Carbohydrate Research 344 (2009) 570–575

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

Efficient synthesis and protein conjugation of β-(1→6)-D-*N*-acetylglucosamine oligosaccharides from the polysaccharide intercellular adhesin

Carmen Leung, Anthony Chibba, Rodolfo F. Gómez-Biagi, Mark Nitz *

Department of Chemistry, University of Toronto, Toronto, ON, Canada M5S 3H6

ARTICLE INFO

Article history:
Received 23 October 2008
Received in revised form 22 December 2008
Accepted 26 December 2008
Available online 3 January 2009

Keywords:
Polysaccharide intercellular adhesin
Glycoconjugates
Neoglycoproteins

ABSTRACT

A wide variety of medically important biofilm forming bacteria produce similar polysaccharide intracellular adhesins (PIAs). The PIA structures consist of partially de-N-acetylated β-(1→6)-*N*-acetylglucosamine polymers. These exopolysaccharides are key components of the bacterial biofilm matrix. Here, we describe the efficient synthesis of PIA oligosaccharides using an acid reversion reaction of *N*-acetylglucosamine in HF·pyridine. The PIA oligosaccharides produced by this reaction can be purified to homogeneity by size exclusion chromatography. Chemistry was developed to conjugate the PIA oligosaccharides to bovine serum albumin using a new heterobifunctional linker containing a thiol and an *N*-methylhydroxylamine functional group. These glycoconjugates may serve as useful precursors for the development of defined conjugate vaccines against PIA producing bacterial strains.

1. Introduction

Surface associated colonies of bacteria, known as biofilms, are the source of approximately 65% of human infections.[1] Biofilm associated infections are particularly challenging to eradicate, because bacteria residing in biofilms have a high tolerance to antibiotics and are more resistant to the host's immune response.[2] The formation of bacterial biofilms requires an extracellular matrix to facilitate the adherence between the bacteria and the surface they colonize.[3] Exopolysaccharides form an essential component of many biofilm matrices. A wide variety of medically important biofilm forming bacterial strains, including *Staphylococcus epidermidis*,[4] *Staphylococcus aureus*,[5] *Escherichia coli*,[6] *Bordetella bronchiseptica*,[7] *Actinobacillus pleuropneumoniae*,[8] and *Yersinia pestis*,[9] generate the same partially de-N-acetylated β-(1→6)-D-*N*-acetylglucosamine homopolymer (polysaccharide intercellular adhesin, PIA) as a key biofilm matrix exopolysaccharide (Fig. 1). The degree of de-N-acetylation along the polymer varies depending on the strain of bacteria from which the PIA has been isolated.

In addition to the importance of PIA in the extracellular matrix of bacterial biofilms, PIA is a promising immunogen for the development of glycoconjugate vaccines. Immunization of mice with high molecular weight PIA isolated from *S. epidermidis* generated antibodies protective against *S. aureus*.[10,11] Diphtheria toxoid-PIA conjugates form the basis of a vaccine, which has shown efficacy against clinically isolated strains of *E. coli* as well as pathogenic strains of *S. aureus* in mouse and rabbit models.[12,13] Chemical de-N-acetylation of the isolated PIA followed by protein conjugation gave a vaccine, which mediated superior antibody dependent opsonic killing of *S. aureus* when compared to vaccines derived from native PIA conjugates.[5,12,13]

The importance of PIA in bacterial biofilms and the promising results obtained with PIA based vaccines warrant the investigation of efficient methods to synthesize chemically defined PIA oligosaccharide glycoconjugates. Defined PIA glycoconjugates will serve as important tools to study the physical properties and biological roles of these medically important oligosaccharides. Furthermore, defined glycoconjugates may also circumvent some of the difficulties inherent in vaccines that are generated from isolated polysaccharides including batch to batch heterogeneity, the potential for biological contamination, and the fact that small synthetic oligosaccharides can be more immunogenic than full polysaccharides.[14]

In this report, we describe an efficient method to synthesize PIA oligosaccharides based on an acid reversion reaction. A new strategy has also been developed to allow for facile conjugation of these synthetic PIA oligosaccharides to the desired probe or protein of interest. The functionalized PIA oligosaccharides can be chemically

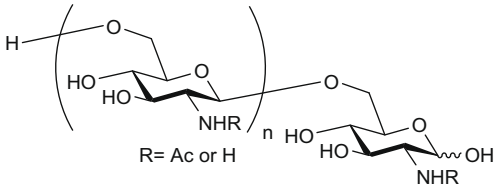


Figure 1. Structure of the polysaccharide intercellular adhesin (PIA).

* Corresponding author. Tel.: +1 416 946 0640.
E-mail address: mnitz@chem.utoronto.ca (M. Nitz).

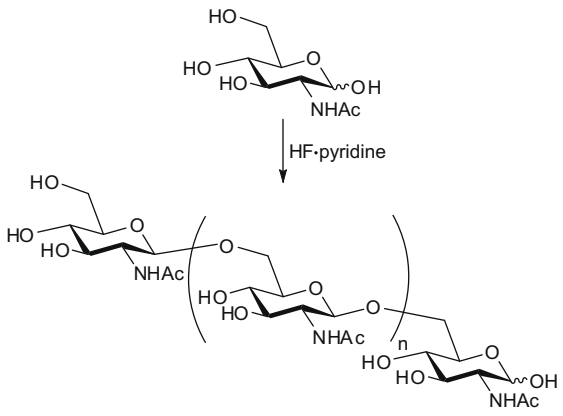
0008-6215/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2008.12.021

de-N-acetylated allowing the importance of de-N-acetylation in the immune response to PIA to be investigated.

2. Results and discussion

Numerous elegant syntheses of β-(1→6)-linked 2-acetamido-2-deoxy-D-glucopyranosides have been developed using a variety of strategies including: one-pot synthesis,[15] solid phase synthesis,[16,17] convergent approaches,[17–20] and polymerization reactions that employ selectively protected monosaccharides.[21,22] We required a simple synthesis which could be easily scaled to produce PIA oligosaccharides for further biochemical analysis and glycoconjugate formation. The most efficient synthesis of β-(1→6)-linked 2-acetamido-2-deoxy-D-glucopyranosyl oligosaccharides was reported by Defaye et al., wherein they describe the stereoselective and regioselective oligomerization of unprotected *N*-acetylglucosamine to the desired β-(1→6)-oligosaccharides in anhydrous hydrogen fluoride (HF).[23] This procedure requires stirring a concentrated suspension of *N*-acetylglucosamine in anhydrous HF and allowing the HF to slowly evaporate over the course of the oligomerization. While the simplicity of this procedure is attractive, it requires special precautions and equipment to be carried out safely. We have found that using a solution of 70% HF·pyridine was similarly effective in carrying out the stereospecific and regiospecific acid reversion reaction of *N*-acetylglucosamine to the desired β-(1→6)-linked oligosaccharide homopolymers (Scheme 1).

The concentration of *N*-acetylglucosamine in the 70% HF·pyridine solution was varied to determine the optimal conditions for the oligomerization. The longest oligosaccharides were obtained using a ratio of *N*-acetylglucosamine to HF·pyridine of 1 g:0.7 mL. This was empirically found to be the minimal amount of solvent required to dissolve the *N*-acetylglucosamine over the course of the reaction. The oligomerization reaction proceeded smoothly at room temperature, but upon heating above 35 °C the production of α-linked glycosides and colored by-products was observed. The reaction was readily carried out in polypropylene tubes, safely quenched with calcium carbonate and, after filtration of the generated calcium fluoride, concentration under reduced pressure gave a crude mixture of PIA oligosaccharides. The oligomerization reaction was monitored over the span of 5 days using HPLC with a Bio-Gel P4 size exclusion column (Table 1). After 5 days, no significant increase in the average oligosaccharide length was observed. The oligosaccharides contained predominately (~95%) the β-(1→6)-linked oligosaccharides, as determined by ^{1}H NMR, and could be purified to homogeneity by size exclusion chromatography (Fig. 2). NMR spectra of the purified oligosaccharides were consistent with those found by Defaye, as well as those measured for the PIA oligosaccharides isolated from bacterial sources.[4,24]



Scheme 1. The acid reversion reaction of *N*-acetylglucosamine to produce oligomers of the polysaccharide intercellular adhesin (PIA).

Table 1
Time course of relative yields (mass %) of various PIA oligosaccharides

PIA oligosaccharide[a]	24 h	48 h	72 h	5 d
Monosaccharide	57	45	41	42
Disaccharide	33	39	31	27
Trisaccharide (**1**)	8	11	16	17
Tetrasaccharide (**2**)	2	4	6	7
Pentasaccharide (**3**)	<1	1	2	2
Hexasaccharide	N/A	N/A	<1	1

[a] Determined by peak integration (A_{215}) from Bio-Gel P4 column.

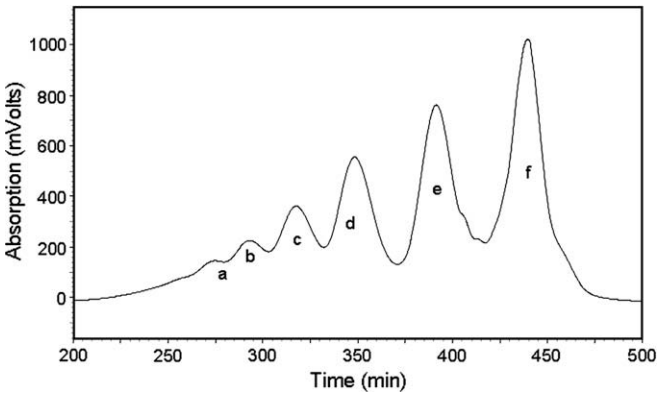


Figure 2. Chromatography trace (A_{215}) obtained with Bio-Gel P4 size exclusion resin from the acid reversion reaction of *N*-acetylglucosamine after 5 days of incubation at room temperature: (a) hexasaccharide; (b) pentasaccharide (**3**); (c) tetrasaccharide (**2**); (d) trisaccharide (**1**); (e) disaccharide; and (f) monosaccharide.

To enable the synthesis of glycoconjugates from the generated PIA oligosaccharides (**1**–**3**), a functional aglycone was installed at the reducing terminus. Initial attempts to form a reducing terminal glycoside employed the addition of a simple alcohol to the oligomerization reaction to terminate the oligosaccharide. Unfortunately, this approach resulted in an anomeric mixture of glycosides at the reducing terminus. The introduction of O-glycosides into the oligomerization reaction as the terminating glycoside also failed to yield the desired functionalized oligosaccharides as the O-glycosides were rapidly hydrolyzed under the reaction conditions. More stable glycosidic linkages were also investigated as chain terminating glycosides. Glycosyl triazoles and thioglycosides proved to be more robust to the reaction conditions, but still gave significant hydrolysis over the course of the 5-day incubation.[25]

Subsequently, our preferred strategy was to functionalize the reducing terminus of the PIA oligosaccharides after their synthesis as *N*-methyl-*O*-alkyl-hydroxylamine glycoconjugates. The use of *N*-methyl-*O*-alkylhydroxylamines in glycoconjugate formation was introduced by Dumy and co-workers, and has shown utility for the formation of a wide variety of useful glycosides.[26–28] In contrast to hydroxylamines, which give acyclic oximes, the *N*-alkylhydroxylamines yield the thermodynamically favored β-pyranosides upon condensation with a free hemiacetal.[28,29] To explore the utility of these conjugates, we have completed a study on the rates of hydrolysis of *N*-methyl-*O*-alkylhydroxylamine conjugates of *N*-acetylglucosamine. These studies have found that the hydrolysis of the conjugates is acid catalyzed, and that the glycoconjugates have half-lives in excess of six months at pH values greater than 6.[30] In addition, these glycosides are stable to the strongly basic conditions necessary for de-N-acetylation of the PIA oligosaccharides.

The heterobifunctional linker **4** was developed to serve as a cross-linker between the PIA oligosaccharides and a thiol reactive probe or protein of interest. The linker (**4**) bears an *N*-methylhydroxylamine for oligosaccharide condensation and a disulfide which can be reduced for reaction with a thiol reactive functional group.[31] The desired linker **4** was synthesized in four steps from commercially available 1-chloro-3-(toluene-*p*-sulfonyloxy)propane. The tosyl ester could be selectively displaced with *t*-butyl hydroxyl (methyl)carbamate to give **5**.[26] Displacement of the primary chloride with potassium thiocyanate followed by deprotection with sodium methoxide gave the *t*-butyl carbamate protected disulfide **6**. Finally, removal of the *t*-butyl carbamate protecting groups with trifluoroacetic acid gave the desired disulfide linker **4** (Scheme 2).

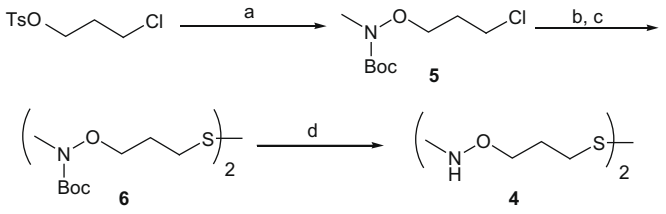
The conjugation of the *N*-alkylhydroxylamine linker (**4**) with the PIA oligosaccharides (**1**–**3**) proceeded to completion under concentrated conditions with mild acid catalysis (DMF/H_2O 1:1, cat. AcOH). It was necessary to reduce the symmetrical disulfide linker **4** to the free thiol prior to conjugation because derivatives of **4** bearing two oligosaccharides were highly insoluble. The desired conjugated PIA oligosaccharides could be isolated by size exclusion chromatography in good yield (Scheme 3).

It was possible to de-N-acetylate the *N*-methylhydroxylamine glycosides (**7**–**9**) via alkaline hydrolysis (10 M NaOH) over the course of 48 h at 37 °C. Previously, isolated PIA polysaccharides were 85% de-N-acetylated by stirring in 5 M NaOH for 24 h.[13] Under the conditions employed here ~90% of the PIA oligosaccharides were de-N-acetylated as determined by ^{1}H NMR. The *N*-alkylhydroxylamine conjugates were stable to these conditions and served as excellent protecting groups for the reducing terminus of the oligosaccharides which would otherwise have been degraded under the strongly alkaline conditions. Under the de-N-acetylation conditions, the oligosaccharides were allowed to oxidize to the disulfide linked oligosaccharides **10**–**12**. Unlike their fully N-acetylated counterparts these derivatives were soluble in aqueous solution. These conjugates (**10**–**12**) could be isolated by gel permeation chromatography in good yield (Scheme 3).

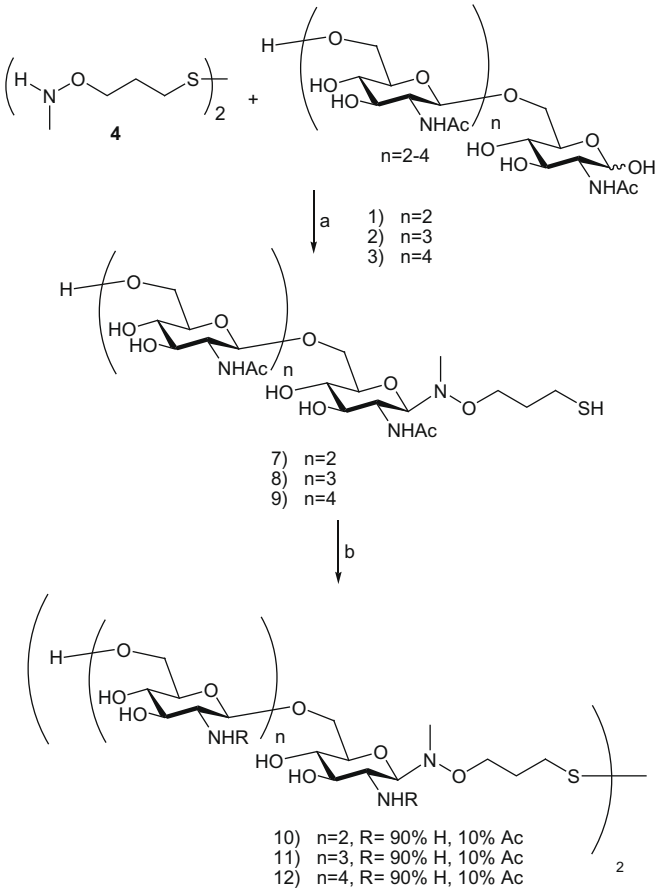
Protein conjugates formed from the fully acetylated and partially de-N-acetylated PIA glycosides (**7**–**12**) were formed with bovine serum albumin (BSA). BSA serves as a convenient protein for developing the conjugation chemistry and provides useful constructs for future ELISAs. Stirring BSA (24 mg/mL) with bromoacetic acid *N*-hydroxysuccinimide ester (~200-fold excess) yielded a range of 15–20 bromoacetamide functional groups per protein as determined by MALDI-MS.[31] The glycosides (**7**–**12**) were reduced in situ to form the free thiol with tris(2-carboxyethyl)phosphine (TCEP), added to bromoacetamide activated BSA, and allowed to stand overnight at room temperature (Scheme 4). Purification by centrifugal filtration gave the desired conjugates with good coupling efficiency. The degree of conjugation was estimated by MALDI-MS (Table 2).

3. Conclusion

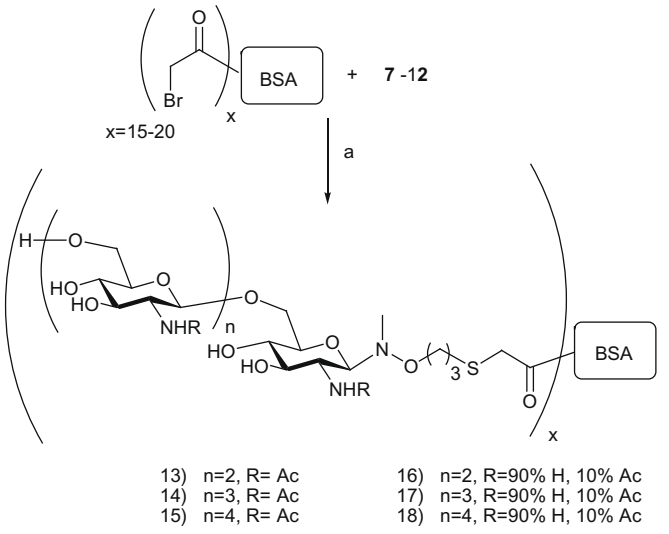
The polysaccharide intercellular adhesin is a crucial part of the extracellular matrix of many medically important bacterial biofilms. The efficient synthesis of this polysaccharide will enable a wide range of studies on the structure and enzymatic processing of PIA. Furthermore, this polysaccharide has been shown to be a promising immunogen for the development of carbohydrate-based vaccines.



Scheme 2. Synthesis of heterobifunctional linker **4**. Reagents and conditions: (a) NaH, DMF, BocN(Me)OH, 61%; (b) KSCN, DMF at 80 °C; (c) 1 M NaOMe, 79% two steps; (d) TFA–H_2O (3:1), 78%.



Scheme 3. Synthesis of glycoconjugates **7–12**. Reagents and conditions: (a) **4** (1.2 M, 2–3 equiv) in DMF/H_2O/AcOH (1:1:0.2), 50–70% isolated yield; (b) 10 M NaOH, 37 °C, 48 h.



Scheme 4. Synthesis of BSA conjugates of the PIA oligosaccharides (See Section 4 for details).

Table 2
Conjugation of **7**–**12** to BSA[a]

Conjugate	Bromoacetyl-BSA (MW)	BSA conjugate (MW)	PIA oligomers conjugated
13	68,000	70,700	~4
14	68,000	75,200	~8
15	68,000	76,000	~7
16	68,000	70,200	~4
17	68,000	74,000	~8
18	68,000	73,300	~6

[a] Masses determined by MALDI-MS using sinapinic acid as matrix.

The clean acid reversion reaction of *N*-acetylglucosamine in HF·pyridine provides an efficient route to produce PIA oligosaccharides. To facilitate the study of these synthetic oligosaccharides in biochemical and immunological experiments, the bifunctional *N*-alkyl hydroxylamine linker (**4**) has been developed to allow for neoglycoconjugate synthesis. This conjugation method should be widely applicable to the conjugation of any free reducing terminal oligosaccharide to a thiol reactive probe or protein. Furthermore, this linker is stable to the strongly basic conditions necessary for de-N-acetylation of the PIA oligosaccharides. These de-N-acetylated glycoconjugates will be useful for studying the immunological properties of the PIA oligosaccharides, which have been previously shown to be de-N-acetylation dependent.

4. Experimental

4.1. General methods

Flash chromatography was performed on Silia-P Flash Silica Gel 60 (40–63 μm particle size, Silicycle). Reactions were monitored by TLC using Silica Gel 60 F_{254} (EMD Science) with detection by quenching of fluorescence and/or by visualization with phosphomolybdic acid in ethanol (0.5% w/v), ethanolic H_2SO_4 (10% v/v) or ninhydrin in ethanol (0.2% w/v). Bio-Gel P4 size exclusion (BioRad) or Prevail Carbohydrate ES (Grace) columns were used for HPLC purification steps. Reagents were obtained from Sigma or Acros Organics and were used without further purification. HPLC was preformed on a Waters 1525 binary HPLC pump with a Waters 2487 dual λ absorbance detector or a Gilson 321 HPLC pump with a Gilson UV–Vis 156 dual λ absorbance detector. ^{1}H and ^{13}C NMR spectra were recorded at 25 °C with a Mercury 300 MHz (ASW-PFG-300 probe), a Varian 400 MHz (AutoX8308-400 probe) or a Varian Unity 500 MHz (Nalorac3-500 probe) spectrometer. The proton and carbon chemical shifts are reported in parts per million (δ scale), and are referenced to the residual NMR solvent signals (^{1}H NMR: D_2O δ 4.79, $CDCl_3$ δ 7.24 and ^{13}C NMR: $CDCl_3$ δ 77.23) or an internal standard (^{1}H NMR: TSP δ −0.015 and ^{13}C NMR: TSP δ −0.12). The assignments of resonances for all compounds were determined by two-dimensional homonuclear and heteronuclear chemical shift correlation experiments. Data are represented as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet), integration, coupling constant (*J*, Hz) and assignment. Mass spectra were obtained from a Waters Micromass Maldi MX Mass spectrometer (MALDI) and high resolution mass spectra were obtained from an ABI/Sciex QStar mass spectrometer (ESI).

4.2. General procedure for preparation of β-(1→6)-linked 2-acetamido-2-deoxy-D-glucopyranosyl oligosaccharides (1–3)

A mixture of *N*-acetyl-D-glucosamine (1 g, 4.5 mmol) and 0.7 mL of HF (~70%) in pyridine (~30%) was stirred in a polypropylene tube for 5 days. The viscous solution was then poured onto a slurry of calcium carbonate (2 g) in ice and the mixture was stirred until the evolution of gas ceased (~30 min). The suspension was filtered through Celite and washed with water. The water was removed under reduced pressure to give a yellow oil. The yellow oil was then resuspended in a minimal volume of water and filtered through a bed of C18 silica (5.0 cm × 2.5 cm), which was subsequently washed with water (100 mL). Evaporation of the water gave a pale yellow oil containing the mixed oligosaccharide lengths of poly-(1→6)-β-*N*-acetyl-D-glucosamine. The oligosaccharides were then separated on a Bio-Gel P4 size exclusion column (5.0 cm I.D. × 70 cm, 45–90 μm particle size) using distilled water as an eluent at 1–1.5 mL/min and the absorbance of the eluent was monitored at 215 nm.

4.2.1. 2-Acetamido-2-deoxy-β-D-glucopyranosyl-(1→6)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→6)-2-acetamido-2-deoxy-D-glucopyranose (1)

The title compound **1** (n = 2) was prepared according to the general procedure (Section 4.2). δ_H (500 MHz, D_2O, TSP) 2.04 (3H, s, N*Ac*), 2.05 (6H, N*Ac*), 3.38–4.21 (18H, m, H-2 to H-6″), 4.51, 4.53, (2H, d, J = 8.4 Hz, 8.3 Hz, H-1′, H-1″), 4.67 and 5.16 (1H, d, J = 8.4 Hz, 3.5 Hz, H-1 β/α); δ_C (100 MHz; D_2O; TSP) 24.6, 24.9(3) and 25.0 (*C*H$_3$), 56.7, 58.1, 58.2, 59.4, 63.4, 71.1, 71.2, 71.4, 72.6(2), 72.7, 72.8, 73.1, 73.4, 76.4(2), 76.6, 77.3(2), 77.5 and 78.6, 93.6 (C1 α), 97.7 (C1 β), 104.2, 104.4 and 104.4 (C1′–C1″) and 177.2(3), 177.3, 177.4(2) (*C*=O); *m/z* (ESI) calcd for $C_{24}H_{41}N_3O_{16}Na$ (M+Na$^+$) 650.2385, found 650.2374.

4.2.2. 2-Acetamido-2-deoxy-β-D-glucopyranosyl-(1→6)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→6)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→6)-2-acetamido-2-deoxy-D-glucopyranose (2)

The title compound **2** (n = 3) was prepared according to the general procedure (Section 4.2). δ_H (500 MHz, D_2O, TSP) 2.04, 2.05(2) and 2.06 (12H, (br)s, N*Ac*), 3.38–4.21 (24H, m, H-2, to 6‴), 4.52–4.56 (3H, m, H-1′, H-1″, H-1‴), 4.68 and 5.16 (1H, d, J = 8.4 Hz, 3.4 Hz, H-1 β/α); δ_C (100 MHz; D_2O) 22.1, 22.3, 22.4(2) and 22.5 (*C*H$_3$), 54.2, 55.6, 55.7, 56.9, 60.9, 68.6(2), 68.7, 68.8, 70.1(2), 70.2, 70.3, 70.5, 70.9, 73.9, 74.1, 74.7(2), 74.8, 74.9, 76.0(2), 91.0 (C1 α), 95.1 (C1 β), 101.7(2), 101.8 and 101.9 (C1′-C1‴) and 174.6, 174.7, 174.8(2), 174.9 (*C*=O); *m/z* (MALDI) 853.1 (M+Na$^+$), 890.0 (M+K$^+$).

4.2.3. 2-Acetamido-2-deoxy-β-D-glucopyranosyl-(1→6)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→6)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→6)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→6)-*O*-2-acetamido-2-deoxy-D-glucopyranose (3)

The title compound **3** (n = 4) was prepared according to general procedure (Section 4.2). δ_H (500 MHz, D_2O, TSP) 2.04–2.06 (15H, m, N*Ac*), 3.38–4.22 (30H, m, H-2-H-6⁗), 4.51–4.56 (4H, m, H-1′, H-1″, H-1‴, H-1⁗), 4.68 and 5.16 (1H, d, J = 8.4 Hz and 3.5 Hz, H-1 β/α); δ_C (100 MHz; D_2O) 22.1, 22.4(4) and 22.5 (2) (*C*H$_3$), 54.2, 55.6, 55.7, 56.9, 60.9, 68.6(2), 68.7(2), 68.8, 70.0, 70.1(2), 70.2, 70.5, 70.9, 73.9, 74.7, 74.8, 74.9 and 76.0, 91.0 (C1 α), 95.1 (C1 β), 101.7(5) (C1′-C1⁗) and 177.6(2), 177.8(2), 177.9 (*C*=O); *m/z* (MALDI) 1056.3 (M+Na$^+$), 1082.3 (M+K$^+$).

4.3. 1,2-Bis(*N*-methyl-*O*-propyl-hydroxylamine)disulfide (4)

Compound **6** (0.9 g, 2.04 mmol) was dissolved in aqueous TFA (3 mL, 75% v/v TFA) solution and was stirred for 30 min. TFA was removed under a stream of N_2 (gas) and the mixture was concentrated under reduced pressure to afford a yellow oil. The crude disulfide was resuspended in DCM (50 mL) and washed with saturated $NaHCO_3$ (3 × 500 mL). The combined organics were concen-

trated and purified by column chromatography on silica using an isocratic elution with Et_2O to give compound **4** as a colorless oil (0.38 g, 78% yield). δ_H (400 MHz; $CDCl_3$) 1.93 (4H, quintet, J = 6.2 Hz, $CH_2CH_2CH_2$), 2.73 (6H, s, NCH_3), 2.68 (4H, t, J = 7.0 Hz, CH_2S) and 3.72 (4H, t, J = 6.2 Hz, CH_2O); δ_C (100 MHz; D_2O) 28.6 ($CH_2CH_2CH_2$), 35.8 (CH_2S), 39.4 (NCH_3) and 71.7 (CH_2O); *m/z* (ESI) calcd for $C_8H_{21}N_2O_2S_2$ ($M+H^+$) 241.1039, found 241.1044.

4.4. *N*-Boc-*N*-methyl-*O*-(3-chloropropyl)-hydroxylamine (5)

To a solution of *N*-Boc-*N*-methyl-hydroxylamine (1.3 g, 8.7 mmol) and 3-chloropropyl tosylate (2.38 g, 9.6 mmol) in DMF (70 mL), sodium hydride (60% dispersion in mineral oil) (0.35 g, 8.7 mmol) was slowly added under N_2 at 0 °C over 1 h. Stirring was continued at room temperature until the reaction was completed as confirmed by TLC. The mixture was then diluted into water (100 mL) and extracted with EtOAc (3 × 150 mL), dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel using Et_2O–pentane (0:1→1:9) to afford compound **5** as a colorless oil (1.19 g, 61% yield). δ_H (300 MHz, $CDCl_3$) 1.45 (9H, s, $C(CH_3)_3$), 2.01 (2H, quintet, J = 6.1 Hz, $CH_2CH_2CH_2$), 3.05 (3H, s, NCH_3), 3.63 (2H, t, J = 6.4 Hz, CH_2Cl) and 3.93 (2H, t, J = 5.9 Hz, CH_2O); δ_C (75 MHz; $CDCl_3$) 28.4 ($C(CH_3)_3$), 31.4 ($CH_2CH_2CH_2$), 36.6 (NCH_3) 41.7 (CH_2Cl), 70.6 (CH_2O), 81.2 ($C(CH_3)_3$) and 157.1 (C=O); *m/z* (ESI) calcd for $C_9H_{18}NO_3ClNa$ ($M+Na^+$) 246.0873, found 246.0880.

4.5. *t*-Butyl 3,3′-disulfanediylbis(propane-3,1-diyl)bis(oxy)bis (methylcarbamate) (6)

A solution of compound **5** (2.26 g, 11.6 mmol) and potassium thiocyanate (5.63 g, 57.9 mmol) in DMF (50 mL) was heated at 80 °C for 16 h. The mixture was diluted into water (100 mL) and extracted with EtOAc (3 × 150 mL). After drying over $MgSO_4$, the organics were concentrated to an oil under reduced pressure. The oil containing the crude thiocyanate was then converted to the disulfide by stirring in a solution of 1 M sodium methoxide (25 mL) in a flask open to the atmosphere until the reaction was complete (~10 h) as judged by TLC. The solvent was then diluted with water (100 mL) and extracted with EtOAc (3 × 150 mL). After concentration, the crude disulfide was purified by column chromatography on silica using a gradient of Et_2O–pentane (1:9→1:4). The product **6** was isolated as a pale yellow oil (1.6 g, 79% yield). δ_H (300 MHz; $CDCl_3$) 1.45 (18H, s, $C(CH_3)_3$), 1.96 (4H, quintet, J = 6.8 Hz, $CH_2CH_2CH_2$), 2.76 (4H, t, J = 7.2 Hz, CH_2S), 3.05 (6H, s, NCH_3), and 3.89 (4H, t, J = 6.0 Hz, CH_2O); δ_C (75 MHz; $CDCl_3$) 27.9 ($CH_2CH_2CH_2$), 28.5 ($C(CH_3)_3$), 35.2 (CH_2S), 36.7 (NCH_3), 72.3 (CH_2O), 81.2 ($C(CH_3)_3$) and 157.0 (C=O); *m/z* (ESI) calcd for $C_{18}H_{36}N_2O_6S_2Na$ ($M+Na^+$) 463.1912, found 463.1905.

4.6. General procedure for condensation of compound 4 with PIA oligosaccharides 1–3 (7–9)

Compound **4** (20 mg) was reduced with dithiothreitol (20 mg) in DMF containing Et_3N (0.1%) (60 μL) at room temperature for 30 min. The reduced linker solution was added into a polypropylene microcentrifuge tube containing PIA oligosaccharide **1** or **2** (20 mg) dissolved in H_2O–AcOH (70 μL, 6:1). These mixtures gave a final reduced linker concentration of ~1.2 M (0.17 mmol in 140 μL). After incubating the solutions at 60 °C overnight, they were quenched with 5 M NH_4OAc (300 μL). The aqueous phase was then washed with EtOAc (5 × 700 μL) to remove excess linker. Conjugates **7** and **8** were purified on a Bio-Gel P4 size exclusion column (1 cm I.D. × 50 cm, 45–90 μm particle size) running at 0.8 mL/min in H_2O.

4.6.1. *N*-Methyl-*O*-(propyl-3-thiol)-*N*-[2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→6)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→6)-2-acetamido-2-deoxy-β-D-glucopyranosyl] hydroxylamine (7)

The title compound was prepared from trisaccharide **1** according to general procedure (Section 4.6) to yield **7** (12.4 mg, 54% yield). δ_H (400 MHz, D_2O) 1.85 (2H, quintet, $CH_2CH_2CH_2$), 2.07 (9H, m, N*Ac*), 2.61 (2H, t, J = 7.1 Hz, CH_2S), 2.73 (3H, s, NCH_3), 3.64 and 3.81 (2H, t, CH_2O), 3.37–4.24 (18H, m, H-2-H-6′′), 4.17 (1H, d, J = 9.7 Hz, H-1), 4.57(2) (2H, d, J = 8.5 Hz and 8.4 Hz, H-1′, H-1′′); δ_C (100 MHz; D_2O) 20.7 ($CH_2CH_2CH_2$), 22.4(2), 22.5 ($COCH_3$), 32.0 (CH_2S), 39.2 (CH_2O), 52.4, 55.6(2), 60.8, 68.6, 68.9, 70.0, 70.1, 70.8, 73.8, 73.9, 74.8 75.7, 76.0, 76.3, 91.8 (C-1), 101.6 (C-1′, C-1′′), 174.2, 174.5, 174.6 (C=O); *m/z* (ESI) calcd for $C_{28}H_{51}N_4O_{16}S$ ($M+H^+$) 731.3022, found 731.3023.

4.6.2. *N*-Methyl-*O*-(propyl-3-thiol)-*N*-[2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→6)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→6)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→6)-2-acetamido-2-deoxy-β-D-glucopyranosyl] hydroxylamine (8)

The title compound was prepared from tetrasaccharide **2** according to the general procedure (Section 4.6) to yield **8** (14.9 mg, 66% yield). δ_H (400 MHz; D_2O) 1.85 (2H, quintet, $CH_2CH_2CH_2$), 2.06–2.08 (12H, m, N*Ac*), 2.61 (2H, t, J = 7.1 Hz, CH_2S), 2.73 (3H, s, NCH_3), 3.64–4.24 (24H, m, H-2-H-6′′′) 3.67 and 3.80 (2H, t, CH_2O), 4.17 (1H, d, J = 9.7 Hz, H-1′), 4.56 (3H, m, H-1′, H-1′′ and H-1′′′); δ_C (100 MHz; D_2O) 20.7 ($CH_2CH_2CH_2$), 22.4(3), 22.5, 23.4 (CH_2S), 31.9 (CH_2O), 39.2, 52.3, 55.6(2), 60.8 68.5, 69.6, 68.9, 70.0, 70.1, 70.9, 73.8, 73.9, 74.7, 75.8, 76.0, 76.3, 91.8 (C1), 101.5, 101.6, 101.7, (C1′, C1′′, C1′′′) and 174.2, 174.5, 174.6(2) (C=O); *m/z* (ESI) calcd for $C_{36}H_{64}N_5O_{21}S$ ($M+H^+$) 934.3815, found 934.3808.

4.6.3. *N*-Methyl-*O*-(propyl-3-thiol)-*N*-[2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→6)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→6)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→6)-2-acetamido-2-deoxy-β-D-glucopyranosyl] hydroxylamine (9)

The title compound was prepared from pentasaccharide **3** (20 mg) according to the general procedure (Section 4.6) with the exception that twice as much water (120 μL) was required to dissolve the oligosaccharide. The amount of compound **4** (40 mg) in the reaction was increased to maintain the high molarity necessary for the conjugation reaction to yield **9** (12.1 mg, 55% yield). δ_H (400 MHz; D_2O) 1.85 (2H, quintet, $CH_2CH_2CH_2$), 2.06–2.08 (15H, m, N*Ac*), 2.62 (2H, t, J = 7.1 Hz, CH_2S), 2.73 (3H, s, NCH_3), 3.66 and 3.81 (2H, t, CH_2O), 3.37–4.24 (24H, m, H-2-H-6′′′′), 4.18 (1H, d, J = 9.7 Hz, H-1), 4.65 (4H, m, H-1′, H-1′′, H-1′′′, H-1′′′′); δ_C (100 MHz; D_2O) 20.6, 22.4, 22.5(3), 32.0, 39.2, 52.4, 55.6(2), 55.7, 60.9, 68.5, 68.6, 68.9, 70.0, 70.0, 70.1, 70.9, 73.8, 74.0, 74.7, 75.7. 76.0, 76.3, 91.8 (C1), 101.7(4) (C1′, C1′′, C1′′′ and C1′′′′) and 174.2, 174.5, 174.6, 174.6(2), 171.6 (C=O); *m/z* (ESI) calcd for $C_{44}H_{77}N_6O_{26}S$ ($M+H^+$) 1137.4609, found 1137.4583.

4.7. General procedure for de-N-acetylation of the oligosaccharide conjugates (10–12)

Compounds **10**, **11**, and **12** were prepared from PIA oligomers **1**, **2**, and **3** (20 mg) according to general procedure for condensation (Section 4.6), and at the end of the incubation period the mixtures were resuspended in water (700 μL) and extracted with EtOAc (~5 mL). The aqueous phase was then lyophilized to a colorless powder. The glycoconjugates **7**, **8**, and **9** were then dissolved in a

solution of dithiothreitol (10 mg) in 10 M NaOH (300 μL) and incubated at 37 °C for 48 h. The mixture was exposed to air for 2 h to allow for disulfide formation and was then quenched in 5 M NH_4Cl (600 μL). Removal of ammonia and solvent was achieved by blowing a stream of air over the mixture for 4 h. After resuspending the mixture in a minimal volume of water (~700 μL), the excess salt was removed by dialysis overnight (100 MWCO). Purification on a Bio-Gel P2 size exclusion column (1 cm I.D. × 50 cm, 45–90 μm particle size) running at 1.2 mL/min in 50 mM $(NH_4)_2CO_3$ gave the de-N-acetylated PIA glycoconjugates **10**, **11,** and **12** in moderate yield.

4.7.1. 1,2-Bis-(*N*-methyl-*O*-propyl-*N*-[2-amino-2-deoxy-β-D-glucopyranosyl-(1→6)-2-amino-2-deoxy-β-D-glucopyranosyl-(1→6)-2-amino-2-deoxy-β-D-glucopyranosyl] hydroxylamine) disulfide (10)

The title compound was prepared from **1** (20 mg) according to the general deacetylation procedure (Section 4.7) to give compound **10**. δ_H (400 MHz; D_2O) 2.0 (4H, quintet, J = 6.5 Hz, $CH_2CH_2CH_2$), 2.1(3) (~1.4H, s, residual $COCH_3$), 2.68–2.74 (4H, m, CH_2S), 2.76 (6H, s, NCH_3), 2.84 (4H, t, J = 7.1 Hz, H-2′, H-2″), 2.92 (2H, J = 9.4 Hz, t, H-2), 3.38–3.96 (34H, m, H-3-H-6″ and CH_2O), 4.12 (2H, d, J = 9.4 Hz, H-1), 4.46 and 4.50 (4H, d, J = 8.1 and 8.2 Hz, H-1′, H-1″); *m/z* (ESI) calcd for $C_{44}H_{88}N_8O_{26}S_2$ ($M+2H^+$) 604.2626, found 604.2686.

4.7.2. 1,2-Bis-(*N*-methyl-*O*-propyl-*N*-[2-amino-2-deoxy-β-D-glucopyranosyl-(1→6)-2-amino-2-deoxy-β-D-glucopyranosyl-(1→6)-2-amino-2-deoxy-β-D-glucopyranosyl-(1→6)-2-amino-2-deoxy-β-D-glucopyranosyl] hydroxylamine)disulfide (11)

The title compound was prepared from **2** (20 mg) according to the general deacetylation procedure (Section 4.7) to give compound **11**. δ_H (400 MHz, D_2O) 2.0 (4H, quintet, J = 6.6 Hz, $CH_2CH_2CH_2$), 2.07 (~3.5H, m, N*Ac*), 2.67–2.75 (4H, m, CH_2S), 2.76 (6H, s, NCH_3), 2.84 (4H, t, J = 7.6 Hz, H-2′, H-2″, H-2‴), 2.90 (2H, t, J = 9.3 Hz, H-2), 3.36–3.97 and 4.17–4.28 (44H, m, H3–H6‴ and CH_2O), 4.11 (2H, d, J = 9.4 Hz, H-1), 4.44–4.50 (6H, m, H-1′, H-1″, H-1‴), 4.54–4.58 (~1.5H, m, residual N-acetylated H-1′, H-1″, H-1‴); *m/z* (ESI) calcd for $C_{56}H_{110}N_{10}O_{34}S_2$ ($M+2H^+$) 765.3314, found 765.3376.

4.7.3. 1,2-Bis-(*N*-methyl-*O*-propyl-*N*-[2-amino-2-deoxy-β-D-glucopyranosyl-(1→6)-2-amino-2-deoxy-β-D-glucopyranosyl-(1→6)-2-amino-2-deoxy-β-D-glucopyranosyl-(1→6)-2-amino-2-deoxy-β-D-glucopyranosyl-(1→6)-2-amino-2-deoxy-β-D-glucopyranosyl] hydroxylamine)disulfide (12)

The title compound was prepared from **3** (20 mg) according to the general deacetylation procedure (Section 4.7) to give compound **12**. δ_H (400 MHz, D_2O) 2.00 (4H, quintet, J = 6.3 Hz, $CH_2CH_2CH_2$), 2.08 (~3.7 H, m, residual N*Ac*), 2.66–2.74 (4H, m, CH_2S), 2.76 (6H, s, NCH_3), 2.84 (6H, t, J = 7.5 Hz, H-2′, H-2″, H-2‴, H-2⁗), 2.89 (2H, t, J = 9.4 Hz, H-2), 3.35–3.98 and 4.16–4.31 (54H, m, H3-H6⁗ and CH_2O), 4.09 (2H, d, J = 9.3 Hz, H-1), 4.44–4.52 (8H, m, H-1′,H-1″, H-1‴, H-1⁗); *m/z* (ESI) calcd for $C_{68}H_{133}N_{12}O_{42}S_2$ ($M+3H^+$) 617.9361, found 617.9435.

4.8. General procedure for preparing BSA conjugates (13–18)

A solution of BSA (360 μM, 60 mg/2.5 mL) in HEPES buffer (50 mM) containing EDTA (1 mM) at pH 7.8 was activated with a solution of bromoacetic acid *N*-hydroxysuccinimide ester in DMF (84.7 mM, 5 mg/250 μL) at room temperature for 30 min. Excess cross-linker was removed by gel filtration (PD-10 Desalting column, Sephadex G-25, 10 mM HEPES, 1 mM EDTA, pH 7.8) and the average number of bromoacetate functional groups per BSA was determined by MALDI-MS (average of 15–20 linkers added). The thiol functionalized glycosides **7–9** and **10–12** (1.45 μmol each) were incubated with TCEP (0.6 and 1.2 equiv, respectively) and $NaHCO_3$ (5 equiv) under N_2 (gas) at 37 °C for 4 h. Activated BSA protein (100 μL) was then added to the reduced thiol functionalized glycosides **7–9** and **10–12,** and allowed to stand overnight at room temperature. Purification of the protein conjugates using centrifugal filtration (MWCO 5000) with buffer (10 mM HEPES, 1 mM EDTA, pH 7.8) gave **13–18**. The average number of oligosaccharides conjugated per BSA was determined by MALDI-MS using sinapinic acid as the matrix.

Acknowledgments

This work was supported by the Natural Science & Engineering Research Council (NSERC) of Canada and the Canadian Institutes of Heath Research (CIHR).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2008.12.021.

References

1. Potera, C. *Science* **1999**, *283*, 1837–1839.
2. Donlan, R. M.; Costerton, J. W. *Clin. Microbiol. Rev.* **2002**, *15*, 167–193.
3. Hall-Stoodley, L.; Costerton, J. W.; Stoodley, P. *Nat. Rev. Microbiol.* **2004**, *2*, 95–108.
4. Mack, D.; Fischer, W.; Krokotsch, A.; Leopold, K.; Hartmann, R.; Egge, H.; Laufs, R. *J. Bacteriol.* **1996**, *178*, 175–183.
5. Cerca, N.; Jefferson, K. K.; Maira-Litran, T.; Pier, D. B.; Kelly-Quintos, C.; Goldmann, D. A.; Azeredo, J.; Pier, G. B. *Infect. Immun.* **2007**, *75*, 3406–3413.
6. Wang, X.; Preston, J. F.; Romeo, T. *J. Bacteriol.* **2004**, *186*, 2724–2734.
7. Sloan, G. P.; Love, C. F.; Sukurnar, N.; Mishra, M.; Deora, R. *J. Bacteriol.* **2007**, *189*, 8270–8276.
8. Izano, E. A.; Sadovskaya, I.; Vinogradov, E.; Mulks, M. H.; Velllyagounder, K.; Ragunath, C.; Kher, W. B.; Ramasubbu, N.; Jabbouri, S.; Perry, M. B.; Kaplan, J. B. *Microb. Pathogenesis* **2007**, *43*, 1–9.
9. Itoh, Y.; Wang, X.; Hinnebusch, B. J.; Preston, J. F.; Romeo, T. *J. Bacteriol.* **2005**, *187*, 382–387.
10. Maira-Litran, T.; Kropec, A.; Abeygunawardana, C.; Joyce, J.; Mark, G.; Goldmann, D. A.; Pier, G. B. *Infect. Immun.* **2002**, *70*, 4433–4440.
11. McKenney, D.; Pouliot, K. L.; Wang, Y.; Murthy, V.; Ulrich, M.; Doring, G.; Lee, J. C.; Goldmann, D. A.; Pier, G. B. *Science* **1999**, *284*, 1523–1527.
12. Cerca, N.; Maira-Litran, T.; Jefferson, K. K.; Grout, M.; Goldmann, D. A.; Pier, G. B. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 7528–7533.
13. Maira-Litran, T.; Kropec, A.; Goldmann, D. A.; Pier, G. B. *Infect. Immun.* **2005**, *73*, 6752–6762.
14. Pozsgay, V. *Adv. Carbohydr. Chem. Biochem.* **2001**, *56*, 153–199.
15. Fridman, M.; Solomon, D.; Yogev, S.; Baasov, T. *Org. Lett.* **2002**, *4*, 281–283.
16. Manabe, S.; Ito, Y.; Ogawa, T. *Synlett* **1998**, 628–630.
17. Melean, L. G.; Love, K. R.; Seeberger, P. H. *Carbohydr. Res.* **2002**, *337*, 1893–1916.
18. Gening, M. L.; Tsvetkov, Y. E.; Pier, G. B.; Nifantiev, N. E. *Carbohydr. Res.* **2007**, *342*, 567–575.
19. Yang, F.; Du, Y. G. *Carbohydr. Res.* **2003**, *338*, 495–502.
20. Yang, F.; He, H. M.; Du, Y. G. *Tetrahedron Lett.* **2002**, *43*, 7561–7563.
21. Kanno, K. I.; Hatanaka, K. *Polym. J.* **1998**, *30*, 678–680.
22. Sadakane, Y.; Hatanaka, Y. *Anal. Sci.* **2006**, *22*, 209–218.
23. Defaye, J.; Gadelle, A.; Pedersen, C. *Carbohydr. Res.* **1989**, *186*, 177–188.
24. Joyce, J. G.; Abeygunawardana, C.; Xu, Q. W.; Cook, J. C.; Hepler, R.; Przysiecki, C. T.; Grimm, K. M.; Roper, K.; Ip, C. C. Y.; Cope, L.; Montgomery, D.; Chang, M.; Campie, S.; Brown, M.; McNeely, T. B.; Zorman, J.; Maira-Litran, T.; Pier, G. B.; Keller, P. M.; Jansen, K. U.; Mark, G. E. *Carbohydr. Res.* **2003**, *338*, 903–922.
25. Wilkinson, B. L.; Bornaghi, L. F.; Poulsen, S. A.; Houston, T. A. *Tetrahedron* **2006**, *62*, 8115–8125.
26. Bohorov, O.; Andersson-Sand, H.; Hoffmann, J.; Blixt, O. *Glycobiology* **2006**, *16*, 21C–27C.
27. Langenhan, J. M.; Peters, N. R.; Guzei, I. A.; Hoffmann, M.; Thorson, J. S. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 12305–12310.
28. Peri, F.; Dumy, P.; Mutter, M. *Tetrahedron* **1998**, *54*, 12269–12278.
29. Liu, Y.; Feizi, T.; Carnpanero-Rhodes, M. A.; Childs, R. A.; Zhang, Y. N.; Muiioy, B.; Evans, P. G.; Osborn, H. M. I.; Otto, D.; Crocker, P. R.; Chai, W. C. *Chem. Biol.* **2007**, *14*, 847–859.
30. Gudmundsdottir, A.V.; Paul, C. E.; Nitz, M., *Carbohydr. Res.* **2009**, **334**, 278–284.
31. Bernatowicz, M. S.; Matsueda, G. R. *Anal. Biochem.* **1986**, *155*, 95–102.