A Reticuloendothelial System-Activating Arabinoxylan from the Bark of *Cinnamomum cassia*

Mieko KANARI, Masashi TOMODA,* Ryōko GONDA, Noriko SHIMIZU, Miyako KIMURA, Masami KAWAGUCHI and Chiori KAWABE

Kyoritsu College of Pharmacy, Shibakōen, Minato-ku, Tokyo 105, Japan. *Received April 3, 1989*

A neutral polysaccharide, named cinnaman AX, was isolated from the dried bark of *Cinnamomum cassia* BLUME. It was homogeneous on electrophoresis and gel chromatography. It is composed of L-arabinose : D-xylose in the molar ratio of 4 : 3, and its molecular weight was estimated to be about 1.0×10^6. Methylation analysis, carbon-13 nuclear magnetic resonance and controlled Smith degradation studies enabled elucidation of its structural features. It showed remarkable reticuloendothelial system-potentiating activity in a carbon clearance test.

Keywords *Cinnamomum cassia*; bark; Cinnamomi Cortex; cinnaman AX; polysaccharide structure; arabinoxylan; methylation analysis; Smith degradation; reticuloendothelial system; immunological activity

As reticuloendothelial system (RES)-activating polysaccharides from Oriental crude drugs, sanchinan A from the root of *Panax notoginseng*[1] and saposhnikovan A from the root and rhizome of *Saposhnikovia divaricata*[2] have recently been reported. In addition, the effect of crude polysaccharide fraction obtained from the dried bark of *Cinnamomum cassia* on RES was reported by Kinoshita *et al.*[3]

The dried bark of *Cinnamomum cassia* BLUME (Lauraceae), the crude drug Cinnamomi Cortex, is frequently used for treatment of inflammation, headache and pyrexia. Many components of this crude drug have been identified, but no pure polysaccharide has been known so far on both structure and biological activity. We have now isolated a pure neutral polysaccharide having high immunological activity on the RES from the water extract of this crude drug. Its properties and structural features are reported here.

The crude polysaccharide fraction was isolated from the bark by hot water extraction followed by precipitation with ethanol, then dissolved again in water. The solution was treated with sodium lauryl sulfate and sodium chloride. After centrifugation, the supernatant obtained was poured into acetone. The precipitate obtained was dissolved in dilute sodium hydroxide and the solution was successively applied to columns of Toyopearl HW-65F and Sephacryl S-500. The high-molecular weight fraction of the eluate was dialyzed and purified by gel chromatography with Cellulofine GCL-25 m, then the eluate was lyophilized.

The purified polysaccharide gave a single spot on glass-fiber paper electrophoresis. Further, it gave a single peak on gel chromatography with Toyopearl HW-75F. The polysaccharide showed a negative specific rotation ($[\alpha]_D^{24}$ $-78.1°$ in H_2O, $c = 0.1$). Gel chromatography using standard dextrans gave a value of 1.0×10^6 for the molecular weight. The name cinnaman AX is proposed for this substance.

As component sugars, L-arabinose and D-xylose were identified. Quantitative analysis showed that the polysaccharide contained 57.5% arabinose and 42.5% xylose, and that their molar ratio was 4 : 3. It contained no nitrogen.

Methylation of the polysaccharide was performed with methylsulfinyl carbanion and methyl iodide in dimethyl sulfoxide.[4] The methylated product was hydrolyzed, then converted into the partially methylated alditol acetates. Gas chromatography-mass spectrometry (GC-MS)[5] revealed derivatives of 2,3,5-tri-*O*-methyl-L-arabinose, 2,3,4-tri-*O*-methyl-L-arabinose, 2,4-di-*O*-methyl-L-arabinose, 2,3-di-*O*-methyl-D-xylose and 2-*O*-methyl-D-xylose in a molar ratio of 2.0 : 0.1 : 2.0 : 1.0 : 2.1.

The carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum of the polysaccharide showed three signals due to anomeric carbons at δ 110.84, 103.74 and 102.07 ppm. The signals at δ 110.84 and 102.07 were assigned to the anomeric carbons of α-L-arabinofuranose and β-L-arabinopyranose residues, respectively.[6] The signal at δ 103.74 was assigned to the anomeric carbon of β-D-xylopyranose.[7]

The polysaccharide was subjected to periodate oxidation followed by reduction with sodium borohydride. The component sugar analysis of the product showed that a half of the arabinose units and two-thirds of the xylose units had survived after periodate oxidation.

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

The controlled Smith degradation[8] of cinnaman AX was performed by mild hydrolysis of the periodate oxidation-reduction product. It afforded five products (SD-A1 to SD-A5), and each of them was composed of L-arabinose and D-xylose in approximately equimolar ratio. Gel chromatography using standard pullulans gave values of 2.0×10^5, 1.6×10^5, 1.3×10^5, 7.7×10^4 and 3.8×10^4 for the molecular weights of SD-A1, -A2, -A3, -A4 and -A5. The ratio of their yields was about 8 : 2 : 2 : 5 : 4. On further controlled Smith degradation of these products, SD-A1 to SD-A5, they gave SD-B1 to SD-B5, respectively. D-Xylose was the only component sugar, and their molecular weights were 2600, 3500, 3500, 3500 and 2600, respectively. On further periodate oxidation, their component sugar units completely disappeared.

Cinnaman AX was partially hydrolyzed with dilute trifluoroacetic acid under a mild condition. After removal of

twenty[a)] α-L-Ara*f* 1→ ten[a)] →4 β-D-Xyl*p* 1→

one[a)] β-L-Ara*p* 1→ twenty-one[a)] →4 β-D-Xyl*p* 1→

3

↑

twenty[a)] →3 β-L-Ara*p* 1→

Chart 1. Component Sugar Residues in the Minimal Repeating Unit in the Structure of Cinnaman AX

a) Number of residues. Ara*f*, arabinofuranose; Ara*p*, arabinopyranose; Xyl*p*, xylopyranose.

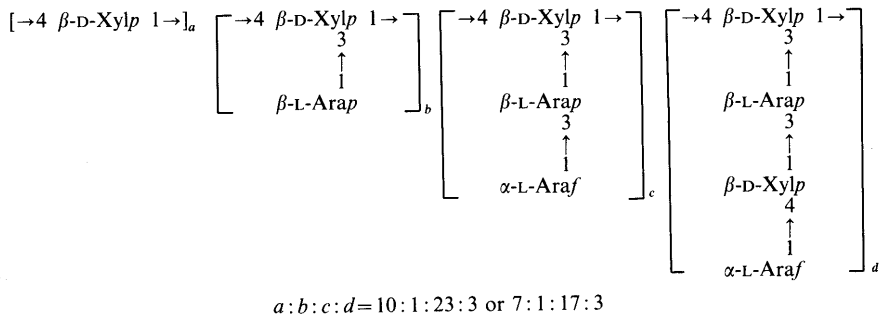

Chart 2. Possible Structural Fragments of Cinnaman AX

the acid, the products were isolated by chromatography with Sephadex G-15. In addition to arabinose, a disaccharide (DPI) and a polysaccharide (DPII) were obtained. The approximate ratio of their yields was 2:1:12. Component sugar analysis and methylation analysis revealed that the disaccharide (DPI) was 3-*O*-D-xylopyranosyl L-arabinose. The polysaccharide (DPII) was composed of L-arabinose and D-xylose in almost equimolar ratio. Methylation analysis of DPII revealed derivatives of 2,3,5-tri-*O*-methyl-L-arabinose, 2,4-di-*O*-methyl-L-arabinose, 2,3-di-*O*-methyl-D-xylose and 2-*O*-methyl-D-xylose in approximately equimolar ratio.

Based on these results, it can be concluded that cinnaman AX possesses a backbone chain composed of β-1→4-linked D-xylopyranose units. About seventy per cent of the D-xylose residues in the backbone carry side chains composed of α-L-arabinofuranosyl-(1→3)-β-L-arabinopyranose and α-L-arabinofuranosyl-(1→4)-β-D-xylopyranosyl-(1→3)-β-L-arabinopyranose units at position 3. From the observed lower stability of arabinofuranoside linkages, it can be presumed that DPI was produced from the trisaccharide side chain units by the mild hydrolysis. These branchings must be continuously present in relatively high degree. The distinguishing decrease of molecular weights of the second controlled Smith degradation products showed that the backbone chain has some limited branches composed of one terminal unit in addition to the major branches composed of disaccharide and trisaccharide side chains. From the values of molecular weight of the second degradation products, SD-B1 to SD-B5, and the results of methylation analysis, it seems likely that there is one branching unit having a terminal β-L-arabinopyranose per twenty or twenty-six branching residues bearing the disaccharide and trisaccharide side chains. Thus cinnaman AX has the structural features shown in Chart 2.

The effect of cinnaman AX on the RES was demonstrated by a modification of the *in vivo* carbon clearance test[9] using zymosan as a positive control. As shown in Fig. 1, the phagocytic index was remarkably increased, suggesting powerful activation of the RES by i.p. injection of the polysaccharide. The other polysaccharide fraction (fr. B, composed of arabinose, xylose, glucose, galactose, rhamnose and galacturonic acid) obtained by chromatography with Toyopearl HW-65F showed almost equivalent activity to that of zymosan (Fig. 1). However, the value was much lower than that of cinnaman AX.

An RES activating polysaccharide named sanchinan A from the root of *Panax notoginseng*[1] has a β-1→3-linked D-galactopyranosyl backbone and β-1→6- and β-1→3-linked

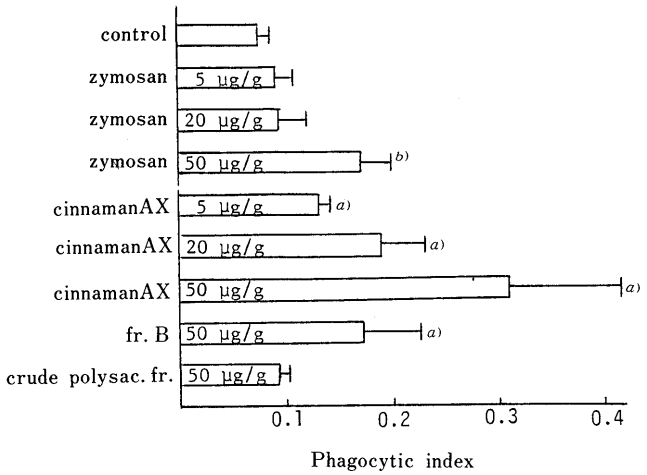

Fig. 1. Effects of Cinnaman AX and Fr. B on Carbon Clearance Index in ICR Mice

Significantly different from the control, *a*) $p<0.05$, *b*) $p<0.01$.

D-galactopyranosyl side chains with α-L-arabinofuranosyl or β-D-galactopyranosyl terminals. We obtained another immunologically active polysaccharide acting on the RES, named saposhnikovan A, from the root and rhizome of *Saposhnikovia divaricata*.[2] This substance possesses a backbone chain consisting of α-1→4-linked D-galacturonic acid residues which exhibit about 35% carboxyl-methyl esterification. Its side chains possessing α-1→5-linked L-arabinofuranosyl 3,6-branched β-D-galactan structure are linked to position 2 or 3 of the backbone D-galacturonic acid residues.

As other examples of polysaccharides having a phagocytosis-enhancing effect from European plants, a complex acidic arabinogalactan (Fb) isolated from *Viscum album* berry,[10] two 4-methylglucuronoxylans (PI and PII) isolated from the herbal part of *Eupatorium cannabinum* and *Eupatorium perfoliatum*,[11] and a fucogalactoxyloglucan (polysaccharide A) isolated from *Echinacea purpurea* cell culture[12] have been reported. Fb has a rhamnogalacturonan backbone and arabino-3,6-galactan type side chains. PI and PII are mainly composed of β-1→4-linked D-xylan having 4-methylglucuronic acid branches at position 2 of some of the xylose units. Polysaccharide A possesses a β-1→4-linked D-glucan backbone and substituent units or chains mainly composed of α-D-xylose residues.

Thus cinnaman AX is a new structural type of polysaccharide having a remarkable activity on the RES. 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 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 performed with a JEOL JMS-GX mass spectrometer.

Isolation of Polysaccharide The crude drug (100 g) was powdered and extracted with water (1000 ml) under stirring in a boiling water bath for 1 h. After centrifugation, the supernatant was poured into two volumes of ethanol. The resulting precipitate was dried *in vacuo*, then dissolved in water (275 ml) under stirring and heating. Sodium lauryl sulfate (5%, 55 ml) and NaCl (3.2 g) were added and dissolved into this solution. After centrifugation, the supernatant was poured into two volumes of acetone. The resulting precipitate was dried *in vacuo*, then dissolved in 0.01 N NaOH (130 ml). A 0.1 N HCl solution was added to this solution to give pH 8.0, and one-fifth of the resulting solution was applied to a column (5 × 82.5 cm) of Toyopearl HW-65F. Elution was carried out with 0.1 M Tris–HCl buffer (pH 8.0) and fractions of 20 ml were collected and analyzed by the phenol–sulfuric acid method.[13] The eluates obtained from tubes 31 to 42 were combined, dialyzed and concentrated. In addition, the eluates obtained from tubes 45 to 69 afforded another polysaccharide fraction (fr. B). The combined solution obtained by double chromatography was applied to a column (5 × 75 cm) of Sephacryl S-500. Elution was carried out with the same buffer and fractions of 20 ml were collected. The eluates obtained from tubes 26 to 33 were combined, dialyzed, concentrated and applied to a column (5 × 67 cm) of Cellulofine GCL-25 m. The column was eluted with water and fractions of 20 ml were collected. The eluates obtained from tubes 22 to 29 were combined, concentrated and lyophilized. Cinnaman AX was obtained as a white powder. Yield, 56 mg from 100 g of the material. Yield of fr. B was 54 mg.

Glass-Fiber Paper Electrophoresis This was carried out as described in a previous report[14] on Whatman GF/C glass-fiber paper at 570 V for 60 min with 0.025 M $Na_2B_4O_7 \cdot 10H_2O$–0.1 N NaOH (10 : 1, pH 9.3) and for 90 min with 0.08 M pyridine–0.04 M acetic acid (pH 5.3). Cinnaman AX gave a single spot at distances of 9.4 cm with the alkaline buffer and of 4.2 cm with the acidic buffer from the origin toward the cathode.

Molecular Weight The sample (3 mg) was dissolved in 0.1 M Tris–HCl buffer (pH 7.0), and applied to a column (2.6 × 95 cm) of Toyopearl HW-75F, preequilibrated and developed with the same buffer. Fractions of 5 ml were collected and analyzed by the phenol–sulfuric acid method. Standard dextrans having known molecular weights were run on the column to obtain a calibration curve. Fraction numbers of the peaks of dextrans 2.0×10^6, 2.7×10^5, 1.5×10^5 and cinnaman AX were 53, 63, 65 and 56.

Qualitative Analysis of Components The sample was hydrolyzed with 3.5 M trifluoroacetic acid in a sealed tube at 100 °C for 6 h followed by evaporation for removal of acid. The hydrolyzate of the sample was subjected to cellulose thin-layer chromatography (TLC) as described in a previous report.[15] The configurations of component sugars were identified by GC of the trimethylsilylated α-methylbenzylaminoalditol derivatives.[16]

Determination of Components The sample was hydrolyzed as described above, and component sugars were analyzed by GC after conversion of the hydrolyzate into alditol acetates as described in a previous report.[2] GC was carried out on a fused silica capillary column (0.53 mm i.d. × 15 m) of SP-2380 (Supelco Co.) with a 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.

Methylation This was performed with methylsulfinyl carbanion and methyl iodide in dimethyl sulfoxide as described in a previous report.[17] The methylation was repeated three times under the same conditions. Yield was 7 mg from 12 mg of the sample.

Analysis of the Methylated Product The product was hydrolyzed with dilute sulfuric acid in acetic acid, then reduced and acetylated in the manner described in a previous report.[18] The partially methylated alditol acetates obtained were analyzed by GC-MS on a fused silica capillary column (0.32 mm i.d. × 30 m) of SP-2330 (Supelco Co.) 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 (100 mg) was oxidized with 0.05 M sodium metaperiodate (50 ml) at 5 °C in the dark. The periodate consumption was measured by a spectrophotometric method.[19] The oxidation was completed after 3 d, and the maximal value of consumption was 0.44 mol per mol of anhydrosugar unit. The reaction mixture was successively treated with ethylene glycol (0.25 ml) at 5 °C for 1 h and sodium borohydride (200 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 × 80.5 cm) of Sephadex G-25. The column was eluted with water, and fractions of 20 ml were collected. The eluates obtained from tubes 30 to 36 were combined, concentrated and lyophilized. Yield, 82.3 mg.

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	0.70	43, 45, 71, 87, 101, 117, 129, 161
1,5-Ac-2,3,4-Me-L-arabinitol	0.81	43, 101, 117, 161
1,3,5-Ac-2,4-Me-L-arabinitol	1.05	43, 87, 113, 117, 233
1,5-Ac-2,3,4-Me-D-xylitol	0.84	43, 101, 117, 161
1,4,5-Ac-2,3-Me-D-xylitol	1.22	43, 87, 101, 117, 129, 189
1,3,4,5-Ac-2-Me-D-xylitol	1.54	43, 117, 261

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-).

Controlled Degradation of the Product The product (52.7 mg) was dissolved in 0.5 N sulfuric acid (6 ml). After standing at 24 °C for 16 h, the solution was neutralized with barium carbonate and filtered. The filtrate was concentrated and applied to a column (2.6 × 95 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 25 were combined, concentrated and applied to a column (2.6 × 90.5 cm) of Toyopearl HW-60F. The column was eluted with 0.1 M Tris–HCl buffer (pH 7.0) and fractions of 5 ml were collected. The eluates obtained were divided into five groups: Frac. 1, tubes 38 to 43; frac. 2, tube 44; frac. 3, tubes 45 to 47; frac. 4, tubes 48 to 50; frac. 5, tubes 51 to 55. After rechromatography on the same Toyopearl column, dialysis, and gel chromatography on a Sephadex G-25 column, SD-A1 to SD-A5 were obtained from fracs. 1 to 5, respectively. The yields were 7.7 mg for SD-A1, 1.9 mg for SD-A2, 1.7 mg for SD-A3, 4.9 mg for SD-A4, and 4.1 mg for SD-A5. The second periodate oxidation and the controlled degradation of these products were performed in the same manner as described above. The products were separately isolated by gel chromatography using a column (2.6 × 93 cm) of Toyopearl HW-60F with 0.1 M Tris–HCl buffer (pH 7.0). Fractions of 5 ml were collected. The eluates from tubes 68 to 72 gave SD-B1 and -B5, and the eluates from tubes 66 to 69 gave SD-B2, -B3 and -B4 after purification on a Sephadex G-25 column.

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. (5, 20 and 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 (Pelikan drawing ink A, 17 black, Pelikan AG, Germany). The ink was diluted eight times with phosphate-buffered saline containing 1% gelatin 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.

Mild Partial Hydrolysis and Isolation of Products The sample (6 mg) was dissolved in 0.5 M trifluoroacetic acid (1.2 ml) and kept at 60 °C for 1 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 (2.6 × 134.5 cm) of Sephadex G-15. The column was eluted with water, and fractions of 5 ml were collected and checked with a Knauer differential refractometer, model 188 : 00. Fraction 1 was obtained from tubes 45 to 49, fr. 2 from tubes 72 to 74, and fr. 3 from tubes 77 to 83. Arabinose was found in fr. 3. Fractions 1 and 2 afforded DPII and DPI, respectively.

Analysis of Products 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. The *Rf* value of DPI was 0.52. The trimethylsilyl (TMS) derivative of reduced DPI 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 at a flow rate of 50 ml per min of helium. The temperature was programmed at 3 °C per min from 180 to 270 °C. The retention time was 18.1 min. Analysis of component sugars and methylation analysis were performed as described above. The results of methylation analysis are also listed in Table I.

References

1) K. Ohtani, K. Mizutani, S. Hatono, R. Kasai, R. Sumino, T. Shiota, M. Ushijima, J. Zhou, T. Fuwa and O. Tanaka, *Planta Medica*, **53**, 166 (1987).
2) N. Shimizu, M. Tomoda, R. Gonda, M. Kanari, N. Takanashi and N. Takahashi, *Chem. Pharm. Bull.*, **37**, 1329 (1989).
3) G. Kinoshita, F. Nakamura and T. Maruyama, *Shoyakugaku Zasshi*, **40**, 325 (1986).
4) S. Hakomori, *J. Biochem.* (Tokyo), **55**, 205 (1964).
5) H. Björndall, B. Lindberg and S. Svensson, *Carbohydr. Res.*, **5**, 433 (1967).
6) J.-P. Joseleau, G. Chambat, M. Vignon and F. Barnoud, *Carbohydr. Res.*, **58**, 165 (1977).
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) 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.
9) G. Biozzi, B. Benacerraf and B. N. Halpern, *Br. J. Exp. Pathol.*, **34**, 441 (1953).
10) H. Wagner and E. Jordan, *Phytochemistry*, **27**, 2511 (1988).
11) A. Vollmar, W. Schäfer and H. Wagner, *Phytochemisty*, **25**, 377 (1986).
12) H. Wagner, H. Stuppner, W. Schäfer and M. Zenk, *Phytochemistry*, **27**, 119 (1988).
13) M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers and F. Smith, *Anal. Chem.*, **28**, 350 (1952).
14) M. Tomoda, Y. Yoshida, H. Tanaka and M. Uno, *Chem. Pharm. Bull.*, **19**, 2173 (1971).
15) M. Tomoda, S. Kaneko, M. Ebashi and T. Nagakura, *Chem. Pharm. Bull.*, **25**, 1357 (1977).
16) R. Oshima, J. Kumanotani and C. Watanabe, *J. Chromatogr.*, **259**, 159 (1983).
17) N. Shimizu, M. Tomoda and M. Adachi, *Chem. Pharm. Bull.*, **34**, 4133 (1986).
18) M. Tomoda, K. Shimada, Y. Saito and M. Sugi, *Chem. Pharm. Bull.*, **28**, 2933 (1980).
19) G. O. Aspinall and R. J. Ferrier, *Chem. Ind.* (London), **1957**, 1216.