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Note

Structural elucidation of a new arabinogalactan from the leaves of *Nerium indicum*

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Abstract

A polysaccharide fraction, NIB-2, was obtained from the 3% aqueous sodium carbonate extract of *Nerium indicum* leaves using anion-exchange chromatography and gel-permeation chromatography. It was found to be composed of rhamnose, arabinose, galactose, in the ratios of 1.0:10.4:4.4, along with 4% of galacturonic acid. The results of methylation analysis, periodate oxidation, partial acid hydrolysis, pectinase treatment, and ^{13}C and ^{1}H NMR spectroscopy indicate that it is mainly an arabinogalactan having a backbone of 1,6-linked β-Gal*p*, with branches at O-3, consisting of terminal, 1,5-, and 1,3,5-linked arabinofuranosyl residues, and a small proportion of galactosyl residues at the termini. Rhamnose and galacturonic acid arose from a contaminating rhamnogalacturonan.

Keywords: *Nerium indicum*; Oleander; Polysaccharide; Arabinogalactan

Oleander is a widely distributed evergreen plant belonging to the family of Apocynaceae. In China there are two types of oleanders: *Nerium indicum* Mill (with white flowers) and *Nerium oderum* (with pink flowers). Their poisonous leaves are found to contain various cardiac glycosides, alkaloids, and carbohydrates.[1] From *Nerium oleander*, the oleander found in Europe, immunologically active acidic pectic polysaccharides have been isolated and characterized.[2] We have previously characterized a polysaccharide fraction NIB-1 isolated from the leaves of *N. indicum* Mill.[3] Arabinogalactans are widespread in higher plants, existing as a component of pectins[4] or linked to proteins, forming glycoproteins (AGP).[5] Arabino-3,6-galactans are often reported to be immunologically active polysaccharides.[6,7] In this paper, we report the isolation, properties, and structure elucidation of an arabinogalactan with new structural features. Preliminary in vitro tests revealed that it could stimulate the mitogen-induced T and B lymphocyte proliferation.

From the 3% Na_2CO_3 extract of *N. indicum* leaves (3.2 kg), a crude polysaccharide (3.3 g, 0.1%) was obtained. After separation using DEAE-Sephadex A-25 anion-exchange chromatography, NIB-A-1 (137 mg) was obtained from the water eluate; NIB-B-1 (745 mg) and NIB-B-2 (545 mg) were obtained from the NaCl eluate. NIB-B-1 (300 mg) was separated

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on à Sephadex G-200 column, giving two fractions: NIB-B-1P1 (182 mg) and NIB-B-1P2 (36 mg). NIB-B-1P1 was further purified on a Sephadex G-200 column, giving NIB-2 (114 mg), which showed a symmetrical narrow peak on high-performance gel-permeation chromatography (HPGPC). Its molecular weight was estimated to be 4.9×10^5 by HPGPC, using a dextran of known molecular weight as standard. The specific rotation was $[\alpha]_D^{20}$ −42.6° (*c* 0.57, water). It did not contain protein according to the Lowry method.[8]

After complete hydrolysis with 2 M trifluoroacetic acid (TFA), TLC showed a large proportion of arabinose and galactose, along with a trace of galacturonic acid. GLC analysis of the alditol acetates showed that NIB-2 was composed of rhamnose, arabinose and galactose in the molar ratios of 1.0:10.4:4.4. The *m*-hydroxydiphenyl method[9] revealed that it contained 4% uronic acid.

The result of methylation analysis on native NIB-2 is shown in Table 1. The nonreducing termini consist of Ara*f* (27.0%) and a small amount of Gal*p* (3.1%), indicating that NIB-2 is highly branched. The branching points are at 1,3,5-linked Ara*f*, and 1,3,6-linked Gal*p*. The intrachain residues are 1,5-linked Ara*f* and 1,3- and 1,6-linked Gal. Rhamnose was also shown to be 1,2,4-linked via methylation analysis, but only in an amount much less than that from the sugar analysis. As 1,2,4-linked rhamnose is a normal component of rhamnogalacturonan (RG-I), it is proposed that rhamnose and galacturonic acid result from a rhamnogalacturonan. This component was partly removed due to β elimination and dialysis, because its backbone was easily degraded by β elimination of the methyl ester of 1,4-linked galacturonosyl residues under the alkaline conditions of repetitive methylation. After treating NIB-2 with pectinase, TLC did not show rhamnose and GalA in the hydrolysate of the nondialysate, proving the presence of a contaminating rhamnogalacturonan.

NIB-2 (38 mg) was partially hydrolyzed with 0.04 M TFA and dialyzed, giving PHA (dialysate), and PHB (nondialysate). PHA was separated into three fractions, PHA-1 (1.1 mg), PHA-2 (2.8 mg), PHA-3 (16 mg), using Sephadex G-25 column chromatography. The major fraction PHA-3 was a mixture of galactose and arabinose in a ratio of 1:40, according to GLC analysis on its alditol acetates. These results indicate that most of the arabinofuranosyl residues were released, and that they are probably distributed in the outer branches. Thus the terminal galactose should be attached directly to the galactan, and not via an arabinosyl residue or it would give a higher proportion in PHA-3. PHA-2 was found to be a noncarbohydrate contaminant. PHA-1 was an oligosaccharide containing arabinose and galactose in a ratio of 2:1; however, its structure was not determined due to the limited quantity of material available. The nondialyzate was separated into the high-MW PHB1 (10.5 mg) and low-MW PHB2 (4.5 mg). After complete hydrolysis, TLC and GLC revealed that both PHB1 and PHB2 are composed of mainly galactose, along with a small

Table 1
Methylation analysis data for NIB-2 and PHB1

Components	Molar ratios		Mass fragments (*m*/*z*) [a]	Linkages
	NIB-2	PHB1		
2,3,5-Me_3-Ara	8.7		117, 129, 101, 45, 161, 87, 71, 145	terminal
2,3-Me_2-Ara	9.2		117, 129, 87, 101, 99, 189, 161	1,5-
2-Me-Ara	4.6		117, 85, 127, 99, 159, 201, 261	1,3,5-
3-Me-Rha	0.5		129, 143, 87, 101, 189, 203	1,2,4-
2,3,4,6-Me_4-Gal	1.0	1.0	101, 117, 145, 129, 45, 161, 87, 205, 71	terminal
2,4,6-Me_3-Gal	1.0		117, 129, 101, 161, 45, 87, 71, 58, 233, 201	1,3-
2,3,4-Me_3-Gal	1.6	3.4	101, 117, 129, 99, 87, 161, 189, 233	1,6-
2,4-Me_2-Gal	5.2	0.7	117, 129, 87, 189, 159, 101, 139, 233	1,3,6-

[a] The *m*/*z* values are arranged in the sequence of decreasing abundance.

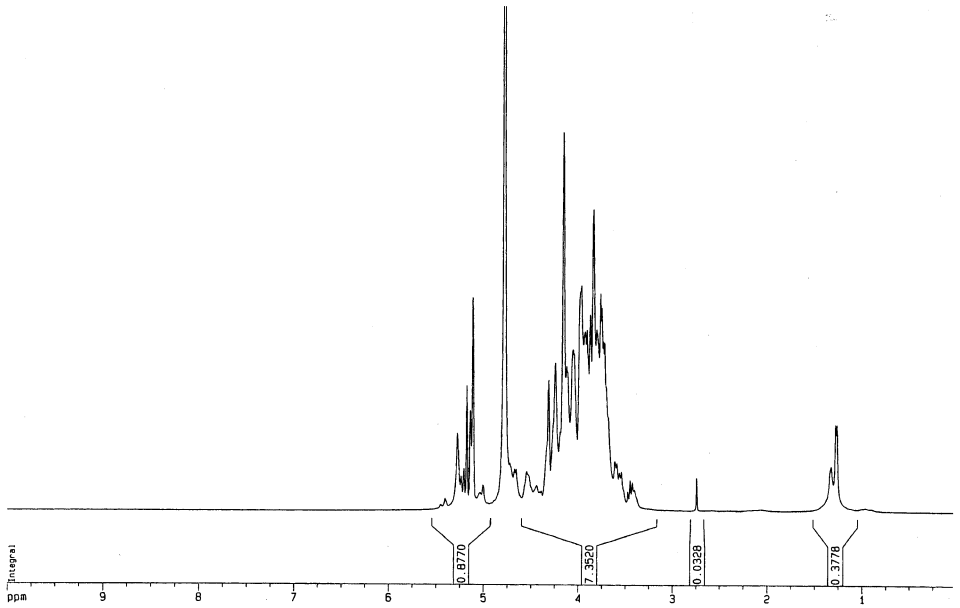

Fig. 1. ^{1}H NMR spectrum of NIB-2.

amount of rhamnose, and a trace of arabinose, all in similar molar ratios. This indicates that they are degraded polysaccharides with similar structure and different molecular weight and that they both contain a galactan backbone. A 1,5-linked arabinan backbone could be excluded because it would not give the nondialysate as PHB1 or PHB2 under mild-acid hydrolysis. Methylation analysis (Table 1) on PHB-1 showed that the proportion of 1,6-linked Gal significantly increased with the expense of 1,3,6-linked Gal, indicating that the polysaccharide had a backbone consisting mainly 1,6-linked galactosyl residues. The 1,3-linked galactosyl residues were removed in PHB-1, indicating that they were distributed in branches.

NIB-2 was oxidized with 0.0156 M sodium metaperiodate ($NaIO_4$) at 5 °C in the dark, and the reaction was complete in 96 h. A total of 0.66 mol of $NaIO_4$ was consumed per mole of sugar residues, based on the averaged molar mass (140) of a glycosyl residue. The formic acid production was 0.10. The oxidized product was reduced and hydrolyzed, and TLC analysis identified galactose, arabinose, and glycerol. These results were in approximate agreement with the expected results based on methylation analysis ($NaIO_4$ consumption: 0.72; formic acid production: 0.08).

The anomeric signals in the ^{1}H NMR spectrum of NIB-2 (Fig. 1) were assigned according to sugar composition and literature data.[10] The strong signals at δ 5.11 and 5.14 were assigned to the nonreducing terminal α-Ara*f* linked to the O-5 and O-3 positions, respectively. The signals at δ 5.18 and 5.27 might arise from 1,5- and 1,3,5-linked α-Ara*f*. The signals at δ 4.67 and 4.55 could be assigned to β-Gal*p*. The H-1 signal for rhamnose was observed at 5.02, indicating an α configuration. This assignment was proved by the ^{1}H NMR spectrum of the partial hydrolyzed product PHB1 in which the signals from arabinose became weaker, while the signals for galactose and rhamnose became stronger. Other signals were assigned according to cross-peaks in the COSY and HMQC spectra or from literature data.[10]

The anomeric signals in the ^{13}C NMR spectrum (Fig. 2) of NIB-2 were mainly assigned according to correlations in the HMQC spectra. The strong signal at δ 110.1 was correlated with the H-1 of terminal arabinofuranose. The resonances at δ 112.0 and 109.6 were then assigned to 1,3,5- and 1,5-linked arabinofuranose. The low-field chemical shifts indicated Ara adopted an α configuration and the furanose form. The comparatively weaker signals at δ 107.1 and 106.0 were attributable to 1,6- and 1,3,6-linked β-Gal*p*, respectively.

The very weak signal at δ 101.2 could be assigned to Rha*p* of the α configuration. The signal at δ 18.99 was undoubtedly assigned to C-6 of rhamnose, while the stronger signal at δ 63.81 was assigned to the C-5 of a terminal arabinose. Three signals that appeared close together were observed at δ 69.01 and corresponded to the C-5 of 1,5- and 1,3,5-linked arabinose, and the substituted C-6 of galactose. Other signals were tentatively assigned in reference to literature values[10,11] or according to HMQC data, and the results are given in Table 2.

According to the results above, we propose that NIB-2 is mainly an arabinogalactan having a backbone consisting of 1,6-linked galactopyranosyl residues, about 75% of which are substituted at O-3 by branches, consisting of terminal, 1,5- and 1,3,5-linked arabinofuranosyl residues. Terminal galactose is attached to O-3 of the backbone or via 1,3-linked galactosyl residues. The presence of 1,3,5-linked arabinose indicates that NIB-2 has a branched $(1\rightarrow3)(1\rightarrow5)$arabinan side chain, consisting mainly 1,5-linked Ara*f*, with an arabinosyl substituent at O-3. A minor rhamnogalacturonan was also found as a minor component of NIB-2.

Most plant arabinogalactans are reported to have a 1,3-linked galactopyranan backbone and arabinofuranan branches substituted at O-6. An arabinogalactan with a 1,6-linked backbone has been reported, but it contains only a small proportion of single arabinosyl branches.[12] NIB-2 has some different structural features by its 1,6-linked backbone, and

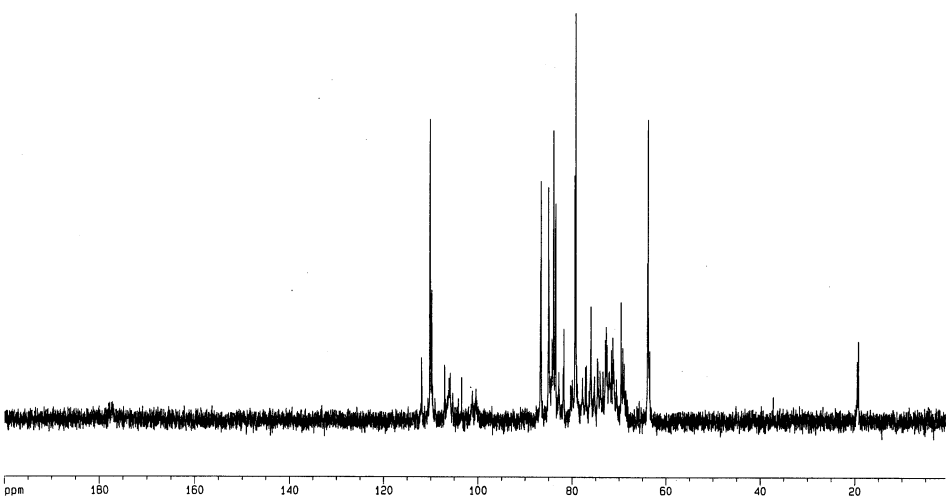

Fig. 2. ^{13}C NMR spectrum of NIB-2.

Table 2
^{1}H and ^{13}C NMR spectral assignments for NIB-2

Residues		1	2	3	4	5	6	
T-α-Ara	H	5.11	4.16	3.97	4.12	3.91 (3.76) [a]		
	C	110.1	83.9	79.3	86.6	63.8		
1,5-α-Ara	H	5.18	4.16	4.06	4.19	3.93 (3.84)		
	C	109.6	84.9	79.4	83.5	69.6		
1,3,5-α-Ara	H	5.27	4.24	4.06	3.84	3.93 (3.84)		
	C	112.0	85.0	86.6	83.5	69.2		
1,6-β-Gal	H	4.67	3.74	3.59	3.72	3.61	3.93 (3.72)	
	C	107.1	75.3	73.4	72.6	76.1	69.6	
1,3,6-β-Gal	H	4.55	3.42	3.61	3.72	3.61	3.93 (3.72)	
	C	106.0	76.1	81.9	72.6	77.0	69.6	

[a] The values inside and outside the brackets denote the chemical shifts of H-6 or H-5 at axial and equatorial positions, respectively.

it is heavily substituted by complex arabinan branches.

1. Experimental

Material.—The leaves of *N. indicum* Mill (3.2 kg) were collected in Shanghai in October, 1993 and immediately immersed in 80% aq EtOH. T-series Dextran, DEAE-Sephadex A-25, and Sephadex G-200 were purchased from Pharmacia Co., and pectinase from E. Merck. All other reagents were of analytical grade.

General methods.—Evaporations were performed below 40 °C under reduced pressure. The optical rotation was measured with a W22-1S automatic polarimeter. IR spectra were determined with a Perkin–Elmer 599B spectrometer. GLC analyses were performed on a Shimadzu GC-14B instrument, equipped with an FID detector and a 5% OV-225 column (2.1 m × 3.2 mm i.d.), and the column temperature was 205 °C for sugar analyses, and 170 °C for methylation analysis. Uronic acid content was determined by the *m*-hydroxydiphenyl method,[9] carbohydrate by the H_2SO_4–phenol method,[13] and protein by the Lowry[8] method.

Separation and purification of NIB-2.—The residue after boiling water extraction was treated with 3% (w/v) Na_2CO_3 at 4 °C for 8 h, then filtered. The filtrate was neutralized to pH 7.0 with 2 M HCl, and then dialyzed against running water for 3 days. The nondialysate was concentrated to a small volume and poured into 3 vols EtOH and centrifuged. The precipitate was washed with EtOH, acetone and ethyl ether and dried in vacuo, giving the crude polysaccharide, NIB.

NIB was dissolved in water (40 mL, 8.2% w/v) and separated using a DEAE-Sephadex A-25 column (90 × 3.0 cm, Cl^-) in two runs, eluting first with water (1 L) and then with a gradient 0–2 M NaCl (polarimetric monitoring). NIB-B-1 was obtained as the first peak of the NaCl eluate. It was purified by twofold gel-permeation chromatography on a Sephadex G-200 column (90 × 2.6 cm).

Homogeneity and molecular weight.—These were detected and determined by HPGPC, which was performed on a Bio-Rad model 1330 pump, equipped with TSK-40, and TSK-50 columns (Bio-Rad) connected in series, and a Shodex RI-51 differential refractive index detector (Showa Denkko). The column was calibrated with T-series Dextran T-2000, T-500, T-110, T-70, T-40 (Phamacia Co.). The sample concentration was 1% (w/v). Sodium hydroxide (0.001 M) was used as the solvent and eluent, and the flow rate was kept at 0.6 mL/min. A 20-μL aliquot was injected for each run.

Composition analysis.—NIB-2 (4 mg) dissolved in 2 M TFA (4 mL) was hydrolyzed at 110 °C for 2 h. The TFA was removed by repeated evaporation with the addition of MeOH. The hydrolysate was analyzed using TLC on a PEI-cellulose plate (E. Merck), developed with 5:5:1:3 EtOAc–pyridine–HOAc–water. The plate was visualized by spraying with *o*-phthalic acid reagent and heating at 100 °C for 5 min. The remaining hydrolysate was reduced with $NaBH_4$ (25 mg) at rt for 3 h, neutralized with AcOH, evaporated to dryness, and then acetylated with Ac_2O (100 °C, 1 h). The resulting alditol acetates were analyzed by GLC.

Methylation analyses.—The polysaccharide (5 mg) was methylated with the modified Ciucanu method as described by Needs.[14] The methylated polysaccharide was recovered by dialysis and freeze-drying. The completeness of methylation was confirmed by the disappearance of the hydroxyl absorption in the IR spectrum (Nujol). Three methylation procedures were performed to achieve complete methylation. The permethylated polysaccharide was hydrolyzed in 90% formic acid (100 °C, 3 h), and then in 2 M TFA (100 °C, 4 h). The partially methylated sugars were reduced and acetylated as described under composition analysis.

Periodate oxidation.—NIB-2 (20 mg) was dissolved in 0.0156 M $NaIO_4$ (20 mL) and kept in the dark at 4 °C. Absorption at 224 nm was determined each day. After the oxidation was complete, ethylene glycol (0.2 mL) was added to the solution with stirring for 0.5 h. The production of formic acid was titrated

with 0.01 N NaOH. The reaction mixture was dialyzed against distilled water, and the nondialysate was reduced with $NaBH_4$ (50 mg, 12 h). The pH was adjusted to 5.0, and the solution was dialyzed. The nondialysate was lyophilized, hydrolyzed with 1 M TFA at 100 °C for 6 h and analyzed by TLC.

Partial acid hydrolysis.—NIB-2 (38 mg) was hydrolyzed with 0.04 M TFA (5 mL) at 100 °C for 1 h. The mixture was evaporated to dryness, and the residue was dialyzed against distilled water (3×250 mL). The dialysate (PHA) was concentrated and separated on Sephadex G-25 (90×1.6 cm), giving three fractions: PHA1, PHA2, and PHA3. PHA3 was analyzed by TLC and GLC as the alditol acetates. PHA-1 and PHA-2 were analyzed by the same procedure as PHA3 after complete hydrolysis. The nondialysate (PHB, 20 mg) was separated on a Sephadex G-25 column (90×1.6 cm), giving two fractions, PHB1 (10.5 mg) and PHB2 (4.5 mg). PHB1 was subject to methylation and ^1H NMR analyses, and PHB2 was subject to sugar analysis.

Pectinase treatment.—NIB-2 (5 mg) was dissolved in water (10 mL), to which 1 mg of pectinase was added. This solution was kept at 37 °C for 16 h, and then dialyzed against distilled water (3×500 mL). The nondialysate was freeze-dried, hydrolyzed with 2 M TFA as for the composition analysis (above), and the hydrolysate was subject to TLC analysis.

NMR spectroscopy.—NIB-2 (45 mg) was dissolved in D_2O (0.5 mL), freeze-dried, and redissolved in D_2O (0.5 mL). ^{13}C and ^1H NMR spectra were recorded at rt with a Bruker AM 400 instrument. All the chemical shifts are reported relative to Me_4Si.

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