Characterization of a Rhamnogalacturonan and a Xyloglucan from *Nerium indicum* and Their Activities on PC12 Pheochromocytoma Cells

Kan Ding,[†] Ji-Nian Fang,*[,†] Tingxia Dong,[‡] Karl Wati-Keung Tsim,[‡] and Houming Wu[§]

Shanghai Institute of Materia Medica, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, People's Republic of China, Department of Biology, The Hong Kong University of Science & Technology, Clear Water Bay Road, Hong Kong, People's Republic of China, and State Key Laboratory of Bioorganic and Natural Products Chemistry, Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences, Shanghai 200032, People's Republic of China

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Two polysaccharides, a rhamnogalacturonan (J1) and a xyloglucan (J2), were isolated and purified from the whole flowers of *Nerium indicum* and characterized by GC, GC−MS, NMR, and ESMS. The rhamnogalacturonan was found to consist of repeating units of a linear trisaccharide with the structure [→4)-α-Gal*p*A(1→2)α-Rha*p*(1→4)-α-Gal*p*A(1→4)−]$_n$, while the xyloglucan consisted of repeating units of the trisaccharide, which was (1→4)-linked β-glucopyranosyl residues as a backbone and substituted at position 6 linked to terminal α-xylopyranose side chains. These two polysaccharides were tested on the proliferation and differentiation on PC12 pheochromocytoma cells and found to have effects similar to nerve growth factor (NGF).

Polysaccharides in organisms, for example, heparin, one of the glycosaminoglycans (GAGs), can potentiate the ability of fibroblast growth factor (FGF) to induce neurite outgrowth in pheochromocytoma (PC12) cells.[1] Heparin and other GAGs such as chondroitin sulfate and dermatan sulfate are glycans that are products of biosynthesis in animal cells.[2] They modulate the actions of a large number of extracellular ligands.[3,4] Herein, we describe a study of the characterization and bioactivity of two plant polysaccharides from *Nerium indicum* Mill. (Apocynaceae). The initial aim of the present study was to investigate if the purified exogenous polysaccharides could affect the behavior of PC12 cells, since this cell line is a model system that has been used for studying the underlying mechanisms of NGF action.[5]

A galacturonan from the plant *Nerium oleander* L. has shown some biological activities for antitumor and immunological systems.[6] Therefore, a second plant in this same genus, *N. indicum*, was investigated. The flowers and leaves of *N. indicum* are used in traditional Chinese medicine and stimulate cardiac muscle, relieve pain, and eliminate blood stasis.[7] Our previous investigations revealed that polysaccharides from the leaves of *N. indicum* could stimulate mitogen-induced T and B lymphocyte proliferation.[8] In the present study two polysaccharides (J1 and J2) were isolated and purified using DEAE-cellulose and gel filtration chromatography. The results obtained show that those polysaccharides can significantly promote the proliferation and differentiation of PC12 cells. This is the first report of exogenous plant-derived polysaccharides with biological effects on nerve cells.

Results and Discussion

The crude polysaccharides were extracted from fresh whole flowers of *N. indicum* and purified on DEAE-cellulose and Sephacryl-300 columns and, in the case of J2, by further treatment with CF_3COOH (TFA). The two polysaccharides obtained, J1 and J2, were shown to each represent a single symmetrical narrow peak by high-performance gel permeation chromatography (HPGPC).

The molecular weight of J1 was estimated to be $>1.0 \times 10^6$ by HPGPC. The specific rotation ($[\alpha]^{20}_D$) was 170.6° (*c* 0.68, H_2O). After complete acidic hydrolysis with 2 M TFA, J1 was shown by TLC to contain a neutral monosaccharide and uronic acid.

There was only one peak for rhamnose shown by GC before J1 was reduced. After this substance was reduced with $NaBH_4$[9] three times, the reduced polysaccharide was revealed to consist of rhamnose and galactose (derived from galacturonic acid) (Table 1), in the molar ratio of 1:1.96. These results suggested that there was no galactose in the original polysaccharide, as confirmed by the ^{13}C NMR DEPT spectrum of J1, which showed no CH_2 peak. Hence, it was deduced that J1 is composed of rhamnose and galacturonic acid (GalA). The methylation analysis of the reduced polysaccharide indicated the presence of a terminal Gal unit, a 1,4-linked Gal unit, and a 1,2-linked rhamnose unit. This is consistent with J1 being a rhamnogalacturonan.

The ^1H NMR (400 MHz, 600 MHz) spectra of J1 showed three signals in the anomeric region (δ 5.20, 5.11, and 5.05) and a signal at δ 1.25, which was assigned to the methyl group of Rha. The ^{13}C NMR spectrum contained three signals attributable to anomeric carbons (101.84, 102.64, 100.48 ppm), a signal for CH_3 of Rha at 19.12 ppm, and two signals for the galacturonic acid COOH groups at 174.99 and 174.86 ppm, respectively. The ^1H NMR spectrum was assigned by a $^1H-^1H$ COSY experiment (Table 2) and comparison of the chemical shifts obtained with those of literature values.[9,10] After all the protons were assigned, an HMQC experiment was used to assign the ^{13}C NMR spectrum of J1. An oligosaccharide (J1O1) was obtained after partial hydrolysis. After partial hydrolysis, the ^{13}C NMR spectrum of the nondialyzed polysaccharide was identical to that of J1. The fragment ions in the ESMS of J1O1 originated from a pseudomolecular ion $[M + 2Na + H]^+$ at *m/z* 916.9, corresponding to the sequential loss of galacturonic acid (*m/z* 740.9), GalA (*m/z* 564.9), and rhamnosyl residues (*m/z* 416.9) (Table 3). The configurations of Rha and GalA were deduced to α by the chemical shifts of their anomeric carbons.[10,11] These results, together with

* To whom correspondence should be addressed. Tel: +86-21-64311833-601. Fax: +86-21-64370269. E-mail: jnfang@mail.shcnc.ac.cn.
[†] Shanghai Institute of Materia Medica, Chinese Academy of Sciences.
[‡] The Hong Kong University of Science & Technology.
[§] Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences.

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Table 1. Glycosyl-Residue Composition and Methylation Analysis of the Polysaccharide J1

residue composition (molar ratio)		methylated sugar		
pre-reduction	post-reduction	post-reduction	mode of linkage	molar ratio
Rha only	Rha: 1	Rha: 3,4-Me2-Rha	→2)-Rha*p*-(1→	1
	Gal: 1.96	Gal: 2,3,4,6-Me4-Gal	Gal*p*-(1→	0.19
		2,3,6-Me3-Gal	→4)-Gal*p*-(1→	2.29

Table 2. ^{1}H and ^{13}C NMR Chemical Shift Assignments of the Polysaccharide J1

	δ_H			δ_C		
position	Rha	GalA[b]	GalA[c]	Rha	GalA[b]	GalA[c]
1	5.20	5.11	5.05	101.84	102.64	100.48
2	4.11	3.76	3.86	79.17	70.51	70.30
3	3.83	4.02	4.13	71.39	70.66	71.97
4	3.43	4.48	4.46	74.26	80.99	80.55
5	3.62	4.65	4.65	72.11	72.88	72.64
6	1.25	*a*		19.12	174.99	174.86

[a] Not present. [b,c] See the position on structure of J1: [→4]-α-[c]Gal*p*A(1→2)α-Rha*p*(1→4)-α-[b]Gal*p*A(1→4)−]$_n$.

Table 3. Mass Spectrometric Analysis of the Hydrolyzed Oligosaccharide J1O1 by Positive-Ion ESMS

nominal mass (*m*/*z*)	type	ion composition
916.9	$[M + 2Na + H]^+$	$GalA_2RhaGalA_2$
740.9	$[M + 2Na + H]^+$	$GalA_1RhaGalA_2$
564.9	$[M + 2Na + H]^+$	$RhaGalA_2$
416.9	$[M + 2Na + H]^+$	$GalA_2$

Table 4. Glycosyl Residue Composition and Methylation Analysis of the Polysaccharide J2

residue	J2	methylated sugar	mode of linkage	J2
Xyl	1	2,3,4-Me_3-Xyl	Xyl*p*-(1→	1
Glc	2.3			
Glc		2,3,6-Me_3-Glc	→4)-Glc*p*-(1→	1.2
Glc		2,3-Me_2-Glc	↑ 6	1.2
			→4)-Glc*p*-(1→	

the methylation data, were consistent with a trisaccharide repeating unit for the J1 (rhamnogalacturonan): [→4)-α-Gal*p*A(1→2)α-Rha*p*(1→4)-α-Gal*p*A(1→4)-]$_n$.[12,13]

The molecular weight of J2 was estimated to be 2.8 × 10^4 by HPGPC. The specific rotation ($[\alpha]^{20}_D$) was 105.8° (*c* 0.677, H_2O). Monosaccharide composition analysis of J2 indicated that the glycosyl residues were Xyl and Glc in a molar ratio of 1:2.3. No uronic acid was observed by TLC analysis. The methylation analysis results showed that J2 consisted of a terminal Xyl unit, a 1,4-linked Glc unit, and a 1,4,6-linked Glc unit (Table 4). This suggested a backbone structure for J2 corresponding to a xyloglucan.[14,15] J2 was branched at C-6, linked to the terminal Xyl, while the molar ratio of the terminal Xyl, the 1,4-linked Glc, and the 1,4,6-linked Glc was 1:1.2:1.2. Hence, in light of published data[14] and methylated analysis data, the structure of J2 was proposed as trisaccharide repeating units of a xyloglucan:

$$[\rightarrow 4)\text{Glc}p^{a}(1\rightarrow 4)\text{-Glc}p(1\rightarrow]_n$$
$$\begin{matrix} 6 \\ \uparrow \\ 1 \\ \text{Xyl}p \end{matrix}$$

The ^{1}H NMR, ^{13}C NMR, and HMQC NMR spectra confirmed the structural sequences of J2. Three signals in the anomeric region, δ 4.983, 4.562, 4.578, in the ^{1}H NMR spectrum of J2 could be assigned, in turn, to one α-Xyl*p* and two *β*-Glc*p* residues. The ^{13}C NMR spectrum of J2 showed three signals at anomeric carbons at 100.78, 104.38, 104.22 ppm, corresponding to one α-Xyl*p* and two *β*-Glc*p* units, respectively. The assignments of the ^{1}H NMR and ^{13}C NMR spectra (Table 5) of J2 refer to previously published reports.[14,16]

The molar ratio of the glycosyl residues of J2 (Xyl:Glc) were established as 1:2.3. On the basis of the above results and the methylation analysis for J2, this substance was assigned as a trisaccharide with the following repeating units:

$$[\rightarrow 4)\text{-}\beta\text{-Glc}p(1\rightarrow 4)\text{-}\beta\text{-Glc}p\ (1\rightarrow]_n$$
$$\begin{matrix} 6 \\ \uparrow \\ 1 \\ \alpha\text{-Xyl}p \end{matrix}$$

After the structures of J1 and J2 were characterized, their bioactivities against PC12 cells were tested. The rat pheochromocytoma PC12 cell line[17] is a nerve growth factor (NGF)-responsive model system that has been useful for studying the underlying mechanisms of NGF actions.[18,19] PC12 cells proliferate in serum-containing culture media and in many respects resemble their non-neoplastic adrenal chromaffin cell counterparts. When exposed to NGF, however, these cells withdraw from the cell cycle and gradually adopt many properties that are characteristic of mature sympathetic neurons. The most dramatic aspect of this response is the extension of long, axon-like neurites with actively motile growth cones. The acquisition of a "neuronal" phenotype by PC12 cells requires both transcription-dependent and -independent cellular responses to NGF. Transcription-dependent events include upregulation of selective cytoskeletal proteins and other neural markers, while relevant transcription-independent actions include regulation of cytoskeletal protein phosphorylation, promotion of growth cone motility, and rapid regeneration of neurites. Stimulation of neuritogenesis by NGF is therefore a complex phenomenon composed of multiple individual responses.[20,21]

PC12 cells were exposed to different concentrations of J1 and J2 in order to examine the potential effects of the isolated J1 and J2 polysaccharides on neurite outgrowth (NGF served as a positive control). The NGF-treated cells showed significantly enhanced neuritogenic potential; over 90% of the NGF-treated PC12 cells showed neurite extension having at least twice the length of the cell body (see Figure 1, NGF). An NGF-like effect could also be detected for both J1 and J2 (Figure 1). The potency was better for J1 because J1 could induce neurite outgrowth at 10 μg/mL, while J2 required a higher concentration up to 20 μg/mL (Figure 1). While the extension of neurites was not determined, the effects of the tested polysaccharides were very similar to NGF (Figure 1) in the experiments that we tested. Since NGF is expensive and difficult to purify, J1 and J2 might represent potential NGF-like neurotrophic factors. Also, the M_r of J1 is very large (estimated size > 1.0×10^6); hence, the molar concentration showing significant bioactivity was less than 1.0×10^{-8}. The mechanism for the promotion of proliferation and differentiation by these two polysaccharides from *N. indicum* is under investigation.

Table 5. ^{1}H and ^{13}C NMR Chemical Shift Assignments of the Polysaccharide J2

	δ_H								δ_C					
residue	H-1	H-2	H-3	H-4	H-5	H-5e	H-6	H-6′	C-1	C-2	C-3	C-4	C-5	C-6
Xyl	4.983	3.557	3.750	3.624	3.581	3.758			100.78	73.39	74.93	71.36	63.46	
Glc	4.562	3.395	3.708	3.693	3.767		3.938	4.022	104.38	74.82	75.92	80.60	75.32	67.99
Glc[a]	4.578	3.439	3.681	3.758	3.657		3.859	4.006	104.22	74.68	76.71	80.77	76.60	61.80

[a] See the assignment of the structure of J2 in the text.

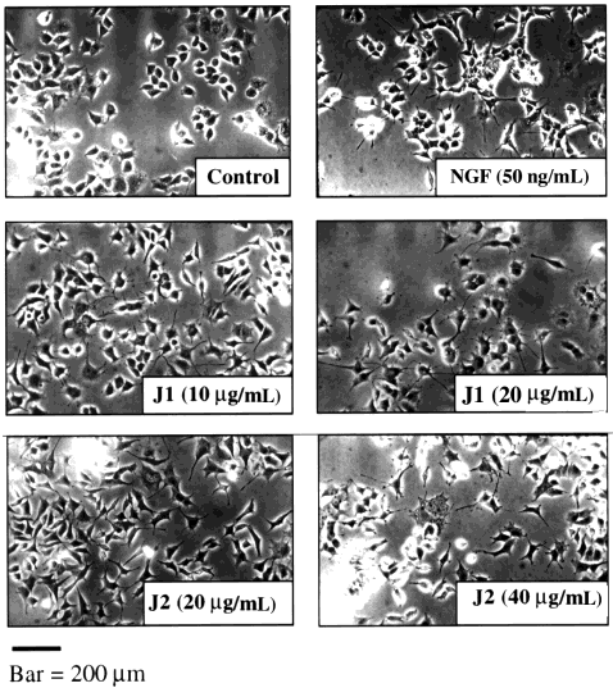

Figure 1. Effects of polysaccharides J1 and J2 on proliferation and differentiation of PC12 cells.

Experimental Section

General Experimental Procedures. Optical rotations were obtained with a Perkin-Elmer PE-24M spectropolarimeter. ^{1}H, ^{13}C, and 2D NMR spectra were recorded on Gemini-300, Bruker AC-400, and Varian Unity Inova-600 NMR spectrometers. Samples were deuterium-exchanged by lyophilization several times from D_2O containing a trace of acetone as internal standard. Positive-mode ESMS was performed using a VG Quattro MS/MS instrument. GC−MS was carried out on a Shimadzu QF-5000 MD 800 system. GLC analysis was performed on a Shimadzu-9A gas chromatograph. Methylation analysis was conducted using a modified NaOH−Me_2SO procedure.[22] The methylation products were then hydrolyzed in 2 M TFA (2 h, 110 °C), reduced at room temperature with 15 mg/mL $NaBH_4$, acetylated with Ac_2O, and extracted with chloroform. The organic layer was subjected to GC−MS analysis. The molecular weights of the pure polysaccharides were 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). Calibration was made with the standard dextrans T-2000, T-110, T-70, T-40, and T-20. Monosaccharide composition analysis[21] of each sample was carried out by hydrolyzing with 2 M TFA at 110 °C for 2 h and then followed by TLC (cellulose plates, Merck) analysis. The major portions of the hydrolyzates were reduced with $NaBH_4$, the resulting alditols were acetylated with Ac_2O, and the alditol acetates were subjected to GC analysis.[23,24] The reduction of the acidic polysaccharide J1 was carried out using *N*-cyclohexyl-*N*′-[2-(-*N*-methylmorpholino)-ethyl]carbodiimide-4-toluenesulfonate (CMC) followed by a $NaBH_4$ reaction three times.[9,25,26] Partial hydrolysis[27] of the rhamnogalacturonan (J1) was performed in 0.2 M TFA at 100 °C for 1 h and yielded the nondialyzed polysaccharide and an oligosaccharide. The oligosaccharide was purified by Sephadex G-10 chromatography. The xyloglucan (J2) was obtained by mild acid hydrolysis (three times) of the original crude xyloglucan using 0.01 M (100 °C, 1.5 h), 0.1 M (100 °C, 1 h), and 0.2 M (100 °C, 1 h) TFA in turn. All of the dialyzed oligosaccharides were separated and purified by Sephadex G-10 and G-25 chromatography. The purity of nondialyzed polysaccharides and oligosaccharides was confirmed by HPGPC. All the nondialyzed polysaccharides and oligosaccharides obtained from partial hydrolysis of the two polysaccharides were subjected to monosaccharide composition and methylation analysis using the usual procedures.

Plant Material. Flowers of *Nerium indicum* were collected in Shanghai, People's Republic of China, in September 1996. The plant material was identified by Prof. P. Li, Department of Pharmacognosy, China Pharmaceutical University, Nanjing, People's Republic of China. A voucher specimen of *N. indicum* (No. 2587) was deposited in the Herbarium of China Pharmaceutical University.

Extraction and Isolation. The fresh whole flowers (9.0 kg) of *N. indicum* were extracted with 95% EtOH for 1 week at room temperature to remove lipid-soluble components. The resultant extract was filtered, and then the residue was dried below 50 °C. To the residue was added 10 volumes of boiling water, and the mixture was maintained at 100 °C for 8 h. After filtration and concentration, a supernatant and a further residue were obtained. The supernatant was precipitated with four volumes of 95% EtOH. After centrifugation, the precipitate was dissolved in water, then 5% (final concentration) cetyltrimethylammonium bromide (CTAB) was added. The resulting mixture was centrifuged, and the supernatant was exhaustively dialyzed (molecular weight cutoff 3000−5000). To the supernatant obtained by centrifugation of the dialyzate was added four volumes of ethanol. The precipitate formed was collected by centrifugation and washed with 10 volumes of ice-cold ethanol. The resulting pellet was a crude rhamnogalacturonan. The crude rhamnogalacturonan was purified by ion-exchange DEAE-cellulose chromatography and eluted by water and 0.2, 0.4, 0.6, 0.8, and 2.0 M NaCl, sequentially. J1 was obtained from the 0.8 M NaCl eluate. It was then purified by 2-fold gel filtration chromatography on a Sephacryl S-300 column (Pharmacia Company). The target fractions were combined, dialyzed, and lyophilized to afford J1 (a pure rhamnogalacturonan). The purity of this compound was confirmed by HPGPC equipped with a RI SE-71 detector and a KS-805 column (Showa Denko K.K.).

After boiling, the flower residue was treated with 1.0 M NaOH followed by 15% trichloroacetic acid. The supernatant was dialyzed and precipitated with four volumes of ethanol. The pellet (a crude xyloglucan) was dried under reduced pressure. The crude xyloglucan was subjected to ion-exchange chromatography on a DEAE-cellulose column. Initial elution was performed with water then followed by elution with 0.2, 0.4, 0.8, and 2.0 M NaCl, sequentially. The fractions eluted with water were purified repeatedly on a DEAE-cellulose colum and by HPGPC gave a narrow symmetrical peak. This polysaccharide was then partially hydrolyzed using, in turn, 0.01 M (100 °C, 1.5 h), 0.1 M (100 °C, 1 h), and 0.2 M (100 °C, 1 h) TFA. The nondialyzed polysaccharide from the 0.2 M hydrolysate was further purified by HPLC using a KS-805 column eluted with 0.001 M NaOH to obtain J2 (a pure xyloglucan), which showed a symmetrical peak by HPGPC.

Cell Culture Assay. PC12 cells (American Type Culture Collection (ATCC), Manassas, VA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 6% horse serum and 6% fetal bovine serum with penicillin (100 U/mL)/streptomycin/(100 μg/mL). The cells were grown on 24-well culture plates and incubated in a humidified atmosphere containing 7.5% CO_2 at 37 °C. The PC12 cells were then seeded at a density of 1×10^4 cells per well and allowed to adhere overnight before treatment. The cells were incubated for 48 h in the presence of polysaccharides J1 and J2 at different concentrations. Nerve growth factor (50 ng/mL) (Sigma, St Louis, MO) served as a positive control, and H_2O as a negative control. After treatment, the cells were washed by PBS, followed by fixing with 2% paraformaldehyde and 5% sucrose in PBS, and then dehydrated by EtOH (50%, 75%, 95%, to 100%). The cells were covered by glycerol and a glass cover slip and subjected to microscopic analysis. Cell morphology was assessed using a phase-contrast microscope (Axiovert 100, Zeiss). The photographs were taken with a camera (MC80, Zeiss) at a magnification of 320×. Experiments were determined in triplicate and in duplicate wells.

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