The Major Pectic Arabinogalactan Having Activity on the Reticuloendothelial System from the Roots and Rhizomes of *Saposhnikovia divaricata*

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The major acidic polysaccharide, named saposhnikovan A, was isolated from the roots and rhizomes of *Saposhnikovia divaricata*. It showed remarkable reticuloendothelial system-potentiating activity in a carbon clearance test. It is composed of L-arabinose: D-galactose: D-galacturonic acid in the molar ratio of 6:15:10, and its molecular weight was estimated to be 54000. About 35% of the D-galacturonic acid residues exist as the methyl esters. Methylation analysis, ^{13}C-nuclear magnetic resonance, and controlled Smith degradation studies indicated that the polysaccharide has the α-1→4-linked D-galacturonan backbone bearing α-1→5-linked L-arabino-β-3,6-branched D-galactan side chains.

Keywords *Saposhnikovia divaricata*; polysaccharide; saposhnikovan A; pectic arabino-3,6-galactan; immunological activity; reticuloendothelial system; methylation analysis; controlled Smith degradation; structural feature

The dried root and rhizome of *Saposhnikovia divaricata* SCHISCHK. (*Ledebouriella seseloides* WOLFF, Umbelliferae) are a well-known Oriental crude drug used as a diaphoretic, an antipyretic and an analgesic in the treatment of cold and its concomitant headache and arthralgia under the name of Fangfeng in China (Japanese name, Boufuh).

As the constituents of this crude drug, nine chromones and five coumarins[1] and three polyacetylene compounds[2] have been reported, and the contents of lead, cadmium, copper, manganese and calcium were determined.[3] However, no study on the water-soluble organic polymers has been reported so far. Recently, we found *myo*-inositol hexaphosphate as a useful indicator for identification of this crude drug.[4] Further, we observed indications of the presence of pectic substances in the electrophoretic pattern of the hot water extract of Saposhnikoviae Radix.[4] We have now isolated a pure acidic polysaccharide from this crude drug. This substance showed immunological activity, and was characteristic of the crude drug in the identification method described above.

The material was sliced, homogenized and extracted with hot water. After treatment with cetyltrimethylammonium bromide in dilute sodium sulfate solution, the resulting precipitate was dissolved in sodium chloride solution. After addition of ethanol, the precipitate obtained was dissolved in water, reprecipitated with ethanol, redissolved in water and applied to a column of Sephadex G-50. The polysaccharide fraction was applied to a column of diethylaminoethyl (DEAE)-Sephadex A-25 (acetate form). After elution with water and 0.2 M acetate buffer, the eluate with 0.5 M acetate buffer was dialyzed and purified by gel chromatography on Sephacryl S-300 followed by dialysis and chromatography on Sephadex G-25.

The purified polysaccharide gave a single band on polyacrylamide gel electrophoresis and gave a single spot on cellulose acetate membrane electrophoresis. Further, it gave a single peak on gel chromatography on Sephacryl S-300 and Cellulofine GCL-2000 m. The polysaccharide showed a positive specific rotation ($[\alpha]_D^{24}+32.1°$ in H_2O, $c=0.5$). Gel chromatography using standard pullulans gave a value of 54000 for the molecular weight. The name saposhnikovan A is proposed for this substance.

As component sugars of it, L-arabinose, D-galactose, and D-galacturonic acid were identified. Quantitative analyses showed that the molar ratio of arabinose: galactose: galacturonic acid is 6:15:10. It contained no nitrogen.

The ^{13}C-nuclear magnetic resonance (^{13}C-NMR) spectrum of the polysaccharide showed a signal at δ 55.499 ppm, suggesting the presence of *O*-methyl groups as carboxylic acid methyl esters. The presence of this group was confirmed by gas chromatography (GC) of the hydrolyzate, and the methoxyl content was determined to be 2.1%. Thus about 35% of the galacturonic acid residues in the polysaccharide exist as methyl esters.

In addition, the ^{13}C-NMR spectrum showed five signals due to anomeric carbons at δ 111.862, 110.081, 106.115, 105.306 and 102.770 ppm. The first two signals were assigned to the anomeric carbons of α-L-arabinofuranose[5,6] and the signals at 106.115 and 105.306 to the anomeric carbons of β-D-galactopyranose residues.[6,7] The signal at 102.770 was assigned to the anomeric carbon of α-D-galactopyranosyluronic acid.[8]

The carboxyl groups of galacturonic acid residues in the polysaccharide were reduced with a carbodiimide reagent and sodium borohydride to give the corresponding neutral sugar residues.[9] Both the original polysaccharide and the carboxyl-reduced derivative were methylated with methylsulfinyl carbanion and methyl iodide in dimethyl sulfoxide.[10] The methylated products were hydrolyzed, then converted into the partially methylated alditol acetates. Methyl ethers of galacturonic acid were removed from the hydrolysis products of the methylated original polysaccharide by treatment with an anion-exchange resin. Gas chromatography–mass spectrometry (GC-MS) revealed derivatives of 2,3,5-tri-*O*-methyl-L-arabinose, 2,3-di-*O*-methyl-L-arabinose, 2,4,6-tri-*O*-methyl-D-galactose, and 2,4-di-*O*-methyl-D-galactose as the products from the methylated original polysaccharide in a molar ratio of 6:6:27:3. The carboxyl-reduced derivative gave 2,3,5-tri-*O*-methyl-L-arabinose, 2,3-di-*O*-methyl-L-arabinose, 2,4,6-tri-*O*-methyl-D-galactose, 2,3,6-tri-*O*-methyl-D-galactose, 3,6-di-*O*-methyl-D-galactose, 2,6-di-*O*-methyl-D-galactose, and 2,4-di-*O*-methyl-D-galactose in a molar ratio of 6:6:27:17:1:2:3.

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

(six)[a] α-L-Araf 1→ (seventeen)[a] →4 α-D-GalpA(Me) 1→
(sic)[a] →5 α-L-Araf 1→
(one)[a] →4 α-D-GalpA(Me) 1→
2
(twenty-seven)[a] →3 β-D-Galp 1→ ↑
(two)[a] →4 α-D-GalpA(Me) 1→
3
(three)[a] →6 β-D-Galp 1→ ↑
3
↑

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

a) Number of residues. Ara*f*, arabinofuranose; Gal*p*, galactopyranose; Gal*p*A, galactopyranosyluronic acid; Gal*p*A(Me), galactopyranosyluronic acid residues having partial methyl esterification.

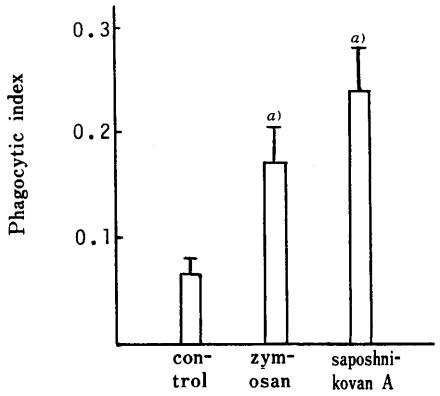

Fig. 1. Effect of Saposhnikovan A on Carbon Clearance Index in ICR Mice

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

The controlled Smith degradation[11] of saposhnikovan A by periodate oxidation and reduction followed by mild hydrolysis yielded a polymer composed of D-galactose and D-galacturonic acid in a molar ratio of 10 : 1. Its molecular weight was 29000. Thus, Smith degradation of saposhnikovan A resulted in complete elimination of L-arabinose residues and the linear parts of D-galacturonic acid residues in the molecule.

Methylation of the controlled smith degradation product was performed as described above, and the methylated product was hydrolyzed and treated with anion-exchange resin. Then the products were converted into partially methylated alditol acetates by reduction and acetylation. GC-MS revealed derivatives of 2,3,4,6-tetra-*O*-methyl-D-galactose, 2,4,6-tri-*O*-methyl-D-galactose, 2,3,4-tri-*O*-methyl-D-galactose, and 2,4-di-*O*-methyl-D-galactose in a molar ratio of 3 : 15 : 1 : 1.

The results of methylation analysis of the polysaccharide and the controlled Smith degradation revealed the presence of a backbone chain composed of α-1→4-linked D-galacturonan. Some of the D-galacturonic acid residues in the backbone carry arabinogalactan side chains at positions 2 and 3. The side chains are mainly composed of β-1→3-linked D-galactopyranosyl units containing 3,6-branched units, and α-1→5-linked L-arabinofuranosyl residues occupy terminal positions.

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

The effect of saposhnikovan A on the reticuloendothelial system (RES) was demonstrated by the *in vivo* carbon clearance test.[12] As shown in Fig. 1, the phagocytic index was remarkably increased, suggesting the activation of RES by i.p. injection of the polysaccharide.

Saposhnikovan A possesses a backbone chain consisting of α-1→4-linked D-galacturonic acid residues which exhibit about 35% carboxyl-methyl esterification. However it has, unlike usual pectins, no interspersed L-rhamnose residues. In typical pectins[13] and many rhamnogalacturonans,[14] α-1→2-linked L-rhamnose residues bear neutral sugar side chains at position 4. In contrast to these groups, side chains composed of arabinogalactan are linked to positions 2 or 3 of α-1→4-linked D-galacturonic acid residues in saposhnikovan A. This is a characteristic feature of this polysaccharide. Based on the molecular weight of the controlled Smith degradation product, it can be presumed that the branching galacturonic acid units are adjacent to each other. In part, terminal L-arabinose or α-1→5-linked L-arabinosyl chain must be directly linked to position 3 of the branching D-galactose unit because of the appearance of 2,3,4-tri-*O*-methyl-D-galactose on methylation analysis of the controlled Smith degradation product.

A reticuloendothelial system-activating arabinogalactan named sanchinan-A was isolated from the roots of *Panax notoginseng*.[15] This substance has a β-1→3-linked D-galactopyranosyl backbone and β-1→6- and β-1→3-linked D-galactopyranosyl side chains with α-L-arabinofuranosyl or β-D-galactopyranosyl terminals. As other examples of plant polysaccharides having a phagocytosis-enhancing effect, two substances were obtained from the herbal part of *Eupatorium cannabium* and *Eupatorium perfoliatum*.[16] They were identified as 4-methylglucuronoxylans, and fur-

backbone chain:
[→4 α-D-GalpA(Me) 1→]$_x$ [→4 α-D-GalpA(Me) 1→]$_y$ [→4 α-D-GalpA(Me) 1→]$_z$
2 3
↑ ↑
side chain side chain

side chains:
α-L-Araf 1→[5 α-L-Araf 1]$_b$
↓
3
[β-D-Galp]$_d$
1
↓
6
α-L-Araf 1→[5 α-L-Araf 1→]$_a$ [3 β-D-Galp 1→]$_c$ 3 β-D-Galp 1→[3 β-D-Galp 1→]$_e$

Chart 2. Structural Units of Saposhnikovan A

$x:y:z=17:1:2$, $a+b=2$, $c+d+e=9$.

ther, an active fucogalactoxyloglucan was isolated from *Echinacea purpurea* cell cultures.[17] In addition, microorganisms produce various macromolecules having immunological activities, such as glucans, mannans, chitins, lipopolysaccharides, and peptidoglycans.[18] Saposhnikovan A is a new structural type of polysaccharide having remarkable activity on the reticuloendothelial system.

Further investigations of the relationship between the biological activities and structural features are in progress.

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 spectrum was recorded on a JEOL JNM-GX 270 FT NMR spectrometer in heavy water containing acetone 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 performed with a JEOL JMS-GX mass spectrometer.

Isolation of Polysaccharide The crude drug (855 g) was sliced and extracted with water (8550 ml) under stirring in a boiling water bath for 30 min. After filtration, the residue was extracted with water (5130 ml) under the same conditions. The filtrates were combined, and 1% sodium sulfate (91 ml) was added to the filtrate (9100 ml), followed by 5% cetyltrimethylammonium bromide (580 ml). The precipitate was separated by centrifugation, then dissolved in 0.5 M sodium chloride (280 ml). The solution was poured into two volumes of ethanol. The resulting precipitate was treated with 80% ethanol (200 ml), and after centrifugation, the precipitate was dried *in vacuo*. The yield of this crude extract was 3.75 g. The crude extract (375 mg) was dissolved in water and applied to a column (5 × 86 cm) of Sephadex G-50. Elution was carried out with water, and fractions of 20 ml were collected. The eluates obtained from tubes 29 to 43 were combined, concentrated and lyophilized. Yield, 173 mg. This high-molecular-weight fraction (1.73 g) 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.[19] After elution with water (1000 ml) and 0.2 M acetate buffer (pH 5.0, 1100 ml), the column was eluted with 0.5 M acetate buffer (pH 5.0, 1160 ml) and 1 M acetate buffer (pH 5.0, 1100 ml). Fractions of 20 ml were collected and analyzed by the phenol–sulfuric acid method.[20] The eluates obtained from tubes 130 to 145 were combined, dialyzed against distilled water, then 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 45 were combined, concentrated and lyophilized. Yield, 353 mg. This fraction (80 mg) was dissolved in 0.1 M Tris–HCl buffer (pH 7.0) and applied to a column (5 × 83 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 37 to 43 were combined, dialyzed, concentrated and applied to a column of Sephadex G-25 as described above. The eluates containing a polysaccharide were combined, concentrated and lyophilized. Saposhnikovan A (31 mg) was obtained as a white powder. It contains no nitrogen.

Polyacrylamide Gel Electrophoresis This was carried out in an apparatus with gel tubes (4 × 130 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 6.3 cm from the origin. Toluidine blue reagent[21] was also used for detection.

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 8.7 cm from the origin towards the anode. Toluidine blue reagent was used for detection.

Gel Chromatography The sample (3 mg) was dissolved in 0.1 M Tris–HCl buffer (pH 7.0), and applied to a column (2.6 × 96 cm) of Sephacryl S-400, pre-equilibrated and developed with the same buffer. A column of Cellulofine GCL-2000-m (2.6 × 94 cm, Seikagaku Kogyo Co.) was also used, and this column was eluted with 0.05 M phosphate buffer containing 0.1 M NaCl (pH 7.5). Fractions of 5 ml were collected and analyzed by the phenol–sulfuric acid method. Standard pullulans (Shodex standard P-82, Showa Denko Co.) having known molecular weights were run on the column to obtain a calibration curve.

Qualitative Analysis of Component Sugars Hydrolysis and cellulose thin-layer chromatography (TLC) of component sugars were performed as described in a previous report.[22] The configurations of component sugars were identified by GC of the trimethylsilylated α-methylbenzylaminoalditol derivatives.[23]

Determination of Component Sugars The sample was hydrolyzed as described above, and after neutralization, the filtrate was reduced with sodium borohydride for 2 h. After neutralization with Dowex 50W-X8 (H^+), boric acid was removed by repeated addition and evaporation of methanol. The product was acetylated with acetic anhydride–pyridine mixture (1 : 1) at 100 °C for 1 h. After evaporation, the residue was dissolved in chloroform–methanol mixture (1 : 1) and subjected to GC. GC was carried out with a fused silica capillary column (0.53 mm i.d. × 15 m) of SP 2380 (Supelco Co.) and with programmed temperature increase of 3 °C per min from 160 to 200 °C at a helium flow of 10 ml per min. Allose was used as an internal standard for the determination of neutral sugars. Galacturonic acid was estimated by a modification of the carbazole method.[24]

Determination of *O*-Methyl Groups in Methyl Esters This was performed by GC after saponification 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 24 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 twice under the same conditions. Yield was 9.8 mg from 10.4 mg of the carboxyl-reduced sample, and 4.8 mg from 10 mg of the original 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.[27] GC-MS of partially methylated alditol acetates was performed with a fused capillary column (0.32 mm i.d. × 30 m) of SP 2330 (Supelco Co.) 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 (122 mg) was oxidized with 0.05 M sodium metaperiodate (60 ml) at 5 °C in the dark. The periodate consumption was measured by a spectrophotometric method.[28] The oxidation was completed after 3 d, and the maximal value of consumption was 0.78 mol per mol of anhydrosugar unit. The reaction mixture was successively treated with ethylene glycol (0.6 ml) at 5 °C for 1 h and sodium borohydride (620 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 (5 × 78 cm) of Sephadex G-25. The column was eluted with water, and fractions of 20 ml were collected. The eluates obtained from tubes 29 to 34 were combined, concentrated and lyophilized. Yield, 100 mg.

Controlled Degradation of the Product The product (91 mg) was dissolved in 0.5 N sulfuric acid (9.1 ml). After standing at 24 °C for 17 h, 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
1,5-Ac-2,3,4,6-Me-D-galactitol	1.09	43, 45, 71, 87, 101, 117, 129, 145, 161, 205
1,3,5-Ac-2,4,6-Me-D-galactitol	1.36	43, 45, 87, 101, 117, 129, 161
1,4,5-Ac-2,3,6-Me-D-galactitol	1.43	43, 45, 87, 99, 101, 113, 117, 233
1,5,6-Ac-2,3,4-Me-D-galactitol	1.58	43, 87, 99, 101, 117, 129, 161, 189
1,3,4,5-Ac-2,6-Me-D-galactitol	1.62	43, 45, 87, 117, 129
1,2,4,5-Ac-3,6-Me-D-galactitol	1.74	43, 45, 87, 99, 113, 129, 189, 233
1,3,5,6-Ac-2,4-Me-D-galactitol	2.00	43, 87, 117, 129, 189

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-).

solution was neutralized with barium carbonate and filtered. The filtrate was concentrated and passed through a column (0.7 × 5 cm) of Dowex 50W-X8 (H^+). The eluate with water was concentrated and applied to a column (5 × 86 cm) of Sephadex G-25. The column was eluted with water, and fractions of 20 ml were collected. The eluates obtained from tubes 31 to 35 were combined, concentrated and applied to a column (5 × 89 cm) of Sephadex G-50. The column was eluted with water, and fractions of 20 ml were collected. The eluates obtained from tubes 28 to 33 were combined, concentrated and lyophilized. Yield, 35 mg.

Methylation Analysis of the Degradation Product This was carried out as described above. 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.

Phagocytic Activity Male mice (ICR-SPF, 25—30 g) were used in groups of five. The sample and a positive control, zymosan, were each dissolved in physiological saline and dosed i.p. (50 μg/g body weight) once a day. At 48 h after a 5 d administration period, mice were injected *via* the tail vein with colloidal carbon (Pelican AG, Germany). The ink was diluted eight times with phosphate-buffered saline containing 1% gelatine before use, and the amount of the resulting solution used was 10 μl/g body weight. Blood samples were drawn from the orbital vein at 0,3,6,9,12, and 15 min. The blood (25 μl) was dissolved in 0.1% sodium carbonate (2 ml) and the absorbance at 660 nm was determined. 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 mice.

References

1) H. Sasaki, H. Taguchi, T. Endo and I. Yosioka, *Chem. Pharm. Bull.*, **30**, 3555 (1982).
2) K. Baba, Y. Tabata, M. Kozawa, Y. Kimura and S. Arichi, *Shoyakugaku Zasshi*, **41**, 189 (1987).
3) Y. Zhu, *Yaowu Fenxi Zazhi*, **3**, 175, 244 (1983) [*Chem. Abstr.*, **99**, 128449*q*, 128452*k* (1983)]; C. Cai and X. Guan, *Zhongcaoyao*, **15**, 61 (1984) [*Chem. Abstr.*, **100**, 145056*r* (1984)].
4) N. Shimizu and M. Tomoda, *Chem. Pharm. Bull.*, **35**, 3918 (1987).
5) J.-P. Joseleau, G. Chambat, M. Vignon and F. Barnoud, *Carbohydr. Res.*, **58**, 165 (1977).
6) N. Shimizu and M. Tomoda, *Chem. Pharm. Bull.*, **35**, 4981 (1987).
7) K. Bock, C. Pedersen and H. Pedersen, "Advances in Carbohydrate Chemistry and Biochemistry," Vol. 42, ed. by R. S. Tipson and D. Horton, Academic Press, Inc., Orland, 1984, pp. 193—214.
8) N. Shimizu and M. Tomoda, *Chem. Pharm. Bull.*, **33**, 5539 (1985).
9) R. L. Taylor and H. E. Conrad, *Biochemistry*, **11**, 1383 (1972).
10) S. Hakomori, *J. Biochem.* (Tokyo), **55**, 205 (1964).
11) I. J. Goldstein, G. W. Hay, B. A. Lewis and F. Smith, "Methods in Carbohydrate Chemistry," Vol. 5, ed. by R. L. Whistler, Academic Press, New York and London, 1965, pp. 361—370.
12) G. Biozzi, B. Benacerraf and B. N. Halpern, *Br. J. Exp. Pathol.*, **34**, 441 (1953).
13) M. Tomoda, M. Ichikawa and N. Shimizu, *Chem. Pharm. Bull.*, **34**, 4992 (1986).
14) A. M. Stephen, "The Polysaccharides," Vol. 2, ed. by G. O. Aspinall, Academic Press, New York and London, 1983, pp. 158—166.
15) K. Ohtani, K. Mizutani, S. Hatono, R. Kasai, R. Sumino, T. Shiota, M. Ushijima, J. Zhou, T. Fuwa and O. Tanaka, *Planta Med.*, **53**, 166 (1987).
16) A. Vollmar, W. Schäfer and H. Wagner, *Phytochemistry*, **25**, 377 (1986).
17) H. Wagner, H. Stuppner, W. Schäfer and M. Zenk, *Phytochemistry*, **27**, 119 (1988).
18) N. Ohno, H. Shinohara and T. Yadomae, *Chem. Pharm. Bull.*, **34**, 5071 (1986).
19) N. Shimizu and M. Tomoda, *Chem. Pharm. Bull.*, **31**, 499 (1983).
20) M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers and F. Smith, *Anal. Chem.*, **28**, 350 (1956).
21) N. Seno, K. Anno, K. Kondo, S. Nagase and S. Saito, *Anal. Biochem.*, **37**, 197 (1970).
22) M. Tomoda, S. Kaneko, M. Ebashi and T. Nagakura, *Chem. Pharm. Bull.*, **25**, 1357 (1977).
23) R. Oshima, J. Kumanotani and C. Watanabe, *J. Chromatogr.*, **259**, 159 (1983).
24) T. Bitter and H. M. Muir, *Anal. Biochem.*, **4**, 330 (1962).
25) M. Tomoda and M. Ichikawa, *Chem. Pharm. Bull.*, **35**, 2360 (1987).
26) N. Shimizu, M. Tomoda and M. Adachi, *Chem. Pharm. Bull.*, **34**, 4133 (1986).
27) M. Tomoda, K. Shimada, Y. Saito and M. Sugi, *Chem. Pharm. Bull.*, **28**, 2933 (1980).
28) G. O. Aspinall and R. J. Ferrier, *Chem. Ind.* (London), **1957**, 1216.