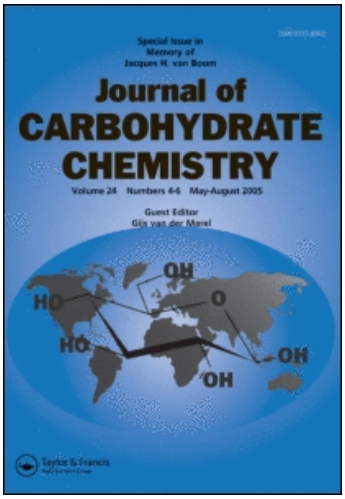

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SYNTHESES OF MODIFIED CARBOHYDRATES WITH GLYCOSIDASES: STEREO- AND REGIOSPECIFIC SYNTHESES OF LACTOSAMINE DERIVATIVES AND RELATED COMPOUNDS[1]

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ABSTRACT

Different lactosamine derivatives, modified in the 2-*N*- and anomeric positions and suitable as intermediates for synthesis of Lewis-x and related compounds, were prepared with high specificity on a multigram scale directly from lactose, employing different D-glucosamine derivatives as acceptors and the abundant β-D-galactosidase from *Bullera singularis* as catalyst. Thus, methyl *O*-β-D-galactopyranosyl-(1→4)-2-azido-2-deoxy-β-D-glucopyranoside, ethyl *O*-β-D-galactopyranosyl-(1→4)-2-deoxy-2-phthalimido-1-thio-β-D-glucopyranoside and ethyl *O*-β-D-galactopyranosyl-(1→4)-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-1-thio-β-D-glucopyranoside were formed in 20-40 % yield as calculated based on added acceptor. The 2-phthalimido derivative was isolated in crystalline form without chromatography (extraction/crystallization procedure). The trisaccharide derivative ethyl *O*-β-D-galactopyranosyl-(1→4)-*O*-β-D-galactopyranosyl-(1→4)-2-deoxy-2-phthalimido-1-thio-β-D-glucopyranoside was also isolated.

The corresponding cellobiosamine derivatives were similarly obtained from cellobiose using the same type of catalyst and acceptors. The β-D-galactosidase from bovine testes was found to catalyze the highly specific formation of the β(1→6)-linked derivative ethyl *O*-β-D-galactopyranosyl-(1→6)-2-deoxy-2-phthalimido-1-thio-β-D-glucopyranoside from lactose and ethyl 2-deoxy-2-phthalimido-1-thio-β-D-glucopyranoside. Ethyl *O*-α-L-fucopyranosyl-(1→4)-2-amino-6-*O*-benzyl-2-deoxy-1-thio-β-D-glucopyranoside, suitable as an intermediate for preparation of Lewis-a and related compounds, was obtained from *p*-nitrophenyl α-L-fucopyranoside and 2-amino-6-*O*-benzyl-2-deoxy-1-thio-β-D-glucopyranoside, employing α-L-fucosidase from bovine testes as catalyst.

INTRODUCTION

Glycosidases are in general abundant enzymes which can be used for large-scale conversions without prior cloning. Moreover, simple substrates can be used, in contrast with nucleotide sugars which are required with alternative enzymes. Glycosidases have been used for the preparation of a broad range of glycosides, oligosaccharides and derivatives employing either reversed hydrolysis or transglycosylation reactions.[2-5] Thus, it has been shown that, e.g., allyl, benzyl and trimethylsilylethyl glycosides[6] as well as glycosylated amino acids or peptides[7,8] readily can be obtained in one-pot reactions directly from simple saccharides, such as lactose or raffinose. The regioselectivity of glycosidase-catalysed oligosaccharide synthesis can be manipulated by employing acceptor glycosides of different anomeric configuration and/or by changing the aglycon structure.[9] This approach has facilitated the preparative-scale synthesis of practically all disaccharide sequences and of several oligosaccharide sequences found in glycoconjugates.[5,10-13] Examples of scaled-up glycosidase-catalysed synthesis employing this approach in our laboratory, are the α-galactosidase-catalysed synthesis of the xeno-antigenic α-D-Gal*p*-(1→3)-D-Gal*p* structure[9] and the β-galactosidase-catalysed synthesis of the TF-antigen, β-D-Gal*p*-(1→3)-α-D-Gal*p*NAc-Ser.[14] The sequential use of glycosidase and glycosyltransferase is useful for synthesis of, e.g., sialylated trisaccharides, and reduces the need for less abundant glycosyltransferases and nucleotide sugars.[15]

It has been demonstrated that partially protected/modified saccharides can be specifically glycosylated employing glycosidases as catalysts to give partially protected disaccharides of interest as intermediates for synthesis of oligosaccharides and analogues.[16,17] Interestingly, the reaction rates and yields in these reactions were similar to those obtained in reactions employing the corresponding non-modified acceptors. Also, the reactions were stereospecific and highly regioselective, and recovery of non-reacted acceptor was achieved by simple extraction/precipitation procedures. These characteristics together with abundant enzymes, are attractive for large-scale work.

There is an ongoing interest in the preparation of Lewis-a, Lewis-x, sialylated or sulfated derivatives thereof, as well as other compounds related to the Lewis-structures, e.g., for the development of potential anti-inflammatory agents.[18] In this report, we give

further examples of the preparative scale syntheses of partially modified disaccharides of interest as intermediates for the preparation of the Lewis-determinants, analogues and related compounds. Thus, the highly specific syntheses of the lactosamine derivatives **1**, **5** - **9**, the β-(1→6)-linked derivative **2**, the trisaccharide derivatives **3** and **4**, the cellobiosamine derivatives **9** - **11**, and the α-(1→4)-linked L-fucosyl-D-glucosamine derivative **12**, employing different glycosidases, are described.

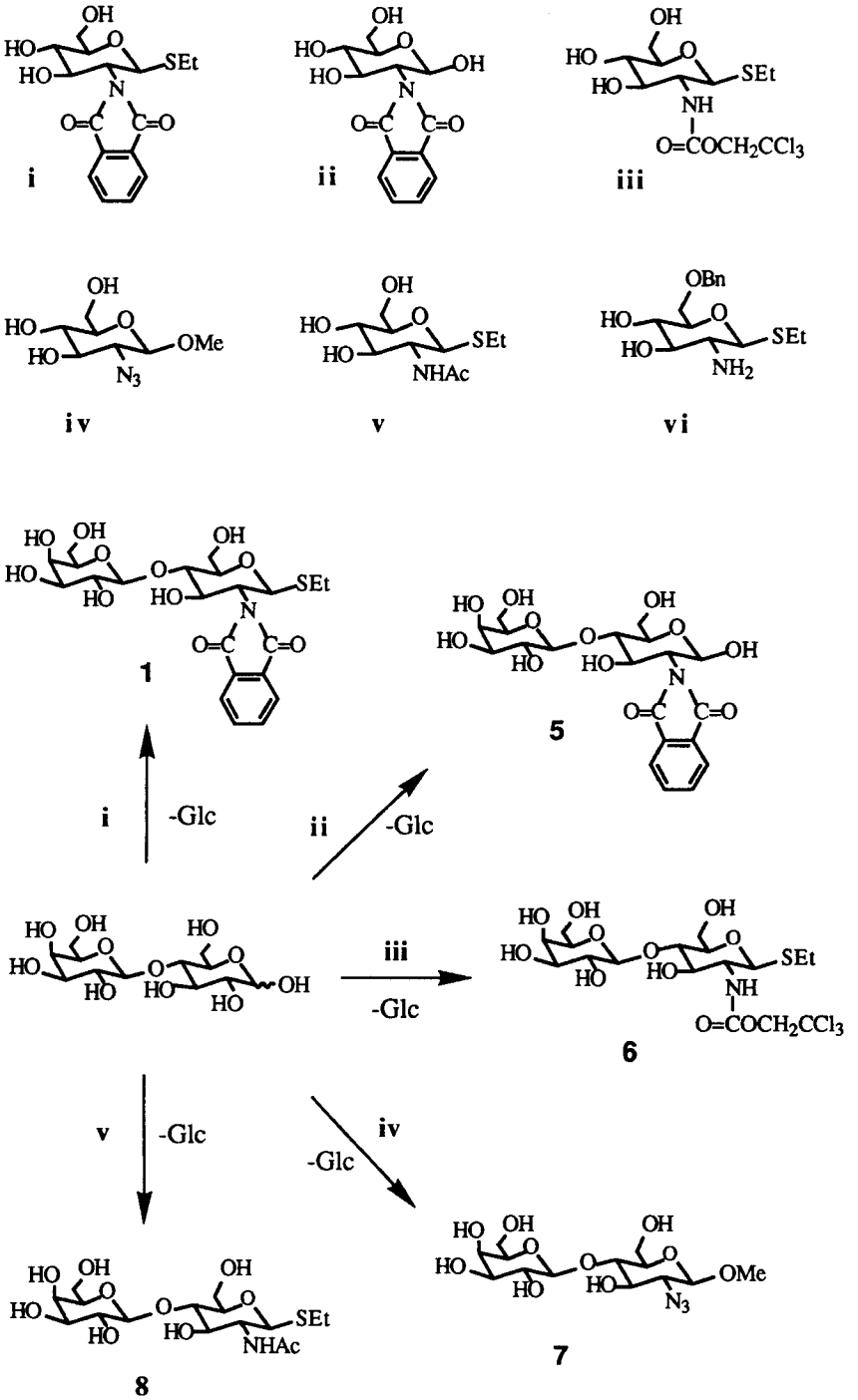
RESULTS AND DISCUSSION

We have previously reported the use of the β-D-galactosidase from the yeast *Bullera singularis* for the specific preparation of lactosamine derivatives directly from lactose and different acceptors.[14,17] Here, the D-glucosamine derivatives **i** - **v** were investigated and compared as acceptors for the preparative syntheses of the lactosamine derivatives **1** and **5** - **8**, employing the β-D-galactosidase activity of *Bullera singularis* (cf. Scheme 1). Acceptor compounds **i** - **v** were found to give the desired compounds stereospecifically, with high regioselectivity and in relatively good yields. No product formation was observed with compound **vi** as acceptor employing the yeast enzyme as catalyst.

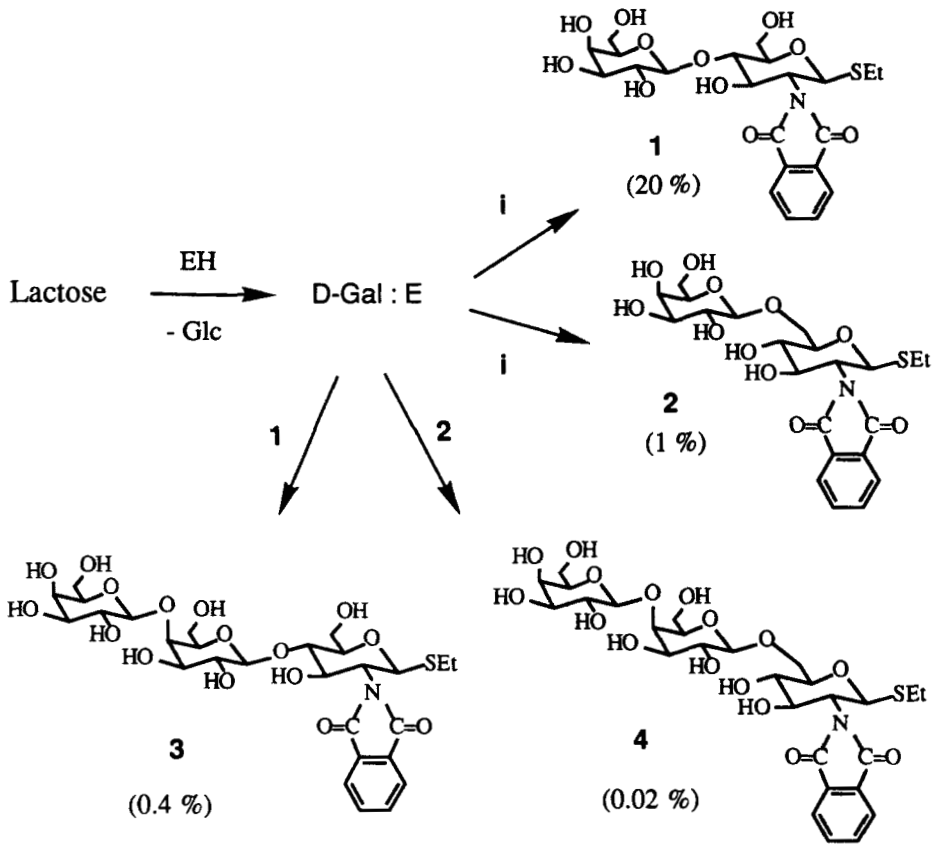
Compound **1**, ethyl *O*-β-D-galactopyranosyl-(1→4)-2-deoxy-2-phthalimido-1-thio-β-D-glucopyranoside, was formed in ca. 20 % yield (from HPLC analysis of the reaction mixture; mol product/mol added acceptor), or in the here reported example, ca. 29 g of product from 100 g of added acceptor. The yield of **1** was similar to that obtained with *N*-acetyl-D-glucosamine as acceptor. In addition to the stereospecificity, the reaction was practically regiospecific and only minor amounts of the β-(1→6)-linked isomer **2** was formed (less than 5 % of the β-(1→4)-isomer). Thus, the enzyme tolerates the relatively bulky phthalimido group in the 2-*N*-position of the acceptor remarkably well.

It is assumed that the reactions proceed via a common galactosyl-enzyme intermediate proposed for other β-galactosidases.[18] Thus, two trisaccharides, **3** and **4**, were formed in small amounts via reaction of the galactosyl-enzyme intermediate with the produced disaccharide derivatives **1** and **2** (cf. Scheme 2).

The minor by-products (**2**, **3** and **4**) did not complicate the purification of the desired product **1**. Thus, the non-reacted acceptor and the product were isolated from the



Scheme 1. Transglycosylation of lactose with β-D-galactosidase from *Bullera singularis*, employing compounds **i** - **v** as acceptors.



Scheme 2. Synthesis of disaccharide and trisaccharide derivatives from lactose with β-D-galactosidase from *Bullera singularis*, employing compound **i** as the initial acceptor.

reaction mixture in a straightforward manner by a) extraction of the reaction mixture with, e.g., ethyl acetate to recover non-reacted acceptor, b) extraction of the reaction mixture with butanol to remove product, followed by c) crystallization (cf. the Experimental part).

Compounds **5**, **6** and **8** were formed with high stereo- and regiospecificity and in similar yields (18 - 20 %) as compound **1**, whereas the 2-azido derivative (compound **7**) was formed in a higher yield, ca. 40 % yield as calculated on added acceptor. The acceptors **ii**, **iii**, **iv** and **v** could be used in a higher concentration than acceptor **i**, meaning that products **5** - **8** were obtained in higher concentrations in the reaction

mixture than compound **1**. Separate experiments showed that organic cosolvents could be used to increase the concentration of the acceptors, but at the expense of a reduced reaction rate.

Purification of compounds **5** - **8** required, at this point, column chromatography, since their extraction from the reaction mixture was less efficient due to the lower hydrophobicity of these products. Their purification by column chromatography (Sephadex^R and/or C-18 silica), was, however, straightforward. For example, the useful *N*-Teoc-lactosamine derivative, ethyl *O*-β-D-galactopyranosyl-(1→4)-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-1-thio-β-D-glucopyranoside (compound **6**), was isolated to homogeneity by one column chromatographic step (Sephadex^R G10; water as eluent), which also recovered non-reacted acceptor in pure form. No other regioisomer was apparently produced in this reaction.

In general, hydrolysis occurs as a side-reaction and limits the yield of glycosidase-catalysed transglycosylation reactions. Thus, galactose was formed in addition to glucose in the above reactions. Moreover, the donor (lactose) may also act as acceptor reacting with the galactosyl-enzyme intermediate, leading to trisaccharide formation. Separate experiments show that this reaction can be suppressed by employing a low concentration of donor (gradual addition of donor during the reaction).

However, none of these side-reactions leading to unmodified saccharides, caused complications in the isolation of the desired product. As reported for other hydrophobic glycosides, e.g., nitrophenyl disaccharide glycosides,[9,20] compounds **1** - **9** were slightly and differently retained on the Sephadex material compared to non-modified saccharides, which facilitated their separation and isolation. Different separation materials may be used (e.g., reversed phase-silica, charcoal-celite), but we found Sephadex to be advantageous, it was repeatedly used with water as eluent without decrease of separation efficiency.

The above method for production of lactosamine derivatives seems attractive for large scale work: lactose is a cheap substrate (currently a few USD/kg), the acceptors are commercially available in larger quantities or can be prepared from glucosamine,[21-24] the products can be easily isolated, the non-reacted acceptor as well as the organic solvents which are used for extraction/precipitation can be recycled, and the yeast cells can be produced on any scale with lactose as carbon source.

The *p*-nitrophenyl β-D-galactopyranoside is often used instead of lactose as glycosyl donor in glycosidase-catalysed transglycosylation reactions.[2,9] This compound could also be used in the above reactions as shown by separate experiments. However, a modified scheme of product purification had to be applied employing this type of donor, due to its higher hydrophobicity and the production of *p*-nitrophenol.

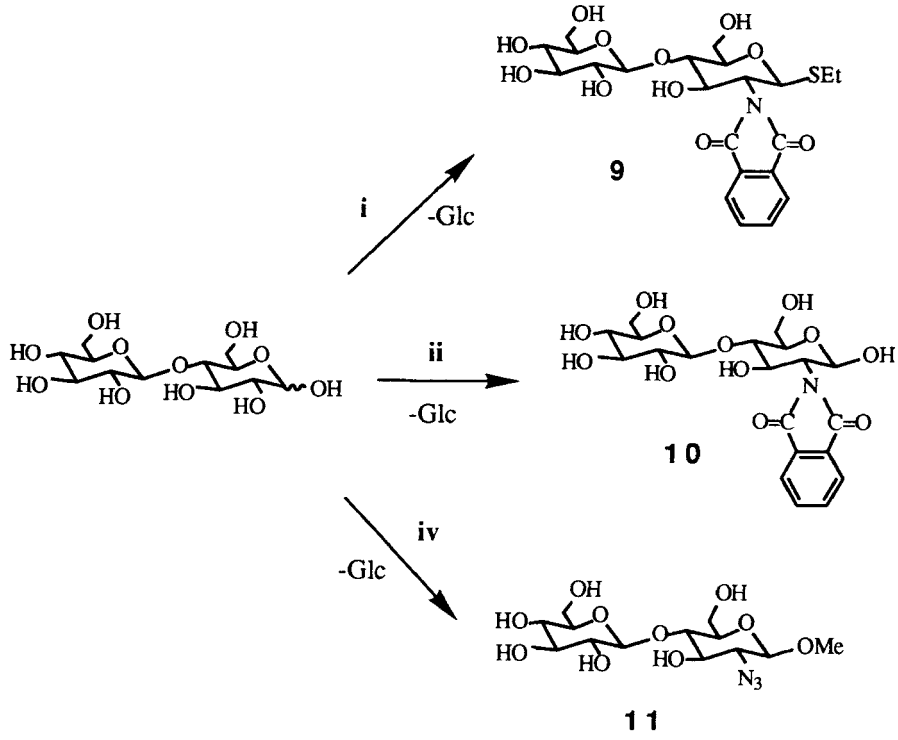
We observed that the yeast enzyme preparation also hydrolysed cellobiose, and it was found that the β-(1→4)-linked compounds **9** - **11** were formed from cellobiose and the acceptors **i**, **ii** and **iv**, respectively (Scheme 3).

Products **9** - **11** were formed with similar high specificity and also in similar yields as the corresponding lactosamine derivatives.

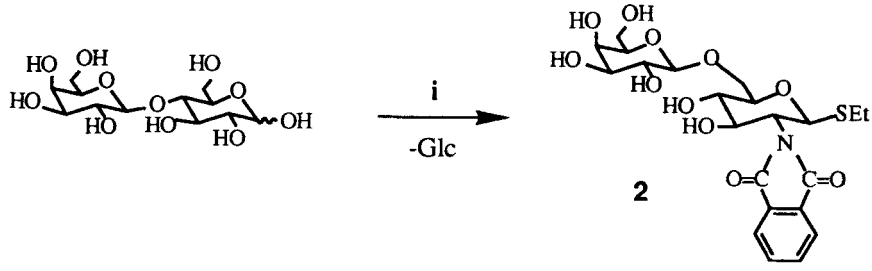
The yeast preparation may contain a β-D-glucosidase with practically identical acceptor specificity as the β-D-galactosidase. However, similar data obtained with the purified enzyme (to be published), indicate that one type of β-glycosidase (or different isozymes of the β-galactosidase) is responsible for production of both the lactosamine and the cellobiosamine derivatives. The ability of the *Bullera singularis* β-galactosidase to tolerate modification of the donor substrate is currentlyinvestigated substrates for the preparation of other β-(1→4)-linked derivatives.

The β-D-galactosidase from bovine testes have been used for the production of various β-(1→3)-linked and β-(1→4)-linked D-Gal*p*-D-Glc*p*NAc glycosides as well as β-(1→3)-linked D-Gal*p*-D-Gal*p*NAc glycosides.[15,25] In these reactions, the β-(1→6)-linked product is obtained in minor amounts (which can be removed by hydrolysis with a second type of β-galactosidase). However, it was shown recently that the bovine testes glycosidase exclusively formed the β-(1→3)-linkage with α-D-Gal*p*NAc-L-serine, or derivatives thereof, as acceptors.[1,14] This shows the importance of the anomeric configuration and/or aglycon structure on the regioselectivity of glycosidase-catalysed transglycosylation reactions.[9] Moreover, exclusive formation of the β-(1→3)-linkage was obtained with ethyl 2-amino-6-*O*-benzyl-2-deoxy-1-thio-β-D-glucopyranoside[26] (**vi**), as acceptor and in relatively good yield (30 %).[17]

However, when different types of D-glucosamine derivatives modified in the 2-amino group, were tested as acceptors with the β-galactosidase from bovine testes, we obtained preponderant formation of the β-(1→6)-linkage in several cases. The use of the phthalimido derivative **i**, gave specific formation of the β-(1→6)-linked regioisomer **2** in relatively good yield (20 % as calculated on the acceptor; Scheme 4).



Scheme 3. Transglycosylation of cellobiose with β-D-galactosidase from *Bullera singularis,* employing compounds **i**, **ii** and **iv** as acceptors.



Scheme 4. Transglycosylation of lactose with β-D-galactosidase from bovine testes, employing compound **i** as acceptor.

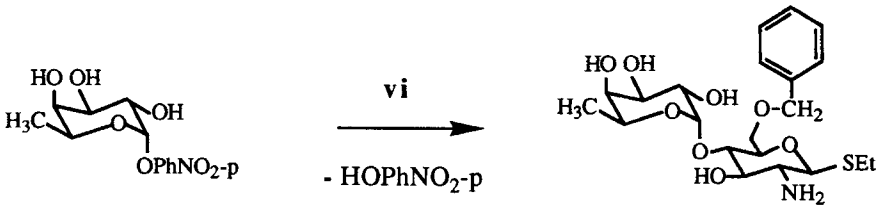
Thus, the β-galactosidase from bovine testes can be manipulated to produce exclusively either β-(1→3)-linked (Lewis-a type) or β-(1→6)-linked D-galactosyl-D-glucosamine-derivatives, depending on the modification of the glucosamine acceptor. This is in contrast with the *Bullera singularis* β-D-galactosidase, which as mentioned above, gave practically exclusive formation of β-(1→4)-linked lactosamine derivatives. Interestingly, no product was obtained with derivative **vi** as acceptor employing the yeast β-galactosidase. This indicates that the 6-*O*-benzyl group is not tolerated by this enzyme.

We have previously reported the stereo- and regiospecific α-L-fucosylation at position 4 of derivative **vi**, employing the α-L-fucosidase from bovine kidney. This allowed for the preparative synthesis of compound **12** in relatively good yield (30 %).[17]

The bovine testes preparation contained substantial amounts of α-L-fucosidase activity. It was found that this enzyme preparation also catalysed the specific formation of **12** from *p*-nitrophenyl α-L-fucopyranoside and **vi** (Scheme 5). Recovery of non-reacted acceptor and isolation of pure product was obtained by extraction and ion-exchange chromatography.

Compound **12** is a useful intermediate for synthesis of Lewis-a type compounds. Moreover, the 6-, 3-, 2-amino- or 1-positions in **12** may be specifically modified. Bovine testes and bovine kidney are waste products which are available in large quantities at low cost, donor and acceptor substrates are commercially available in larger quantities and compound **12** may thus be produced on a large scale.

Compounds **1**, and **5** - **8** are of interest for preparation of Lewis-x type compounds. Thus, they may, for example, be sialylated with sialyltransferases or sulphated in the 3'-position or other positions before or after other modifications. The protection/modification of the 2-*N*-position with the common azido,[21] phthalimido[22] or Teoc groups,[23] and of the 1-position with the thioethyl group,[24] give the opportunity to specifically modify the products with a number of other chemical groups. This facilitates the construction of a broad range of carbohydrate-based compounds or to use the products as building blocks in oligosaccharide synthesis. It may be noted that compound **1**, when conventionally treated with acetic anhydride in pyridine, gave an acylated compound in which the 3-OH group was non-acylated (ca. 50 % yield), which gives the opportunity to use this group for further synthesis (to be published).



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Scheme 5. Transglycosylation of *p*-nitrophenyl α-L-fucopyranoside with α-L-fucosidase from bovine testes, employing compound **vi** as acceptor.

In conclusion, β-(1→3)-, β-(1→6)-, or β-(1→4)-linked D-galactosyl-D-glucosamine derivatives can be prepared with high specificity, directly from lactose employing different D-glucosamine derivatives as acceptors and with abundant glycosidases as catalysts. The *Bullera singularis* enzyme catalyses the highly specific synthesis of a range of lactosamine and cellobiosamine derivatives employing lactose or cellobiose, respectively, as glycosyl donors. This enzyme can also be employed for the specific β-(1→4)-galactosylation of other glucosamine compounds, such as β-D-Glc*p*NAc-OPhNO$_2$-*p* and β-D-Glc*p*NAc-L-serine derivatives.[1,14] Since the reagents used in the above reactions are available at low cost and in bulk quantities, non-reacted acceptors can be recovered and as products are easy to isolate, the above glycosidase-reactions may be used for bulk scale syntheses.

EXPERIMENTAL

D-(+)-Cellobiose (98 %; crystalline; Sigma, St. Louis, USA), α-lactose (monohydrate; Sigma, St. Louis, USA), *p*-nitrophenyl α-L-fucopyranoside (97 %; Glycorex AB, Lund, Sweden) and acceptor compounds (**i** - **vi**; 95 %; Glycorex AB, Lund, Sweden), were used as supplied. Sephadex^R G10 was obtained from Pharmacia, Uppsala, Sweden and silica gel (Kieselgel 60; 0.040 - 0.063 mm) from E. Merck, Darmstadt, Germany. The ammonium sulphate preparation containing β-D-galactosidase and α-L-fucosidase from bovine testes, were from Glycorex AB, Lund, Sweden. Yeast

cells from *Bullera singularis* were obtained by fermentation with lactose as carbon source as described.[14] Yeast cells were stored frozen before use or used directly after production by fermentation. The crude β-D-galactosidase preparation from *Bullera singularis* (a 10 liter fermentation gave ca. 500 g of crude preparation) were obtained as described.[14] Double distilled water, analytical grade organic solvents and buffer salts were used for all reactions and purifications. NMR spectra were recorded at ambient temperature with a JEOL 400 Hz instrument, using solvent peaks as internal standards (CD_3OD, δ_C 49.0, δ_H 3.30; Me_2SO-d_6, δ_C 39.5, δ_H 2.50) and TMSNa as internal standard with D_2O as solvent. Chemical shifts were assigned by H,H and H,C COSY and long range H,H and H,C experiments. Beckman Gold^R HPLC-system was used for HPLC-analyses, and a Büchi preparative HPLC-system was used for preparative reversed phase (C18-silica) chromatography. Melting points were determined with an Electrothermal 1A 9200 apparatus and are uncorrected. Optical rotations were measured with a Perkin-Elmer 241 polarimeter.

Ethyl *O*-β-D-Galactopyranosyl-(1→4)-2-deoxy-2-phthalimido-1-thio-β-D-glucopyranoside (1). Lactose (500 g, 1.4 mol) and ethyl 2-deoxy-2-phthalimido-1-thio-β-D-glucopyranoside (**i**; 100 g, 0.28 mol) were dissolved in sodium acetate buffer, (pH 6.0, 10 L) and yeast cells (250 g) were added. The reaction was allowed to proceed at 30 °C for three days, when an additional amount of donor (300 g) and yeast cells (250 g) were added. After 3 additional days, the reaction was terminated by separation of the yeast cells, the reaction medium was concentrated to 5 L and extracted with ethyl acetate (3 x 2.5 L) and 1-butanol (3 x 2.5 L). The ethyl acetate and butanol phases were concentrated to give crude, unreacted acceptor (90 g; contained also minor amount of **1**) and crude **1** (70 g; contained also minor amounts of trisaccharide and possibly salts), respectively. Crystallization from methanol:water (1:1; v/v) followed by recrystallization from water gave **1** (14 g, 9.7 %): mp 258.7-259.7 °C; $[\alpha]_D$ +24.8° (*c* 0.5, Me_2SO); NMR data (Me_2SO-d_6): Selected ^1H, δ 5.17 (d, 1H, $J_{1,2}$ = 10.2 Hz, H-1), 3.90 (t, 1H, $J_{2,3}$ = 10.3 Hz, H-2), 4.21 (t, 1H, $J_{3,4}$ = 9.3, H-3), 4.26 (d, 1H, $J_{1,2}$ = 6.8 Hz, H-1'), 1.08 (t, 3H, $J_{1,2}$ = 7.3 Hz, $SCH_2C\underline{H}_3$), 2.55 - 2.62 (m, 2H, $SC\underline{H}_2CH_3$), 7.81 - 7.90 (m, 4H, phthalimido group); ^{13}C, δ 80.32 (C-1), 55.65 (C-2), 70.21 (C-3), 79.61 (C-4), 75.64 (C-5), 60.48 (C-6), 103.93 (C-1'), 70.74 (C-2'), 73.24 (C-3'), 68.16 (C-4'), 80.74 (C-5'), 60.48 (C-6'); phthalimido group: 123.33,

123.61, 130.76, 130.96, 135.02, 135.09, and 167.39 167.63 (2 C=O); SEt group: 14.92 and 23.27.

Anal. Calcd for $C_{22}H_{29}NO_{11}S$ (515.53): C, 51.2; H, 5.6; N, 2.7, S, 6.2. Found: C, 50.5; H, 5.5; N, 3.0, S, 6.0.

The supernatant from the above product precipitation was further purified by Sephadex G10 chromatography (water as eluent) to give additional **1** (10 g, 7 %), which was pure according to NMR. This chromatographic step also gave the β-(1→6)-linked regioisomer **2** (1.18 g, 0.8 %) and the trisaccharides **3** (408 mg, 0.2 %) and **4** (20 mg, 0.01 %) in pure form.

Ethyl *O*-β-D-Galactopyranosyl-(1→6)-2-deoxy-2-phthalimido-1-thio-β-D-glucopyranoside (2). This compound (obtained as described above) had the following data: $[\alpha]_D$ +5.8° (*c* 0.4, H_2O); NMR data (CD_3OD): Selected ^1H, δ 5.34 (d, 1H, $J_{1,2}$ = 10.6 Hz, H-1), 4.08 (t, 1H, $J_{2,3}$ = 10.3 Hz, H-2), 4.38 (t, 1H, $J_{3,4}$ = 10.1, H-3), 4.39 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1'), 1.16 (t, 3H, $J_{1,2}$ = 7.3 Hz, $SCH_2\underline{CH_3}$), 2.58 - 2.75 (m, 2H, $SC\underline{H_2}CH_3$), 7.82 - 7.89 (m, 4H, phthalimido group); ^{13}C, δ 82.43 (C-1), 57.45 (C-2), 73.45 (C-3), 72.21 (C-4), 81.28 (C-5), 69.96 (C-6), 105.25 (C-1'), 72.48 (C-2'), 74.77 (C-3'), 70.20 (C-4'), 76.56 (C-5'), 62.41 (C-6'); phthalimido group: 124.09, 124.40, 132.67, 132.92, 135.58, 135.65, and 169.35, 169.63 (2 C=O); SEt group: 15.39 and 24.97.

Anal. Calcd for $C_{22}H_{29}NO_{11}S \cdot H_2O$ (533.54): C, 49.5; H, 5.8; N, 2.6, S, 6.0. Found: C, 49.3; H, 5.8; N, 2.6, S, 5.7.

Compound **2** was also prepared employing the following procedure: Lactose (50 g, 0.14 mol) and ethyl 2-deoxy-2-phthalimido-1-thio-β-D-glucopyranoside (**i**; 10 g, 28 mmol) were dissolved in buffer (25 mM sodium phosphate; pH 4.5; 400 mL volume) and crude bovine testes β-D-galactosidase preparation (2 g) was added at 30 °C. The reaction was carried out at 30 °C and stopped after 67 h when HPLC indicated that ca. 3 g product had been formed. The unreacted acceptor and product were removed by ethyl acetate extraction (2 x 1 L) and butanol extraction (3 x 1 L), respectively. Product **2** did not crystallize as described for the β-(1→4)-linked product **1** above, and, therefore, **2** was further purified by column chromatography (silica gel; CH_2Cl_2:MeOH:H_2O; 75:25:5, v/v/v). This gave after removal of solvent, 2 g of dry powder, which was > 90 % pure (NMR). The crude product was dissolved in water and extracted with ethyl acetate and butanol to give **2** (1.5 g, 10 %) which was pure according to NMR.

Ethyl *O*-β-D-Galactopyranosyl-(1→4)-β-D-galactopyranosyl-(1→4)-2-deoxy-2-phthalimido-1-thio-β-D-glucopyranoside (3). Compound **3** had the following data: $[\alpha]_D$ +13.5° (*c* 0.8, H_2O); NMR data (CD_3OD): Selected ^1H, δ 5.32 (d, 1H, $J_{1,2}$ = 10.4 Hz, H-1), 4.09 (t, 1H, $J_{2,3}$ = 10.5 Hz, H-2), 4.39 (dd, 1H, $J_{3,4}$ = 10.4 Hz, H-3), 4.42 (d, 1H, $J_{1,2}$ = 7.6 Hz, H-1'), 4.45 (d, 1H, $J_{1,2}$ = 7.7 Hz, H-1''), 1.17 (t, 3H, $J_{1,2}$ = 7.3 Hz, $SCH_2C\underline{H}_3$), 2.58 - 2.77 (m, 2H, $SC\underline{H}_2CH_3$), 7.82 - 7.89 (m, 4H, phthalimido group); ^{13}C, δ 82.43 (C-1), 57.04 (C-2), 72.06 (C-3), 81.34 (C-4), 80.95 (C-5), 62.03 (C-6), 105.01 (C-1'), 73.13 (C-2'), 75.07 (C-3'), 79.55 (C-4'), 76.91 (C-5'), 62.49 (C-6'), 106.77 (C-1''), 73.23 (C-2''), 75.13 (C-3''), 70.26 (C-4''), 76.18 (C-5''), 61.83 (C-6''); phthalimido group: 124.17, 124.47, 132.80, 132.97, 135.65, 135.69, and 169.27, 169.56 (2 C=O); SEt group: 15.30 and 24.90.

Anal. Calcd for $C_{28}H_{39}NO_{16}S \cdot 2H_2O$ (713.69): C, 47.1; H, 6.0; N, 2.0, S, 4.5. Found: C, 47.2; H, 6.1; N, 2.0, S, 4.4.

Ethyl *O*-β-D-Galactopyranosyl-(1→4)-β-D-galactopyranosyl-(1→6)--2-deoxy-2-phthalimido-1-thio-β-D-glucopyranoside (4). This compound had the following data: $[\alpha]_D$ +14.0° (*c* 0.35, H_2O); NMR data (CD_3OD): Selected ^1H, δ 5.31 (d, 1H, $J_{1,2}$ = 10.7 Hz, H-1), 4.05 (t, 1H, $J_{2,3}$ = 10.4 Hz, H-2), 4.26 (dd, 1H, $J_{3,4}$ = 10.1 Hz, H-3), 3.45 (dd, 1H, $J_{4,5}$ = 9.8 Hz, H-4), 4.36 (d, 1H, $J_{1,2}$ = 7.6 Hz, H-1'), 4.48 (d, 1H, $J_{1,2}$ = 7.6 Hz, H-1''), 1.16 (t, 3H, $J_{1,2}$ = 7.3 Hz, $SCH_2C\underline{H}_3$), 2.58 - 2.74 (m, 2H, $SC\underline{H}_2CH_3$), 7.81 - 7.87 (m, 4H, phthalimido group); ^{13}C, δ 82.52 (C-1), 57.60 (C-2), 73.54 (C-3), 72.31 (C-4), 81.44 (C-5), 70.20 (C-6), 105.31 (C-1'), 73.23 (C-2'), 75.21 (C-3'), 79.41 (C-4'), 75.70 (C-5'), 61.47 (C-6'), 106.85 (C-1''), 73.23 (C-2''), 75.21 (C-3''), 70.38 (C-4''), 77.02 (C-5''), 62.59 (C-6''); phthalimido group: 124.11, 124.42, 132.13, 132.87, 135.58, 135.65, and 169.35, 169.63 (2 C=O); SEt group: 15.42 and 25.04.

Anal. Calcd for $C_{28}H_{39}NO_{16}S \cdot 2H_2O$ (713.69): C, 47.1; H, 6.0; N, 2.0, S, 4.5. Found: C, 47.6; H, 6.0; N, 2.1, S, 4.1.

***O*-β-D-Galactopyranosyl-(1→4)-2-deoxy-2-phthalimido-β-D-glucopyranose (5):** Lactose (50 g, 0.14 mol) and 2-deoxy-2-phthalimido-β-D-glucopyranose (**ii**; 40.5 g, 0.13 mol) were dissolved in 1 L of 50 mM sodium acetate buffer, pH 6.0, and crude *Bullera singularis* β-D-galactosidase preparation (48 g) was

added. The reaction was allowed to proceed with shaking (120 rpm) at 27 °C for two days, when additional lactose (30 g) was added. After one additional day (totally 66 h reaction) the reaction was terminated by centrifugation (removal of the crude enzyme preparation) followed by brief heat treatment of the supernatant (80 °C for 5 min). The reaction mixture was extracted with ethyl acetate-butanol (94:4; v/v; 5 x 1 L) which removed most (ca. 18 g) of the unreacted acceptor, followed by precipitation of lactose, other simple saccharides and salts, employing a mixture of methanol:2-propanol (400 mL:8 mL; -20 °C). The product was isolated from the supernatant by chromatography (Sephadex G10; 19 L column volume; elution with water) and a repeated Sephadex G10 chromatography on a smaller column (1.5 L), which gave **5** (6 g, 9.5 %) after freeze-drying. (Another fraction contained a mixture of **5**, 0.9 g, and acceptor, ca. 10 g). $[\alpha]_D$ +33.3° (*c* 0.75, H_2O); NMR data (CD_3OD): Selected ^1H, δ 5.37 (d, 1H, $J_{1,2}$ = 8.3 Hz, H-1), 3.93 (dd, 1H, $J_{2,3}$ = 10.8 Hz, H-2), 4.40 (dd, 1H, $J_{3,4}$ = 10.7, H-3), 4.40 (d, 1H, $J_{1,2}$ = 7.3 Hz, H-1'), 7.79 - 7.84 (m, 4H, phthalimido group); ^{13}C, δ 93.84 (C-1), 59.22 (C-2), 71.09 (C-3), 81.46 (C-4), 76.93 (C-5), 62.06 (C-6), 105.14 (C-1'), 72.65 (C-2'), 74.80 (C-3'), 70.40 (C-4'), 77.16 (C-5'), 62.63 (C-6'); phthalimido group: 124.04, 124.27, 133.05 (2C), 135.50 (2C) and 169.76 (2 C=O groups).

Anal. Calcd for $C_{20}H_{25}NO_{12}\cdot H_2O$ (489.41): C, 49.0; H, 5.5; N, 2.9. Found: C, 48.6; H, 5.4; N, 2.9.

Ethyl *O*-β-D-Galactopyranosyl-(1→4)-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-1-thio-β-D-glucopyranoside (6). Lactose (20 g, 55.5 mmol) and ethyl 2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-1-thio-β-D-glucopyranoside (**iii**; 10 g, 25 mmol) were dissolved in 400 mL 50 mM sodium acetate, pH 6.0. Yeast cells were added (20 g) and the reaction was carried out at 30 °C with gentle agitation at pH 6.0 for three days. Column chromatography (Sephadex G10; 4 L column volume; water as eluent), gave pure non-reacted acceptor (7.9 g) and **6** (2.8 g after crystallization from ethanol; 19 %); mp 176.7-177.5 °C; $[\alpha]_D$ -20.0° (*c* 0.4, MeOH); NMR data (D_2O-CD_3OD; 1:1): Selected ^1H, δ 4.62 (d, 1H, $J_{1,2}$ = 10.6 Hz, H-1), 3.83 and 3.96 (dd, 2H, $J_{5,6a}$ = 4.9 Hz, $J_{5,6b}$ = not resolved, $J_{6a,6b}$ = 12.5 Hz, H-6a,6b), 4.45 (d, 1H, $J_{1,2}$ = 7.6 Hz, H-1'), 3.62 (dd, 1H, $J_{2,3}$ = 10.1 Hz, $J_{3,4}$ = 3.4 Hz, H-3'), 4.72 and 4.91 (d, 2H, $J_{a,b}$ = 12.2 Hz, -O$\underline{CH_2}CCl_3$), 1.25 (t, 3H, $J_{1,2}$ = 7.4

Hz, SCH_2CH_3), 2.67 - 2.79 (m, 2H, SCH_2CH_3); ^{13}C, δ 85.28 (C-1), 57.39 (C-2), 74.80 (C-3), 79.67 (C-4), 79.87 (C-5), 61.34 (C-6), 104.07 (C-1'), 72.08 (C-2'), 73.78 (C-3'), 69.67 (C-4'), 76.45 (C-5'), 62.06 (C-6'); Teoc-group: 157.03 (C=O), 75.26 (CH_2), 96.31 (CCl_3).

Anal. Calcd for $C_{17}H_{28}Cl_3NO_{11}S \cdot H_2O$ (578.83): C, 35.2; H, 5.2; N, 2.4, S, 5.5. Found: C, 35.6; H, 5.2; N, 2.5, S, 5.3.

Methyl *O*-β-D-Galactopyranosyl-(1→4)-2-azido-2-deoxy-β-D-glucopyranoside (7). Lactose (1.8 g, 5 mmol) and methyl 2-azido-2-deoxy-β-D-glucopyranoside (**iv**; 640 mg, 2.9 mmol) were dissolved in 26 mL 50 mM sodium acetate, pH 6.0. The crude *Bullera singularis* β-D-galactosidase preparation was added (2.1 g) and the reaction was carried out at 30 °C with gentle agitation at pH 6.0 for two days. Purification (non-optimized) was achieved by column chromatography with preparative HPLC (C18-silica; 300 mL; water-methanol; 100 to 50 % gradient) followed by Sephadex G10 (400 ml; water as eluent) to give after freeze-drying, pure acceptor (0.30 g) and **7** (150 mg, 13 % yield). $[\alpha]_D$ -7.9° (*c* 0.85, H_2O); NMR data (D_2O): Selected ^1H, δ 4.48 (d, 1H, $J_{1,2}$ = 8.8 Hz, H-1), 4.46 (d, 1H, $J_{1,2}$ = 9.3 Hz, H-1'), 3.54 (dd, 1H, $J_{2',3'}$ = 9.8 Hz, H-2'), 3.61 (s, 3H, OMe); ^{13}C NMR δ 102.90 (C-1), 66.03 (C-2), 73.44 (C-3), 79.12 (C-4), 75.70 (C-5), 60.87 (C-6), 103.87 (C-1'), 71.84 (C-2'), 74.02 (C-3'), 69.45 (C-4'), 76.25 (C-5'), 61.90 (C-6'), 58.11 (OMe).

Anal. Calcd for $C_{13}H_{23}N_3O_{10} \cdot 0.5H_2O$ (390.35): C, 40.0; H, 6.2; N, 10.8. Found: C, 40.4; H, 6.2; N, 10.6.

Ethyl *O*-β-D-Galactopyranosyl-(1→4)-2-acetylamino-2-deoxy-1-thio-β-D-glucopyranoside (8). Lactose (39 g, 0.11 mol) and methyl 2-acetylamino-2-deoxy-1-thio-β-D-glucopyranoside (**v**; 90 g, 0.34 mol) were dissolved in buffer (50 mM sodium phosphate; pH 6.0, 780 mL). Yeast cells (78 g) were added and the reaction was carried out at 35 °C at 130 rpm in a water-bath. Additional lactose was added after 2 and 3 days (40 g each time). The reaction was stopped after 6 days when HPLC indicated that ca. 20 % product (corresponding to ca. 28 g) had been formed. The mixture was centrifuged and applied to a column packed with active carbon (1.5 kg; Merck, p.a quality), which was eluted with water to remove other sugars and the product was eluted with 20 % aqeuous methanol. The solvent was removed from the product

containing fractions and the material was crystallized from 95 % ethanol to give **8** (16 g, 10.6 %); mp 210.6-212.0 °C; $[\alpha]_D$ -31.1° (*c* 0.8, H_2O-MeOH, 1:1); NMR data (D_2O): Selected ^1H, δ 4.49 (d, 1H, $J_{1,2}$ = 10.3 Hz, H-1), 3.65 (dd, 1H, $J_{2,3}$ = 11.3 Hz, H-2), 1.89 (s, 3H, Ac), 4.32 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1'), 1.09 (t, 3H, $J_{1,2}$ = 7.3 Hz, $SCH_2\underline{C}H_3$), 2.49 - 2.56 (m, 2H, $SC\underline{H}_2CH_3$); ^{13}C, δ 84.87 (C-1), 55.28 (C-2), 74.68 (C-3), 79.29 (C-4), 76.29 (C-5), 61.18 (C-6), 23.14 ($\underline{C}H_3C$=O), 175.32 (C=O), 103.78 (C-1'), 71.90 (C-2'), 73.45 (C-3'), 69.50 (C-4'),79.64 (C-5'), 61.95 (C-6'), 15.24 and 25.41 (SEt group).

Anal. Calcd for $C_{16}H_{29}NO_{10}S \cdot H_2O$ (445.51): C, 43.1; H, 7.0; N, 3.1, S, 7.2. Found: C, 43.7; H, 7.0; N, 3.3, S, 7.4.

Ethyl *O*-β-D-Glucopyranosyl-(1→4)-2-deoxy-2-phthalimido-1-thio-β-D-glucopyranoside (9). Cellobiose (9 g, 25 mmol) and ethyl 2-deoxy-2-phthalimido-1-thio-β-D-glucopyranoside (**i**; 1.35 g, 3.8 mmol) were dissolved in 112 mL 25 mM sodium phosphate buffer, pH was adjusted to 6.0, and crude β-D-galactosidase preparation from *Bullera singularis* was added (11 g). The reaction was allowed to proceed for 6 h at 30 °C. The reaction mixture was treated with methanol (precipitation of excess donor) followed by column chromatography (Sephadex G10; water as eluent) and concentration of product-containing fractions. Crystallization from water gave **9** (150 mg; 7.7 %); mp 241.7-243.0 °C; $[\alpha]_D$ +5.5° (*c* 0.4, H_2O-MeOH; 1:1); NMR data (CD_3OD): Selected ^1H, δ 5.39 (d, 1H, $J_{1,2}$ = 10.7 Hz, H-1), 4.16 (t, 1H, $J_{2,3}$ = 10.4 Hz, H-2), 4.48 (t, 1H, $J_{3,4}$ = 10.0, H-3), 4.58 (d, 1H, $J_{1,2}$ = 7.9 Hz, H-1'), 1.08 (t, 3H, $J_{1,2}$ = 7.3 Hz, $SCH_2\underline{C}H_3$), 2.70 - 2.80 (m, 2H, $SC\underline{H}_2CH_3$), 7.85 - 7.91 (m, 4H, phthalimido group); ^{13}C, δ 82.1 (C-1), 56.8 (C-2), 71.9 (C-3), 80.9 (C-4), 77.5 (C-5), 62.0 (C-6), 104.1 (C-1'), 74.6 (C-2'), 77.1 (C-3'), 70.9 (C-4'), 80.5 (C-5'), 61.6 (C-6'), 15.4, 25.0 (SEt group), 124.3, 124.6, 132.0, 132.2, 135.9, 169.7, 169.9 (phthalimido group).

Anal. Calcd for $C_{22}H_{29}NO_{11}S$ (515.53): C, 51.2; H, 5.6; N, 2.7, S, 6.2. Found: C, 50.7; H, 5.7; N, 2.8, S, 6.1.

***O*-β-D-Glucopyranosyl-(1→4)-2-deoxy-2-phthalimido-β-D-glucopyranose (10).** Cellobiose (8 g, 22 mmol) and 2-deoxy-2-phthalimido-β-D-glucopyranose (**ii**; 4 g, 12.9 mmol) were dissolved in 100 mL 25 mM sodium phosphate buffer, pH was adjusted to 6.0, and crude β-D-galactosidase preparation from *Bullera*

singularis (7.5 g) was added. The reaction was allowed to proceed for 4 h at 30 °C. The reaction mixture was treated with methanol (precipitation of excess donor) followed by column chromatography (Sephadex G10; C18-silica; Sephadex G-15) which gave **10** (400 mg, 6.3 %); $[\alpha]_D$ +25.1° (*c* 0.8, H_2O); NMR data (D_2O): Selected ^1H, δ 5.51 (d, 1H, $J_{1,2}$ = 9.5 Hz, H-1), 4.03 (t, 1H, $J_{2,3}$ = 10.3 Hz, H-2), 4.58 (d, 1H, $J_{1,2}$ = 8.3 Hz, H-1'), 7.80 - 7.90 (m, 4H, phthalimido group); ^{13}C, δ 95.4 (C-1), 59.6 (C-2), 72.9 (C-3), 82.7 (C-4), 79.5 (C-5), 64.1 (C-6), 106.0 (C-1'), 76.7 (C-2'), 79.0 (C-3'), 73.0 (C-4'), 78.6 (C-5'), 63.6 (C-6'), 127.0, 127.2, 134.2 (2C), 138.4 (2C), 173.5 (2C) (phthalimido group).

Anal. Calcd for $C_{20}H_{25}NO_{12}\cdot H_2O$ (489.42): C, 49.0; H, 5.5; N, 2.9. Found: C, 49.4; H, 5.6; N,3.0.

Methyl *O*-β-D-Glucopyranosyl-(1→4)-2-azido-2-deoxy-β-D-glucopyranoside (11). Cellobiose (800 mg, 2.2 mmol) and methyl 2-azido-2-deoxy-β-D-glucopyranoside (**iv**; 360 mg, 1.6 mmol) were dissolved in 10 mL 25 mM sodium phosphate buffer, pH was adjusted to 6.0, and crude β-D-galactosidase preparation from *Bullera singularis* was added (1 g). The reaction was allowed to proceed for 30 h at 30 °C. The reaction mixture was treated with methanol (precipitation of excess donor) followed by column chromatography (Sephadex G10; C-18) and crystallization from EtOH-MeOH (3:1) which gave **11** (50 mg, 8 %); mp 218.3-218.8 °C; $[\alpha]_D$ -24.5° (*c* 0.5, H_2O); NMR data (D_2O): Selected ^1H, δ 4.47 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1), 3.72 and 3.92 (dd, 2H, $J_{5,6a}$ = 4.6 Hz, $J_{5,6b}$ = 2.3 Hz, $J_{6a,6b}$ = 11.9 Hz, H-6a,6b), 4.51 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1'), 3.32 (dd, 1H, $J_{2,3}$ = 9.6 Hz, H-2'), 3.82 and 3.98 (dd, 2H, $J_{5,6a}$ = 4.4 Hz, $J_{5,6b}$ = 2.3 Hz, $J_{6a,6b}$ = 11.4 Hz, H-6a,6b), 3.95 (s, 3H, OMe); ^{13}C, δ 105.4 (C-1), 68.7 (C-2), 76.6 (C-3), 82.0 (C-4), 79.0 (C-5), 64.1 (C-6), 106.1 (C-1'), 76.5 (C-2'), 78.2 (C-3'), 72.9 (C-4'), 79.5 (C-5'), 63.4 (C-6'), 60.7 (OMe).

Anal. Calcd for $C_{13}H_{23}N_3O_{10}\cdot 0.5H_2O$ (390.35): C, 40.8; H, 6.2; N, 10.8. Found: C, 40.3; H, 6.3; N, 10.1.

Ethyl *O*-α-L-fucopyranosyl-(1→4)-2-amino-6-*O*-benzyl-2-deoxy-1-thio-β-D-glucopyranoside (12). *p*-Nitrophenyl α-L-fucopyranoside (86 mg, 0.3 mmol) and ethyl 2-amino-6-*O*-benzyl-2-deoxy-1-thio-β-D-glucopyranoside (**vi**; 0.33 g, 1 mmol) were dissolved in 10 mL 20 mM sodium acetate buffer, pH was adjusted to

6.0, and crude α-L-fucosidase from bovine testes was added (0.2 g; 0.1 U). The reaction proceeded for 52 h at 30 °C and was stopped by brief heat treatment. Non-reacted substrates and product **12** were isolated by extraction and ion-exchange chromatography (sodium acetate as eluent) as described previously.[14] The isolated product (acetate form; 56 mg, 33 % yield) was pure according to NMR. NMR data (CD_3OD): Selected ^1H NMR δ 4.32 (d, 1H, $J_{1,2}$ = 10.1 Hz, H-1), 3.38 (dd, 1H, $J_{3,4}$ = 9.8 Hz, H-3), 3.57 (dd, 1H, $J_{4,5}$ = 10.1, H-4), 3.81 and 3.92 (dd, 2H, $J_{5,6a}$ = 3.7 Hz, $J_{5,6b}$ = 1.8 Hz, $J_{6a,6b}$ = 11.3 Hz, H-6a,6b), 4.57 (d, 2H, $J_{1,2}$ = 5.1 Hz, OCH_2), 3.68 (d, 1H, $J_{2',3'}$ = 1.0 Hz, H-2'), 1.18 (d, 3H, $J_{5',6'}$ = 6.8 Hz, H-6'), 1.28 (d, 3H, $J_{1,2}$ = 7.3 Hz, $SCH_2\underline{C}H_3$), 2.66 - 2.71 (m, 2H, $S\underline{C}H_2CH_3$), 7.24 - 7.36 (m, 5H, C_6H_5); ^{13}C NMR δ 87.95 (C-1), 57.91 (C-2), 77.57 (C-3), 79.47 (C-4), 80.07 (C-5), 69.81 (C-6), 101.18 (C-1'), 71.29 (C-2'), 73.62 (C-3'), 70.00 (C-4'), 68.28 (C-5'), 16.58 (C-6'), 74.33 ($\underline{C}H_2C_6H_5$), 128.59, 128.88 (2C), 129.28 (2C), 139.65 (C_6H_5 group), 15.77 and 25.15 (SEt group).

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