Reticuloendothelial System-Activating Polysaccharides from Rhizomes of *Panax japonicus*. I. Tochibanan-A and -B

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From rhizomes of *Panax japonicus* (Araliaceae), two polysaccharides named tochibanan-A and -B, which show reticuloendothelial-potentiating activity in the carbon clearance test in mice, were isolated. The structure of tochibanan-A (molecular mass: 23000) was elucidated as a linear β-1,4-D-galactan. Tochibanan-B (molecular mass: 40000) consists of α-galactose (87.1%), l-arabinose, D-glucose and D-galacturonic acid and has a β-D-(1→4)-linked galactopyranosyl backbone possessing GalA-(1→6)-Gal, Ara-(1→5)-Ara, Gal, and Glc side chains. The structure around the branching points and the repeating unit were investigated and a possible structure of tochibanan-B is proposed.

Keywords *Panax japonicus*; chikusetsu-ninjin; Araliaceae; polysaccharide; reticuloendothelial system activity; tochibanan-A; tochibanan-B; β-1,4-D-galactan

As a part of our serial studies on active principles of *Panax ginseng* C. A. MEYER and the congeners, the isolation and structure elucidation of a reticuloendothelial system (RES)-activating arabinogalactan named sanchinan-A from Sanchi-ginseng (=roots of *P. notoginseng* (BURK.) F. H. CHEN, Araliaceae) have been reported.1 *Panax japonicus* C. A. MEYER (Japanese name: chikusetsu-ninjin or tochiba-ninjin 竹節人参) grows wild in Japan and China and the rhizomes have been used as a traditional medicine. Shoji and co-workers2) have studied the saponins of this crude drug extensively, reporting the isolation and structure determination of several oleane and dammarane saponins. Comparative studies on the saponin composition of specimens collected in South Kyushu and China have recently been reported by our group.3) However, the active principles of this drug other than saponins have not been investigated as yet. The present paper describes the isolation and structure investigation of RES-activating polysaccharides from this drug.

RES potentiation was followed by use of the carbon clearance (CC) test in mice according to Halpern *et al.*4)

![Graph](image-url)

**Fig. 1.** Ion-Exchange Chromatography of the Ethanol Precipitates of Water Extract from Rhizomes of *Panax japonicus* on a DEAE-Toyopearl 650 M Column

The eluate was collected in 15 ml fractions and analyzed for total carbohydrate.

using zymosan as a positive control. The dried rhizomes were washed with hot methanol to remove saponins, etc. and then extracted with hot water. An excess of ethanol was added to an aqueous solution of the water extract, and the resulting precipitate, which exerted the activity, was subjected to chromatography on a DEAE-Toyopearl 650M column to give five fractions, tentatively designated as JN, J1, J2, J3 and JA, in the order of elution (Fig. 1). Among these fractions, the CC activity was observed strongly for JN, J1 and J2, weakly for J3 and not at all for JA (Fig. 2).

Fraction JN was chromatographed again on a DEAE-Toyopearl 650M column using another elution system to give two active compounds, JN-1 and -3, together with an inactive substance, JN-2 which was hydrolyzed completely with amyloligosidase to give glucose (Figs. 3, 4). The proton and carbon-13 nuclear magnetic resonance (1H- and 13C-NMR) spectra and the result of high performance gel permeation chromatography (HP-GPC) suggested that JN-2 is a mixture of amyllopectin-like α-glucans with molecular masses of 23000 and 40000.

Homogeneity of each of JN-1 and -3, which are named tochibanan-A and -B, respectively, was substantiated by ion-exchange chromatography under various conditions and also by analytical HP-GPC. Further chemical investigation of other active fractions such as J1 and J2 will be reported in a forthcoming paper.

![Graph](image-url)

**Fig. 2.** Effects of JN, J1, J2, J3 and JA on Clearance Rate of Carbon Particles from Blood Circulation in ICR Mice

The values are means ± standard errors of 6 mice. Significantly different from the control, a) p < 0.01, b) p < 0.05 or c) p < 0.001.

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The molecular mass of JN-1 was estimated to be 23000 by analytical HP-GPC comparison with authentic pullulans. Acid treatment as well as treatment with β-D-galactosidase hydrolyzed this polysaccharide to give D-galactose exclusively. The methylation analysis revealed the presence of only 4-linked galactopyranosyl units, and the 13C-NMR spectrum of JN-1 consisted of signals due to a 4-O-glycosylated β-galactopyranoside unit. Based on these results, JN-1 was concluded to be a linear β-1,4-galactan.

The molecular mass of JN-3 was estimated to be 40000 by the same procedure as above, and acid hydrolysis afforded D-arabinose (7.9%), d-galactose (87.1%), D-glucose (1.6%) and D-galacturonic acid (3.5%). The galacturonide units of JN-3 were reduced to galactosides by the Taylor–Conrad method to give a product named JN-3R. The methylation analysis of JN-3 and JN-3R is summarized in Table I. The presence of 48.4% of 4-linked galactoside units indicated that JN-3 is composed of a (1→4)-linked galactopyranosyl backbone. The increase of terminal galactoside unit on going from JN-3 to JN-3R by ca. 3.2% proved that most of the galacturonide units are located at the terminal of the side chains.

The so-called β-elimination reaction, selectively decomposes a 4-O-substituted uronide ester and a 6-oxoglycoside unit, has been utilized for the selective cleavage of the glycosidic linkages of these units in structure studies on polysaccharides containing uronide moieties. The structure of the side chain containing galacturonic acid of JN-3 was elucidated by means of this β-elimination reaction coupled with methylation analysis. Permethylated JN-3 was subjected to β-elimination with dimethylsulfoxyl carbamion to give a product named JN-3B. Comparison of the results of methylation analyses of JN-3B and JN-3 revealed the disappearance of 6-linked galactoside units and a corresponding increase of terminal galactoside units in JN-3B (Table I). This indicates that the galacturonide units are connected to the 6-position of galactosyl units of the side chain. The unsubstituted 6-hydroxyl groups of the galactosyl units of JN-3B which were formed as the result of the β-elimination reaction, were oxidized to aldehyde with Cl2-dimethyl sulfoxide (DMSO) complex and the product was further subjected to the β-elimination reaction under mild alkaline conditions to give a compound named JN-3BO1. The sequencing analysis of JN-3BO1 revealed a decrease of 3,4,6-linked galactoside unit and the formation of 3,4-linked galactoside units which were not present in JN-3 and JN-3B. This indicates that the GalA-(1→6)-Gal- units are located on the 6-hydroxyl group of the 3,4,6-linked galactoside units in the main chain.

The further oxidation of unsubstituted 6-hydroxyl groups of galactoside units of JN-3BO1 followed by the β-

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**Fig. 3.** Ion-Exchange Chromatography of the JN on a DEAE-Toyopearl 650 M Column
The eluate was collected in 15 ml fractions and analyzed for total carbohydrate.

**Fig. 4.** Effects of JN-1, -2 and -3 on Clearance Rate of Carbon Particles from Blood Circulation in ICR Mice
The values are means ± standard errors of 6 mice. Significantly different from the control, a) p<0.01 or b) p<0.001.

**Table I. Methylation Analysis of JN-3 and Its Derivatives**

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<tr>
<th>Glucose</th>
<th>O-Methyl groups</th>
<th>Deduced linkages</th>
<th>Mol. portions (%)</th>
<th>JN-3</th>
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<th>JN-3B</th>
<th>JN-3BO1</th>
<th>JN-3P</th>
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| α         | 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylglucitol. |

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Table II. Sequencing Analysis of JN-3BO2R and JN-3BO2RM

<table>
<thead>
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<th>Glycose</th>
<th>O-Methyl groups</th>
<th>Deduced linkages</th>
<th>Mol. portion (%)</th>
<th>( t_R^{a} )</th>
<th>Main fragments (m/z)</th>
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\( t_R^{a} \), retention time relative to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylglucitol.

elimination reaction resulted in a remarkable lowering of the molecular mass (cleavage of the main sugar chain) to give fragments named JN-3A and JN-3BO2.

Fragment JN-3A was reduced with NaBH\(_4\) and then ethylated with C\(_2\)H\(_5\)I by Hakomori’s method. The product, on sequencing analysis, gave equivalent quantities of 1,4-di-O-ethyl-2,3-di-O-methylarabinobitol and 2,3,5-tri-O-methylarabinobitol. It follows that JN-3A can be formulated as 2,3-di-O-methyl-5-O-(2,3,5-tri-O-methylarabinobiofurano)arabinofuranose.

Fragment JN-3BO2 obtained from JN-3BO1 (vide supra) was reduced with NaBH\(_4\) to give JN-3BO2R, which was methylated to yield JN-3BO2RM. From the sequencing analysis\(^5\) (Table II), on going from JN-3BO2R to JN-3BO2RM, the appearance of methyl 4-linked galactoside units and 2,3-linked galactoside units was observed together with the disappearance of 2,3,4-linked galactoside unit as well as a decrease of 4-linked galactoside units. These results disclosed that the arabinofuranobioside side chain is attached to the 3-hydroxy group of a galactoside unit of the main chain on which the GalA-(1→6)-Gal chain is located. It is also apparent that vicinal to this 3,6-branched galactose unit, there exists a 2,3-branched unit, leading to the partial structure \( A \) illustrated in Chart 1.

On mild acid hydrolysis, JN-3 afforded a partially hydrolyzed product (JN-3P), the methylation analysis of which showed the decrease of terminal and 5-linked arabinofuranoside units and 3,4-linked galactoside units together with an increase of 4,6-linked galactoside units. This supports the attachment of the arabinofuranobiosyl side chain in the partial structure, \( A \).

The sequencing analyses of JN-3BO2R and JN-3BO2RM also suggest that JN-3BO2 is composed of a repeating block of about twenty 1,4-linked galactoside units, of which three compose the partial structure \( A \) and another three have a galactopyranosyl, glucopyranosyl or arabinobiosyl unit side chain (side chains).

The controlled Smith’s degradation\(^12\) of JN-3R using NaBH\(_4\)-\(d_4\) afforded two products, JN-3R-SD1 and JN-3R-SD2, the structures of which were elucidated by methylation analysis as shown in Chart 2. Formation of JN-3R-SD1 supports the partial structure \( A \) and that of JN-3R-SD2 indicates the presence of isolated branching points. No formation of galactotriosyl-threitol excluded the structure in which three branching points are adjacent to each other. The anomeric configuration of galactopyranoside units to be proved as \( \beta \) by the coupling constant of the anomeric proton signal.

It follows that a possible structure of JN-3 is as illustrated in Chart 1.

**Experimental**

Optical rotations were measured with a Union PM-101 digital polarimeter. The infrared (IR) spectra were measured with a Shimadzu IR-408 spectrometer. \(^1\)H- (100 MHz) and \(^13\)C-NMR (25.00 MHz) spectra were obtained with a JEOL FX-100 spectrometer at 85 °C in D\(_2\)O [internal dioxane, 3.7 (H) and 67.4 ppm (C)] relative to the signal for tetramethylsilane. HP-GPC was carried out with a CCPM (Tosoh). Detection was accomplished with a differential refractometer (RI-8000, Tosoh).
General Methods  The carbon clearance test in ICR mice was carried out in the same way as in the previous paper.13 The molecular masses of polysaccharides were determined by HP-GPC on columns of TSKgel G-3000PWxL and G-5000PWxL (8.0 mm x 30 cm each) placed successively: mobile phase, 0.5 M NaCl; flow rate, 0.8 ml/min; column temperature, 60 °C. The molecular weight of the polysaccharide was calculated using pullulans as standards (molecular mass = 1000). 14 The total carbohydrate, uronic acid and protein contents were assayed by the phenol-sulfuric acid15 16 method and Lowry methods,17 respectively, using galactose, galacturonic acid and bovine serum albumin as standards. Nitrogen content was measured by elemental analysis.

Examples of Polysaccharides  Powder of rhizomes of Panax japonicus C. A. Meyer (180 g), purchased in Hiroshima, was extracted with methanol, and the residue was extracted with water on a boiling water bath. The water extract (32.6 g) was dissolved in water and an excess of ethanol was added to this solution to form precipitates, which were collected by centrifugation, washed with ethanol, and dried to afford a brownish powder (12.5 g). The polysaccharide (5 g) was dissolved in 50 ml of 25 mM phosphate buffer (pH 7.2) and applied to a column of DEAE-Toyopearl 650M (Toso Co., Ltd., 50.0 x 80 cm), which had been equilibrated with the same buffer. Elution was performed with the buffer, and then the buffer containing 0.1, 0.2, 0.3 M NaCl, and finally with 0.5 M NaCl. The eluents were collected in 15 ml fractions and carbohydrate composition was monitored by the phenol-sulfuric acid method to separate into five fractions (JN-1, JN-2, JN-3, JN-4, JN-5) as shown in Fig. 1. JN-1 (4 g) was applied to a DEAE-Toyopearl 650M column equilibrated with 25 mM borate buffer (pH 8.0) and linear gradient elution (0 to 0.3 M) with NaCl in the buffer gave JN-1 (60 mg), JN-2 (200 mg) and JN-3 (60 mg) as shown in Fig. 1. JN-1 was eluted from the column by HP-GPC on columns of TSKgel G-4000PWxL (7.5 mm x 30 cm) equilibrated with 0.3 M NaCl and also by ion-exchange column chromatography on DEAE-Toyopearl 650M (1.2 x 50 cm) by a linear gradient elution with NaCl (0 to 0.3 M in 25 mM phosphate buffer (pH 6.8), 25 mM borate buffer (pH 8.0) or 25 mM Tris-HCl buffer (pH 7.0)).

Determination of Neutral Sugar Components  Polysaccharides were hydrolyzed with 1 M H2SO4 for 4 h. After being neutralized with BaCO3, the resulting sugars were converted into alditoic acetates18 and analyzed by gas liquid chromatography (GLC). GLC was performed on a Shimadzu GC-6A; glass column (2.6 mm x 20 m) packed with 5% ECNSS-F. W, column temperature 180 °C; N2 flow rate 40 ml/min; detection hydrogen flame ionization detector (FID). The molar ratios were calculated by applying the E.c.r. theory.19

Methylation Analysis20 Step 1: 2 mM dimethyl sulfoxide (2 ml) was added to a solution of polysaccharide (10 mg) in a mixture of DMSO and 1,1,3,3-tetramethylethylene (1: 1, 2 ml).20 The solution was agitated ultrasonically for 1 h, and then methyl iodide was added. After ultrasonication for 30 min of the reaction mixture, ice-cooled water was added. The solution was dialyzed against running water for 24 h and then against distilled water for 12 h, and the non-dialysate was lyophilized. The product was purified by GPC on a column of Sephadex LH-20 eluted with CHCl3-acetone (2: 1). No hydroxy group was observed by IR in this product. Step 2: The fully or partially methylated product (3 mg) was treated with 17% formic acid (2 ml) at 100 °C for 1 h, and then the reaction mixture was concentrated to dryness. The residue was treated with 0.13 M H2SO4 at 100 °C for 16 h. After being neutralized with BaCO3, the hydrolysate was concentrated to dryness and reduced with NaBD4 in 95% ethanol. The resulting methylated alditoic acetates were analyzed by GLC and gas chromatography mass spectrum (GC-MS). GLC was performed on a Shimadzu GC-6A: 2.5% Silicone SE-60 on Chromosorb W (2.6 mm x 20 m); N2 flow rate 40 ml/min; column temperature 180 °C; detection hydrogen flame ionization detector (FID). GC-MS was carried out with a Shimadzu GC-7000: glass column packed with 2.5% Silicone SE-60 on Chromosorb W (2.6 mm x 20 m); He flow rate 30 ml/min; column temperature 155-245 °C (1 °C/min); ionization voltage 70 eV. Peaks were identified on the basis of relative retention times and fragmentation patterns,21 and molar ratios of the partially methylated alditoic acetate was determined on the basis of E.c.r. theory.22

Reduction of Carboxyl Groups in JN-3  Carboxyl group of uronic acid in the polysaccharide (50 mg) was reduced with 1-cyclohexyl-3-(4'-morpholinophenyl)carbodiimide metho-p-toluenesulfonate and NaH并购 according to the method of Taylor and Conrad,23 and the reaction mixture was dialyzed and lyophilized to give JN-3R (45 mg).

Acid Hydrolysis of JN-3  A solution (5 ml) of JN-3 (10 mg) in 0.1 N HCl was heated on a boiling water bath for 20 min. After neutralization and concentration, the products was chromatographed on Toyopearl HW-55F (2.6 x 100 cm) eluted with water to give the high molecular fraction (JN-3P).

Controlled Smith's Degradation24 of JN-3R  JN-3R (20 mg) was oxidized with 50 mM sodium periodate (7 mol) at 4 °C in the dark. After 6 days, ethylene glycol was added, decomposing the excess periodate. The reaction mixture was dialyzed against running water for 24 h and then against distilled water for 12 h. The non-dialyzed solution was concentrated to 10 ml and NaBD4 (25 mg) was added to this solution. After standing at room temperature for 2 h, acetic acid was added to destroy excess reagent, and then the solution was dialyzed running water for 24 h and then against distilled water for 12 h. The non-dialyzed was lyophilized and treated with 0.1 N H2SO4 (5 ml) at 25 °C for 24 h. After neutralization, the products was chromatographed on a TSKgel Toyopearl HW-40F column (2.6 x 100 cm) eluted with water, detected by RI to give JN-3S-RSD1 (2 mg) and JN-3S-RSD2 (4.6 mg).

Amino-Based β-Elimination25 of Permethylated JN-3  The permethylated polysaccharide (20 mg) and p-toluenesulfonic acid (300 μl) were dissolved in 4 ml of a 19:1 (v/v) mixture of DMSO and 2,2-dimethoxypropane. The solution was ultrasonicated for 30 min at 25 °C under a nitrogen atmosphere, and then 1 ml of 2 M dimethyl sodium was added. The mixture was agitated ultrasonically at 25 °C for 30 min. After sonication, the mixture was standing at room temperature for 20 min. After concentration, the aqueous solution was dialyzed against distilled water for 24 h, and then lyophilized. The product was purified by GPC on a column of Toyopearl HW-55F eluted with CHCl3-acetone (2: 1). A CHCl3 solution (1 ml) of oxidized polysaccharide was treated with sodium ethoxide in ethanol (1 M, 0.5 ml) at 15 °C for 2 h, neutralized with acetic acid, and concentrated to dryness. The residue was treated with 17% formic acid (1 ml) and overnight boiling water bath for 4 h, cooled, concentrated to dryness, and purified by GPC to give JN-3BOI (16 mg). By the same procedure as above, JN-3BOI (20 mg) gave JN-3BOII (13 mg) and JN-3A (2 mg).

Sequencing Analysis of JN-3A  On sequencing analysis, JN-3A gave 1,4-di-O-acetyl-2,3-di-O-methylarabinitol [m/z 204, 0.8; main fragments (m/z) 189, 129, 117, 101, 99, 87 43] and 1,4-di-O-acetyl-2,3,5-tri-O-methylarabinitol [m/z, 0.4: main fragments (m/z) 219, 206, 175, 174, 160, 159, 148, 131, 116, 105, 80, 40, 37] and 1,5-O-acetyl-2,3,4,tri-O-methylarabinitol.

Sequencing Analysis of Reduced and Ethylated JN-3A  JN-3A was reduced with NaBD4, and then the partially O-methylated products were ethylated in the same way as for the methylation analysis (step 1) with the exception of using C2H5I instead of CH3I (vide supra). On sequencing analysis, the resulting material gave equivalent quantities of 5-O-acetyl-1,4-di-O-ethyl-2,3-di-O-methylarabinitol [m/z 424: 2 3; main fragments (m/z) 219, 206, 175, 174, 160, 159, 148, 131, 116, 105, 80, 40, 37] and 1,5-O-acetyl-2,3,4,tri-O-methylarabinitol.

Acknowledgement  This study was financially supported by a Grant-in-Aid for Developmental Scientific Research (No. 60807078) from the Ministry of Education, Science and Culture, Japan, in 1982 to O. Tanaka.


5) The methylation analysis involves permethylation (step 1), then acid hydrolysis, reduction with NaBH₄ (or NaBD₄) and acetylation, followed by GC-MS analysis of the resulting mixture of methyl ether acetates of alditols (step 2). In this paper, the procedures after the initial permethylation (*i.e.*, step 2) are designated as the sequencing analysis.


