Common sialylated glycan in Actinobacillus suis

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A sialylated oligosaccharide was identified in four representative strains of the Gram-negative swine pathogen, Actinobacillus suis. As characterized, the glycan consists of a free oligosaccharide with a N-acetyllactosamine-like backbone decorated with sialic acid, phosphoethanolamine (PEA) and O-acetyl units: 9-O-Ac-Neu5Ac-(2→6)-β-d-Galp-(1→4)-β-d-6-O-Ac-GlcNAc-(1→3)-[PEA-6]-β-d-Galp-(1→3)-β-d-GlcNAc-(1→2)-[9-O-Ac-Neu5Ac-(2→6)]-β-d-Galp-(1→4)-β-d-6-O-Ac-GlcNAc-(1→3)-[PEA-6]-β-d-Galp-(1→3)-d-GlcNAc. The ubiquitous expression of this sialylated glycan suggests that this carbohydrate may play an important role in the survival of A. suis in the host.

Keywords: Actinobacillus suis/phosphoethanolamine/sialic acid/sialylated glycan

Introduction

Actinobacillus suis is a facultative anaerobic Gram-negative bacterium and a common commensal in the upper respiratory tract of swine (MacInnes et al. 2008). A. suis was traditionally reported to cause sporadic cases of septicemia and sudden death, mostly in young suckling and recently weaned piglets (Bilkei et al. 1995). During the last two decades, however, A. suis emerged as a swine pathogen in adult pigs causing a variety of diseases including skin lesions, pleuropneumonia, septicemia, metritis, meningitis, arthritis, abortion and sudden death especially in high-health status pigs (Mniats et al. 1989; Yaeger 1996; Sanford 1998; MacInnes and Desrosier 1999).

At present, there is no commercial vaccine available against this pathogen and autogenous bacterins have been used with mixed success. Although many strains of A. suis are sensitive to antibiotics, disease may be underway before treatment can be initiated and there is growing resistance to the use of antibiotics in food animals (MacInnes and Desrosier 1999). To control A. suis diseases and the attendant economic losses, an effective vaccine is needed. This need may be fulfilled by an appropriate vaccine based on the carbohydrates produced by A. suis.

Previous work in our laboratories has laid the ground work for an O/K serotyping system based on the specificity for O-antigen polysaccharides (PS) and capsular polysaccharide (CPS), referred to as O and K serotypes, respectively. The O1 and K1 antigens comprised (1→6)-β-d-glucan that is similar in structure to a key cell wall component in yeasts such as Saccharomyces cerevisiae and Candida albicans (Monteiro et al. 2000). The O2 antigen PS is built up by the repeats of a branched tetrasaccharide block [Gal-[Gal→]Glc→GlcNAc]6m (Rullo et al. 2006). Epidemiological and direct challenge experiments suggest that O2 strains have greater pathogenic potential than O1 strains (Slavić, Toffner et al. 2000; Slavić, DeLay et al. 2000); however, development of a simple serotyping method has thus far been elusive due to pervasive cross-reactivity (Van Ostaaijen et al. 1997). Several lines of evidence suggested that a common carbohydrate antigen might be responsible for the observed cross-reactivity. Accordingly, the structure of a common oligosaccharide expressed by several A. suis strains (VSB 3714 — rough/K1, ATCC 15557 — O1/K1, H91-0380 — O2/K2, C84 — O1/K2) was identified and it is described here.

Results

Determination of sugar composition and linkage types

Monosaccharide composition analysis by the alditol acetate method revealed the presence of galactose (Gal) and N-acetylglucosamine (GlcNAc) in equimolar ratios. The glycosylations with 2-(S)- and 2-(R)-butanol provided evidence that the observed sugar residues were present as D enantiomers. Sugar linkage-type analysis revealed four Gal linkage types: terminal Gal [Gal(1→)], 3-substituted Gal [→3]-Gal(1→), 6-substituted Gal [→6]-Gal(1→) and 2,6-substituted Gal [→2,6]-Gal(1→); and two GlcNAc linkage types: 3-substituted GlcNAc [→3]-GlcNAc(1→) and 4-substituted GlcNAc [→4]-GlcNAc(1→).

Ring proton assignments

The 1-D 1H-nuclear magnetic resonance (NMR) (Figure 1A) and the corresponding 2-D 1H–1H correlation spectroscopy (COSY) and 1H–1H total correlation spectroscopy (TOCSY) NMR spectra (not shown) of the intact glycan yielded a highly convoluted anomic and ring proton resonance regions, which did not allow for proton assignments to be made with any degree of confidence. A sole α-anomeric resonance at...
δH 5.18 (J1,2 3.6 Hz) was identified with a corresponding H-2 and C-2 at δH 4.06 and δC 53.0 in the two dimensional heteronuclear spectra (1H-13C heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC), not shown). A C-2 resonance at δC 53.0 is characteristic of N-acetyl-hexosamines, which suggested that the H-1 resonance at δH 5.18 belonged to a GlcNAc unit. This H-1 residue was no longer present after reduction of the intact glycan with NaBD4 (Figure 2), which suggested that this GlcNAc may be the reducing end unit, and that all the nonreducing units present possessed the β-anomeric configuration. A triplet at δH 1.72 and a doublets-of-doublet at δH 2.66 strongly suggested the presence of a neuraminic acid type of moiety and the sharp singlets at δH 2.14, 2.16 and 2.19 were indicative of O-acetylation.

The 1H-NMR spectrum of the desialylated glycan (Figure 1B) showed that the sialic acid moiety was removed, but the region of the anomeric protons remained complicated. The high degree of overlapping of anomeric proton resonances dissipated after de-O-acetylation of the desialylated sample (Figure 1C). In the desialylated/de-O-acetylated sample, one α-anomeric proton resonance was observed at δ 5.24 (J1,2 3.6 Hz) (residue A) and 8 β-anomeric proton resonances at δ 4.89 (J1,2 8.4 Hz) (residue B), δ 4.73 (J1,2 7.8 Hz) (residue C), δ 4.74 (J1,2 7.2 Hz) (residue D), δ 4.72 (J1,2 7.2 Hz) (residue D’), δ 4.60 (J1,2 7.8 Hz) (residue E), δ 4.55 (J1,2 6.0 Hz) (residue F), δ 4.49 (J1,2 7.8 Hz) (residue G) and δ 4.46 (J1,2 7.8 Hz) (residue H). Thorough analysis and cross-referencing of the 2-D 1H-1H COSY, 1H-1H TOCSY and 1H-13C HSQC NMR spectra (not shown) of the desialylated/de-O-acetylated glycan allowed the assignment of the proton and carbon chemical shifts for each residue (Table I).

The desialylated glycan was composed of terminal, 2- and 3-substituted Gal units and 3- and 4-substituted GlcNAc residues. Two dimensional 1H-13C HMBC experiment (Figure 3) performed on the desialylated/de-O-acetylated sample was then used to furnish information about the monosaccharide sequence of the glycan backbone. The spectrum showed connectivities between H-1 of D and C-3 of E for β-GlcNAc-(1→3)-β-Gal, H-1 of F and C-4 of D for β-Gal-(1→4)-β-GlcNAc, H-1 of B and C-2 of F for β-GlcNAc-(1→2)-β-Gal, H-1 of H and C-3 of B for β-Gal-(1→3)-β-GlcNAc, H-1 of C and C-3 of H for β-GlcNAc-(1→3)-β-Gal, H-1 of G and C-4 of C for β-Gal-(1→4)-β-GlcNAc and H-1 of D’ and C-3 of A for β-GlcNAc-(1→3)-α-Gal. These results revealed that the previously observed reducing end GlcNAc residue of the intact and desialylated glycans was eliminated during the alkaline treatment, which now exposed a reducing end Gal unit (anomeric resonances A and E in Figure 1C). This observed degradation of saccharides at the reducing end in an alkaline environment, known as the “peeling” reaction, occurs at elevated temperatures and it can lead to the complete degradation of the saccharide chain in a stepwise manner (Sasisekharan et al. 2003).

**Determination of monosaccharide sequence of the desialylated/de-O-acetylated glycan backbone**

A subsequent gas–liquid chromatography mass spectrometry (GLC-MS) of the permethylated alditol acetates revealed that the desialylated glycan was composed of terminal, 2- and 3-substituted Gal units and 3- and 4-substituted GlcNAc residues.
These data allowed for a proposed glycan sequence (shown below), which also accounted for the linkage-type units previously observed by GLC-MS.

\[
\begin{array}{cccccccc}
\text{Residue} & \text{H-1/C-1} & \text{H-2/C-2} & \text{H-3/C-3} & \text{H-4/C-4} & \text{H-5/C-5} & \text{H-6,6′/C-6} \\
\hline
\rightarrow 3) \alpha \text{Gal} (1 \rightarrow) & 5.24 & 3.86 & 3.93 & 4.26 & 3.89 & 4.03 \\
A & 95.2 & 71.5 & 81.7 & 71.5 & 76.1 & 67.4 \\
\rightarrow 3) \beta \text{GlcNAc} (1 \rightarrow) & 4.89 & 3.86 & 3.81 & 3.52 & 3.45 & 3.93, 3.75 \\
B & 104.0 & 57.8 & 85.2 & 71.5 & 78.1 & 63.7 \\
\rightarrow 4) \beta \text{GlcNAc} (1 \rightarrow) & 4.73 & 3.81 & 3.74 & 3.75 & 3.59 & 3.97, 3.85 \\
C & 105.3 & 58.0 & 75.0 & 80.9 & 77.3 & 62.6 \\
\rightarrow 4) \beta \text{GlcNAc} (1 \rightarrow) & 4.72 & 3.80 & 3.74 & 3.72 & 3.59 & 4.06, 3.97 \\
D & 105.3 & 58.0 & 75.0 & 81.9 & 77.3 & 62.6 \\
\rightarrow 3) \beta \text{Gal} (1 \rightarrow) & 4.74 & 3.80 & 3.74 & 3.72 & 3.59 & 4.06, 3.97 \\
D′ & 105.3 & 58.0 & 75.0 & 81.9 & 77.3 & 62.6 \\
\rightarrow 3) \beta \text{Gal} (1 \rightarrow) & 4.60 & 3.54 & 3.73 & 4.20 & 3.89 & 4.03 \\
E & 99.3 & 73.7 & 85.0 & 70.9 & 76.1 & 67.4 \\
\rightarrow 2) \beta \text{Gal} (1 \rightarrow) & 4.55 & 3.72 & 3.87 & 3.94 & 3.73 & 3.78 \\
F & 104.2 & 80.2 & 71.5 & 71.2 & 78.1 & 63.7 \\
\beta \text{Gal} (1 \rightarrow) & 4.49 & 3.54 & 3.68 & 3.94 & 3.73 & 3.78 \\
G & 105.6 & 73.7 & 75.2 & 71.2 & 78.1 & 63.7 \\
\rightarrow 3) \beta \text{Gal} (1 \rightarrow) & 4.46 & 3.58 & 3.74 & 4.21 & 3.89 & 4.03 \\
H & 106.2 & 72.5 & 84.5 & 70.8 & 76.1 & 67.4 \\
\end{array}
\]

**Characterization of sialic acid and its linkage site**

The proton and carbon resonances of a sialic acid (Neu5Ac) residue were assigned from cross-referenced 2-D $^1$H-$^1$H COSY, $^1$H-$^1$H TOCSY, $^1$H-$^1$C HSQC and $^1$H-$^1$C HMBC spectra (data not shown) of the intact oligosaccharide ($^\delta_{\mathrm{C-1}}$ 173.9, $^\delta_{\mathrm{C-2}}$ 100.4, $^\delta_{\mathrm{C-3}}$ 40.3, $^\delta_{\mathrm{H-3ax}}$ 1.72, $^\delta_{\mathrm{H-3eq}}$ 2.67, $^\delta_{\mathrm{C-4}}$ 68.5, $^\delta_{\mathrm{H-4}}$ 3.82, $^\delta_{\mathrm{C-5}}$ 72.7, $^\delta_{\mathrm{C-6}}$ 3.74, $^\delta_{\mathrm{C-7}}$ 68.8, $^\delta_{\mathrm{H-7}}$ 3.16, $^\delta_{\mathrm{H-8}}$ 69.5, $^\delta_{\mathrm{H-9}}$ 4.11, $^\delta_{\mathrm{H-9}}$′ 4.41, $^\delta_{\mathrm{H-9}}$′′ 4.21). The linkage site of the sialic acid was determined by comparing the permethylated alditol acetate derivatives obtained from the intact glycan with those from the desialylated sample. The absence of 6-, and 2,6-substituted Gal and the presence of a new 2-substituted Gal after the removal of the sialic acid collectively suggested that the sialic acid is attached to the glycan at the O-6 position of the 6-, and 2,6-substituted Gal. This Neu5Ac-Gal connection was also detected as a cross peak between $^\delta_{\mathrm{H}}$ 3.99 (H-6 of Gal) and $^\delta_{\mathrm{C}}$ 100.4 (C-2 of Neu5Ac) in the $^1$H-$^1$C HMBC spectrum of the intact material. The large downfield shift of the resonances of H-9 and H-9′ strongly suggested that C-9 of the sialic acid may be one of the sites of O-acetylation. This observation was supported by the correlation of the H-9,9′ protons with the carbonyl carbon of an O-acetyl group at $^\delta_{\mathrm{C}}$ 174.8 in the 2-D $^1$H-$^1$C HMBC spectrum of the intact glycan.

**Characterization of phosphoethanolamine and its linkage site**

The $^3$P NMR spectrum of the intact oligosaccharide showed a single resonance at $^\delta_{31P}$ 0.4 which in a 2-D $^1$H-$^3$P HMBC spectrum appeared to be correlated to the characteristic methylene proton resonances of H$_2$N-CH$_2$– ($^\delta_{\mathrm{H}}$ 3.26, $^\delta_{\mathrm{C}}$ 40.3) and –CH$_2$O– ($^\delta_{\mathrm{H}}$ 4.10, $^\delta_{\mathrm{C}}$ 62.5) of a phosphoethanolamine (PEA) group (Drzewiecka et al. 2002; Senchenkova et al. 2004; Katzenellenbogen et al. 2008). The $^1$H-$^3$P HMBC spectrum also allowed the detection of a correlation between the phosphorous resonance and proton resonances at around $^\delta_{31P}$ 4.03 attributable to H-6/6′ of the 3-substituted Gal residues. Thus, the correlations seen in the $^1$H-$^3$P HMBC spectrum placed the PEA at the O-6 position of the 3-substituted Gal.

**Placement of O-acetyl groups**

The 2-D $^1$H-$^1$C HSQC spectrum (not shown) of the intact sample showed two sets of methylene protons in the range where C-6 resonances are usually expected ($^\delta_{\mathrm{C-6a}}$ 63.9, $^\delta_{\mathrm{H-6a}}$ 4.65, $^\delta_{\mathrm{C-6b}}$ 63.1, $^\delta_{\mathrm{H-6b}}$ 4.52, $^\delta_{\mathrm{C-6b}}$ 63.7). However, the significant downfield shift of the corresponding protons suggested that these primary hydroxyls might be the additional sites of the O-acetylation. The absence of 6-, and 2,6-substituted Gal and the presence of a new 2-substituted Gal after the removal of the sialic acid collectively suggested that the sialic acid is attached to the glycan at the O-6 position of the 6-, and 2,6-substituted Gal. This Neu5Ac-Gal connection was also detected as a cross peak between $^\delta_{\mathrm{H}}$ 3.99 (H-6 of Gal) and $^\delta_{\mathrm{C}}$ 100.4 (C-2 of Neu5Ac) in the $^1$H-$^1$C HMBC spectrum of the intact material. The large downfield shift of the resonances of H-9 and H-9′ strongly suggested that C-9 of the sialic acid may be one of the sites of O-acetylation. This observation was supported by the correlation of the H-9,9′ protons with the carbonyl carbon of an O-acetyl group at $^\delta_{\mathrm{C}}$ 174.8 in the 2-D $^1$H-$^1$C HMBC spectrum of the intact glycan.

**Fig. 3.** $^1$H-$^1$C HMBC spectrum of the desialyated/de-O-acetylated common oligosaccharide.
acetylation. This assignment was confirmed by correlations of the H-6,6′ resonances with δC 174.4 and δC 174.7 in the 1H-13C HMBC spectrum of the intact glycan. These proton resonances mentioned above also furnished correlations with a GlcNAc residue in a 1H-1H TOCSY experiment. A careful comparison of our data with published carbohydrate structures containing 3-substituted and/or 4-substituted GlcNAc units (Breg et al. 1988; Gronberg et al. 1989; Strecker et al. 1989; Gronberg et al. 1992; Haeuw-Fievre et al. 1993; Dasgupta et al. 1994) strongly suggested that the observed GlcNAc residue carrying the O-acetyl moiety was the 4-substituted GlcNAc.

MALDI-TOF-MS of the intact oligosaccharide

To corroborate the predictions above, the molecular weight of the intact glycan had to be determined. The matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) spectrum of the intact glycan displayed an ion at m/z 2431, which corresponds to [M-H] of the above elucidated sialylated glycan:

\[ \text{Ac} \quad \text{PEA} \quad \text{Ac} \]
\[ \downarrow \quad \downarrow \quad \downarrow \]
\[ 6 \quad 6 \quad 6 \]
\[ \beta\text{Gal(1→4)}\beta\text{GlcNAc(1→3)}\beta\text{Gal(1→3)}\beta\text{GlcNAc(1→2)}\beta\text{Gal(1→6)}\beta\text{GlcNAc(1→3)}\beta\text{Gal(1→3)}\alpha\beta\text{GlcNAc} \]
\[ 6 \quad 6 \]
\[ 1 \quad 1 \]
\[ 2 \]
\[ \text{NeuSNAc-9-Ac} \]
\[ \text{NeuSNAc-9-Ac} \]

Molecular model of oligosaccharide

In order to gain insight into the spatial positioning of the monosaccharides, especially the sialic acid units, we carried out computerized molecular mechanics modeling (in water) of the oligosaccharide using AMBER10 (Case et al. 2008) and force field parameters of GLYCAM06 (Kirschner et al. 2008) and the Generalized AMBER Force Field. Figure 4 shows the molecular model of the above elucidated oligosaccharide, in which both sialic acid moieties appear well exposed to the environment, and the PEA and O-Ac moieties decorate the glycan’s surface.

Discussion

Here we described the detailed analysis of an oligosaccharide identified in several representative strains of the swine pathogen, A. suis. As characterized, this glycan was present as a free oligosaccharide. The structure of this oligosaccharide is similar to glycosaminoglycans (GAG), frequently produced by vertebrates, with a backbone formed by alternating units of Gal and GlcNAc. The glycan was identified in both virulent and non-virulent A. suis strains and it likely plays an important role in the initial colonization of the host as observed previously with other microbes that produce GAG chains to deceive the host’s immune system (DeAngelis 2002), however, that remains to be established. An operon (containing seven genes that are predicted to be involved in the synthesis of this sialylated oligosaccharide) has been detected in all strains tested, but to date, it has not been possible to create a null knockout (Daraban 2009). No glycosyl units found in this oligosaccharide were observed to be present in the growth medium.

The exact biological function of the described A. suis oligosaccharide is not known. However, the presence of O-acetylxy, sialic acids and PEA residues may give this oligosaccharide an anionic character. The ionic sites of the glycan chain may be involved in the attraction and transport of charged molecules through the bacterial cell wall (Schauer 1982).
Moreover, bacterial strains expressing CPS or lipopolysaccharides (LPS) expressing sialic acid side branches tend to be more virulent, in that the bulky acidic monosaccharide can hide neighboring constituents from immune recognition (Schauer 2000). Also, in some cases, terminal sialic acid in a Neu5Ac-Gal structure appears to shield the otherwise recognizable Gal unit (Eserstam et al. 2002; Gonzalez-Outeiro et al. 2005). The microbial application of sialic acid shield is most likely due to its wide occurrence in various tissues, predetermining this compound to be an ideal bacterial camouflage. Protection against host defenses by this glycan might be further fortified with an O-acetyl protective group in the sialic acid at C-9, as it has been reported that this functionality is able to reduce the rate of desialylation by approximately 50% (Corfield et al. 1981).

Materials and methods

Bacterial strains and growth conditions

The origin, biotype, toxin type and restriction endonuclease fingerprinting profile of the four A. suis strains used in this study have been described previously (van Ostaaijen et al. 1997). The type strain, A. suis ATCC 15557, was originally recovered from a healthy animal after exposure to atomic radiation, strain H91-0380 was originally isolated from a pig with septicemia, strain C84 was cultured from a healthy animal at a slaughterhouse and the rough strain (no O-chain PS) VSB 3714 was a clinical isolate, but details of the disease presentation have been described previously (van Ostaaijen et al. 1997). The type strain, A. suis ATCC 15557, was originally recovered from a healthy animal after exposure to atomic radiation, strain H91-0380 was originally isolated from a pig with septicemia, strain C84 was cultured from a healthy animal at a slaughterhouse and the rough strain (no O-chain PS) VSB 3714 was a clinical isolate, but details of the disease presentation have been described previously (van Ostaaijen et al. 1997). These strains were first grown on sheep blood agar plates overnight at 37°C in an atmosphere of 5% CO2 then three well-isolated colonies were used to inoculate 2 L of brain heart infusion broth (Difco Laboratories, Detroit, MI). After growth at 37°C for 24 h with agitation at 200 r/min, cells were harvested by centrifugation (8000 × g, 20 min).

Extraction and purification of the oligosaccharide

Bacterial cell pellets were extracted using the hot water–phenol procedure (70°C, 5 h) (Westphal and Jann 1965), and the aqueous layer was subjected to dialysis against deionized water. Most of the insoluble LPS material was removed by high-speed centrifugation (15,000 rpm, 8 h, 4°C), and the supernatant was passed through a Bio-Gel P-6 column. The glycan described here was collected as a lower molecular weight fraction. All strains examined here expressed the low molecular weight glycan described here; however, since the rough strain VSB 3714 provided the oligosaccharide sample with the greatest purity, most of our efforts were directed at extracting the glycan from this strain.

Sugar composition and linkage analyses

Sugar composition analysis was performed using the alditol acetate method (Sawardeker et al. 1965). The hydrolysis was carried out in 4 M trifluoroacetic acid at 105°C for 5 h followed by overnight reduction with NaBD₄ in H₂O at room temperature. The dried product was then peracetylated with acetic anhydride at 105°C for 2 h. The alditol acetate derivatives were analyzed with gas chromatography (GC)-MS in the electron impact mode using a PolarisQ MS with Trace Ultra GC system (Thermo Electron Corporation, Austin, Texas) equipped with a DB-17MS capillary column (isothermally at 190°C for 60 min). The absolute configurations of the sugar residues were assigned after characterization of the but-2-yl glycosides in GC-MS (Leontine et al. 1978). Samples were also permethylated with CH₃I in NaOH/DSMO followed by hydrolysis, reduction (NaBD₄) and acetylation (Ciucanu and Kerek 1984). The permethylated alditol acetates were then characterized by GC-MS.

Desialylation and de-O-acetylation

The glycan was desialylated with 1% acetic acid at 100°C for 2 h followed by purification by gel permeation chromatography (Bio-Gel P2). De-O-acetylation was accomplished with 10% NH₄OH at 50°C for 3 h followed by purification by gel permeation chromatography (Bio-Gel P2).

MS

MALDI-TOF-MS was carried out on a Reflex III mass spectrometer (Bruker, Germany) equipped with a 337 nm nitrogen laser. The sample was mixed directly with matrix solution (2 mg of 3,4-dihydroxybenzoic acid in 20% of ethanol) in an analyte:matrix 1:2 ratio (v/v), and 1 μL was spotted on the MALDI sample target and allowed to dry at room temperature. The sample was analyzed in reflectron and negative ion modes scanning from m/z 1500 to 4000 and using ion suppression up to m/z 150.

NMR spectroscopy

1-D ¹H, ¹³C and ³¹P NMR spectra and the 2-D ¹H-¹H COSY, ¹H-¹³C HSQC and ¹H-¹³C HMBC spectra were recorded on a Bruker NSC 600 MHz spectrometer while the 2-D ¹H-³¹P HMBC spectra were recorded on a Bruker Mac 600 MHz spectrometer. The temperature was kept at 295 K in all experiments. Prior to performing the NMR experiments, the samples were lyophilized three times with D₂O (99.9%). 3-(Trimethylsilyl)propionic acid (dH 0.00) in D₂O was used to aid in the reference of the monodeuterated water signal. Orthophosphoric acid (dH 0.0) was used as the external reference for the ³¹P NMR experiments.

Computational methods

The oligosaccharide was built from the GLYCAM library (Kirschner et al. 2008) of monosaccharide residues using the XLEaP module of AMBER10 (Case et al. 2008). The OAc and PEA moieties were added to the oligosaccharide using XLEaP. Force field parameters were obtained using GLYCAM06 (Kirschner et al. 2008) and the Generalized AMBER Force Field. The complete system was immersed in a preequilibrated box of 4419 TIP3P water molecules with box dimensions of 18.774 × 18.774 × 18.774 Å. Nonbonded van der Waals and electrostatic scaling factors for 1–4 interactions were set to 1.2 and 2.0, respectively. Long range electrostatic interactions were computed with a nonbond cutoff distance of 100.0 Å. Energy minimization was carried out using implicit solvation minimization over 500 cycles of steepest descent followed by another 500 cycles of conjugate gradient minimization for a total of 1000 cycles.
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Conflict of interest statement
None declared.

Abbreviations
PEA, phosphoethanolamine; PS, polysaccharide; LPS, lipopolysaccharide; GAG, glycosaminoglycan; NMR, nuclear magnetic resonance; COSY, correlation spectroscopy; TOCSY, total correlation spectroscopy; HSQC, heteronuclear single quantum coherence; HMBC, heteronuclear multiple bond correlation; GC, gas chromatography; GLC, gas–liquid chromatography; MS, mass spectrometry; MALDI, matrix-assisted laser desorption/ionization; TOF, time of flight.

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