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Structural elucidation of the polysaccharide moiety of a glycopeptide (GLPCW-II) from *Ganoderma lucidum* fruiting bodies

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Abstract—A water-soluble glycopeptide (GLPCW-II) was isolated from the fruiting bodies of *Ganoderma lucidum* by DEAE-Sepharose Fast-Flow and Sephacryl S-300 High Resolution Chromatography. The glycopeptide had a molecular weight of 1.2×10^4 Da (determined by HPLC), and consisted of ~90% carbohydrate and ~8% protein as determined using the phenol-sulfuric acid method and the BCA™ protein assay reagent kit, respectively. The polysaccharide moiety was composed mainly of D-Glc, L-Fuc, and D-Gal in the ratio of 1.00:1.09:4.09. To facilitate structure–activity studies, the structure of the GLPCW-II polysaccharide moiety was elucidated using ^{1}H and ^{13}C NMR spectroscopy including COSY, TOCSY, HMBC, HSQC, and ROESY, combined with GC–MS of methylated derivatives, and shown to consist of repeating units with the following structure:

α-L-Fuc*p*
1
↓
2
6)-α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→3)-α-D-Glc*p*-(1→6)-α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→

Keywords: *Ganoderma lucidum*; Glycopeptide; NMR spectroscopy; Polysaccharide moiety; Structural analysis

1. Introduction

Usage of *Ganoderma lucidum* (known in China as Lingzhi) in traditional Chinese medicine dates back more than two thousand years.[1] Polysaccharides and glycoconjugates isolated from this fungus have been reported to stimulate the proliferation of mouse spleen lymphocytes,[2] and to exhibit various other bioactivities including anti-HIV,[3] antiherpetic,[4] antiviral,[5] immunomodulating and antitumor[6] properties. These effects have prompted numerous structural studies on *G. lucidum* and other fungal polysaccharides/glycoconjugates, and several conformations have already been determined.[7,8] However, in many cases, the in vitro–in vivo effects of these polysaccharides/glycoconjugates remain unclear, due in large part to a lack of structural data.

Experiments in our laboratory have shown that a newly isolated glycopeptide from *G. lucidum* fruit bodies, GLPCW-II, stimulated the proliferation of mouse spleen lymphocytes (to be published). Several glycoconjugates of similar molecular weight had been isolated previously from *G. lucidum*,[9,10] although their structures have not yet been fully determined. We have now elucidated the structure of the polysaccharide moiety of GLPCW-II using methylation analysis, combined with ^{1}H and ^{13}C NMR spectroscopy, including COSY,

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TOCSY, HMBC, HSQC, and ROESY experiments, as a prelude to investigating structure–activity relationships.

2. Results and discussion

Glycopeptide GLPCW-II, purified according to the procedure outlined in Figure 1, appeared as a single symmetrical peak on HPLC and had an estimated molecular weight of 1.2×10^4 Da. Total carbohydrate and protein contents were estimated to be ~90% and ~8%, respectively.

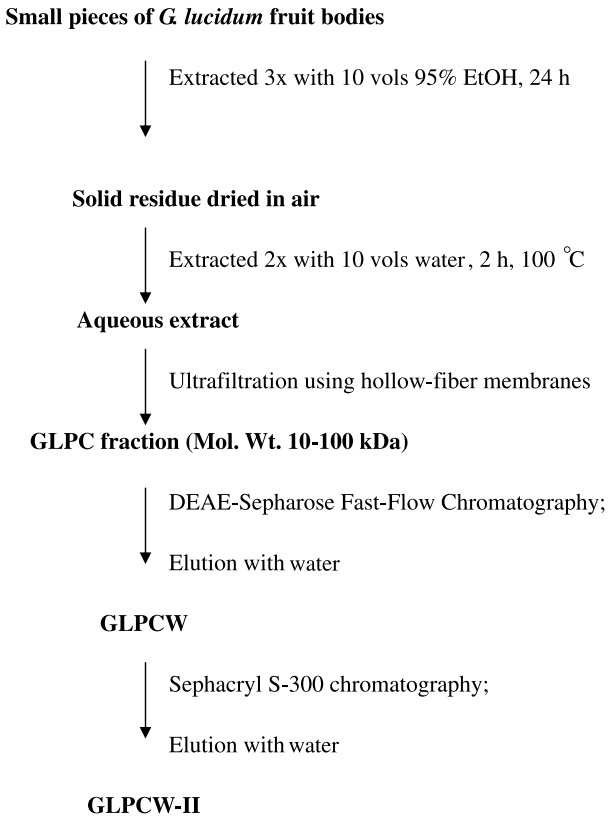


Figure 1. Protocol for the purification of glycopeptide, GLPCW-II, from *G. lucidum*.

The monosaccharides in GLPCW-II, identified by high performance anion exchange chromatography (HPAEC) after hydrolysis with trifluoroacetic acid (TFA), were glucose, fucose, and galactose in the molar ratio of 1.00:1.09:4.09, along with small amounts of mannose. Absolute configuration analysis[11,12] showed that both the galactose and glucose residues had the D configuration and the fucose residue had the L configuration. Methylation analysis detected 2,4,6-tri-*O*-methylglucose, 2,3,4-tri-*O*-methylfucose, 2,3,4-tri-*O*-methylgalactose, and 3,4-di-*O*-methylgalactose in a ratio of ca. 1:1:3:1, together with minor amounts of 3,4,6-tri-*O*-methylgalactose and 2,3,4-tri-*O*-methylmannose. HPAEC also revealed that the protein moiety of GLPCW-II was composed mainly of Arg, Gly, and Thr in a molar ratio of ca. 4:3:1.

On the basis of GC–MS data, the polysaccharide moiety was tentatively thought as a branched structure with a fucopyranose residue at one terminal, with 1,2,6-trisubstituted galactose, 1,3-disubstituted glucose, and 1,6-disubstituted galactose residues constituting the main chain, and possibly a galactose or a peptide residue at the other terminal. Since a 6-mer repeating unit has a MW of ca. 1000, we propose that one glycan chain probably consists of 11–12 repeating units. HPAEC data indicating that the three major amino acids, Arg, Gly, and Thr, are present in a ratio of 4:3:1, respectively, matches well with the molecular weight of the peptide moiety (ca. 960 Da) calculated on the basis of the estimated percentage (~8%) of protein in the glycopeptide. Since peptides are linked to polysaccharides by either Ser or Thr residues,[13] we propose that attachment to the polysaccharide moiety of GLPCW-II is via Thr.

The ^{1}H NMR (600 MHz) spectrum (Fig. 2) contained signals for four anomeric protons at δ 5.18, 5.15, 5.10, 5.10, corresponding to the signals at δ 104.2, 100.7, 101.1, 100.7, respectively, in the ^{13}C NMR spectrum (Fig. 3), and indicated that the repeating unit of the

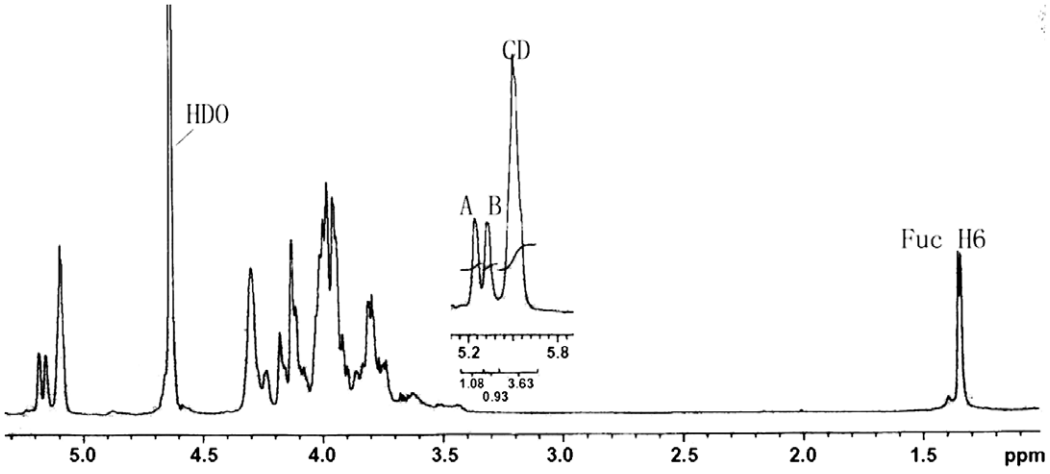


Figure 2. ^{1}H NMR spectrum of *G. lucidum* glycopeptide GLPCW-II.

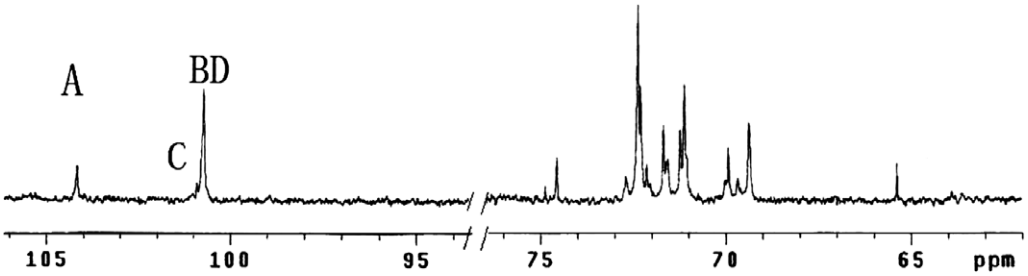


Figure 3. ^{13}C NMR spectrum of *G. lucidum* glycopeptide GLPCW-II.

polysaccharide moiety was made up mainly of these residues. One CH_3–C group at δ 1.35 corresponded to the chemical shift of H-6 of Fuc. The sugar residues were designated A, B, C, and D according to the decreasing chemical shifts of the anomeric protons, and the peak areas of A, B, and C (D) in the ^{1}H NMR spectrum were 1.00:0.93:3.63 (Fig. 1). All the four low-field signals (A, B, C, and D) appeared as singlets ($^3J_{H\text{-}1,H\text{-}2} \sim 3$ Hz) and represented sugars having the α-anomeric configuration.[14] The ^{13}C NMR spectrum of the polysaccharide moiety contained a signal at δ 18.6, which was assigned to a CH_3–C group (C-6 of Fuc). Signals were detected in the δ 57.7–64.7 region indicating that some C-6 positions were non-glycosylated.[15] The absence from the ^{13}C NMR spectrum of signals within the δ 82–88 region suggested that all the sugar residues were in the pyranose form.[16]

2.1. Identification of residue A with α-L-Fuc*p*-(1→

Residue A had an anomeric signal at δ 5.18 and a very small $^3J_{H\text{-}1,H\text{-}2}$ value, indicating an α-linked residue. The ^{1}H resonances for H-2, H-3, and H-4 of this residue were assigned from COSY, TOCSY, and ROESY spectra. H-5 and H-6 were assigned from the ^{1}H–^{1}H COSY spectrum. The cross-peak of H-6 and C-4 in the HMBC spectrum unambiguously showed that H-5 and H-6 were located on residue A. The proton chemical shift for the methyl group at δ 1.35 indicated an α-Fuc residue. On the basis of the proton assignments, the chemical shifts of the carbon atoms of residue A were readily obtained from the HSQC spectrum (Fig. 4, Table 1). Both carbon and proton chemical shifts were typical of 6-deoxyhexopyranose, and residue A can only be fucose since this sugar was the only deoxyglycose identified by GC–MS analysis. Except for the downfield shift of C-1 (δ 104.2), no carbon signal was evident in the δ 76–82 range indicating that residue A was a 1-linked α-L-fucopyranose.

2.2. Identification of residue B with →2,6)-α-D-Gal*p*-(1→

Residue B had an anomeric signal at δ 5.15 and a small $^3J_{H\text{-}1,H\text{-}2}$ value, indicating an α-linked residue. The cross-peak δ 5.15/3.94 was detected in the COSY spectrum and, since δ 5.15 corresponded to H-1, δ 3.94 was assigned to H-2. The ^{1}H resonances for H-3 and H-4 were assigned from the cross-peaks in the COSY and TOCSY spectra. The H-5 resonance was assigned

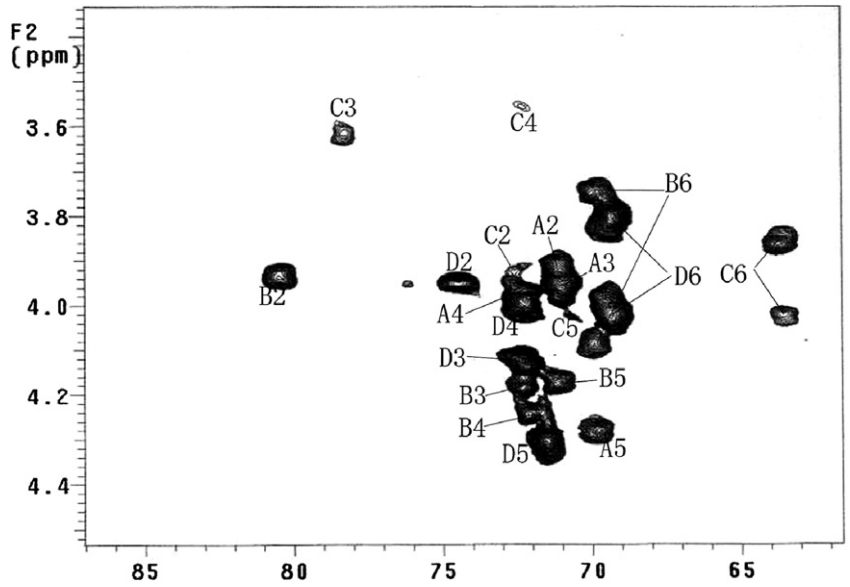


Figure 4. Part of the HSQC spectrum of the polysaccharide moiety of *G. lucidum* glycopeptide GLCPW-II.

Table 1. ^{1}H and ^{13}C NMR chemical shift data (δ, ppm) for the polysaccharide moiety of *G. lucidum* glycopeptide GLPCW-II

Residue	$^{1}H/^{13}C$						
	1	2	3	4	5	6a	6b
(A) α-L-Fuc*p*-(1→	5.18	3.93	3.98	3.96	4.28	1.35	
	104.2	71.2	72.3	71.1	69.9	18.6	
(B) →2,6)-α-D-Gal*p*-(1→	5.15	**3.94**	4.17	4.19	4.24	**3.75**	**4.09**
	100.7	**80.7**	71.1	72.3	72.0	**70.1**	
(C) →3)-α-D-Glc*p*-(1→	5.10	3.92	**3.63**	3.58	4.02	3.86	4.04
	101.1	72.3	**78.5**	72.1	70.6	63.8	
(D) →6)-α-D-Gal*p*-(1→	5.10	3.96	4.13	4.00	4.32	**3.82**	**4.03**
	100.7	74.5	72.1	72.3	71.6	**69.4**	

Values shown in bold font indicate linkage positions.

from the H-3/H-4 and H-4/H-5 cross-peaks in the ROESY spectrum. The H-5 and the H-6a and H-6b resonances were then obtained from the COSY spectrum. A self-spin system from H-1 to H-3, the large coupling constant value $J_{H\text{-}2,H\text{-}3}$ (~8.2 Hz), and the small coupling constant value $J_{H\text{-}4,H\text{-}5}$ (<3.0 Hz) indicated that residue B was galactopyranose. The carbon signals from C-1 to C-6 of residue B were identified from the HSQC spectrum (Fig. 4). The downfield shifts of the C-1 (δ 100.7), C-2 (δ 80.7), and C-6 (δ 70.1) carbon signals indicated that residue B was a 1,2,6-linked α-D-galactopyranose.

2.3. Identification of residue C with →3)-α-D-Glc*p*-(1→

The anomeric signal at δ 5.10 and the small $^{3}J_{H\text{-}1,H\text{-}2}$ value indicated that residue C was an α-linked residue. The proton chemical shifts from H-2 to H-6 were assigned from two-dimensional NMR, including COSY, TOCSY, ROESY, HMBC, and HSQC spectra. The large $J_{H\text{-}2,H\text{-}3}$ and $J_{H\text{-}3,H\text{-}4}$ coupling constants (~8 Hz) and the typical H-1, H-2, and H-4 intra-correlations in the ROESY spectrum indicated that residue C was D-glucopyranose.[7] On the basis of the proton assignments, the carbon chemical shifts of residue C were determined from the HSQC spectrum. The downfield shifts of C-1 (δ 101.1) and C-3 (δ 78.5) carbon signals indicated that residue C was a 1,3-linked α-D-glucopyranose.

2.4. Identification of residue D with →6)-linked D-Gal*p*-(1→

The anomeric signal at δ 5.10 and the small $^{3}J_{H\text{-}1,H\text{-}2}$ value indicated that residue D was an α-linked residue. The cross-peak δ 5.10/3.96 was detected in the COSY spectrum and, since δ 5.10 corresponded to H-1, the δ 3.96 signal was assigned to H-2. The ^{1}H resonances for H-3 and H-4 were assigned from the cross-peaks in the COSY and TOCSY spectra. The H-5 resonance was assigned from the H-3/H-4 and H-4/H-5 cross-peaks in ROESY spectrum, and from the COSY spectrum. The H-6a and H-6b resonances were obtained from the COSY spectrum. A self-spin system from H-1 to H-3, the large coupling constant value $J_{H\text{-}2,H\text{-}3}$ (~8.2 Hz), and the small coupling constant value $J_{H\text{-}4,H\text{-}5}$ (<3.0 Hz) indicated that residue D was a galactopyranose. The C-1 to C-6 carbon signals of residue D were identified from the HSQC spectrum with reference to the documented NMR data.[17] The downfield shifts of the C-1 (δ 100.7) and C-6 (δ 69.4) carbon signals indicated that residue D was a 1,6-linked α-D-galactopyranose.

The chemical shifts of all four residues (A, B, C and D) are shown in Table 1. Assignment of the peak signal at δ 65.4 (Fig. 3) is more problematic since no cross-peak was found between δ 3.00 and δ 5.90 in the HSQC spectrum. This peak could be assigned to C-6 of the →2)-α-D-Glap-(1→ residue, or to a mannose-containing residue present at low levels in the polysaccharide moiety of GLPCW-II. Small amounts of mannose were detected by HPAEC. Alternatively, the signal may relate to the Thr residue of the peptide moiety; two of the carbon chemical shifts of Thr are located at δ 61.5 and δ 67.1.[18]

The sequence of the residues in the repeating unit was established from the cross-peaks of anomeric protons and carbons observed in the HMBC spectrum (Table 2). Inter-residue connectivities were observed between

Table 2. ^{1}H–^{13}C intra- and inter-residue correlations for the polysaccharide moiety of *G. lucidum* glycopeptide GLPCW-II

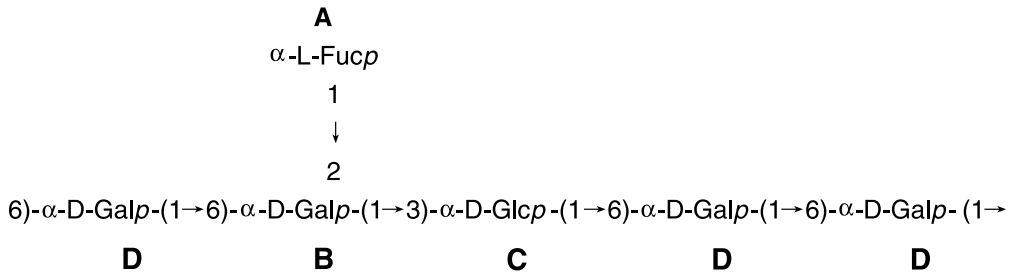
Residue	Proton	Correlation
(A) α-L-Fuc*p*-(1→	H-1 (δ 5.18)	AC-3 (δ 72.3), AC-5 (δ 69.9), BC-2 (δ 80.7)
(B) →2,6)-α-D-Gal*p*-(1→	H-1 (δ 5.15)	BC-3 (δ 71.1), BC-5 (δ 72.0), CC-3 (δ 78.5)
	H-2 (δ 3.94)	BC-4 (δ 72.3)
(C) →3)-α-D-Glc*p*-(1→	H-1 (δ 5.10)	CC-2 (δ 72.3), CC-5 (δ 70.0), DC-6 (δ 69.4)
	H-3 (δ 3.63)	BC-1 (δ 100.7), CC-1 (δ 101.1), CC-5 (δ 63.8)
(D) →6)-α-D-Gal*p*-(1→	H-1 (δ 5.10)	DC-5 (δ 71.6), DC-6 (δ 69.4), BC-6 (δ 70.1)

Table 3. NOE data for the polysaccharide moiety of *G. lucidum* glycopeptide GLPCW-II

Residue	Proton	NOE to proton
(A) α-L-Fuc*p*-(1→	H-1 (δ 5.18)	AH-2 (δ 3.93), BH-2 (δ 3.94)
(B) →2,6)-α-D-Gal*p*-(1→	H-1 (δ 5.15)	BH-2 (δ 3.94), BH-3 (δ 4.18), BH-5 (δ 4.17), CH-3 (δ 3.63)
	H-2 (δ 3.94)	AH-1 (δ 5.18), BH-1 (δ 5.15)
(C) →3)-α-D-Glc*p*-(1→	H-1 (δ 5.10)	CH-2 (δ 3.92), CH-4 (δ 3.58), DH-6a (δ 3.82), DH-6b (δ 4.03)
	H-3 (δ 3.63)	CH-5 (δ 4.02), BH-1 (δ 5.15)
(D) →6)-α-D-Gal*p*-(1→	H-1 (δ 5.10)	BH-6a (δ 3.75), DH-2 (δ 3.96), DH-6a (δ 3.82), DH-6b (δ 4.03)

H-1 of residue A and C-2 of residue B, between H-1 of residue B and C-3 of residue C, between H-1 of residue C and C-6 of residue D, and between H-1 of residue D and C-6 of residue B. The ROESY spectrum (Table 3) confirmed these inter-residue connections and revealed intra-residue connections.

Based on the analysis of the experimental data presented above, it is proposed that the residues in the repeating unit occur in the following sequence:



Although several polysaccharide/polysaccharide–protein structures from *G. lucidum* have been reported recently, the polysaccharide moiety of GLPCW-II represents a previously undocumented novel structure. Research is ongoing in our laboratory to further characterize the nature of the glycopeptide and to investigate structure–activity relationships.

3. Materials and methods

3.1. Materials

Artificially cultivated *G. lucidum* fruit bodies were provided by the Institute of Edible Fungi, Shanghai Academy of Agricultural Sciences. DEAE-Sepharose Fast Flow and Sephacryl S-300 were purchased from Amersham Pharmacia Company. Dextrans and the monosaccharide standards, D-Gal, D-Glc, D-Ara, L-Fuc, L-Rha, D-Man, and D-Xyl were from Sigma–Aldrich. All other reagents were from Chinese sources and of AR grade.

3.2. Isolation and purification of glycopeptide GLPCW-II

Air-dried *G. lucidum* fruit bodies (~5.0 kg) were cut into small pieces and extracted with 10 vol 95% (v/v) EtOH for three 24 h periods to remove lipid material. The residue was air-dried, extracted twice with 10 vol of distilled water for 2 h at 100 °C, and the combined aqueous extracts were then separated on the basis of molecular weight into four fractions by ultrafiltration using hollow fiber membranes. The 10–100 kDa fraction, GLPC, was concentrated under diminished pressure at 40 °C using a rotary evaporator and freeze-dried. Crude GLPC extract (17.5 g, 0.35% yield) was dissolved in 300 mL of distilled water, applied to a DEAE-Sepharose Fast-Flow column (XK26 × 100 cm), and a single peak (fraction GLPCW) was eluted with filtered (0.45 μm membrane) distilled water. Polysaccharide in the eluate was detected using the phenol-sulfuric acid method.[19] Fraction GLPCW was further purified by gel permeation chromatography on a High Resolution Sephacryl S-300 column (XK26 × 100 cm) using filtered distilled water as the eluent. Two polysaccharide peaks were detected using a refractive index detector (RID-10A, Shimadzu, Japan), and the fractions forming the second peak were collected and designated GLPCW-II.

3.3. Determination of purity and molecular weight

Homogeneity and the molecular weight of GLPCW-II were determined by HPLC using a Waters 2695 HPLC system fitted with serially linked TSK PWXL 4000 and 3000 gel filtration columns, a Model 2695 pump, a Waters 2410 RI detector, a Waters 2487 dual wavelength absorbance detector, and an on-line de-gasser. Column and RI detector temperatures were set at 35 °C. Columns injected with 10 μL of GLPCW-II (2 mg/mL) were eluted with buffer consisting of 0.1 M NaH_2PO_4 and 0.3 M $NaNO_3$ (adjusted to pH 7.0 with 0.1 M NaOH) at a flow rate of 0.5 mL/min, and

calibrated using Dextrans T-5, 12, 25, 50, 80, 150, and 270.

3.4. Sugar analysis

GLPCW-II (2 mg) was hydrolyzed with 2 M trifluoroacetic acid (TFA) at 110 °C for 3 h, and the monosaccharide composition was determined by high-performance anion-exchange chromatography (HPAEC) using a Dionex LC30 equipped with a CarboPac^{TM} PA20 column (3 mm × 150 mm). The column was eluted with 2 mM NaOH (0.45 mL/min) and the monosaccharides were monitored using a pulsed amperometric detector (Dionex).[20] Monosaccharide components and percentage composition were determined using D-Gal, D-Glc, D-Ara, L-Fuc, L-Rha, D-Man, and D-Xyl standards. To identify all the component monosaccharides of GLPCW-II, sugars in the hydrolysates were converted into their alditol acetates[21] by reduction with $NaBH_4$, and analyzed by GC–MS using a DB-5MS column (30 m × 0.25 mm × 0.25 μm) and a temperature program consisting of 180–270 °C at 20 °C/min, and held at 270 °C for 25 min. The absolute configurations of the monosaccharides were determined as described by Vliegenthart and co-workers[11,12] using (+)-2-butanol.

3.5. Amino acid composition

GLPCW-II (10 mg) was hydrolyzed with 10 mL of 6 M HCl at 110 °C for 24 h under nitrogen, the hydrolysate was evaporated to dryness in a Speed-Vac, and the amino acid composition determined by HPAEC using a Dionex LC30 Amino Pac® PA-10 column (2 × 25 mm). The column was eluted with a solution consisting of MilliQ water, 250 mM NaOH, and 1 M sodium carbonate at a flow rate of 0.22 mL/min according to the Amino Pac® PA-10 product manual, and the eluate was monitored using a pulsed amperometric detector (Dionex). Data were analyzed with the Chromeleon chromatography management system software.

3.6. Methylation analysis

The vacuum-dried sample (2 mg) was dissolved in Me_2SO (1 mL) and methylated with a solution of NaOH/Me_2SO (1 mL) and CH_3I (0.5 mL) according to the method of Anumula and Taylor.[22] The reaction mixture was extracted with $CHCl_3$, the organic phase was washed 3× with MilliQ water, and the solvent was then removed by evaporation. Complete methylation was confirmed by the disappearance of the OH band (3200–3700 cm^{-1}) in the IR spectrum. The permethylated polysaccharide was hydrolyzed by treatment with HCO_2H (88%, 0.5 mL), MilliQ water (0.1 mL), and TFA (0.05 mL) for 16 h at 100 °C. The partially methylated sugars in the hydrolysate were reduced with $NaBH_4$, acetylated by Ac_2O, and the methylated alditol acetates (PMMAs) were extracted into an equal volume of chloroform. The organic phase was washed 4× with MilliQ water and evaporated to dryness under diminished pressure. PMAAs were re-dissolved in chloroform to ~1 nmol/μL concentration and 2 μL samples were analyzed by GC–MS using the same conditions as described in Section 3.4.

3.7. Nuclear magnetic resonance (NMR) spectroscopy

Samples of GLPCW-II (30 mg) were deuterium-exchanged three times by lyophilization from D_2O. ^1H NMR, ^{13}C NMR, COSY, DEPT, TOCSY, ROESY, HSQC, and HMBC spectra were recorded using a Varian INOVA 600 NMR spectrometer. ^1H chemical shifts were referenced to residual HDO at δ 4.63 ppm (50 °C) as internal standard. ^{13}C chemical shifts were acquired in relation to DSS (δ 0.00 ppm) calibrated externally. The COSY spectrum was recorded with a relaxation delay time of 1.00 s using standard pulse sequences, and the TOCSY spectrum was recorded with a mixing time of 0.08 s and a delay time of 1.00 s. Delay and mixing times in the ROESY experiment were 1.00 s and 0.21 s, respectively. HSQC and HMBC data were recorded after a 50 ms delay to allow for the evolution of long-range couplings.

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