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β-1,4-Galactosyltransferase-catalyzed Synthesis of the Branched Tetrasaccharide Repeating Unit of *Streptococcus pneumoniae* Type 14

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Abstract—A chemoenzymatic approach is described towards the branched tetrasaccharide repeating unit, β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→6)-[β-D-Gal*p*-(1→4)]-β-D-Glc*p*NAc, of *Streptococcus pneumoniae* type 14 in a form suitable for conjugation. The linear trisaccharide acceptor, β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→6)-β-D-Glc*p*NAc-(1→O)$CH_2CH=CH_2$, was synthesized by coupling of peracetylated lactosyl trichloroacetimidate to a suitably protected glucosamine building block and subsequent deprotection steps. The obtained derivative was found to be a good acceptor for bovine milk β-1,4-galactosyltransferase, and the resulting branched tetrasaccharide β-allyl glycoside was isolated and characterized by NMR spectroscopy and FAB mass spectrometry. Reaction of the anomeric allyl function with cysteamine under UV-irradiation gave the β-aminoethylthio-extended glycoside suitable for further coupling of the tetrasaccharide to protein carriers.

Introduction

Pneumococcal infections like otitis media, pneumonia and meningitis are still significant causes of morbidity and mortality throughout the world.[1,2] Disease prevention by vaccination is of great interest due to increasing resistance of pneumococci to penicillin and other antibiotics.[3] The currently used polyvalent vaccines[4] Pneumovax 23® (Merck, Sharp & Dohme) and Pnu-Immune 23 (Lederle-Praxis) which contain capsular polysaccharides of 23 out of the 90 known serotypes,[5] offer 90% protection in immunocompetent adults but are due to the poor immunogenicity of polysaccharide antigens of limited use for people at highest risk. Polysaccharides are thymus-independent antigens, stimulating mainly IgM antibodies with weak memory, readily induced tolerance and poor immune response in infants up to the age of two, elderly people and immunodeficient patients.[6] Conjugation of carbohydrate antigens to protein carriers represents an approach to convert T-independent antigens to more immunogenic T-dependent antigens through the addition of T-helper cell epitopes.[7,8]

For immunological binding studies and the development of more efficacious synthetic glycoconjugate vaccines we have been investigating the synthesis of well-defined oligosaccharide fragments corresponding to the capsular polysaccharides of *Streptococcus pneumoniae* type 2,[9] 6A,[10–12] 6B,[10–15] 8,[16] 7F,[9] 14,[17] 18C,[18,19] 22F,[9] and 23F.[9,20,21] Here, we report on a chemoenzymatic approach towards the branched tetrasaccharide repeating unit of *S. pneumoniae* type 14 containing a spacer for subsequent coupling to protein carriers.

The capsular polysaccharide of *S. pneumoniae* type 14, which is identical with the *asialo* core antigen of type III group B *Streptococcus* (GBS),[22] consists of a linear repeating trisaccharide backbone bearing a β-D-galactopyranose side chain attached to the *N*-acetyl-β-D-glucosamine residue:[23]

Key words: *Streptococcus pneumoniae* type 14; chemoenzymatic oligosaccharide synthesis; β-1,4-galactosyltransferase; tetrasaccharide allyl glycoside.

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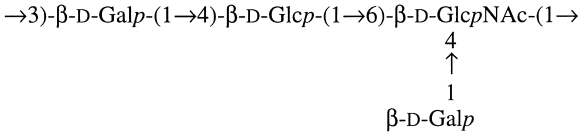


Although several oligosaccharide fragments related to the capsular polysaccharide of *S. pneumoniae* type 14 have been synthesized,[17,24–30] immunological studies on oligosaccharide-protein conjugates have up to now been limited to oligosaccharide fragments derived by degradation of the isolated pneumococcal polysaccharide.[31–33] For an easier access to well-defined synthetic oligosaccharide–protein conjugates, a glycosyltransferase-mediated chemoenzymatic approach towards spacer-linked branched-chain oligosaccharides using commercially available β-1,4-galactosyltransferase was investigated.

In biosynthetic pathways β-1,4-galactosyltransferase (E.C. 2.4.1.22) catalyzes the transfer of β-D-galactopyranosyl groups from UDP-galactose to the 4-position of terminal *N*-acetyl-β-D-glucosamine residues.[34] The transferase has been extensively studied with regard to synthetic applications and substrate specificity,[35–37] and it has been shown that the enzyme allows various modifications at the GlcNAc acceptor residues. Diverse aglycons,[38,39] a variety of *N*-acyl groups[40,41] and substitutions at the 3- and 6-position are tolerated. With respect to the 6-position, 6-*O*-acetyl,[42,43] 6-*O*-methyl[38] and 6-*O*-sulfate[44] groups are accepted. The enzyme, commonly regarded as synthesizing terminal *N*-acetyllactosamine sequences, can moreover use 6-*O*-glycosylated compounds, such as α-L-Fucp-(1→6)-β-D-Glc*p*NAc and α-Neu5Ac(OMe)-(2→6)-β-D-Glc*p*NAc, as substrates.[38]

In order to evaluate the feasibility of an enzymatic approach to branched-chain oligosaccharide structures of *S. pneumoniae* type 14 we have synthesized the 6-*O*-lactose substituted β-D-GlcNAc allyl glycoside **7** and investigated its conversion into the branched tetrasaccharide allyl glycoside **10** by use of bovine milk β-1,4-galactosyltransferase. Conversion of the anomeric allyl group into an amino-derivatized spacer via light-induced free-radical addition of cysteamine[45] was intended for the subsequent coupling of the tetrasaccharide to carrier proteins.

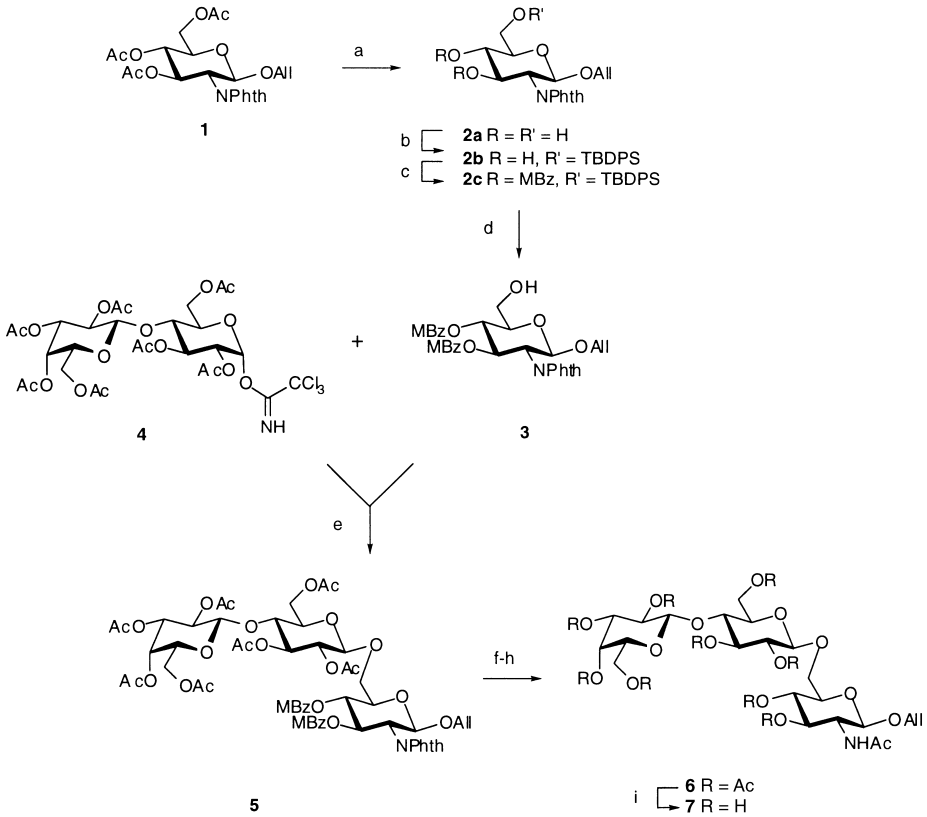
Results and Discussion

The synthetic strategy for the preparation of the precursor of compound **7**, trisaccharide derivative **5**, involved the selective temporary protection of the primary HO-6 group of allyl glycoside **2a** as *tert*-butyldiphenylsilyl ether, selective de-*O*-silylation in the presence of *p*-methylbenzoyl blocking groups, and subsequent coupling of peracetylated lactosyl donor **4** to the unprotected 6-position of **3** (Scheme 1).

Allyl glycoside **1**[46] was deacetylated (→**2a**) and selectively silylated at the primary hydroxyl group with *tert*-butyldiphenylsilyl chloride in the presence of 4-dimethylaminopyridine and triethylamine yielding **2b** in 89% yield. The *tert*-butyldiphenylsilyl[47] (TBDPS) protecting group is considerably more stable than the *tert*-butyldimethylsilyl[48] (TBDMS) group, and was found to be more suitable for the subsequent introduction of *p*-methylbenzoyl groups at the 3- and 4-position of **2b** affording the completely protected glucosamine derivative **2c** (89%). Whereas selective cleavage of secondary silyl ether groups with tetrabutylammonium fluoride often gives complicated mixtures attributable to extensive acyl migration,[49] clean deprotection of the silyl ether function in the presence of *p*-methylbenzoyl groups could be achieved by reaction with hydrogen chloride in methanol[49] prepared in situ from acetyl chloride and methanol (→**3**, 86%). The trisaccharide derivative **5** was obtained by coupling of peracetylated lactosyl trichloroacetimidate **4**[17] to the glucosamine derivative **3** in dichloromethane in the presence of trimethylsilyl trifluoromethanesulfonate (63%). Deprotection of trisaccharide derivative **5** by deacylation/dephthaloylation with ethylenediamine in butanol[50] at 80 °C followed by re-*N*,*O*-acetylation gave besides the *N*-acetyl protected glycoside **6** (62%), the corresponding *N*-*p*-methylbenzoylated derivative as by-product in 32% yield. Therefore, trisaccharide **5** was first de-*p*-methylbenzoylated and deacetylated with sodium methoxide in methanol. Subsequent dephthaloylation with ethylenediamine in butanol at 80 °C and re-*N*,*O*-acetylation with acetic anhydride in pyridine gave **6** after chromatography in 98% overall yield. After de-*O*-acetylation with sodium methoxide in methanol and subsequent purification on Sephadex LH-20 with methanol as eluent, the unprotected trisaccharide allyl glycoside **7** was obtained in 94% yield.

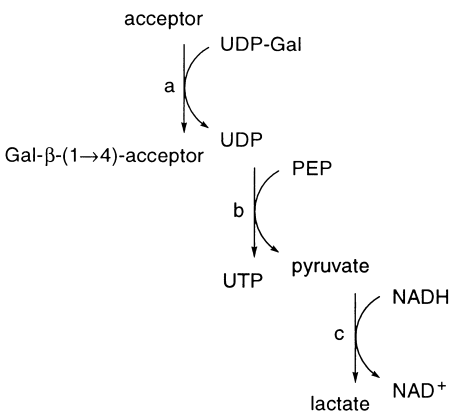
The rate of galactose transfer to the synthetic trisaccharide acceptor **7** using UDP-Gal and bovine milk β-1,4-galactosyltransferase was tested with a coupled enzyme assay for UDP (Scheme 2)[51] by measuring spectrophotometrically the decrease in absorbance at 340 nm.

In order to investigate both the influence of the anomeric allyl group and the 6-*O*-lactosyl substitution, initial reaction rates were determined under standard conditions for *N*-acetylglucosamine **8** (assigned relative rate of 100), the corresponding β-allyl glycoside **9**[52,53] and trisaccharide allyl glycoside **7** (Table 1). Both compounds **7** and **9** were found to retain at a 10 mM



Scheme 1. Reagents and conditions: (a) NaOMe, MeOH; (b) *tert*-butylchlorodiphenylsilane, Et_3N, 4-dimethylaminopyridine, pyridine; (c) MBzCl, pyridine; (d) acetyl chloride, MeOH, toluene; (e) TMSOTf, CH_2Cl_2; (f) NaOMe, MeOH; (g) $H_2NCH_2CH_2NH_2$, *n*-butanol, 80 °C; (h) Ac_2O, pyridine; (i) NaOMe, MeOH. TBDPS = *tert*-butyldiphenylsilyl. MBz = *p*-methylbenzoyl.

acceptor concentration good acceptor activity with relative rates of 35% and 29%, respectively. The substitution at the 6-position of β-allyl glycoside **7** by lactose is thus tolerated by the enzyme and decreases the relative rate of galactosyl transfer only slightly (83% compared with the non-substituted glycoside). The kinetic parameters V_{max} and K_m for trisaccharide **7** were determined to be 5.2 nmol/min and 5.4 mM, respectively.

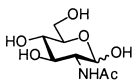
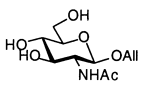
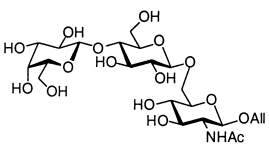


Scheme 2. Reagents: (a) β-1,4-galactosyltransferase; (b) pyruvate kinase; (c) L-lactate dehydogenase.

Then, the galactosyltransferase reaction with UDP-Gal as donor and trisaccharide acceptor **7** was performed on a preparative scale. To prevent feedback inhibition by released UDP,[54] alkaline phosphatase was added to the incubation mixture[55] permitting a near quantitative conversion of the acceptor to tetrasaccharide **10** (Scheme 3).

The progress of the reaction could be followed by TLC (butanol:acetic acid:water, 2:1:1) showing the disappearance of **7** (R_f 0.35) and the appearance of a new more polar product (R_f 0.25). HPLC analysis on Lichrospher-NH_2 with 70:30, acetonitrile:water as eluent showed retention times of 8.9 min for trisaccharide **7** and 12.7 min for tetrasaccharide **10** at a flow rate of 1.2 mL/min. The product could be isolated by removal of excess UDP-Gal on Dowex 1X8 (Cl^- form) and subsequent size-exclusion chromatography on Toyopearl

Table 1. Relative rates of galactopyranosyl transfer to substituted GlcNAc residues at a 10 mM acceptor concentration

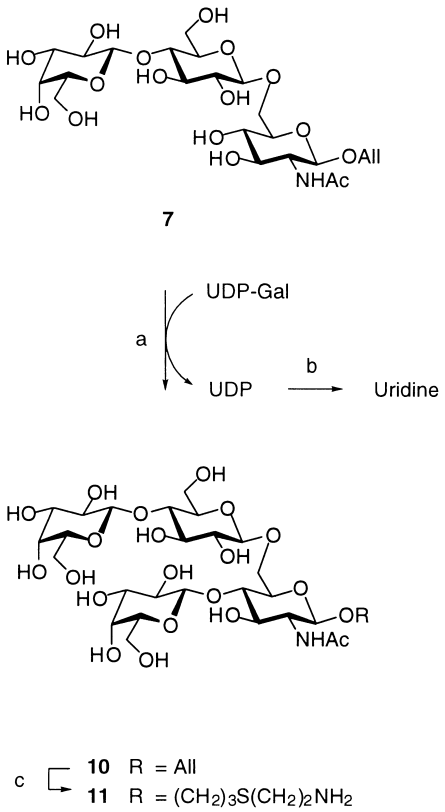
Compd		Rel. rate (%)
8		100
9		35
7		29

HW-40S in 70% yield. FABMS confirmed the molecular mass of the isolated product **10** ($[M+H]^+$ at m/z 748), and the structure of the tetrasaccharide was confirmed by 2-D ^{1}H COSY, TOCSY and ROE spectroscopy (Table 2). The signal for the anomeric proton of the new galactose residue appeared at δ 4.54 as a doublet with a coupling constant of 8.5 Hz. The ROE connectivity between Gal H-1 and GlcNAc H-4 confirmed the transfer of galactose to the 4-position of the non-terminal *N*-acetyl-β-D-glucosamine residue.

The allyl glycoside **10** was converted into the 3-(2-aminoethylthio)propyl glycoside **11** by reaction with cysteamine[45] under UV-irradiation. The radical addition of 2-aminoethanethiol to the double bond could be followed by TLC showing the formation of a new compound (R_f 0.65 → R_f 0.48). Excess of cysteamine and traces of unreacted allyl glycoside were removed by size-exclusion chromatography on Toyopearl HW-40S, and **11** was obtained as an amorphous white powder in 71% yield.

Conclusion

A combined chemoenzymatic synthesis of the tetrasaccharide repeating unit of *S. pneumoniae* type 14 could be established. The trisaccharide **7** was synthesized using a convenient approach by combination of *p*-methylbenzoyl and *tert*-butyldiphenylsilyl protecting groups. β-1,4-Galactosyltransferase from bovine milk was found to utilize the synthetic trisaccharide **7** as acceptor. The branched-chain tetrasaccharide **10** was prepared on a milligram scale and the transfer of galactose to the non-terminal *N*-acetylglucosamine residue was confirmed by NMR spectroscopy. The introduction of an amino-derivatized spacer for the subsequent coupling of the tetrasaccharide to protein carriers could be achieved by irradiation of the anomeric allyl group in the presence of cysteamine.



Scheme 3. Reagents: (a) β-1,4-galactosyltransferase; (b) alkaline phosphatase; (c) cysteamine hydrochloride.

Experimental

General methods

Reactions were monitored by TLC on Silica Gel 60 F_{254} (Merck) with detection by UV light and/or charring with aq 50% H_2SO_4 or 0.2% orcinol in 20% methanolic H_2SO_4. Evaporations were conducted under reduced pressure at $<40\,°C$. Column chromatography was performed on Silica Gel 60 (0.063–0.200 mm, Merck). Optical rotations were measured with a Perkin–Elmer 241 polarimeter. ^{1}H NMR spectra (300 MHz) were recorded with a Bruker AC 300 spectrometer. Two-dimensional double-quantum filtered ^{1}H-^{1}H correlated spectra (2-D DQF ^{1}H-^{1}H COSY), two-dimensional TOCSY spectra with 100 ms and 150 ms mixing sequences, and 2-D ^{1}H ROE spectra (300 ms mixing

Table 2. ^{1}H NMR data (COSY, TOCSY, ROESY) of **10**

Proton (δ_H in ppm)	GlcNAc	Glc	Gal[a]	Gal[b]
H-1	4.59 (8.5)[c]	4.56 (8.5)	4.45 (7.3)	4.53 (8.5)
H-2	3.76	3.39 (8.5)	3.55 (9.8)	3.54 (9.8)
H-3	3.69	3.68	3.64	3.64
H-4	3.84	3.65	3.93	3.93
H-5	3.71	3.61	3.74	3.72
H-6a	4.29 (11.0)	3.98 (11.0)	n.d.[d]	n.d
H-6b	3.96 (3.7)	3.81	n.d.	n.d.
$OCH_2CH=CH_2$	4.33, 4.16 (2m, each 1H)			
$OCH_2=CH=CH_2$	5.9 (m, 1H)			
$OCH_2=CH=CH_2$	5.32–5.25 (m, 2H)			
$NHCOCH_3$	2.03 (s, 3H)			

[a]Gal-(β1-4)-Glc.
[b]Gal-(β1-4)-GlcNAc.
[c]^{1}H-^{1}H coupling constants (Hz) were determined from a 500 MHz 1-D spectrum.
[d]n.d. = not determined.

sequence) were recorded at 300 K using a Bruker AMX 500 spectrometer. Chemical shifts (δ) are given in ppm relative to the signal for internal Me_4Si (δ 0, $CDCl_3$) or acetone (δ 2.225, D_2O). ^{13}C NMR spectra (75.5 MHz) were recorded with a Bruker AC 300 spectrometer; δ (ppm) values are given relative to the signal for $CDCl_3$ (δ 76.9) or internal acetone (δ 31.08). Fast-atom bombardment mass spectrometry (FABMS) was carried out on a JEOL JMS SX/SX 102A four-sector mass spectrometer, equipped with a JEOL MS-FAB 10 D FAB gun. Size-exclusion chromatography was performed on Sephadex® LH-20 (2.5×35 cm) or Toyopearl® HW-40S (2.0×60 cm), and ion-exchange chromatography on Dowex 1X8 (200–400 mesh, Cl^- form) or Dowex 1X8 (200–400 mesh, OH^- form). HPLC analysis was carried out on a Lichrospher® NH_2 (250 mm, I.D. 4.6 mm) column, using 70:30, CH_3CN:H_2O as eluent at a flow rate of 1.2 mL/min. Elemental analyses were carried out by H. Kolbe Mikroanalytisches Laboratorium (Mülheim an der Ruhr, Germany).

Materials

Bovine milk β-1,4-galactosyltransferase (E.C. 2.4.1.22), UDP-galactose, β-NADH, phospho(enol)pyruvate, pyruvate kinase (E.C. 2.7.1.40, type III from rabbit muscle), L-lactate dehydrogenase (E.C. 1.1.1.27, type XI from rabbit muscle), and alkaline phosphatase (E.C. 3.1.3.1, type I from bovine intestine) were obtained from Sigma. Toyopearl HW®-40S was supplied by Supelco. Cysteamine hydrochloride was bought from Fluka.

Measurement of β-1,4-galactosyltransferase activity. Initial reaction rates were determined under standard conditions at 20 °C in 500 μL 100 mM sodium cacodylate buffer (pH 7.5), containing 10 mM $MnCl_2$, 50 mM KCl, 0.2 mM UDP-galactose, 1 mM phospho(enol)pyruvate, 0.3 mM NADH, 25 U pyruvate kinase, 25 U L-lactate dehydrogenase, 0.25–10 mM acceptor and 20 mM β-1,4-galactosyltransferase. UDP-formation was followed by monitoring the decrease in absorbance at 340 nm. Kinetic parameters V_{max} and K_m were determined from the initial rate data using a millimolar extinction coefficient of 6.22 $mM^{-1}cm^{-1}$ for NADH absorbance and rate data were fit to the Michaelis–Menten equation using SigmaPlot.

Allyl 6-*O*-*tert*-butyldiphenylsilyl-2-deoxy-3,4-di-*O*-*p*-methylbenzoyl-2-phthalimido-β-D-glucopyranoside (2c). To a solution of allyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranoside (**1**)[46] (1.43 g, 3.0 mmol) in dry MeOH (35 mL) was added 0.2 M NaOMe in dry MeOH (3 mL). After stirring for 2 h at room temperature, the solution was neutralized with Dowex 50W-X8 (H^+ form), filtered, and concentrated (→**2a**). The residue was dissolved in dry CH_2Cl_2 (15 mL) and dry pyridine (1 mL), and after the addition of 4-dimethylaminopyridine (30 mg), Et_3N (480 μL) and *tert*-butylchlorodiphenylsilane (900 μL), the solution was stirred overnight at room temperature. The mixture was poured onto ice-water, extracted with CH_2Cl_2, and the organic layer was washed with satd $NaHCO_3$, then concentrated. Column chromatography (toluene: EtOAc, 3:2) of the residue gave amorphous **2b** (1.58 g, 89%). To a solution of **2b** (1.58 g, 2.69 mmol) in dry pyridine (10 mL) was added at 0 °C a solution of 4-methylbenzoyl chloride (0.9 mL, 6.7 mmol) in dry CH_2Cl_2 (15 mL). The mixture was stirred overnight at room temperature, diluted with CH_2Cl_2, poured onto ice-water, extracted with CH_2Cl_2, and the organic layer was washed with satd $NaHCO_3$, dried ($MgSO_4$), and concentrated. Column chromatography (30:1, toluene:EtOAc) of the residue gave **2c** (1.98 g, 89%). TLC (10:1, toluene:EtOAc): R_f 0.49 (**2b**), 0.56 (**2c**). $[\alpha]_D^{20}$

+17° (*c* 1; $CHCl_3$). ^1H NMR ($CDCl_3$) δ 7.76–7.01 (m, 22H, Phth, 2 $COC_6H_4CH_3$, and 2 Ph), 6.21 (dd, 1H, $J_{2,3}=10.7$ Hz, $J_{3,4}=9.2$ Hz, H-3), 5.79 (m, 1H, $OCH_2CH=CH_2$), 5.62 (t, 1H, $J_{4,5}=9.5$ Hz, H-4), 5.58 (d, 1H, $J_{1,2}=8.4$ Hz, H-1), 5.24–5.06 (m, 2H, $OCH_2CH=CH_2$), 4.58 (dd, 1H, H-2), 4.33 and 4.12 (2m, each 1H, $OCH_2CH=CH_2$), 3.99–3.83 (m, 3H, H-5,6a,6b), 2.33 and 2.24 (2s, each 3H, 2 $COC_6H_4CH_3$), 1.05 (s, 9H, $C(CH_3)_3$); ^{13}C NMR ($CDCl_3$) δ 165.7 and 164.9 (2 $COC_6H_4CH_3$), 117.5 ($OCH_2CH=CH_2$), 97.0 (C-1), 75.0, 71.3, and 69.6 (C-3,4,5), 62.9 and 60.3 (C-6 and $OCH_2CH=CH_2$), 54.9 (C-2), 26.5 ($C(CH_3)_3$), 21.5 ($COC_6H_4CH_3$), 19.1 ($C(CH_3)_3$). Anal. calcd for $C_{49}H_{49}NO_9Si$: C, 71.42; H, 5.99 %. Found: C, 71.40; H, 6.08 %. FABMS calcd for $C_{49}H_{49}NO_9Si$ (M+Na): 846.3; found: 846.5.

Allyl 2-deoxy-3,4-di-*O*-*p*-methylbenzoyl-2-phthalimido-β-D-glucopyranoside (3). To a solution of acetyl chloride (6.5 mL) in dry MeOH (100 mL) was added at room temperature a solution of **2c** (830 mg, 1.0 mmol) in dry toluene (100 mL). The mixture was stirred overnight at room temperature, then neutralized with Et_3N, and concentrated. The residue was dissolved in EtOAc, and the solution washed twice with H_2O, dried ($MgSO_4$), and concentrated. Column chromatography (5:1, then 3:1, toluene:EtOAc) of the residue gave amorphous **3** (504 mg, 86%). TLC (toluene:EtOAc, 5:1): R_f 0.72 (**2c**), 0.30 (**3**). $[\alpha]_D^{20}$ +6° (*c* 1; $CHCl_3$). ^1H NMR ($CDCl_3$) δ 7.84–7.03 (m, 12H, Phth and 2 $COC_6H_4CH_3$), 6.30 (dd, 1H, $J_{2,3}=10.8$ Hz, $J_{3,4}=9.2$ Hz, H-3), 5.76 (m, 1H, $OCH_2CH=CH_2$), 5.58 (d, 1H, $J_{1,2}=8.4$ Hz, H-1), 5.48 (t, 1H, $J_{4,5}=9.5$ Hz, H-4), 5.16 and 5.08 (2m, each 1H, $OCH_2CH=CH_2$), 4.52 (dd, 1H, H-2), 4.33 and 4.13 (2m, each 1H, $OCH_2CH=CH_2$), 3.89–3.71 (m, 3H, H-5,6a,6b), 2.34 and 2.27 (2s, each 3H, 2 $COC_6H_4CH_3$); ^{13}C NMR ($CDCl_3$) δ 166.0 and 165.5 (2 $COC_6H_4CH_3$), 117.5 ($OCH_2CH=CH_2$), 97.2 (C-1), 74.3, 70.4, and 69.8 (C-3,4,5), 70.1 and 61.2 (C-6 and $OCH_2CH=CH_2$), 54.7 (C-2), 21.4 ($COC_6H_4CH_3$). Anal. calcd for $C_{33}H_{31}NO_9$: C, 67.68; H, 5.34%. Found: C, 67.95; H, 5.60%. FABMS calcd for $C_{33}H_{31}NO_9$ (M+Na): 608.2; found: 608.3.

Allyl (2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-(2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl)-(1→6)-2-deoxy-3,4-di-*O*-*p*-methylbenzoyl-2-phthalimido-β-D-glucopyranoside (5). To a solution of **3** (58 mg, 0.10 mmol) and peracetylated lactosyl trichloroacetimidate **4**[17] (62 mg, 0.079 mmol) in dry CH_2Cl_2 (0.5 mL) was added powdered 4 Å molecular sieves (150 mg), and the suspension was stirred for 1 h at room temperature. At 0 °C trimethylsilyl trifluoromethanesulfonate (25 μL, 0.14 mmol) was added, and the reaction mixture stirred at room temperature for 3 h. Then the solution was neutralized with Et_3N, diluted with CH_2Cl_2, filtered through Celite, and concentrated. Column chromatography (toluene:EtOAc, 3:2) of the residue gave amorphous **5** (66 mg, 63%). TLC (toluene: EtOAc, 2:3): R_f 0.76 (**3**), 0.52 (**4**), 0.64 (**5**). $[\alpha]_D^{20}$ −5° (*c* 1; $CHCl_3$). ^1H NMR ($CDCl_3$) δ 7.80–7.02 (m, 12H, Phth and 2 $COC_6H_4CH_3$), 6.19 (dd, 1H, $J_{2,3}=10.7$ Hz, $J_{3,4}=9.2$ Hz, H-3), 5.76 (m, 1H, $OCH_2CH=CH_2$), 5.53 (d, 1H, $J_{1,2}=8.4$ Hz, H-1), 5.37 (t, 1H, $J_{4,5}=9.5$ Hz, H-4), 5.34 (d, 1H, $J_{3'',4''}=4.0$ Hz, H-4″), 5.12 and 5.08 (2m, each 1H, $OCH_2CH=CH_2$), 4.63 (d, 1H, $J_{1',2'}=7.7$ Hz, H-1′), 4.51 (dd, 1H, H-2), 4.47 (d, 1H, $J_{1'',2''}=7.8$ Hz, H-1″), 2.34 and 2.27 (2s, each 3H, 2 $COC_6H_4CH_3$), 2.14–1.94 (m, 21H, 7 Ac); ^{13}C NMR ($CDCl_3$) δ 170.2, 170.1, 170.0, 169.9, 169.6, 169.4, and 168.9 (7 $COCH_3$), 165.5 and 165.2 (2 $COC_6H_4CH_3$), 133.1 ($OCH_2CH=CH_2$), 117.7 ($OCH_2CH=CH_2$), 101.0 and 100.3 (C-1′,1″), 97.0 (C-1), 76.3, 73.9, 72.8, 72.4, 71.5, 70.9, 70.8, 70.6, 69.9, 69.0, and 66.5 (C-3, 4, 5, 2′, 3′, 4′, 5′, 2″, 3″, 4″, 5″), 69.9, 68.1, 62.1, and 60.7 (C-6, 6′, 6″ and $OCH_2CH=CH_2$), 54.7 (C-2), 21.5 and 21.4 (2 $COC_6H_4CH_3$), 20.6–20.3 ($COCH_3$). Anal. calcd for $C_{59}H_{65}NO_{26}$: C, 58.85; H, 5.44%. Found: C, 58.74; H, 5.50%. FABMS calcd for $C_{59}H_{65}NO_{26}$ (M+Na): 1226.4; found: 1226.5.

Allyl (2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-(2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl)-(1→6)-2-acetamido-3,4-di-*O*-acetyl-2-deoxy-β-D-glucopyranoside (6). A solution of **5** (108 mg, 0.09 mmol) in MeOH (15 mL) was stirred with NaOMe at pH 8–9 for 3 h at room temperature. The solution was neutralized with Dowex 50W-X8 (H^+ form), filtered, and concentrated. The residue was dissolved in butanol (15 mL) and 1,2-diaminoethane (3 mL), and the mixture heated under Ar for 24 h at 80 °C, then co-concentrated with toluene. TLC (butanol:HOAc:H_2O, 2:1:1): R_f 0.35. A solution of the residue in pyridine (30 mL) and Ac_2O (15 mL) was stirred overnight at room temperature, then co-concentrated with toluene. Column chromatography (CH_2Cl_2:acetone, 2:1) of the residue gave amorphous **6** (86 mg, 98%). TLC (CH_2Cl_2:acetone, 2:1): R_f 0.44. $[\alpha]_D^{20}$ −14° (*c* 1; $CHCl_3$). ^1H NMR ($CDCl_3$) δ 5.86 (m, 1H, $OCH_2CH=CH_2$), 5.58 (d, $J_{2,NH}=8.7$ Hz, $NHCOCH_3$), 4.68 and 4.58 (2d, each 1H, $J=8.3$ and 7.6 Hz, H-1,1′), 4.50 (d, 1H, $J_{1'',2''}=7.8$ Hz, H-1″), 4.37 and 4.32 (2m, each 1H, $OCH_2CH=CH_2$), 2.14, 2.12, 2.06, 2.05, 2.04, 2.03, 2.02, 2.01, 1.96, and 1.94 (10s, each 3H, 10 Ac); ^{13}C NMR ($CDCl_3$) δ 170.6–168.8 ($COCH_3$), 133.3 ($OCH_2CH=CH_2$), 117.6 ($OCH_2CH=CH_2$), 100.9, 100.0, and 99.3 (C-1,1′,1″), 76.0, 73.1, 72.7, 72.5, 72.2, 71.4, 70.8, 70.4, 69.1, 69.0, and 66.5 (C-3, 4, 5, 2′, 3′, 4′, 5′, 2″, 3″, 4″, 5″), 69.6, 67.9, 61.9, and 60.6 (C-6, 6′, 6″ and $OCH_2CH=CH_2$), 54.6 (C-2), 23.1 ($NHCOCH_3$), 20.6–20.3 ($COCH_3$). FABMS calcd for $C_{41}H_{57}NO_{25}$ (M+Na): 986.3; found: 986.5.

Allyl (β-D-galactopyranosyl)-(1→4)-(β-D-glucopyranosyl)-(1→6)-2-acetamido-2-deoxy-β-D-glucopyranoside (7). To a solution of **6** (54 mg, 0.056 mmol) in dry MeOH (5 mL) was added NaOMe (33 mg), and the solution was stirred overnight at room temperature. Then, H_2O (1 mL) was added, and after stirring overnight at room temperature, the mixture was neutralized with Dowex 50W-X8 (H^+ form), filtered, and concentrated. The residue was purified on Sephadex LH-20 with MeOH as eluent to give **7** (30 mg, 94%). TLC (butanol:HOAc:H_2O, 2:1:1): R_f 0.35. $[\alpha]_D^{20}$ −25° (*c* 1; MeOH). ^{1}H NMR (MeOD) δ 5.87 (m, 1H, $OCH_2CH{=}CH_2$), 5.26 and 5.12 (2m, each 1H, $OCH_2CH{=}CH_2$), 4.44, 4.42, and 4.36 (3d, each 1H, J = 8.3, 7.7 and 7.4 Hz, H-1, 1′,1″), 4.31 and 4.06 (2m, each 1H, $OCH_2CH{=}CH_2$), 1.96 (s, 3H, $NHCOCH_3$); ^{13}C NMR (MeOD) δ 173.7 ($NHCOCH_3$), 135.6 ($OCH_2CH{=}CH_2$), 117.0 ($OCH_2CH{=}CH_2$), 105.1, 104.6, and 101.9 (C-1, 1′, 1″), 80.6, 77.1, 77.0, 76.5, 76.4, 75.9, 74.8, 74.7, 72.5, 72.0, and 70.3 (C-3, 4, 5, 2′, 3′, 4′, 5′, 2″, 3″, 4″, 5″), 70.9, 69.8, 62.5, and 61.9 (C-6, 6′, 6″ and $OCH_2CH{=}CH_2$), 57.3 (C-2), 22.9 ($NHCOCH_3$). FABMS calcd for $C_{23}H_{39}NO_{16}$ (positive-ion mode) (M + H): 586.6; found: 586.2. (M + Na): 608.6; found: 608.2. (negative-ion mode) (M-H): 584.6; found: 584.4.

Allyl (β-D-galactopyranosyl)-(1→4)-(β-D-glucopyranosyl)-(1→6)-[(β-D-galactopyranosyl)-(1→4)]-2-acetamido-2-deoxy-β-D-glucopyranoside (10). To a solution of trisaccharide **7** (6.3 mg, 11 μmol) in 50 mM sodium cacodylate buffer pH 7.5 (600 μL) containing 5 mM $MnCl_2$, bovine serum albumin (0.5 mg) and NaN_3 (0.02%), were added alkaline phosphatase (4 U), UDP-galactose (6.5 mg, 11 μmol) and β-1,4-galactosyltransferase (1 U). The reaction mixture was incubated at 37 °C. After 4 h, another batch of UDP-galactose (4.6 mg, 8 μmol) was added and the incubation was continued for 16 h at 37 °C. Then, H_2O (100 mL) was added and UDP-galactose was removed using a Dowex 1X8 (Cl^- form) column with H_2O as eluent. The eluate was concentrated, and the residue applied on a Toyopearl HW-40S column, eluted with 5 mM NH_4HCO_3 at a flow rate of 13 mL/h. The appropriate fractions were freeze-dried to give **10** (5.6 mg, 70%). TLC (butanol:HOAc:H_2O, 2:1:1): R_f 0.35 (**7**), 0.25 (**10**). $[\alpha]_D^{20}$ −1° (*c* 1; D_2O). For ^{1}H NMR data (D_2O, 500 MHz), see Table 2; ^{13}C NMR (D_2O) δ 175.4 ($NHCOCH_3$), 134.2 ($OCH_2CH{=}CH_2$), 119.0 ($OCH_2CH{=}CH_2$), 103.8, 103.6, 103.2, and 101.0 (C-1, 1′, 1″, 1‴), 79.3, 78.7, 76.2, 76.1, 75.5, 75.1, 74.4, 73.5, 73.4 (2 C), 73.2, 71.8 (2 C), and 69.4 (2 C) (C-3, 4, 5, 2′, 3′, 4′, 5′, 2″, 3″, 4″, 5″, 2‴, 3‴, 4‴, 5‴), 71.4, 68.2, 61.9, and 60.9 (2 C) (C-6, 6′, 6″, 6‴ and $OCH_2CH{=}CH_2$), 55.9 (C-2), 23.0 ($NHCOCH_3$). FABMS calcd for $C_{29}H_{49}NO_{21}$ (positive-ion mode) (M + H): 748.7; found: 748.3. (M + Na): 770.7; found: 770.3. (negative-ion mode) (M-H): 746.7; found: 746.3.

3-(2-Aminoethylthio)propyl (β-D-galactopyranosyl)-(1→4)-(β-D-glucopyranosyl)-(1→6)-[(β-D-galactopyranosyl)-(1→4)]-2-acetamido-2-deoxy-β-D-glucopyranoside (11). The allyl glycoside **10** (6.2 mg, 8 μmol) was dissolved in a solution of cysteamine hydrochloride (4.5 mg, 0.04 mmol) in H_2O (200 μL) and the mixture was irradiated in a quartz vial under UV-light for 3 days at room temperature. The product was purified twice by size-exclusion chromatography on a Toyopearl HW-40S column, eluted with 0.1 M NH_4OAc at a flow rate of 13 mL/h. Product-containing fractions were lyophilized and deionized on a Dowex 1X8 (OH^- form) column with H_2O as eluent to give **11** (4.7 mg, 71%). TLC (isopropanol:HOAc:H_2O, 2:1:1): R_f 0.65 (**10**), 0.48 (**11**). ^{1}H NMR (D_2O) δ 4.60–4.51 (m, 3H, H-1, 1′, 1‴), 4.46 (d, 1H, $J_{1'',2''}$ = 7.7 Hz, H-1″), 4.29 (d, 1H, $J_{5,6}$ = 11.2 Hz, H-6), 3.01, 2.74, and 2.61 (3t, each 2H, $CH_2CH_2SCH_2CH_2NH_2$), 2.04 (s, 3H, $NHCOCH_3$), 1.85 (m, 2H, $CH_2CH_2CH_2S$); ^{13}C NMR (D_2O) δ 180.0 ($NHCOCH_3$), 103.8, 103.6, 103.3, and 102.2 (C-1, 1′, 1″, 1‴), 79.3, 78.7, 76.2, 76.1, 75.6, 75.1, 74.7, 73.5, 73.4 (2 C), 73.1, 71.8 (2 C), and 69.4 (2 C) (C-3, 4, 5, 2′, 3′, 4′, 5′, 2″, 3″, 4″, 5″, 2‴, 3‴, 4‴, 5‴), 69.6, 68.3, 61.9 (2 C), and 60.9 (C-6, 6′, 6″, 6‴ and $OCH_2CH{=}CH_2$), 55.9 (C-2), 39.8, 31.8, 29.4, and 28.0 ($OCH_2CH_2CH_2SCH_2CH_2$), 23.0 ($NHCOCH_3$).

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