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Structural characterization of a 2-*O*-acetylglucomannan from *Dendrobium officinale* stem

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Abstract—A heteropolysaccharide obtained from an aqueous extract of dried stem of *Dendrobium officinale* Kimura and Migo by anion-exchange chromatography and gel-permeation chromatography, was investigated by chemical techniques and NMR spectroscopy, and is demonstrated to be a 2-*O*-acetylglucomannan, composed of mannose, glucose, and arabinose in 40.2:8.4:1 molar ratios. It has a backbone of (1→4)-linked β-D-mannopyranosyl residues and β-D-glucopyranosyl residues, with branches at *O*-6 consisting of terminal and (1→3)-linked Man*p*, (1→3)-linked Glc*p*, and a small proportion of arabinofuranosyl residues at the terminal position. The acetyl groups are substituted at *O*-2 of (1→4)-linked Man*p* and Glc*p*. The main repeating unit of the polysaccharides is reported.

Keywords: *Dendrobium officinale* Kimura and Migo; Polysaccharide; Glucomannan; Structure

1. Introduction

Herba Dendrobii, prepared from the dried stem of *Dendrobium* species, is a Chinese tonic and an antipyretic herbal medicine. Research focused on its basic constituents and pharmacological activity has shown several active constituents, such as alkaloids, stilbenoids, glycosides, and polysaccharides.[1] Polysaccharides are widespread in many medicinal plants, such as Konjac glucomannan[2] and *Hedysarum polybotrys* polysaccharides,[3] or are linked to proteins as glycoproteins or proteoglycans.[4,5] Polymers having branches of (1→3)-linked or (1→6)-linked glycosyl residues are often reported to be pharmacologically active.[6,7] *Dendrobium officinale* Kimura and Migo is one of the most valuable *Dendrobium* species. Its pharmacological activity for immune enhancement and hypoglycemic effect has been verified in animal models.[8,9] In this paper, we report the isolation, purification, properties, and structure elucidation of a new glucomannan.

2. Results and discussion

Powdered stems of *D. officinale* (10 g) was pre-extracted for 24 h in a Soxhlet system with acetone (100 mL) and subsequently for 24 h with MeOH (100 mL) to inactivate the enzymes, and remove pigments and low-molecular-weight substances. Exhaustive extraction of the residues with hot distilled water (100 mL×3) gave a crude *D. officinale* polysaccharide (DOP) (1.502 g, 15.0%). After removal of insoluble impurities by centrifugation (1000 rpm, 30 min) and subsequent removal of proteins by the Sevag method[10] and separation by anion-exchange chromatography on DEAE-cellulose (NH_2^-), gave DOP-1 (0.984 g, 65.5%) from the distilled water eluate (500 mL); and DOP-2 (0.036 g, 2.4%), DOP-3 (0.119 g, 7.9%), DOP-4 (0.029 g, 1.9%), DOP-5, and

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DOP-6 were obtained in turn from an aqueous NaCl gradient (0.05, 0.1, 0.2, 0.3, 0.5 M). The last two components were in trace amount (Fig. 1). DOP-1 (0.500 g) was separated on a Sephacryl S-400 column, giving two fractions: DOP-1-A (0.316 g) and DOP-1-B (0.042 g). DOP-1-A was purified on a Sephacryl S-200 column, giving DOP-1-A1, which presented a symmetrical, narrow peak on high-performance gel-permeation chromatography (HP-GPC) as shown in Figure 2. Its weight-average molar mass was estimated to be 1.3×10^5 by HP-GPC, using dextrans of known molecular weight as standards. The optical rotation was $[\alpha]_D^{20}$ −32.5 (*c* 0.40, H_2O). It was partially acetylated, as determined by a titrative method[11] and from the NMR signals at δ 2.05–2.08 ppm/^{1}H and δ 173.2–173.8 ppm/^{13}C. The polysaccharide was free of proteins as determined by the Lowry method.[12]

Complete hydrolysis of DOP-1-A1 with 2 M trifluoroacetic acid (TFA) and examination by thin-layer chromatography (TLC) showed a large proportion of mannose and glucose, with a trace of arabinose. The glycoses released were converted into their corresponding alditols by treatment with $NaBH_4$ and quantified by gas–liquid chromatography (GLC) analysis. The result indicated mannose, glucose, and arabinose in the molar ratios of 40.2:8.4:1. Mannose and glucose were assigned the β-pyranose form from characteristic peaks of 814, 873, 897 cm^{-1} in Fourier-transform infrared (FTIR) spectra,[13] and from anomeric signals in the ^{1}H and ^{13}C NMR spectra; ^{13}C NMR signals at δ 82–88 ppm (characteristic of furanoses) were absent. GLC analysis of the acetylated glycosides with (+)-2-octanol[14] confirmed that all the monosaccharides were in the D configuration; the *m*-hydroxydiphenyl method[15] revealed that uronic acid was absent.

DOP-1-A1 was oxidized with 0.02 M sodium metaperiodate ($NaIO_4$) at 4 °C in the dark for 5 days. A total of 0.624 mol $NaIO_4$ was consumed per mole of sugar residues, based on the averaged molar mass (160) of a glycosyl residue. The production of formic acid was 0.04 mol. It was thus deduced that the nonreducing terminal residues amounted to 4.0%, with (1→2)-/(1→4)-linked and (1→3)-linked glycosyl bonds amounting to 54% and <42%, respectively. The oxidized product was reduced and hydrolyzed. TLC and GLC–MS analysis identified the presence of mannose, glucose, erythritol, and glycerol in the ratio of 6.2:1.4:11.2:1, indicating that (1→4)-linked glycosyl residues existing in DOP-1-A1, the nonreducing terminal, and (1→3)-linked mannose and glucose were in the molar ratio of 11.2:1:6.2:1.4.

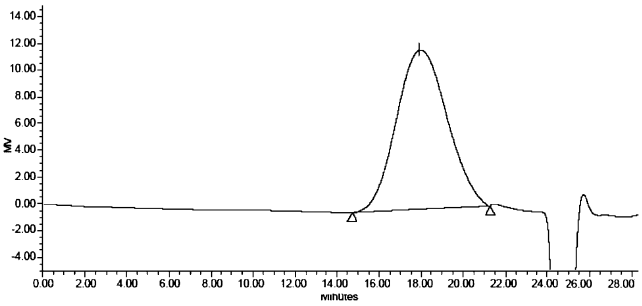

Figure 2. High-performance gel-permeation chromatogram of DOP-1-A1.

Methylation analysis of DOP-1-A1 (Table 1) indicated that the nonreducing termini had minor amounts of Man*p* (3.0%) and Ara*f* (2.5%). Branch points were at (1→4)(1→6)-linked Man*p* (3.5%) and Glc*p* (1.5%), indicating limited branching in DOP-1-A1. The intrachain residues were (1→4)-linked Man*p* (64.4%) and Glc*p* (14.3%) as well as (1→3)-linked Man*p* (8.6%), and Glc*p* (2.2%). Compared with the results of periodate oxidation, (1→3)-linked Man*p* and Glc*p* decreased by 72.5 and 69.0%, respectively. It was proposed that acetyl groups joined to C-2 or C-3 position of (1→4)-linked mannopyranose and glucopyranose, forming a different glycosyl residue: 2/3-*O*-acetyl-(1→4)-linked Man*p* and Glc*p*, of which the periodate oxidation results were the same as (1→3)-linked Man*p* and Glc*p*, respectively. Furthermore the components were converted into (1→4)-linked glycosyl residues, because of the removal of the acetyl groups under the strongly alkaline conditions in the course of repetitive methylation.

DOP-1-A1 (0.100 g) was partially hydrolyzed with 0.3 M TFA and dialyzed. Further fractionation of the

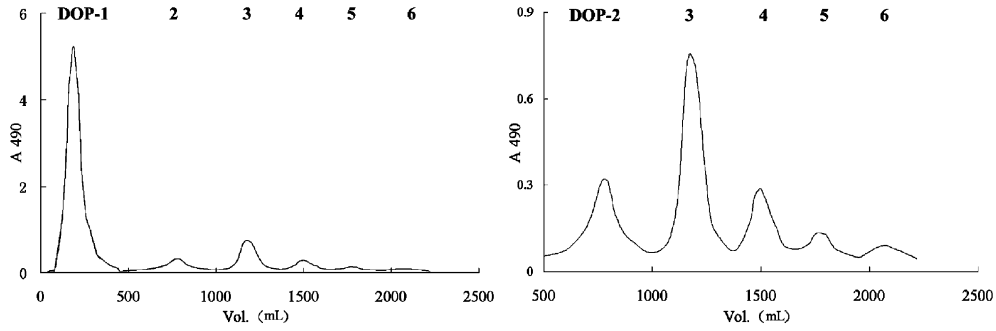

Figure 1. DEAE-cellulose column chromatogram of DOP by NaCl gradient elution. The right figure is an enlargement in the 500–2500 mL range.

Table 1. Methylation analysis data for DOP-1-A1

Methylated sugar	Mass fragments (*m/z*)	Molar ratios	Linkage types
2,3,4,6-Me_4-Man[a]	43,45,71,87,101,117,129,145,161,205	1.2	Terminal
2,3,5-Me_3-Ara	43,45,71,87,101,117,129,161	1	Terminal
2,4,6-Me_3-Glc	43,45,58,71,87,101,117,129,161,201,233	0.9	3→)Glc-(→1
2,4,6-Me_3-Man	43,45,58,71,87,101,117,129,161,201,233	3.5	3→)Man-(→1
2,3,6-Me_3-Man	43,45,71,87,99,101,113,117,129,161,173,233	26.1	4→)Man-(→1
2,3,6-Me_3-Glc	43,45,71,87,99,101,113,117,129,161,173,233	5.8	4→)Glc-(→1
2,3-Me_2-Man	43,85,87,101,117,127,142,159,161,201,261	1.4	4,6→)Man-(→1
2,3-Me_2-Glc	43,85,87,101,117,127,142,159,161,201,261	0.6	4,6→)Glc-(→1

[a] 2,3,4,6-Me4-Man = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-mannitol, etc.

dialyzate (0.025 g) by Sephadex G-25 column chromatography, gave Frac-1 (0.013 g) and Frac-2 (0.008 g). Frac-2 was a mixture of monosaccharides including mannose, glucose, and arabinose in the ratio of 5:1:1.3, according to GLC analysis. Frac-1 was shown to be a mixture too, containing oligosaccharides of various length, and consisted of mannose, glucose, and arabinose in the ratio of 10.3:1:0.8 as shown by complete hydrolysis and GLC analysis. The retentate (0.061 g), purified by Sephacryl S-200 column chromatography, was shown by TLC and GLC analysis to be mannose and glucose in 6:1 ratio. Further methylation analysis showed that the majority of linkage types were (1→4)-linked Man*p* and Glc*p*, along with a trace amount of (1→4)(1→6)-linked Man*p*, inferring that the proportion of (1→4)-linked Man*p* increased in the expense of (1→3)-linked Man*p* in the retentate. The overall results suggested that the polysaccharide had a backbone consisting of (1→4)-linked Man*p* and Glc*p*. (1→3)-Linked mannosyl and glucosyl residues were distributed in branches, for they decreased greatly in the retentate. Terminal arabinose groups were attached to (1→3)-linked Glc*p*, as the ratio between them were relatively constant in Frac-1 and Frac-2. Considering that mannose accounted for a large proportion (85%) in Frac-1 that was released from branches, terminal mannose was thus considered to join to (1→3)-lined Man*p*, otherwise glucose would be in higher proportion.

The NMR sample used for structural studies was viscous and gave relatively broad signals. Assignments of signals and identification of the sugar residues were done by combinations of two-dimensional techniques and comparison of the chemical shifts with published data on similarly substituted sugar residues. The anomeric signals in the ^{1}H NMR spectrum of DOP-1-A1 (Fig. 3) at δ 4.10–4.86 were assigned by comparison with the NMR data reported.[16–23] Signal at δ 4.65, 4.77, 4.10 were assigned to (1→4)-linked, (1→3)-linked, and (1→4)(1→6)-linked β-D-Man*p*, respectively; the signal at δ 4.41 was to (1→4)-linked β-D-Glc*p*. The highest-field signals, at δ 2.05–2.08, belonged to Me of an acetyl group. Combined with the low-field shift of the anomeric hydrogens (δ 4.86 *vs* 4.65), the lowest-field signal at δ 5.41 was assigned to H-2 of 2-*O*-acetyl-(1→4)-linked Man*p*. It was 1.4 ppm to low field as compared with H-2 (δ 4.01) of (1→4)-linked Man*p*. Detailed assignments of other signals are in Table 2 according to HMQC spectra as well as literature data.[16–23]

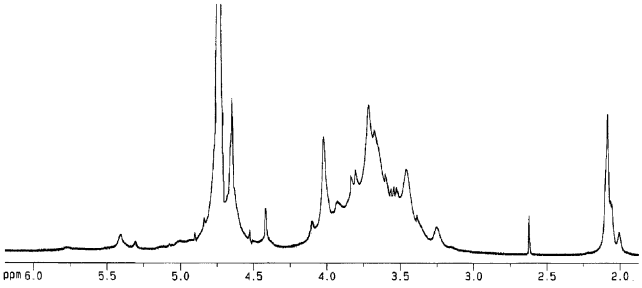

Figure 3. ^{1}H NMR spectrum of DOP-1-A1, obtained at 50 °C.

The ^{13}C NMR spectrum of DOP-1-A1 (Fig. 4) showed five signals for anomeric carbons at δ 99.5–102.9. Signals at δ 100.5, 101.7, 100.3 belonged to (1→4)-linked, (1→3)-linked, and (1→4)(1→6)-linked β-D-Man*p*, respectively; a signal at δ 102.9 was to (1→4)-linked β-D-Glc*p*. Me and CO of acetyl groups were at δ 20.6–21.0 and δ 173.2–173.8, respectively. The signal at δ 99.5 was assigned to C-1 of 2-*O*-acetyl-(1→4)-linked Man*p* according to Ref. 20, which was shifted upfield (1.0 ppm) significantly as compared with nonacetylated Man*p*. Likewise the signal of C-1 of 2-*O*-acetyl-(1→4)-linked Glc*p* was at δ 101.7, which overlapped with that of (1→3)-linked Man*p*. The rest of the observed signals (Table 2) were assigned by reference to HMQC and relevant literature data.[16–23]

The water-soluble heteropolysaccharide DOP-1-A1 extracted from the stem of *D. officinale* Kimura and Migo, and investigated by chemical technique and NMR spectroscopies, was thus shown to be a 2-*O*-acetylglucomannan having a backbone consisting of (1→4)-linked β-D-Man*p* and β-D-Glc*p* (in 6:1 ratio). The two glycosyl residues were substituted at O-2 by the acetyl groups to the extent of ~27.5 and ~31%, respectively, and the acetylated residues are distributed randomly along the backbone. The branches consist of (1→3)-linked β-D-Man*p* and β-D-Glc*p*, which are

Table 2. ^{1}H and ^{13}C NMR chemical shifts (ppm) for DOP-1-A1

Sugar residues		1	2	3	4	5	6
→4)-β-D-Man*p*-(1→	H	4.65	4.01	3.71	3.79	3.64	3.92 (3.79)[a]
	C	100.5	70.9	71.8	76.9	75.4	60.9
→4) β-D-Glc*p*-(1→	H	4.41	3.26	3.60	3.53	3.51	3.82 (3.66)
	C	102.9	73.1	74.4	78.9	75.2	60.7
→4)-2-*O*-acetyl-β-D-Man*p*-(1→	H	4.86	5.41	4.01	3.79	3.60	3.92 (3.79)
	C	99.5	73.3	70.6	76.9	75.4	60.9
→3)-β-D-Man*p*-(1→	H	4.77	n.d.	3.68	3.71	3.39	3.79 (3.79)
	C	101.7	69.1	80.7	65.0	74.4	60.9
→4,6)-β-D-Man*p*-(1→	H	4.10	n.d.[b]	3.92	n.d.	n.d.	n.d.
	C	100.3	70.2	71.7	76.9	74.0	67.0

[a] The values inside and outside the brackets denote the chemical shifts of H-6a and H-6b.
[b] n.d. = not determined.

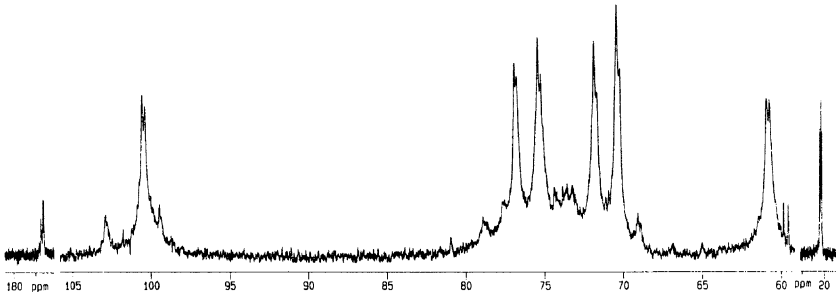

Figure 4. ^{13}C NMR spectrum of DOP-1-A1, obtained at 50 °C.

β-D-Man*p*-(1→3)-β-D-Man*p*-(1→3)-β-D-Man*p* D-Ara*f*-(1→3)-β-D-Glc*p*

OAc 1 OAc 1

↓ ↓ ↓ ↓

2 6 2 6

[→4)-β-D-Man*p*-(1→4)-β-D-Man*p*-(1→4)-β-D-Man*p*-(1→4)-β-D-Glc*p*-(1→4)-β-D-Man*p*-(1→4)-β-D-Glc*p*-(1→4)-β-D-Man*p*-(1→ 4)-β-D-Man*p*-(1→ 4)-β-D-Man*p*-(1→4)-β-D-Man*p*-(1→4)-β-D-Man*p*-(1→]

Figure 5. Repeating units of the *D. officinale* polysaccharide (DOP-1-A1).

attached to O-6 of the backbone. Terminal mannose and arabinose join, respectively, to (1→3)-linked Man*p* and Glc*p*. The repeating units of this *D. officinale* polysaccharides (DOP-1-A1) are indicated in Figure 5.

Glucomannan, a viscous polysaccharide, is not widely found in medicinal plants, but it plays an important role in specific pharmacological activities.[24] The polymer extracted from *D. officinale* represents certain structural characteristics: it is rich in mannose and acetyl groups, has a high degree of polymerization, and a limited degree of branching.

3. Experimental

3.1. Materials and source of sample

The stems of *D. officinale* Kimura and Migo, collected in Yunnan province in May, 2000, were crushed into a powder after being dried in an oven. Sephacryl S-200/S-400 and Sephadex G-25 were purchased from Amersham Pharmacia Co., and Dextrans from Fluka. All other reagents were of analytical grade.

3.2. General methods

Evaporation was performed at ~40 °C under reduced pressure, and products dried by lyophilization. Optical rotations were measured with a 341LC polarimeter as solutions in H_2O. FTIR spectra were recorded on a Nexus 670 spectrometer. GLC was performed on a Shimadzu GC-9A instrument equipped with a hydrogen flame-ionization detector and a 3% OV-225 column (0.25 mm×28 m i.d.); the temperature was 200 °C for sugar analysis. Analysis by GLC–MS of methylated sugars was performed on a Trace GC2000/Trace MS chromatograph fitted with a fused silica capillary column (0.25 μm×0.25 mm×30 m) of HP-5. The column oven was first cooled to 50 °C for 3 min, and then the temperature was raised to 250 °C at 10 °C/min and maintained for 20 min. The hydrogen flow rate was 20 mL/

min and the ion-source temperature was 150 °C. Uronic acid was determined by the *m*-hydroxydiphenyl method,[15] carbohydrate content by the H_2SO_4–phenol method,[25] acetyl groups by the titrative method,[11] and proteins by the Lowry method.[12]

3.3. Separation and purification of DOP-1-A1

The powdered material (10 g) was pre-extracted for 24 h in a Soxhlet system with acetone and subsequently for another 24 h with MeOH. The extract was were discarded. The residue was dried at 40 °C and extracted three times with 100 mL hot distilled water and 1.0 g polyvinylpyrrolidone. The combined extracts were concentrated to ~70 mL and centrifuged at a speed of 1000 rpm for 30 min; the supernatant was concentrated to 60 mL. The solution was poured into 240 mL of 95% EtOH and centrifuged, and the precipitate was dissolved in 60 mL of H_2O: This process was repeated three times. The precipitate was then washed with Sevag reagent (isoamyl alcohol and $CHCl_3$ in 1:4 ratio)[10] and dried in a vacuum, giving the crude polysaccharide, DOP.

Dissolved DOP (1.5%, w/v) was fractionated on a DEAE-cellulose anion-exchange column (3.6×70 cm, NH_2^-) in an LKB-2023 liquid-chromatographic system, first with water, and then with a gradient (0.05–0.5 M NaCl). DOP-1 was obtained by water elution. DOP-1-A was separated from DOP-1 by gel-permeation chromatography on a Sephacryl S-400 column (2.6×50 cm), which was fixed to a Äkta Explorer 100 (Pharmacia.) chromatographic system with a Frac-900 automated collector, UV-900 detector, and RID-10A detector, and DOP-1-A1 was purified via a Sephacryl S-200 column (2.6×50 cm).

3.4. Homogeneity and molecular weight

Samples were detected and determined with an HP-GPC (Waters-2690) system, equipped with Ultrahydrogel™ 2000 and Ultradydrogel™ 250 columns (7.8 mm×30 cm i.d.) connected in series. The temperature of the columns was kept at 34 °C and the sensitivity of the Waters-2410 refractive index detector was 4. The linear regression was calibrated with Dextrans 31418, 31420, 31422, 31424, 31425. The sample concentration was 1% (w/v), and its injection volume was 10 μL. The eluent was phosphate buffer (0.1 M), at a flow rate of 0.8 mL/min.

3.5. Monosaccharide analysis

DOP-1-A1 (5 mg), dissolved in 2 M TFA (2 mL), was hydrolyzed at 110 °C for 2 h. The hydrolyzate was repeatedly co-concentrated with MeOH. The residue was analyzed by TLC on microcrystalline TLC-PET cellulose plates (Sigma) developed with (6:4:3) butanol–pyridine–H_2O, and detection with aniline-*o*-phthalatic acid reagent with heating at 110 °C for 5 min. The remaining hydrolyzate was reduced with $NaBH_4$ (10 mg) at room temperature for 1.5 h, neutralized with AcOH, evaporated to dryness below 40 °C, and then acetylated with 1:1 pyridine–Ac_2O (100 °C, 1 h). It was finally converted into alditol acetates, which were analyzed by GLC. In addition, the absolute configuration of monosaccharides was established by the GLC of the acetylated glycosides formed with (+)-2-octanol.[14]

3.6. Methylation analysis

DOP-1-A1 (12 mg) was methylated by the modified Ciucanu method as described in the literature.[26] The methylated polysaccharide was recovered by two-phase extract ion and freeze drying. The procedures were repeated three times to attain complete methylation, indicated by the absence of hydroxyl group IR absorption. The permethylated polysaccharide was then hydrolyzed with 90% (v/v) formic acid (100 °C, 6 h), and with 2 M TFA (100 °C, 6 h). The partially methylated sugars were reduced and acetylated as already described.

3.7. Periodate oxidation

DOP-1-A1 (16 mg) was treated with 0.02 M $NaIO_4$ (5 mL) and kept in the dark at 4 °C. Consumption of oxidant was monitored by spectrophotometry. Ethylene glycol (0.2 mL) was added to the solution with agitation for 0.5 h after complete oxidation. The production of formic acid was determined by titration with 0.01 N NaOH. The reaction mixture was dialyzed against distilled water (3×500 mL). The retentate was reduced with $NaBH_4$ (20 mg, 24 h) and dialyzed after adjusting the pH to 5.0, then lyophilized and hydrolyzed with 1 M TFA at 100 °C for 6 h, and finally analyzed by TLC and GLC–MS.[27]

3.8. NMR spectroscopy

DOP-1-A1 (30 mg) was deuterium-exchanged by freeze drying three times from 99.9% D_2O (Euriso-Top) and then examined as solutions in 0.5 mL 99.9% D_2O in 5-mm tubes. NMR spectra were recorded at 50 °C on a Bruker Avance™DXM 500 MHz three-channel NMR spectrometer equipped with an inverse 5-mm triple-resonance probe. Chemical shifts are reported in ppm, using acetone (δ_H 2.19 ppm and δ_C 30.5 ppm) as the internal reference. Proton–carbon correlated spectra (HMQC) were obtained with decoupling. Data processing was performed using standard Bruker XWIN-NMR software.

3.9. Partial acid hydrolysis

DOP-1-A1 was hydrolyzed with 0.3 M TFA at 100 °C for 20 min, and dried with N_2. The hydrolyzate was dialyzed against distilled water (3×500 mL). The dialyzate was then concentrated and separated on Sephadex G-25 (1.6×30 cm), giving two fractions: Frac-1 and Frac-2. Both of them were analyzed by GLC as the alditol acetates. The retentate was purified on a Sephacryl S-200 column, and subjected to TLC and GLC analysis, and further methylated analysis.[28] The chromatographic conditions were the same as those for DOP-1-A1.

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