petterson, J. Knowles and A. M. Gronenborn, *Biochemistry*, 1989, **28**, 7241; J. Rouvinen, T. Bergfors, T. Teeri, J. K. C. Knowles and T. A. Jones, *Science*, 1990, **249**, 380.
14 H.-L. Lai, L. G. Butler and B. Axelrod, *Biochem. Biophys. Res. Commun.*, 1974, **60**, 635.
15 R. J. Whistler and M. L. Wolfrom, *Methods in Carbohydrate Chemistry*, Academic Press, New York, 1963, vol. 3, pp. 215–220.
16 G. Zemplen and A. Gerecs, *Ber. Dtsch. Chem. Ges.*, 1930, **63**, 2720.
17 R. G. Edwards, L. Hough and A. C. Richardson, *Carbohydr. Res.*, 1977, **55**, 129.
18 G. M. Bebault and G. F. S. Dutton, *Carbohydr. Res.*, 1974, **37**, 309.
19 C. D. Hurd and W. A. Brunner, *J. Org. Chem.*, 1946, **11**, 50.
20 T. J. Tewson and M. J. Welch, *J. Org. Chem.*, 1978, **43**, 1090.
21 J. C. Irvine and A. Hynd, *J. Chem. Soc.*, 1909, **95**, 1222; D. J. Loder and W. E. Lewis, *J. Am. Chem. Soc.*, 1932, **54**, 1044.
22 W. P. Shyluck and T. E. Timell, *Can. J. Chem.*, 1956, **34**, 571.
23 T. Mukaiyama, Y. Murai and S. Shoda, *Chem. Lett.*, 1981, 431.
24 P. Brougham, M. S. Cooper, D. A. Cummerson. H. Heaney and N. Thompson, *J. Chem. Soc., Chem. Commun.*, 1987, 1015.
25 V. Wray, *J. Chem. Soc., Perkin Trans. 2*, 1976, 1598.
26 R. U. Lemieux and J. D. Stevens, *Can. J. Chem.*, 1965, **43**, 2061.

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