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Detailed structural analysis of the O-polysaccharide expressed by *Burkholderia thailandensis* E264

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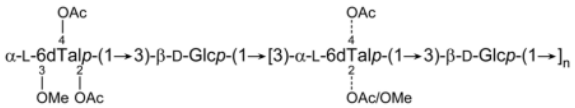
Abstract

O-polysaccharide (OPS) was isolated from purified *Burkholderia thailandensis* E264 lipopolysaccharide by mild-acid hydrolysis and gel-permeation chromatography. Glycosyl composition and methylation analyses along with 1D and 2D ^{1}H and ^{13}C NMR spectroscopy experiments revealed that the OPS antigen was an unbranched heteropolymer with the following structure:



Collectively, these results suggest that *B. thailandensis* OPS is structurally more complex than *B. pseudomallei* OPS and provide evidence of the signal used by *B. thailandensis* to terminate chain elongation.

Keywords

Burkholderia thailandensis; *Burkholderia pseudomallei*; Lipopolysaccharide; O-polysaccharide; NMR; Structure

Burkholderia thailandensis is a Gram-negative, environmental saprophyte that can be readily isolated from moist soils and stagnant waters throughout Southeast Asia and northern Australia.[1–3] Prior to its identification and classification during the late 1990s, *B. thailandensis* was often mistaken for *B. pseudomallei* (etiologic agent of melioidosis) due to similarities between their biochemical, morphological and antigenic profiles.[1,4,5] Recent studies, however, have now identified a number of important genotypic and phenotypic traits that enable these organisms to be easily differentiated from one another.[6–9] Important amongst these are the ability of *B. thailandensis* to assimilate L-arabinose and the inability

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to synthesize a →3)-2-*O*-acetyl-6-deoxy-β-D-*manno*-heptopyranose-(1→ capsule as well as the relative avirulence of the organism towards humans and animals.[2,10–13] Curiously, *B. thailandensis* has been shown to produce several homologues of known virulence factors expressed by *B. pseudomallei*. Some of these include lipopolysaccharide (LPS), virulence-associated type III and type VI secretion systems as well as complex quorum sensing systems.[14–18] In addition, *B. thailandensis* is capable of surviving and replicating within a number of eukaryotic cell lines, polymerizing host-cell actin to facilitate intra- and intercellular spread as well as stimulating multinucleated giant cell formation.[16,19–23] Because of these attributes, *B. thailandensis* is considered to represent a tractable model system for studying specific aspects associated with the pathogenesis of melioidosis.[14,16,18,24]

Lipopolysaccharides, commonly referred to as endotoxins, are a major component of Gram-negative cell envelopes.[25] Bacterial strains expressing a 'smooth' phenotype synthesize LPS antigens that are composed of three covalently linked domains: a lipid A moiety, a core region and an O-polysaccharide (OPS).[26] Previous studies by Perry et al have shown that the predominant OPS serotype expressed by *B. pseudomallei* is an unbranched polymer consisting of disaccharide repeats having the structure →3)-β-D-glucopyranose-(1→3)-6-deoxy-α-L-talopyranose-(1→ in which ~33% of the 6-deoxy-α-L-talopyranose (6dTal*p*) residues possess 2-*O*-methyl (2-*O*-Me) and 4-*O*-acetyl (4-*O*-Ac) substitutions while the remainder of the 6dTal*p* residues bear only 2-*O*-acetyl (2-*O*-Ac) modifications.[27] Additionally, studies in our lab and others have demonstrated that *B. pseudomallei* OPS is both a virulence factor and a protective antigen.[28–32] As a result, this carbohydrate moiety has become an important component of the various glycoconjugate vaccines that we are developing for immunization against melioidosis. Recently, studies have suggested that the predominant OPS serotype expressed by *B. thailandensis* is structurally similar to that expressed by *B. pseudomallei*.[4,14,33,34] Because of this, we are interested in determining whether or not *B. thailandensis* OPS might serve as a safe and cost-effective source of OPS antigen for melioidosis vaccine development. To investigate this possibility, we describe for the first time a detailed structural analysis of the OPS expressed by *B. thailandensis* E264.

Glycosyl composition analysis of the purified *B. thailandensis* E264 OPS by GC-MS of the TMS-methyl glycosides detected the presence of glucose (Glc) and 6-deoxytalose (6dTal) as the major constituents in a 0.8:1 ratio, along with minor, non-stoichiometric amounts of methyl 6dTal, rhamnose (Rha), quinovosamine (QuiN), glucosamine (GlcN), heptose (Hep) and a trace of galactose (Gal). The methylation analysis showed 3-linked Glc*p* and 6dTal*p* as major linkages, but also detected several other minor linkages including terminal 6dTal*p*, Glc*p*, Gal*p* and Hep*p* along with 3-linked *N*-acetylglucosamine (Glc*p*NAc), *N*-acetylquinovosamine (Qui*p*NAc) and Rha*p*, as well as 7-linked and 3,7-linked Hep*p*. GC-MS of the TMS-derivatized 2-butylglycosides showed the absolute configuration of Glc and GlcNAc to be D and of Rha to be L, but standards of 6dTal and QuiNAc were not available. Nevertheless, the absolute configurations of these two sugars could be determined by NMR (see below). We did not determine the stereochemistry of the Hep residue as it likely was a part of the inner core oligosaccharide, which was not the focus of this study.

It has been previously shown that *B. thailandensis* OPS possesses the same basic repeating unit structure as that of *B. pseudomallei*.[14,27] The considerable complexity of the 1D proton NMR spectrum of the native *B. thailandensis* E264 OPS sample analyzed in this study, however, suggested that the antigen might have additional structural features that were not detected in previous studies (Figure 1B).[14] To reduce spectral complexity and to aid the interpretation of the spectra of the native sample, we also acquired NMR of de-*O*-acetylated OPS (Figure 1A). We analyzed the COSY, TOCSY, and HSQC spectra of the de-*O*-acetylated OPS (data not shown) to identify each of the monosaccharide spin systems by

their proton and carbon chemical shifts and determined the positions involving glycosidic linkage based on the downfield displacement of their carbon chemical shifts (glycosylation shifts; Table 1).[35] These analyses identified 3-linked α-6dTal and 3-linked β-Glc, as well as 3-linked 2-*O*-Me-6dTal as the main constituents of the polysaccharide, which was in agreement with previous results.[14] However, we also detected several other, minor spin systems that were not discovered previously, including 3-linked Rha, 3-linked QuiNAc, and 3-linked GlcNAc. A 3-linked 2-*O*-Me-6dTal residue that was still acetylated on *O*-4 was also detected in the de-*O*-acetylated sample.

NOESY and HMBC of de-*O*-acetylated E264 OPS (data not shown) connected the anomeric protons of all the 6dTal residues with the 3-position of Glc, confirming that the E264 OPS consisted mainly of a disaccharide repeating unit comprised of →3)-α-L-6dTal*p*-(1→3)-β-D-Glc*p*-(1→. About one third of the 6dTal residues were methylated on *O*-2, the same proportion as was reported for the OPS of *B. pseudomallei*.[27] Under the de-*O*-acetylation conditions used, one of the acetyl groups was not completely removed, namely the one in the 4-position of 2-*O*-Me-6dTal*p*. We were able to differentiate the Glc residues (F, G, and H in Table 1) glycosylating the three main 6dTal forms: 2-*O*-methylated (A), unsubstituted (B), and 2-*O*-methylated and 4-O-acetylated (C), respectively. However, the H/C-3 signals of the various Glc residues were too similar to determine their respective glycosylating sugars. NOESY demonstrated and HMBC confirmed that 3-α-Rha was glycosylated on *O*-3 with 3-β-GlcNAc, but we could not ascertain whether or how this disaccharide sequence was connected to the main polysaccharide. Likewise, the NMR analysis of the de-*O*-acetylated sample revealed the atypical monosaccharide, 3-β-Qui*p*NAc, but did not show how this moiety was connected to the polysaccharide.

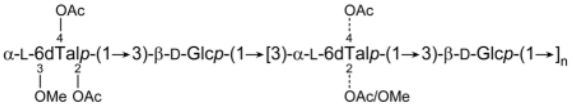
The absolute configuration of the residues for which standards were not available were determined by measuring their glycosylation shifts relative to the unsubstituted monosaccharides. The 6dTal residue showed a large α-effect of +7.5 ppm and a large β-effect of −3.2 ppm, and similar effects had also been observed in *B. pseudomallei* OPS which comprised L-6dTal.[27] Therefore, we conclude that 6dTal in *B. thailandensis* OPS also has the L-configuration. The QuiNAc residue also showed a large β-effect of −1.8 ppm, which is consistent with it having the opposite absolute configuration of the 6dTal, which it glycosylates, indicating that it has the D-configuration.[35]

Having established the main sequence of the polysaccharide, we went on to determine the positions of *O*-acetylation using 2D NMR analysis of the native sample. *O*-acetylation is associated with a considerable downfield displacement of chemical shifts of the protons in the acetylated positions, but does not greatly affect the corresponding carbon chemical shifts. Tracing the connectivities in the COSY (Figure 2, black spectrum) and TOCSY (data not shown) spectra, we found five different *O*-substitution patterns among the 6dTal monosaccharides (Table 2). Three of these (A, B, and C in Table 2) had been characterized before as part of *B. pseudomallei* OPS.[27,36] According to the HSQC spectrum (Figure 3, black spectrum), the non-acetylated species (Residue B) was characterized by an anomeric carbon that resonated further downfield than the 6dTal species with 2-*O*-substitution. Acetylation in the 2-position (Residue C) was associated with a >1 ppm downfield displacement of H-2 and >2 ppm upfield displacement of C-1, and 2-*O*-methylation (Residue A) led to a ~8 ppm downfield displacement of C-2, a ~0.4 ppm upfield displacement of H-2, a >0.1 ppm downfield displacement of H-1, and a >3 ppm upfield displacement of C-1. The fourth species (Residue D) was 3-linked 2,4-di-*O*-Ac-6dTal, which had not been detected in previous studies.[14,27] The additional acetyl group in the 4-position led to an increase of the chemical shifts of H-4 by 1.4 ppm and of H-3 by 0.2 ppm, relative to the 2-*O*-acetylated residue. The C-3 chemical shift moved upfield by about 2 ppm. The chemical shifts of H-5 and C-5 were similarly affected by 4-*O*-acetylation. The

fifth species (Residue E) was similar to Residue D, except for H-3, which was 0.5 ppm further upfield, and C-2, which was 1.7 ppm further upfield. These significant differences are explained by 3-*O*-methyl substitution instead of glycosylation in the 3-position. The methylation in this position was confirmed by HMBC (Figure 3, gray spectrum) between the methyl protons and C-3 and the methyl carbon and H-3 of Residue E. The presence of this residue, together with the absence of any unsubstituted sugars, suggests that the OPS chains are terminated at the non-reducing end by a 3-*O*-methylated 6dTal residue. Integration of the anomeric signals in the HSQC spectrum gave approximate percentages of each 6dTal residue relative to the sum of all 6dTal residues. Thus, the majority of 6dTal was acetylated only on *O*-2 (40 %), followed by 2-*O*-Me-4-*O*-Ac-6dTal (28 %), 3-*O*-Me-2,4-di-*O*-Ac-6dTal (14 %) and 2,4-di-*O*-Ac-6dTal (9 %). Only 9 % of 6dTal did not carry any non-carbohydrate substituents. As in the de-*O*-acetylated sample, three different Glc residues (F, G, and H in Table 2) could be distinguished by NOESY (Figure 2, gray spectrum) and HMBC (Figure 3, gray spectrum), according to which form of 6dTal was linked to its *O*-1.

The minor residues Rha, QuiNAc, and GlcNAc have also not been previously detected in OPS samples from *B. pseudomallei* and *B. thailandensis*. The Rha residue was acetylated on *O*-2, but no other acetylations were observed in these three residues. Like in the de-*O*-acetylated polysaccharide, NOESY (Figure 2, gray spectrum) and HMBC (Figure 3, gray spectrum) showed that Rha was glycosylated on *O*-3 by 3-linked β-GlcNAc, but the connection of this disaccharide sequence, as well as of the QuiNAc residue to the polysaccharide could not be established by NMR. It is possible that the three minor residues are from a different polysaccharide that co-elutes with the major OPS or that they are substituted for a small portion of the major monosaccharides in the OPS, β-QuiNAc and β-GlcNAc for β-Glc and α-Rha for 6dTal. Alternatively, these residues may represent components of the core. Studies to confirm whether or not these residues constitute part of the OPS are ongoing in our laboratories.

In the present study, we have shown that *B. thailandensis* E264 expresses an unbranched OPS antigen with the following structure:



This is consistent with previous findings that *B. thailandensis* produces an OPS antigen that is structurally similar to the predominant OPS serotype expressed by *B. pseudomallei*. [14,27,33,36] Interestingly, however, identification of the 3-*O*-Me-2,4-di-*O*-Ac-6dTal and 2,4-di-*O*-Ac-6dTal residues in this study indicates that *B. thailandensis* OPS may be structurally more complex than *B. pseudomallei* OPS. Further analysis of OPS antigens from both of these species will likely be required to reconcile this issue. Additionally, identification of the 3-*O*-Me-2,4-di-*O*-Ac-6dTal residues at the non-reducing termini of the *B. thailandensis* OPS moieties provides evidence of the signal used to terminate chain elongation during LPS biosynthesis. [37] Studies are ongoing to further investigate this observation as well as determine whether or not this unique glycosyl residue may also be used by other closely related *Burkholderia* species to terminate OPS chain elongation. [27,38]

1. Experimental

1.1. Bacterial strains, growth conditions and reagents

B. thailandensis E264 (ATCC 700388; type strain) was grown at 37°C on Luria Bertani-Lennox (LBL) agar or in LBL broth. Bacterial stocks were maintained at −80°C as 20 % glycerol suspensions.

1.2 LPS and OPS purification

LBL broth in 2 L baffled Erlenmeyer flasks was inoculated with *B. thailandensis* E264 and incubated overnight at 37°C with vigorous shaking. Cell pellets were obtained by centrifugation and extracted using a modified hot aqueous-phenol procedure.[27] Purified LPS and OPS antigens were then obtained essentially as previously described.[33]

1.3 Glycosyl composition analysis

Glycosyl composition analysis was performed essentially as previously described.[39] Briefly, the sample (200 μg) was methanolyzed (80°C, 16 h), re-N-acetylated with acetic anhydride/pyridine and per-O-trimethylsilylated with Tri-Sil® (80°C, 20 min). The resulting TMS-methyl glycosides were analyzed by GC-MS in electron impact ionization mode.

1.4 Absolute configuration

The dry sample (800 μg) was mixed with 400 μL 2 M TFA and incubated at 121°C in a sealed glass tube. After 2 h, the mixture was dried down under a stream of dry nitrogen and lyophilized. After re-N-acetylation with acetic anhydride (100 μL) in methanol/pyridine (2:1, 300 μL), the sample was dried under nitrogen. The sample was then treated with 200 μL S-(+)-2-butanol and 30 μL acetyl chloride and heated to 80°C for 16 h. Re-N-acetylation, TMS derivatization, and GC-MS analysis were performed as described above. D-Glc, L-Rha, D-GlcNAc were derivatized likewise with both S-(+)-2-butanol and R-(−)-2-butanol to provide standards. The absolute configurations of QuiN and 6dTal were determined by using the glycosylation shifts observed in HSQC-MR.[35]

1.5 Methylation analysis

Methylation analysis was performed essentially as previously described.[39] Briefly, the sample (800 μg) was dissolved in 300 μL DMSO and permethylated by three consecutive additions of 400 μL NaOH base, followed by 100 μL methyl iodide. After quenching with water and removal of methyl iodide, the permethylated polysaccharide was extracted with dichloromethane. The permethylated material was hydrolyzed with 2 M TFA (121°C, 2 h) and reduced with $NaBD_4$ in 1 M NH_4OH. After borate removal, the mixture was O-acetylated, and the resulting PMAAs were analyzed by GC-MS in electron impact ionization mode. Separation was performed on a 0.1 μm Rtx-2330 capillary column (30 m × 0.25 mm; Restek) for neutral sugars and a 0.25 μm EC-1 capillary column (30 m × 0.25 mm; Alltech) for amino sugars.

1.4 NMR spectroscopy

The samples were deuterium exchanged by dissolving in D_2O and lyophilizing and then were dissolved in 0.27 mL D_2O containing 1 μL acetone. 1D Proton and 2D gradient-enhanced COSY (gCOSY), TOCSY, NOESY, gHSQC, and gHMBC spectra were obtained on a Varian Inova-600 MHz spectrometer at 50°C using standard Varian pulse sequences. The spectral width was 3.17 kHz in the ^1H dimension and 18.1 kHz in the ^{13}C dimension. The number of scans and increments was 4 and 400 for gCOSY, 8 and 200 for TOCSY and NOESY, 64 and 128 for gHSQC, and 128 and 200 for gHMBC. Acquisition times were 2 s for 1-D ^1H, 137 ms for gCOSY, TOCSY, and NOESY, 150 ms for gHSQC, and 128 ms for

gHMBC. Mixing times for TOCSY and NOESY experiments were 120 and 300 ms, respectively. Proton chemical shifts were measured relative to internal acetone (δ_H=2.218 ppm, δ_C=33.0 ppm).[40]

Acknowledgments

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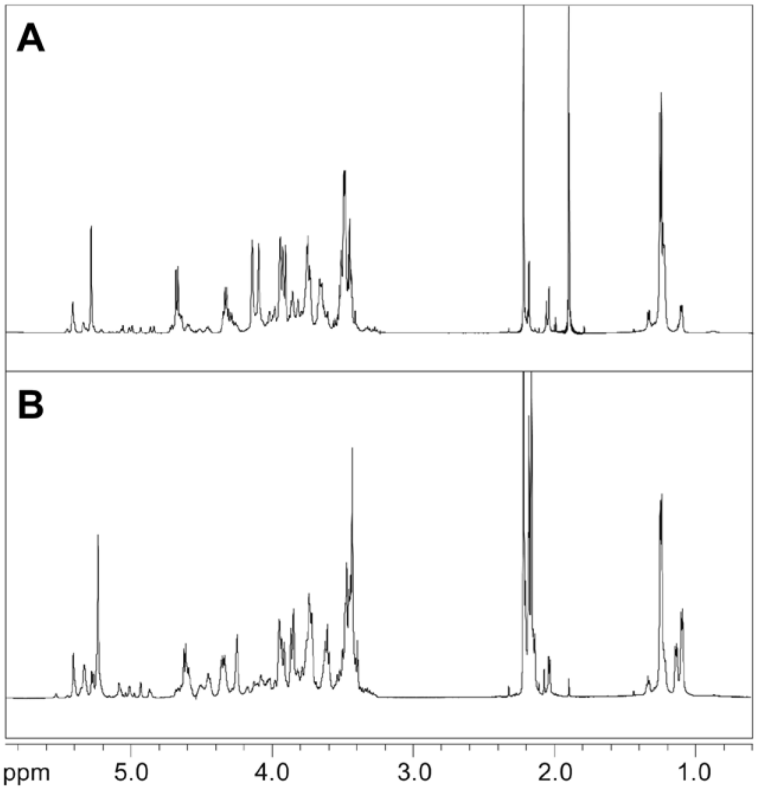

Figure 1.
1D ^{1}H NMR spectra of (A) de-*O*-acetylated and (B) native *B. thailandensis* E264 OPS.

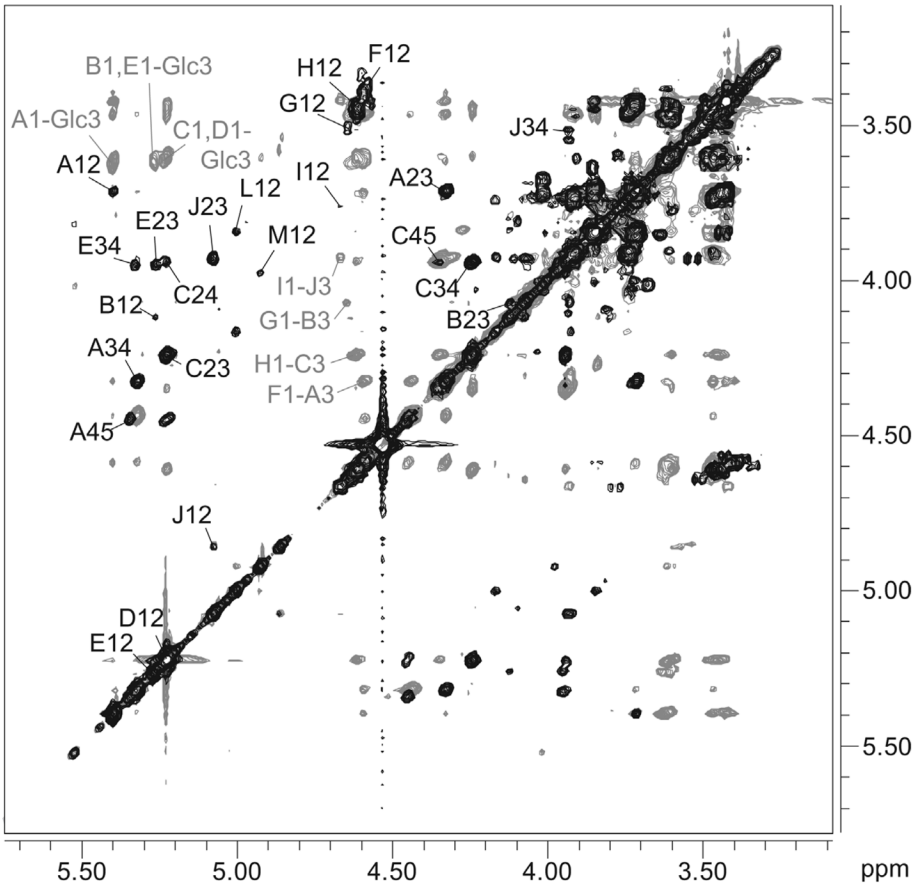

Figure 2.
Overlaid partial 2D COSY (black) and NOESY (gray) NMR spectra of native *B. thailandensis* E264 OPS.

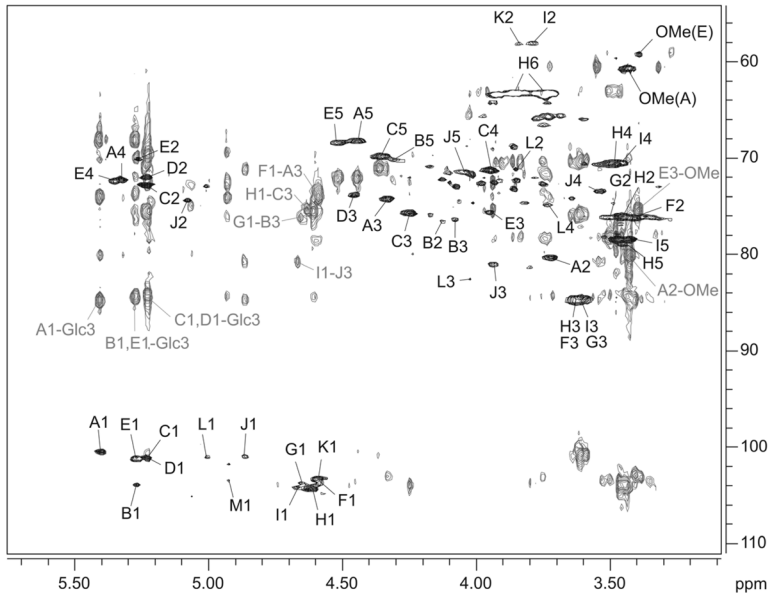

Figure 3.

Overlaid partial 2D HSQC (black) and HMBC (gray) NMR spectra of native *B.* thailandensis E264 OPS.

Table 1

Chemical shift assignments of de-*O*-acetylated *B. thailandensis* E264 OPS.

	Residue	Chemical shift (ppm)						NOE HMBC
		1	2	3	4	5	6	
A	→3)-2-*O*-Me-α-L-6dTal*p*-(1→	5.40	3.81	4.14	3.85	4.28	1.22	Glc-3*
		100.5	81.3	76.6	71.9	70.5	19.0	Glc-3*
B	→3)-α-L-6dTal*p*-(1→	5.28	4.14	4.09	3.94	4.32	1.24	Glc-3*
		103.8	72.4	76.2	72.4	70.1	18.0	Glc-3*
C	→3)-2-*O*-Me-4-*O*-Ac-α-L-6dTal*p*-(1→	5.40	3.74	4.35	5.33	4.45	1.10	Glc-3*
		100.5	80.4	74.3	72.4	68.2	17.6	Glc-3*
F	→3)-β-D-Glc*p*-(1→ **A**	4.64	3.49	3.65	ND	ND	ND	A-3
		104.3	76.2	84.6	ND	ND	ND	A-3
G	→3)-β-D-Glc*p*-(1→ **B**	4.67	3.51	3.66	3.49	3.47	3.74/3.92	B-3
		103.3	76.2	84.6	70.5	78.5	63.5	B-3
H	→3)-β-D-Glc*p*-(1→ **C**	4.60	3.39	3.63	3.47	ND	ND	C-3
		103.3	76.2	84.6	70.5	ND	ND	C-3
I	→3)-β-D-Glc*p*NAc	4.70	3.83	3.55	3.46	3.46	ND	H-3
		104.3	58.3	84.6	70.5	78.5	ND	H-3
J	→3)-α-L-Rha*p*	4.86	3.98	3.77	3.47	4.00	1.22	
		103.8	71.7	83.2	73.3	71.4	19.0	
K	→3)-β-D-Qui*p*NAc	4.59	3.83	3.55	3.27	3.50	1.33	
		102.9	58.3	84.6	76.6	74.7	19.4	

*Correlations to one of the β-D-Glc*p* residues; ND - not determined. *O*-methyl signals: 3.45/57.9 (**A**); 3.44/60.9 (**C**)

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Table 2

Chemical shift assignments of native *B. thailandensis* E264 OPS.

	Residue	Chemical shift (ppm)						NOE HMBC
		1	2	3	4	5	6	
A	→3)-2-*O*-Me-4-*O*-Ac-α-L-6dTal*p*-(1→	5.40	3.71	4.33	5.32	4.44	1.09	Glc-3*
		100.5	80.4	74.3	72.4	68.2	17.8	Glc-3*
B	→3)-α-L-6dTal*p*-(1→	5.27	4.12	4.08	3.93	4.31	1.24	Glc-3*
		103.8	72.2	76.4	72.6	70.2	18.0	Glc-3*
C	→3)-2-*O*-Ac-α-L-6dTal*p*-(1→	5.23	5.23	4.25	3.95	4.36	1.24	Glc-3*
		101.2	72.9	75.7	71.2	69.8	18.0	Glc-3*
D	→3)-2,4-di-*O*-Ac-α-L-6dTal*p*-(1→	5.23	5.23	4.46	5.35	4.52	1.13	Glc-3*
		101.2	71.9	73.8	72.4	68.4	17.6	Glc-3*
E	3-*O*-Me-2,4-di-*O*-Ac-α-L-6dTal*p*-(1→	5.27	5.27	3.96	5.32	4.52	1.13	Glc-3*
		101.2	70.2	75.8	72.2	68.4	17.6	Glc-3*
F	→3)-β-D-Glc*p*-(1→ **A**	4.59	3.40	3.62	3.46	3.43	3.85/3.73	A-3
		103.3	76.2	84.8	70.5	78.5	63.3	A-3
G	→3)-β-D-Glc*p*-(1→ **B**	4.65	3.50	3.62	3.46	3.43	3.85/3.73	B-3
		103.8	75.9	84.8	70.7	78.5	63.3	B-3
H	→3)-β-D-Glc*p*-(1→ **C**	4.61	3.46	3.61	3.48	3.45	3.85/3.73	C-3
		104.3	76.2	84.6	70.5	78.5	63.3	C-3
I	→3)-β-D-Glc*p*NAc	4.67	3.79	3.61	3.45	3.42	3.85/3.79	J-3
		104.3	58.1	84.8	70.5	78.5	63.3	J-3
J	→3)-2-*O*-Ac-α-L-Rha*p*	4.86	5.07	3.93	3.53	4.06	1.24	
		101.0	74.5	81.1	73.6	71.5	19.0	
K	→3)-β-D-Qui*p*NAc	4.59	3.84	3.54	3.27	3.49	1.32	
		103.8	58.2	84.6	76.4	74.7	19.4	
L	→3)-α-D-Gal*p*	5.00	3.84	4.00	4.17	ND	ND	
		101.0	71.2	82.5	71.0	ND	ND	
M	Hep*p*	4.92	3.98	3.64	3.86	ND	ND	
		103.5	72.6	74.3	ND	ND	ND	

* Correlations to one of the β-D-Glc*p* residues; ND - not determined. *O*-methyl signals: 3.43/60.7 (**A**); 3.40/59.3 (**E**)