An Acidic Polysaccharide Having Activity on the Reticuloendothelial System from the Roots and Rhizomes of *Saposhnikovia divaricata*

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An acidic polysaccharide, named saposhnikovan C, was isolated from the roots and rhizomes of *Saposhnikovia divaricata* SCHISCHK. It was homogeneous as judged by electrophoresis and gel chromatography, and showed remarkable reticuloendothelial system-potentiating activity in a carbon clearance test. It is composed of D-galacturonic acid : L-rhamnose : L-arabinose : D-galactose in a molar ratio of 27 : 7 : 8 : 8, and its molecular mass was estimated to be 132000. About 30% of the D-galacturonic acid residues exist as the methyl esters. *O*-Acetyl groups were identified, and the content amounted to 3.3%. Methylation analysis, carbon-13 nuclear magnetic resonance, and controlled Smith degradation studies indicated the structural features. It has a pectin-like rhamnogalacturonan backbone with branched arabinan and galactan side chains.

Keywords *Saposhnikovia divaricata*; Saposhnikoviae Radix; polysaccharide; reticuloendothelial system; immunological activity; saposhnikovan C; methylation analysis; controlled degradation; structural feature

Recently, we have obtained a reticuloendothelial system (RES)-activating polysaccharide named saposhnikovan A from the roots and rhizomes of *Saposhnikovia divaricata* SCHISCHK. (*Ledebouriella seseloides* WOLFF, Umbelliferae).[1] The dried root and rhizome of this plant are used as the crude drug Saposhnikoviae Radix, which is a well-known Oriental crude drug employed as a diaphoretic, an antipyretic and an analgesic in the treatment of cold and concomitant headache and arthralgia. Saposhnikovan A was isolated as a major acidic polysaccharide having immunological activity. The present paper describes the isolation and structural features of another RES-activating polysaccharide from this drug.

The extraction of acidic components with hot water followed by successive treatments with cetyltrimethylammonium bromide in dilute sodium sulfate solution, sodium chloride solution and ethanol have been described previously.[1] The polysaccharide fraction was obtained by gel chromatography with Sephadex G-50, then it was applied to a column of diethylaminoethyl (DEAE)-Sephadex A-25 (acetate form). After elution with water, 0.2 M and 0.5 M acetate buffer, the eluate with 1 M acetate buffer was dialyzed and purified by gel chromatography on Sephacryl S-300 followed by dialysis, chromatography on Sephadex G-25 and lyophilization.

The purified polysaccharide gave a single spot on cellulose acetate membrane electrophoresis and gave a single band on polyacrylamide gel electrophoresis. In addition, it gave a single peak on gel chromatography with Toyopearl HW-65F. Gel chromatography using standard pullulans gave a value of 132000 for the molecular mass. It showed a positive specific rotation ($[\alpha]_D^{24}$ +85.7° in H_2O, $c=0.3$). The name saposhnikovan C is proposed for this substance.

The eluate obtained from the DEAE-Sephadex A-25 column with 0.5 M acetate buffer afforded a minor polysaccharide named saposhnikovan B in addition to the major polysaccharide, saposhnikovan A. It was composed of D-galacturonic acid, L-arabinose, D-galactose, acetyl and methoxyl groups in a molar ratio of 27 : 4 : 3 : 4 : 17. Because of its low yield, however, further chemical investigation of saposhnikovan B has not been performed.

The effect of saposhnikovan C on the RES was examined by using the *in vivo* carbon clearance test[2] with zymosan as a positive control. As shown in Fig. 1, the phagocytic index was remarkably increased, suggesting the activation of the RES by i.p. injection of the polysaccharide.

Quantitative analysis showed that saposhnikovan C contained 54.0% D-galacturonic acid, 11.5% L-rhamnose, 11.9% L-arabinose, 13.9% D-galactose, 3.3% acetyl and 2.7% methoxyl groups, and their molar ratio was approximately 27 : 7 : 8 : 8 : 7 : 8. It contained no nitrogen.

The carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum of the polysaccharide showed signals at δ 21.69 and 178.21 ppm, suggesting the presence of *O*-acetyl groups. In addition, the ^{13}C-NMR spectrum showed a signal at δ 55.55 ppm, suggesting the presence of *O*-methyl groups as carboxylic acid methyl esters. The presence of these groups was confirmed by gas chromatography (GC) of the hydrolyzate. The value of methoxy content showed that about 30% of the galacturonic acid residues in the polysaccharide exist as methyl esters.

Further, the ^{13}C-NMR spectrum showed four signals due to anomeric carbons at δ 101.12, 102.80, 106.36 and 110.22 ppm. The first and the second were assigned to

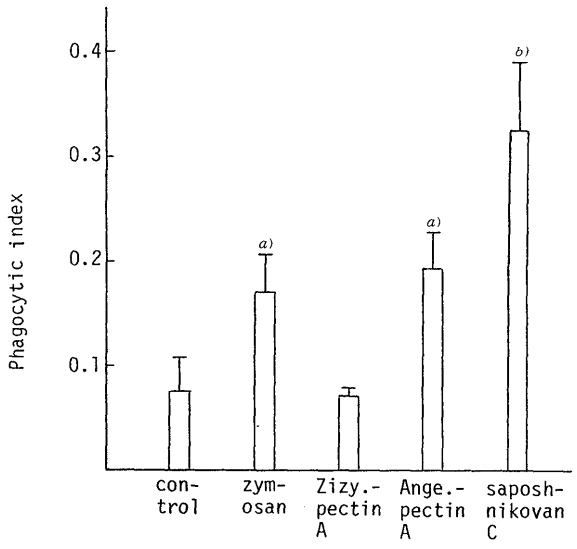

Fig. 1. Effects of Saposhnikovan C and Two Pectins on Carbon Clearance Index in ICR Mice

Significantly different from the control: *a*) $p<0.01$, *b*) <0.001.

the anomeric carbons of α-D-galactopyranosyluronic acid and α-L-rhamnopyranose, respectively.[3] The signal at δ 106.36 ppm was assigned to the anomeric carbon of β-D-galactopyranose,[4] and the signal at δ 110.22 ppm was assigned to the anomeric carbon of α-L-arabinofuranose.[5]

The carboxyl groups of galacturonic acid residues in the polysaccharide were reduced to give the corresponding neutral sugar residues.[6] Both the original polysaccharide and the carboxyl-reduced derivative were methylated with methylsulfinyl carbanion and methyl iodide in dimethylsulfoxide.[7] The methylated products were hydrolyzed, and the hydrolyzates were converted into the partially methylated alditol acetates. Hexuronic acid methyl ethers were removed from the hydrolyzate by treatment with an anion-exchange resin. Gas chromatography-mass spectrometry (GC-MS)[8] revealed derivatives of 2,3,5-tri-*O*-methyl-L-arabinose, 2,3-di-*O*-methyl-L-arabinose, 2-*O*-methyl-L-arabinose, 3,4-di-*O*-methyl-L-rhamnose, 3-*O*-methyl-L-rhamnose, 2,3,4,6-tetra-*O*-methyl-D-galactose, 2,3,6-tri-*O*-methyl-D-galactose and 2,6-di-*O*-methyl-D-galactose as the products from the methylated original polysaccharide in a molar ratio of 4:2:2:4:3:5:1:2. Derivatives of 2,3,5-tri-*O*-methyl-L-arabinose, 2,3-di-*O*-methyl-L-arabinose, 2-*O*-methyl-L-arabinose, 3,4-di-*O*-methyl-L-rhamnose, 3-*O*-methyl-L-rhamnose, 2,3,4,6-tetra-*O*-methyl-D-galactose, 2,3,6-tri-*O*-methyl-D-galactose and 2,6-di-*O*-methyl-D-galactose were identified from the carboxyl-reduced product in a molar ratio of 4:2:2:4:3:5:26:4.

These results indicated that the minimal unit of the polysaccharide is composed of ten kinds of component sugar units, as shown in Chart 1.

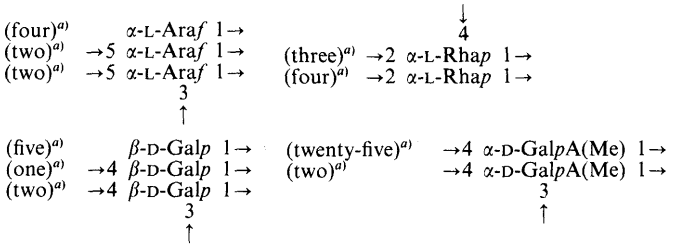

Chart 1. Component Sugar Residues in the Minimal Unit in the Structure of Saposhnikovan C

a) Number of residues. Araf, arabinofuranose; Galp, galactopyranose; Rhap, rhamnopyranose; GalpA, galactopyranosyluronic acid; GalpA(Me), galactopyranosyluronic acid residues having partial methyl esterification.

Both the original polysaccharide and the *O*-deacetylated product were separately subjected to periodate oxidation followed by reduction with sodium borohydride. The periodate oxidation–reduction product from the original sample contained 4.4% galacturonic acid, 4.9% rhamnose, 3.0% arabinose and 3.6% galactose. The deacetylated product gave a similar result to that of the original sample. These results suggested that the *O*-acetyl groups must be located at terminal arabinose and/or galactose units or branching component sugar residues. Controlled degradation[9] of the periodate oxidation–reduction product from the polysaccharide yielded a polymer composed of D-galacturonic acid, L-rhamnose and D-galactose in a molar ratio of 2:3:2. Its molecular mass was 3400. From these results, it can be presumed that the branching galacturonic acid, rhamnose and galactose are adjacent to one another.

The polysaccharide was partially hydrolyzed with dilute trifluoroacetic acid. After removal of the acid, an aqueous solution of the residue was applied to a column of DEAE-Sephadex A-25 (formate form). In addition to all arabinose, galactose, partially liberated rhamnose and galacturonic acid, two oligosaccharides (OS-I and OS-II) were obtained by stepwise elution with dilute formic acid. Component sugar analysis, a comparison of chromatographic properties with those of an authentic sample,[10] and methylation analysis of the reduction products revealed that they were 2-*O*-α-(D-galactopyranosyluronic acid)-L-rhamnopyranose and *O*-α-(D-galactopyranosyluronic acid)-(1→4)-*O*-α-(D-galactopyranosyluronic acid)-(1→2)-L-rhamnopyranose. This result has substantiated the presence of a rhamnogalacturonan backbone.

Based on the accumulated evidence described above, it can be concluded that saposhnikovan C has the structural features shown in Chart 2.

Saposhnikovan C has a backbone chain consisting of α-1→4-linked D-galacturonic acid residues with interspersed α-1→2-linked L-rhamnose residues. The hexuronic acid residues exhibit about 30% carboxyl-methyl esterification. These structural features are similar to those of pectic substances.[11] In addition, in analogy with the pectins from Zizyphi Fructus[12] and Angelicae Radix,[13] the polysaccharide possesses 3,5-branched α-L-arabinofuranosyl side chains. Zizyphus-pectin A[12] showed no RES activity and Angelica-pectin A[13] showed weak activity (Fig. 1). In

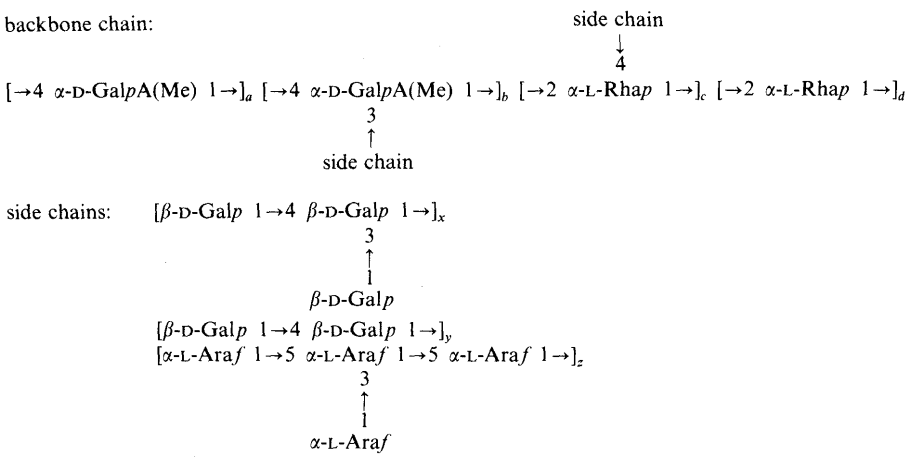

Chart 2. Possible Structural Units of Saposhnikovan C

$a:b:c:d=25:2:3:4$, $x:y:z=2:1:2$.

contrast to these pectins, the arabinan and galactan side chains are linked to both position 3 of α-1→4-linked D-galacturonic acid and position 4 of α-1→2-linked L-rhamnose residues in saposhnikovan C. Further, 3,4-branched β-D-galactopyranosyl side chains as seen in this polysaccharide were not found in the pectins. These characteristic features and the presence of many neutral sugar residues in saposhnikovan C may contribute to the activity.

Saposhnikovan A, the major RES-activating polysaccharide from Saposhnikoviae Radix,[1] possesses a backbone chain consisting of partially methyl-esterified α-1→4-linked D-galacturonic acid residues. It has no rhamnose units, and arabino-3,6-branched β-D-galactan side chains are linked to positions 2 or 3 of the backbone in this polysaccharide.

An RES-activating arabinogalactan named sanchinan A from the root of *Panax notoginseng*[14] has a β-1→3-linked D-galactopyranosyl backbone and α-L-arabino-3,6-β-D-galactan side chains. Most recently, we obtained an RES-activating arabinoxylan named cinnaman AX from the bark of *Cinnamomum cassia*.[15] This polysaccharide possesses a β-1→4-linked D-xylopyranosyl backbone and α-L-arabinofuranosyl-(1→3)-β-L-arabinopyranose side chains.

As other examples of plant polysaccharides having a phagocytosis-enhancing effect, two 4-methylglucuronoxylans isolated from the herbal part of *Eupatorium cannabinum* and *Eupatorium perfoliatum*,[16] a fucogalactoxyloglucan isolated from *Echinacea purpurea* cell culture,[17] and an acidic polysaccharide having a rhamnogalacturonan backbone and arabino-3,6-galactan type side chains isolated from *Viscum album* berry[18] have been reported. Thus, saposhnikovan C is a RES-activating polysaccharide of a new structural type.

Experimental

Solutions were concentrated at or below 40 °C with rotary evaporators under reduced pressure. Optical rotation was measured with a JASCO DIP-140 automatic polarimeter. NMR spectra were recorded on a JEOL JNM-GX 270 FT NMR spectrometer in heavy water containing 2,2-dimethyl-2-silapentane-5-sulfonate as an internal standard at 30 °C. Infrared (IR) spectra were measured with a JASCO IRA-2 infrared spectrophotometer. GC was carried out on a Shimadzu GC-7AG gas chromatograph equipped with a hydrogen flame ionization detector. GC-MS was done on a JEOL JMS-GX mass spectrometer.

Isolation of Polysaccharides The crude extract (3.75 g) was obtained from the crude drug (855 g) by extraction with hot water followed by successive treatments with cetyltrimethylammonium bromide, sodium chloride solution and ethanol, then the polysaccharide fraction (1.73 g) was isolated from the extract by gel chromatography with Sephadex G-50 as described in a previous report.[1] This polysaccharide fraction was dissolved in water and applied to a column (5 × 47 cm) of DEAE-Sephadex A-25. DEAE-Sephadex was used as the acetate form in the manner described in a previous report.[12] After elution with water (1000 ml), 0.2 M acetate buffer (pH 5.0, 1100 ml) and 0.5 M acetate buffer (pH 5.0, 1160 ml), the column was eluted with 1 M acetate buffer (pH 5.0, 1100 ml). Fractions of 20 ml were collected and analyzed by the phenol–sulfuric acid method.[19] The eluates obtained from tubes 189 to 200 were combined, dialyzed, concentrated and applied to a column (5 × 84 cm) of Sephadex G-25. The column was eluted with water and fractions of 20 ml were collected. The eluates obtained from tubes 32 to 41 were combined, concentrated and lyophilized. Yield, 128 mg. A half of this fraction was dissolved in 0.1 M Tris–HCl buffer (pH 7.0) and applied to a column (5 × 86.5 cm) of Sephacryl S-300. Elution was carried out with the same buffer and fractions of 20 ml were collected. The eluates obtained from tubes 29 to 35 were combined, dialyzed, concentrated and applied to a column of Sephadex G-25 as described above. The eluates obtained from tubes 32 to 38 were combined, concentrated and lyophilized. Saposhnikovan C (24 mg) was obtained as a white powder. On the other hand, the eluates obtained from tubes 130 to 145 of the DEAE-Sephadex A-25 column described above were combined, dialyzed and treated with a column of Sephadex G-25. The fraction (50 mg) obtained was dissolved in 0.1 M Tris–HCl buffer (pH 7.0) and applied to a column (5 × 83 cm) of Sephacryl S-300 as described above. The eluates obtained from tubes 27 to 31 were combined, dialyzed, concentrated and applied to a column of Sephadex G-25. The eluates containing a polysaccharide were combined, concentrated and lyophilized. Yield, 14 mg. Saposhnikovan B was purified from this fraction by high-performance liquid chromatography (HPLC) using a column (0.76 × 50 cm) of Asahipak GS-320 with water elution.

Cellulose Acetate Membrane Electrophoresis This was performed with Separax (Fuji Film Co., 6 × 21 cm long) using a buffer of 0.08 M pyridine and 0.04 M acetic acid (pH 5.4) at 420 V for 30 min. The sample was applied in a line at a distance of 7 cm from the cathode and gave a single spot at a distance of 5.2 cm from the origin towards the anode. Toluidine blue reagent was used for detection.

Polyacrylamide Gel Electrophoresis This was carried out in an apparatus with gel tubes (4 × 145 mm each) and 0.005 M Tris–glycine buffer (pH 8.3) at 5 mA/tube for 40 min. Gels were stained using the periodate-Schiff (PAS) procedure. The sample gave a clear band at a distance of 11.2 cm from the origin. Toluidine blue reagent was also used for detection.

Molecular Mass The sample (3 mg) was dissolved in 0.1 M Tris–HCl buffer (pH 7.0), and applied to a column (2.6 × 93 cm) of Toyopearl HW-65F, preequilibrated and developed with the same buffer. Fractions of 5 ml were collected and analyzed by the phenol–sulfuric acid method. Standard pullulans having known molecular masses were run on the column to obtain a calibration curve.

Phagocytic Activity This was measured as described in a previous report.[1] The phagocytic index, K, was calculated by means of the following equation:

$$K = (\ln OD_1 - \ln OD_2)/(t_2 - t_1)$$

where OD_1 and OD_2 are the optical densities at times t_1 and t_2, respectively. Results were expressed as the arithmetic mean ± S.D. of five male mice (ICR-SPF).

Qualitative Analysis of Component Sugars Hydrolysis and cellulose thin-layer chromatography (TLC) of component sugars were performed as described in a previous report.[20] The configurations of component neutral sugars were identified by GC of the trimethylsilylated α-methylbenzylaminoalditol derivatives.[21]

Determination of Component Sugars Neutral sugars were analyzed by GC after conversion of the hydrolyzate into alditol acetates as described in a previous report.[1] Rhamnose was also estimated by the thioglycolic acid method.[22] Galacturonic acid was determined by a modification of the carbazole method.[23]

Determination of *O*-Acetyl Groups The sample was hydrolyzed with 0.2 N hydrochloric acid and analyzed by GC using propionic acid as an internal standard as described in a previous report.[24]

Determination of *O*-Methyl Groups in Methyl Esters This was performed by GC after saponification using ethanol as an internal standard as described in a previous report.[13]

Reduction of Carboxyl Groups This was carried out with 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate and sodium borohydride as described in a previous report.[25] The reduction was repeated three times under the same conditions. Yield was 18 mg from 50 mg of the sample.

Methylation This was performed with methylsulfinyl carbanion and methyl iodide in dimethyl sulfoxide as described in a previous report.[26] The methylation was repeated three times under the same conditions. Yield was 9.8 mg from 10 mg of the original sample, and 4.6 mg from 5 mg of the carboxyl-reduced sample.

Analysis of the Methylated Products The products were hydrolyzed with dilute sulfuric acid in acetic acid, then reduced and acetylated in the manner described in a previous report.[10] The partially methylated alditol acetates obtained were analyzed by GC-MS with a fused silica capillary column (0.32 mm i.d. × 30 m) of Supelco SP-2330 and with a programmed temperature increase of 4 °C per min from 160 to 220 °C at a helium flow of 1 ml per min. The relative retention times of the products with respect to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol in GC and the main fragments in MS are listed in Table I.

Periodate Oxidation The sample (29 mg) was dissolved in 0.01 N sodium hydroxide (6 ml) and kept for 10 min at room temperature, then the solution was neutralized with 1 M acetic acid. The resulting solution was diluted with water up to 8 ml. After addition of 0.1 M sodium metaperiodate (8 ml), the solution was kept at 5 °C in the dark. The

Table I. Relative Retention Times on GC and Main Fragments in MS of Partially Methylated Alditol Acetates

	Relative retention time[a]	Main fragments (*m/z*)
1,4-Ac-2,3,5-Me-L-arabinitol	0.69	43, 45, 71, 87, 101, 117, 129, 161
1,4,5-Ac-2,3-Me-L-arabinitol	1.13	43, 87, 101, 117, 129, 189
1,3,4,5-Ac-2-Me-L-arabinitol	1.40	43, 117, 261
1,2,5-Ac-3,4-Me-L-rhamnitol	0.96	43, 89, 129, 131, 189
1,2,4,5-Ac-3-Me-L-rhamnitol	1.29	43, 87, 101, 129, 143, 189, 203
1,5-Ac-2,3,4,6-Me-D-galactitol	1.09	43, 45, 71, 87, 101, 117, 129, 145, 161, 205
1,4,5-Ac-2,3,6-Me-D-galactitol	1.44	43, 45, 87, 99, 101, 113, 117, 233
1,3,4,5-Ac-2,6-Me-D-galactitol	1.64	43, 45, 87, 117, 129

a) Relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. Abbreviations: Ac = acetyl; Me = methyl (*e.g.*, 1,4-Ac-2,3,5-Me- = 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methyl-).

periodate consumption was measured by a spectrophotometric method.[27] The oxidation was completed after 3 d, and the maximal value of consumption was 0.77 mol per mol of anhydrosugar unit. The reaction mixture was successively treated with ethylene glycol (0.16 ml) at 5 °C for 1 h and sodium borohydride (150 mg) at 5 °C for 16 h, then adjusted to pH 5 by addition of acetic acid. The solution was concentrated and applied to a column (2.6 × 97 cm) of Sephadex G-25. The column was eluted with water, and fractions of 10 ml were collected. The eluates obtained from tubes 22 to 26 were combined, concentrated and lyophilized. Yield, 25.8 mg.

Controlled Degradation of the Product The product (19 mg) was dissolved in 0.5 N sulfuric acid (1.9 ml). After standing at 22 °C for 16 h, the solution was neutralized with barium carbonate and filtered. The filtrate was concentrated and passed through a column (0.7 × 2 cm) of Dowex 50W-X8 (H^+). The eluate with water was concentrated and applied to a column (2.6 × 92 cm) of Sephadex G-25. The column was eluted with water, and fractions of 5 ml were collected. The eluates obtained from tubes 40 to 43 were combined, concentrated and lyophilized. Yield, 2 mg.

Partial Hydrolysis and Isolation of Oligosaccharides The sample (6 mg) was dissolved in 2 M trifluoroacetic acid (1 ml) and heated at 100 °C for 3 h in a sealed tube. The solution was evaporated for the removal of acid, then the residue was dissolved in water and applied to a column (1 × 5 cm) of DEAE-Sephadex A-25 (formate form). The column was eluted successively with water (20 ml), 0.1 M formic acid (40 ml) and 0.2 M formic acid (30 ml). Fractions of 10 ml were collected and analyzed by TLC. Fraction 1 was obtained from tubes 1 and 2, fr. 2 from tube 4, fr. 3 from tube 5, and fr. 4 from tubes 8 and 9. Neutral component sugars were found in fr. 1, and galacturonic acid was present in fr. 3. Fractions 2 and 4 afforded OS-I and OS-II, respectively.

Analysis of Oligosaccharides TLC was carried out on Merck precoated Kieselgel 60 plates using *n*-butanol : acetic acid : water (2 : 1 : 1, v/v) as a developing solvent. *Rf* values of OS-I and OS-II were 0.39 and 0.32, respectively. The trimethylsilyl (TMS) derivative of OS-I was prepared in the usual way, and GC of the derivative was performed with a column (3 mm × 1 m long spiral glass) packed with 2% OV-17 on Gaschrom Q (80 to 100 mesh) and with a flow of 50 ml per min of helium. The programmed temperature was increased at 3 °C per min from 180 to 270 °C. Retention times were 17.3 and 18.0 min. Analysis of component sugars and methylation analysis of the reduction product[28] were performed as described above. The results are also listed in Table I.

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