Enzymatic Synthesis of Galactose-Containing Disaccharides Employing β-Galactosidase from *Bacillus circulans*

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Galβ1→4 disaccharide structures are vital core units of the oligosaccharide components of glycoconjugates. β-Galactosidase from *Bacillus circulans* (E.C.3.2.1.23) catalyses the transfer of galactose from a donor structure such as GalβO*p*NP to various GlcNAc and galactose derivatives, forming β1→4 linkages. The synthesis of several biologically relevant disaccharides {Galβ1→4GlcNAcαOAll (**3**), Galβ1→4GlcNAcβOAll (**5**), Galβ1→4GalαOAll (**10**), Galβ1→4GalβOAll (**12**), Galβ1→4GalβSPh (**14**), Galβ1→4GalβO*p*NP (**16**) and the trisaccharide Galβ1→4Galβ1→4GalβO*p*NP (**18**)} has been achieved in 30–66 % yield by application of this enzyme.

Introduction

The oligosaccharide components of glycoproteins and glycolipids play key roles in many vital biological processes, such as cell−cell recognition and interaction, cell communication and growth regulation.[1] Oligosaccharides with Galβ1→4 linkages are fundamental constituents of various biologically important glycoconjugates. For example, *N*-acetyllactosamine (Galβ1→4GlcNAc) is a typical terminal sequence of N-linked oligosaccharides and a core unit of carbohydrates isolated from human milk. It has been found in sialooligosaccharides, such as sialyl Lewis^x, as well as in ligands of various lectins.[2][3] Biologically active Galβ1→4Gal structures[4] can be isolated by partial hydrolysis of several of the polysaccharides obtained from the white birch (*Betula papyrifera*).[5][6] The need to investigate such compounds further is a powerful incentive to develop a synthesis of such oligosaccharides, and their analogues, inexpensively and in sufficient quantities.

The isolation of Galβ1→4-linked oligosaccharides from natural sources is both labour-intensive and difficult. Because of their low concentrations in complex natural product mixtures, such an approach is uneconomic on a large scale. Access to the desired Galβ1→4-linked structures by classical organic synthetic methodologies is often restricted by the need to perform laborious protective-group chemistry, which requires multistep syntheses resulting in low overall yields. In recent years, the use of enzymes has been investigated as an alternative to classical synthesis, following the biochemical pathways of carbohydrate metabolism.[7−9]

Two groups of enzymes can be used for the synthesis of glycosidic bonds: glycosyltransferases and glycosylhydrolases. Glycosyltransferases[10−12] act as in vivo biocatalysts and attach monosaccharide units to sugar chains, via the activated nucleotide components, with high stereo- and regiospecificity. To date, the application of glycosyl transferases to oligosaccharide synthesis has been restricted by their sensitivity, rarity, the requirement for expensive nucleotide donors, and cofactors, including cofactor regeneration.

In nature, glycosylhydrolases (glycosidases) usually cleave oligosaccharides to yield monosaccharides, but under unnatural conditions the equilibrium of the reversible cleavage can be shifted.[10−13] Thus, glycosylhydrolases can be used to form interglycosidic linkages under appropriate reaction conditions (transglycosylation). Glycosidase-catalysed reactions have the advantage of high enzyme stability and simple reaction conditions, and are relatively low-cost.

β-Galactosidase from *Bacillus circulans*[14−23] catalyses the transfer of galactose from GalβO*p*NP or lactose predominantly to the OH-4 position of Glc, GlcNAc, Gal, and GalNAc. We report here the enzymatic synthesis of the following di- and trisaccharides using β-galactosidase from *Bacillus circulans*: Galβ1→4GlcNAcαOAll (**3**); Galβ1→4GlcNAcβOAll (**5**); Galβ1→4GalαOAll (**10**); Galβ1→4GalβOAll (**12**); Galβ1→4GalβSPh (**14**); Galβ1→4GalβO*p*NP (**16**); Galβ1→4Galβ1→4GalβO*p*NP (**18**).

Results and Discussion

The chemoenzymatic synthesis of Galβ1→4 structures was achieved by transglycosylation using β-D-galactosidase from *Bacillus circulans* (E.C.3.2.1.23). The donor *p*-nitrophenyl β-D-galactopyranoside (GalβO*p*NP) was transferred to the 4-position of the appropriate glycosyl acceptor unit, resulting in the required β1→4 linkage. The enzymatic glycosylation was successfully applied to the following acceptors: GlcNAcαOAll[24] (**1**), GlcNAcβOAll[24] (**2**), GalαOAll[24] (**7**), GalβOAll[24] (**8**) and GalβSPh[25] (**9**).

In order to optimise the reaction conditions, the effect of the donor-acceptor ratio, buffer pH, reaction time and temperature were examined in a comparison with the yield of the desired disaccharide product. The optimised reaction conditions were found to involve a 1:7.5 donor/acceptor mixture which was incubated in a 1:1 sodium phosphate buffer/acetonitrile mixture with 0.7−1.0 U β-D-galactosid-

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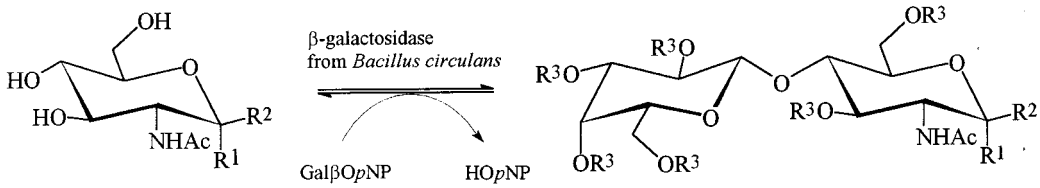


1: R^1 = OAll, R^2 = H

2: R^2 = OAll, R^1 = H

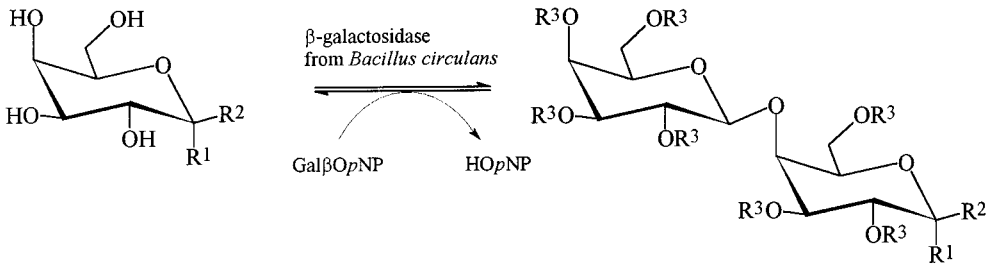
3: R^1 = OAll, R^2 = R^3 = H

4: R^1 = OAll, R^2 = H, R^3 = Ac

5: R^2 = OAll, R^1 = R^3 = H

6: R^2 = OAll, R^1 = H, R^3 = Ac

Scheme 1. Galactosylation of GlcNAc derivatives with β-galactosidase from *Bacillus circulans*



7: R^1 = OAll, R^2 = H

8: R^2 = OAll, R^1 = H

9: R^2 = SPh, R^1 = H

10: R^1 = OAll, R^2 = R^3 = H

11: R^1 = OAll, R^2 = H, R^3 = Ac

12: R^2 = OAll, R^1 = R^3 = H

13: R^2 = OAll, R^1 = H, R^3 = Ac

14: R^2 = SPh, R^1 = R^3 = H

15: R^2 = SPh, R^1 = H, R^3 = Ac

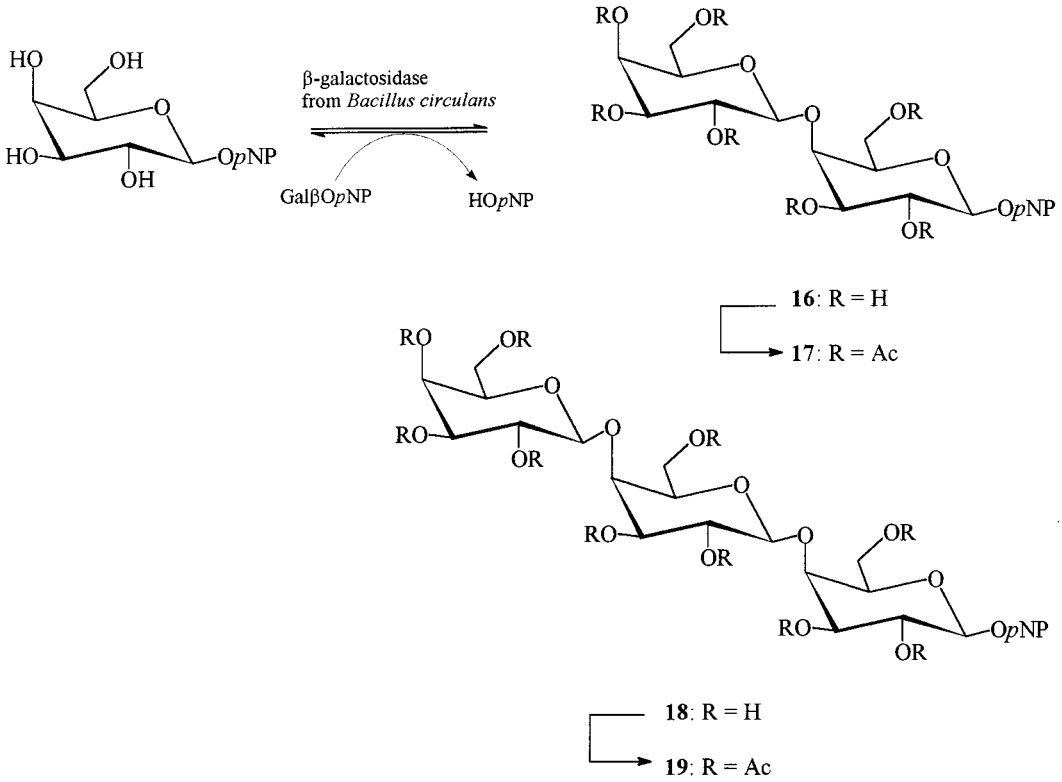
Scheme 2. Galactosylation of galactose derivatives with β-galactosidase from *Bacillus circulans*

ase (E.C.3.2.1.23) for 72 h at 30°C. The reaction was terminated by heating to 90°C for 10 min, and worked up by extraction with ethyl acetate to remove p-nitrophenol followed by lyophilisation and purification by column chromatography (Biogel P2). After lyophilisation the residue was acetylated, the product was isolated and purified by silica gel chromatography. The disaccharides **3**, **5**, **10**, **12** and **14** were isolated in 66, 30, 55, 49, and 63% yields, respectively. These yields are more than satisfactory, especially in light of the simplicity of the method.

In the course of optimising the reaction, other donor-acceptor ratios were examined. When a 3:2 donor/acceptor system was employed, the disaccharide Galβ1→4Galβ-O*p*NP (**16**) was formed (< 4% yield). This self-galactosylation is attributed to the high donor concentration, and was investigated further by using GalβO*p*NP both as donor and acceptor. Employing a high GalβO*p*NP concentration (664 mM) and an increased amount of enzyme (25 U), formation of the disaccharide (**16**) and trisaccharide Galβ1→4Galβ1→4GalβO*p*NP (**18**) was observed in 23% and 10% yields, respectively. The reaction was stopped after 2 h and the products were isolated and purified as above.

Conclusion

β-D-galactosidase from *Bacillus circulans* (E.C.3.2.1.23) has proved to be an excellent catalyst for the formation of Galβ1→4-linked structures. An efficient method has been developed for the one-step synthesis of *N*-acetyllactosamine and analogous Galβ1→4Gal oligosaccharides in excellent (30−66%) yields. The allyl glycosides thus synthesised (**3**, **5**, **10**, **12**) can easily be coupled to BSA or other proteins to form the corresponding glycoprotein. The thiophenyl ga-



Scheme 3. Galactosylation with β-galactosidase from *Bacillus circulans* using GalβO*p*NP as both donor and acceptor substrate

lactoside **14** and *p*-nitrophenyl galactosides **16** and **18** can be used for the construction of larger oligosaccharides. It is to be expected that the methodology developed herein can be applied to the synthesis of any number of analogous Galβ1→4GlcNAc and Galβ1→4Gal structures.

Experimental Section

General Remarks: The reactions were monitored by TLC analysis with silica gel plates (Kieselgel 60 F_{254}, Merck). Compounds were visualised by spraying with 20% sulfuric acid in ethanol, followed by charring at 150°C and/or UV irradiation. − Column chromatography was performed on silica gel 60 M (0.040−0.063 mm, Merck) or Biogel P2. − Optical rotations were determined at room temperature with a Perkin−Elmer 241 and 341 polarimeter. − NMR spectra were accumulated with a Bruker AMX 400 spectrometer. Chemical shifts are given in ppm (δ). − Mass spectra were recorded with a Bruker MALDI-TOF mass spectrometer (with N_2 laser operating at 337 nm and 5 μL of 2,5-dihydroxybenzoic acid as matrix) and FAB with a VG 7035 mass spectrometer. − β-Galactosidase from *Bacillus circulans* (E. C. 3. 2. 1. 23) was purchased from "Biolacta" Daiwa Kasei Co., Ltd., Osaka, Japan, and *p*-nitrophenyl β-D-galactopyranoside from Sigma, Germany.

Enzyme Assay: Enzyme solution (0.2 mL, 0.12 g enzyme powder/mL) was added to *p*-nitrophenyl β-D-galactopyranoside {0.8 mL of a 12.2 mM solution in 2 M sodium acetate buffer (pH = 6.0)} and the mixture was incubated at 37°C for 15 min. The reaction was stopped by adding 1 mL of 0.1 M Na_2CO_3 solution and the liberated *p*-nitrophenol was determined spectrophotometrically. The absorbance was measured in cells with 1 cm light path at 420 nm. One unit of activity was defined as the amount of the enzyme releasing 1μmol *p*-nitrophenol per min.

Allyl β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-α-D-glucopyranoside (3): To compound **1**[24] (81 mg, 0.31 mmol) in a 1:1 solution of 50 mM sodium phosphate buffer (pH = 7.0) and acetonitrile (0.8 mL) was added *p*-nitrophenyl β-D-galactopyranoside (14 mg, 0.046 mmol). The mixture was incubated with β-galactosidase from *Bacillus circulans* (0.7 U) at 30°C. After 72 h the reaction was stopped by heating at 90°C for 10 min. The solution was extracted with ethyl acetate to remove *p*-nitrophenol and lyophilised. The residue was applied to a Biogel P2 column and eluted with water to give compound **3** (13 mg, 66%). − TLC (ethyl acetate/methanol/water, 7:3:1): R_f = 0.59. − $[\alpha]_D^{20}$ = + 93.3 (*c* = 0.15, H_2O). − ^{1}H NMR (400 MHz, D_2O): δ = 5.75 (m, 1 H, CH=), 5.10 (dd, 2 H, CH_2=), 4.74 (d, 1 H, $J_{1,2}$ = 3.1 Hz, 1-H), 4.28 (d, 1 H, $J_{1',2'}$ = 7.6 Hz, 1′-H), 4.03 (dd, 1 H, CH_2), 3.84 (dd, 1 H, CH_2), 3.48 (dd, 1 H, 3′-H), 3.35 (dd, 1 H, 2′-H), 1.84 (s, 3 H, CH_3CONH). − MALDI-TOF MS: $C_{17}H_{29}NO_{11}$ (423.42): *m/z* 424 $[M + H]^+$.

Allyl (2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-2-acetamido-3,6-di-*O*-acetyl-2-deoxy-α-D-glucopyranoside (4): Compound **3** (13 mg, 0.03 mmol) was dissolved in pyridine (1 mL), and acetic anhydride (1 mL) and DMAP (10 mg) were added. The reaction mixture was stirred at room temperature for 12 h and dried in vacuo. The residue was co-evaporated with toluene, concentrated, and purified by column chromatography (dichloromethane/acetone, 85:15) to yield compound **4** (13 mg, 63%). − $[\alpha]_D^{20}$ = + 33.3 (*c* = 0.13, $CHCl_3$). − ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.80 (m, 1 H, CH=), 5.62 (d, 1 H, NH), 5.28−5.15 (m, 4 H, 4′-H, CH_2=, 3-H), 5.06 (dd, 1 H, $J_{2',3'}$ = 7.6 Hz, 2′-H), 4.89 (dd, 1 H, $J_{3',4'}$ = 3.6 Hz, 3′-H), 4.74 (d, 1 H, $J_{1,2}$ = 3.6 Hz, 1-H), 4.45 (d, 1 H, $J_{1',2'}$ = 8.1 Hz, 1′-H), 4.39−4.36 (dd, 1 H, 6a′-H,), 4.17 (m, 1 H, $J_{2,3}$ = 10.7 Hz, 2-H), 4.10 (m, 1 H, CH_2), 4.11−3.99 (m, 3 H, 6a,b-H, 6b′-H), 3.90 (m, 1 H, CH_2), 3.83−3.78 (m, 2 H, 5-H, 5′-H), 3.70 (m, 1 H, 4-H), 2.08−1.88 (m, 21 H, 6 CH_3CO, CH_3CONH). − ^{13}C NMR (100.62 MHz, $CDCl_3$): δ = 169.9−168.1 (C=O), 132.04

(CH=), 117.48 (CH_2=), 100.20 (1′-C), 95.19 (1-C), 75.34 (4-C), 70.61 (3-C), 70.00 (3′-C), 69.57 (5-C), 68.16 (2′-C), 67.71 (CH_2), 67.49 (5′-C), 65.55 (4′-C), 60.96 (6-C), 59.71 (6′-C), 51.00 (2-C), 22.2−19.5 (CH_3).

Allyl (2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranoside (6): To compound **2**[24] (120 mg, 0.46 mmol) in a 1:1 solution of 50 mM sodium phosphate buffer (pH = 7.0) and acetonitrile (0.6 mL) was added *p*-nitrophenyl β-D-galactopyranoside (21 mg, 0.07 mmol). The mixture was incubated with β-galactosidase from *Bacillus circulans* (0.9 U) at 30°C. After 72 h the reaction was stopped by heating at 90°C for 10 min. The solution was extracted with ethyl acetate to remove *p*-nitrophenol and lyophilised. The residue was applied to a Biogel P2 column and eluted with water to give allyl β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside **5** (9 mg, 30%). − TLC (ethyl acetate/methanol/water, 7:3:1): R_f = 0.58. − Compound **5** (9 mg, 0.02 mmol) was dissolved in pyridine (1 mL), and acetic anhydride (1 mL) and DMAP (10 mg) were added. The reaction mixture was stirred at room temperature for 12 h and dried in vacuo. The residue was co-evaporated with toluene, concentrated, and purified by column chromatography (dichloromethane/acetone, 85:15) to yield compound **6** (9 mg, 63%). − $[\alpha]_D^{20}$ = − 25 (*c* = 0.5, $CHCl_3$). − ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.80 (m, 1 H, CH=), 5.55 (d, 1 H, NH), 5.29 (d, 1 H, 4′-H), 5.22−5.10 (m, 2 H, CH_2=), 5.08−4.98 (m, 2 H, 2′-H, 3-H), 4.91 α-D(dd, 1 H, $J_{3',4'}$ = 3.6 Hz, 3′-H), 4.45 (m, 1 H, 6b-H), 4.43 (d, 1 H, $J_{1',2'}$ = 8.1 Hz, 1′-H), 4.42 (d, 1 H, $J_{1,2}$ = 7.1 Hz, 1-H), 4.24 (m, 1 H, CH_2), 4.09−3.94 (m, 5 H, 2-H, 6a-H, 6′a,b-H, CH_2), 3.81 (m, 1 H, 5′-H), 3.72 (t, 1 H, 4-H), 3.55 (m, 1 H, 5-H), 2.08−1.90 (m, 21 H, 6 CH_3CO, C*H*$_3$CONH). − ^{13}C NMR (100.62 MHz, $CDCl_3$): δ = 169.2−168.3 (C=O), 132.48 (CH=), 116.59 (CH_2=), 99.94 (1′-C), 98.70 (1-C), 74.53 (4-C), 71.57 (5-C), 71.15 (3-C), 69.78 (3′-C), 69.73 (5′-C), 69.59 (CH_2), 68.05 (2′-C), 65.60 (4′-C), 61.37 (6′-C), 59.80 (6-C), 51.96 (2-C), 22.2−19.5 (CH_3). − MALDI-TOF MS: $C_{29}H_{41}NO_{17}$ (675.64): *m/z* 677 $[M + H]^+$.

Allyl β-D-galactopyranosyl-(1→4)-α-D-galactopyranoside (10): To compound **7**[24] (55 mg, 0.25 mmol) in a 1:1 solution of 50 mM sodium phosphate buffer (pH = 7.0) and acetonitrile (0.6 mL) was added *p*-nitrophenyl β-D-galactopyranoside (10 mg, 0.033 mmol). The solution was incubated with β-galactosidase from *Bacillus circulans* (0.7 U) at 30°C. After 72 h the reaction was stopped by heating at 90°C for 10 min. The solution was extracted with ethyl acetate to remove *p*-nitrophenol and lyophilised. The residue was applied to a Biogel P2 column and eluted with water to give compound **10** (7 mg, 55%). − TLC (ethyl acetate/methanol/water, 7:3:1): R_f = 0.50. − ^{1}H NMR (400 MHz, D_2O): δ = 6.03 (m, 1 H, CH=), 5.40 (m, 2 H, CH_2=), 5.08 (d, 1 H, $J_{1,2}$ = 3.6 Hz, 1-H), 4.65 (d, 1 H, $J_{1',2'}$ = 7.6 Hz, 1′-H).

Allyl (2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-α-D-galactopyranoside (11): Compound **10** (7 mg, 0.018 mmol) was dissolved in pyridine (1 mL), and acetic anhydride (1 mL) and DMAP (10 mg) were added. The reaction mixture was stirred at room temperature for 12 h and dried in vacuo. The residue was co-evaporated with toluene, concentrated, and purified by column chromatography (dichloromethane/acetone, 94:6) to yield compound **11** (7 mg, 56%). − $[\alpha]_D^{20}$ = + 54.1 (*c* = 0.4, $CHCl_3$). − ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.86 (m, 1 H, CH=), 5.35 (d, 1 H, 4′-H), 5.28 (m, 1 H, CH_2=), 5.25−5.13 (m, 4 H, 3-H, 2′-H, 2-H, CH_2=), 5.00 (d, 1 H, $J_{1,2}$ = 3.6 Hz, 1-H), 4.96 (dd, 1 H, 3′-H), 4.38 (d, 1 H, $J_{1',2'}$ = 8.1 Hz, 1′-H), 4.32 (dd, 1 H, 6a-H), 4.18−4.13 (m, 3 H, 4-H, 6b-H, CH_2), 4.09−4.07 (m, 3 H, 6′a,b-H, 5-H), 4.00 (m, 1 H, CH_2), 3.82 (m, 1 H, 5′-H), 2.15−1.97 (m, 21 H, 7 CH_3CO). − ^{13}C NMR (100.62 MHz, $CDCl_3$): δ = 168.8−168.3 (C=O), 132.3 (CH=), 116.8 (CH_2=), 100.8 (1′-C), 94.3 (1-C), 74.1 (4-C), 69.7 (3′-C), 69.7 (5′-C), 69.3 (3-C), 67.6 (2′-C), 67.4 (CH_2), 66.7 (5-C), 66.6 (2-C), 65.9 (4′-C), 62.7 (6-C), 60.4 (6′-C), 19.8−19.5 (CH_3). FAB-MS: $C_{29}H_{40}O_{18}$ (676.63): *m/z* 678 $[M + H]^+$.

Allyl β-D-galactopyranosyl-(1→4)-β-D-galactopyranoside (12): To compound **8**[24] (42 mg. 0.19 mmol) in a 1:1 solution of 50 mM sodium phosphate buffer (pH = 7.0) and acetonitrile (0.6 mL) was added *p*-nitrophenyl β-D-galactopyranoside (8 mg, 0.026 mmol). The solution was incubated with β-galactosidase from *Bacillus circulans* (0.7 U) at 30°C. After 72 h the reaction was stopped by heating at 90°C for 10 min. The solution was extracted with ethyl acetate to remove *p*-nitrophenol and lyophilised. The residue was applied to a Biogel P2 column and eluted with water to give compound **12** (5 mg, 49%). − TLC (ethyl acetate/methanol/water, 7:3:1): R_f = 0.50. − ^{1}H NMR (400 MHz, D_2O): δ = 5.82 (m, 1 H, CH=), 5.17 (m, 2 H, CH_2=), 4.42 (d, 1 H, $J_{1,2}$ = 7.6 Hz, 1-H), 4.30 (d, 1 H, $J_{1',2'}$ = 7.6 Hz, 1′-H), 4.23−4.06 (m, 2 H, CH_2).

Allyl (2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-β-D-galactopyranoside (13): Compound **12** (5 mg, 0.013 mmol) was dissolved in pyridine (1 mL), and acetic anhydride (1 mL) and DMAP (10 mg) were added. The reaction mixture was stirred at room temperature for 12 h and dried in vacuo. The residue was co-evaporated with toluene, concentrated, and purified by column chromatography (dichloromethane/acetone, 94:6) to yield compound **13** (5 mg, 57%). − $[\alpha]_D^{20}$ = − 8.1 (*c* = 0.3, $CHCl_3$). − ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.79 (m, 1 H, CH=), 5.30 (d, 1 H, 4′-H), 5.23−5.09 (m, 3 H, $J_{2',3'}$ = 10.7 Hz, 2′-H, CH_2=), 5.04 (dd, 1 H, $J_{2,3}$ = 10.2 Hz, 2-H), 4.94 (dd, 1 H, 3′-H), 4.82 (dd, 1 H, 3-H), 4.40 (d, 1 H, $J_{1,2}$ = 8.1 Hz, 1-H), 4.37 (d, 1 H, $J_{1',2'}$ = 7.6 Hz, 1′-H), 4.30 (dd, 1 H, 6′a-H), 4.25 (m, 1 H, CH_2), 4.18 (dd, 1 H, 6′b-H), 4.07−4.00 (m, 4 H, 4-H, 6a,b-H, CH_2), 3.77 (m, 1 H, 5-H), 3.62 (m, 1 H, 5′-H), 2.10−1.92 (m, 21 H, 7 CH_3CO). − ^{13}C NMR (100.62 MHz, $CDCl_3$): δ = 169.6−168.3 (C=O), 132.6 (CH=), 116.4 (CH_2=), 100.8 (1′-C), 98.5 (1-C), 73.3 (4-C), 72.3 (3-C), 70.8 (5′-C), 69.7 (3′-C), 69.6 (5-C), 68.3 (CH_2), 68.1 (2-C), 67.5 (2′-C), 65.8 (4′-C), 62.3 (6-C), 60.3 (6′-C), 19.9−19.5 (CH_3). − FAB-MS:$C_{29}H_{40}O_{18}$ (676.63): *m/z* 678 $[M + H]^+$, 700 $[M + Na]^+$.

Phenyl β-D-galactopyranosyl-(1→4)-1-thio-β-D-galactopyranoside (14): To compound **9**[25] (68 mg, 0.25 mmol) in a 1:1 solution of 50 mM sodium phosphate buffer (pH = 7.0) and acetonitrile (0.6 mL) was added *p*-nitrophenyl β-D-galactopyranoside (10 mg, 0.033 mmol). The solution was incubated with β-galactosidase from *Bacillus circulans* (0.7 U) at 30°C. After 72 h the reaction was stopped by heating at 90°C for 10 min. The solution was extracted with ethyl acetate to remove *p*-nitrophenol and lyophilised. The residue was applied to a Biogel P2 column and eluted with water to give compound **14** (9 mg, 63%). − TLC (ethyl acetate/methanol/water, 7:3:1): R_f = 0.52. − ^{1}H NMR (400 MHz, D_2O): δ = 7.60 (m, 2 H, Ph), 7.35 (m, 3 H, Ph), 4.65 (d, 1 H, $J_{1,2}$ = 9.7 Hz, 1-H), 4.52 (d, 1 H, $J_{1',2'}$ = 7.6 Hz, 1′-H).

Phenyl (2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-1-thio-β-D-galactopyranoside (15): Compound **14** (9 mg, 0.021 mmol) was dissolved in pyridine (1 mL), and acetic anhydride (1 mL) and DMAP (10 mg) were added. The reaction mixture was stirred at room temperature for 12 h and dried in vacuo. The residue was co-evaporated with toluene, concentrated, and purified by column chromatography (dichloromethane/acetone, 95:5) to yield compound **15** (9 mg, 60%). − $[\alpha]_D^{20}$ = + 2.7 (*c* = 0.5, $CHCl_3$). − ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.42 (m, 2 H, Ph), 7.23 (m, 3 H, Ph), 5.29 (d, 1 H, 4′-H), 5.15 (dd, 1 H, $J_{2',3'}$ =

10.7 Hz, 2′-H), 5.06 (dd, 1 H, $J_{2,3}$ = 9.7 Hz, 2-H), 4.93 (dd, 1 H, 3′-H), 4.86 (dd, 1 H, 3-H), 4.57 (d, 1 H, $J_{1,2}$ = 10.2 Hz, 1-H), 4.36 (d, 1 H, $J_{1',2'}$ = 8.1 Hz, 1′-H), 4.29−4.18 (m, 2 H, 6a,b-H), 4.08 (d, 1 H, 4-H), 4.02 (d, 2 H, 6′a,b-H), 3.77 (m, 1 H, 5′-H), 3.68 (m, 1 H, 5′-H), 2.10−1.93 (m, 21 H, 7 CH_3CO). − ^{13}C NMR (100.62 MHz, $CDCl_3$): δ = 169.6−168.2 (C=O), 131.8, 131.07, 127.9, 126.8 (Ph), 100.81 (1′-C), 85.3 (1-C), 74.8 (5-C), 73.3 (4-C), 73.2 (3-C), 69.7 (5′-C), 69.7 (3′-C), 67.5 (2′-C), 66.5 (2-C), 65.9 (4′-C), 62.7 (6-C), 60.3 (6′-C), 19.85−19.51 (CH_3). − FAB-MS: $C_{32}H_{40}O_{17}S$ (728.72): *m/z* 730 [M + H]+, 745 [M + Na]$^+$.

***p*-Nitrophenyl β-D-galactopyranosyl-(1→4)-β-D-galactopyranoside (16) and *p*-Nitro-phenyl β-D-galactopyranosyl-(1→4)-β-D-galactopyranosyl-(1→4)-β-D-galactopyranoside (18):** *p*-Nitrophenyl β-D-galactopyranoside (200 mg, 0.66 mmol) was incubated with β-galactosidase from *Bacillus circulans* (25 U) in 1 mL of 50 mM sodium acetate buffer (pH = 5.0) at 30°C. After 2 h the reaction was stopped by heating at 90°C for 10 min. The reaction mixture was lyophilised, the residue was applied to a Biogel P2 column and eluted with water to give compounds **16** (36 mg, 23%) and **18** (15 mg, 10%).

16: $[\alpha]_D^{20}$ = − 24.5 (*c* = 0.1, H_2O); ^{1}H NMR (400 MHz, D_2O): δ = 8.11 (d, 2 H, Ph), 7.13 (d, 2 H, Ph), 4.94 (d, 1 H, $J_{1,2}$ = 7.6 Hz, 1-H), 4.40 (d, 1 H, $J_{1',2'}$ = 7.6 Hz, 1′-H).

Compound **18** was identified and characterised only after acetylation.

***p*-Nitrophenyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-β-D-galactopyranoside (17):** Compound **16** (30 mg, 0.065 mmol) was dissolved in pyridine (1 mL), and acetic anhydride (1 mL) and DMAP (10 mg) were added. The reaction mixture was stirred at room temperature for 12 h and dried in vacuo. The residue was co-evaporated with toluene, concentrated, and purified by column chromatography (dichloromethane/acetone, 95:5) to yield compound **17** (40 mg, 82%). − $[\alpha]_D^{20}$ = + 9.1 (*c* = 0.4, $CHCl_3$). − ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.15 (d, 2 H, Ph), 7.00 (d, 2 H, Ph), 5.31 (d, 1 H, 4′-H), 5.32 (dd, 1 H, 2-H), 5.20 (dd, 1 H, 2′-H), 5.04 (d, 1 H, $J_{1,2}$ = 7.6 Hz, 1-H), 4.95 (m, 2 H, 3-H, 3′-H), 4.40 (d, 1 H, $J_{1',2'}$ = 8.1 Hz, 1′-H), 4.32 (dd, 1 H, 6a-H), 4.22 (dd, 1 H, 6b-H), 4.12 (d, 1 H, 4-H), 4.04 (m, 2 H, 6′a,b-H), 3.86 (m, 1 H, 5-H), 3.80 (m, 1 H, 5′-H), 2.13−1.94 (m, 21 H, 7 CH_3CO). − ^{13}C NMR (100.62 MHz, $CDCl_3$): δ = 169.53−168.04 (C=O), 160.30 (Ph) 142.10 (*p*-Ph), 124.69, 124.35 (*m*-Ph), 118.53, 115.71 (*o*-Ph), 100.97 (1′-C), 97.27 (1-C), 72.92 (4-C), 71.87 (3-C), 71.67 (5-C), 69.79 (5′-C), 69.60 (3′-C), 67.59 (2-C), 67.54 (2′-C), 65.84 (4′-C), 62.31 (6-C), 60.31 (6′-C), 19.8−19.6 (CH_3). − FAB-MS: $C_{32}H_{39}NO_{20}$ (757.66): *m/z* 759 [M + H]$^+$.

***p*-Nitrophenyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-β-D-galactopyranoside (19):** Compound **18** (15 mg, 0.024 mmol) was dissolved in pyridine (1 mL), and acetic anhydride (1 mL) and DMAP (10 mg) were added. The reaction mixture was stirred at room temperature for 12 h and dried in vacuo. The residue was co-evaporated with toluene, concentrated, and purified by column chromatography (dichloromethane/acetone, 9:1) to yield compound 19 (21 mg, 84%). − $[\alpha]_D^{20}$ = + 25 (*c* = 0.5, $CHCl_3$). − ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.25 (d, 2 H, Ph), 7.05 (d, 2 H, Ph), 5.29 (m, 2 H, 2-H, 4′′-H), 5.14−5.04 (m, 2 H, 2′-H, 2′′-H), 5.02 (d, 1 H, $J_{1,2}$ = 8.1 Hz, 1-H), 4.96−4.90 (m, 2 H, 3-H, 3′′-H), 4.83 (dd, 1 H, 3′-H), 4.43−4.38 (m, 2 H, $J_{1'',2''}$ = 8.1 Hz, 1′′-H, 6′a-H), 4.33 (d, 1 H, $J_{1',2'}$ = 8.1 Hz, 1′-H), 4.31−4.26 (dd, 1 H, 6a-H), 4.22−4.17 (dd, 1 H, 6′b-H), 4.13−4.07 (m, 2 H, 4-H, 6b-H), 4.05−3.97 (m, 3 H, 4′-H, 6′′a,b-H), 3.84 (m, 1 H, 5′-H), 3.76 (m, 1 H, 5′′-H), 3.61 (m, 1 H, 5-H), 2.13−1.91 (m, 30 H, 10 CH_3CO). − ^{13}C NMR (100.62 MHz, $CDCl_3$): δ = 169.70−168.05 (C=O),160.20 (Ph), 142.07 (*p*-Ph), 124.70, (2 C, *m*-Ph), 118.26,115.88 (*o*-Ph), 100.76 (1′′-C), 100.61 (1′-C), 97.3 (1-C), 72.79 (4-C), 72.73 (4′-C), 71.87 (3-C, 3′-C, 5-C), 71.27 (5′′-C), 69.79 (5′-C), 69.63 (3′′-C), 67.84 (2′-C), 67.53 (2′′-C), 67.37 (2-C), 65.84 (4′′-C), 62.46 (6′′-C, 6′-C), 60.27 (6-C), 19.8−1955 (CH_3). − FAB-MS: $C_{44}H_{55}NO_{28}$ (1045.91): *m/z* 1069 [M + Na]$^+$.

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