Biliary Excretion of Metabolites of Baicalin and Baicalein in Rats[1)]

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Biliary excretion of metabolites of baicalin, present in Scutellariae Radix, was investigated using rats. The bile of rats administered baicalin orally was shown to contain five major metabolites, which were identified as baicalein 6-*O*-β-glucopyranuronoside (M1), 6-*O*-methyl-baicalein 7-*O*-β-glucopyranuronoside (oroxylin A 7-*O*-β-glucuronide (M2)), baicalein 7-*O*-β-glucopyranuronoside (M3), 6-*O*-β-glucopyranuronosyl-baicalein 7-*O*-sulfate (M4), and baicalein 6,7-di-*O*-β-glucopyranuronoside (M5) on the basis of chemical and spectroscopic evidence.

The bile of rats treated with baicalein also contained the above metabolites. Slower biliary excretion of the metabolites after baicalin administration suggested that it was absorbed as baicalein after hydrolysis in the gastrointestinal tract.

The total cumulative amounts of the five metabolites excreted in the bile during 30 h after oral administration of baicalin and that of baicalein were approximately 54% and 40% of the doses, respectively. In addition the bilary metabolites of both drugs were shown to be mainly composed of M5 and M4, which have high polarity and large molecular weight.

Keywords baicalin; baicalein; Scutellariae Radix; metabolite; sulfate conjugate; glucuronide conjugate; methylate conjugate; biliary excretion; rat

Baicalin is one of the major flavonoids present in Scutellariae Radix, a crude drug widely used in traditional Chinese formulations, and has been shown to possess anti-anaphylactic[2)] and anti-inflammatory[3)] activities. However, little information is available on the absorption, metabolism and excretion of baicalin in animals, except for a study on its urinary excretion following oral administration in human.[4)]

In the present study, we isolated and characterized the major biliary metabolites of baicalin and also its aglycone baicalein administered orally, and examined their excretion rates in rats.

Baicalin was orally administered to rats with bile-duct cannulation, through which the bile samples were collected. Reversed-phase high-performance liquid chromatography (HPLC) of the bile revealed the presence of five distinct peaks attributable to the metabolites of baicalin, which were tentatively designated as M1, M2, M3, M4 and M5 in order of increasing polarity (Fig. 1).

The isolation of M1—5 from the bile was performed by chromatographic separation on a Sephadex LH-20 column and repeated preparative HPLC, as shown in Chart 1.

M1—5 were all positive to the Mg–HCl color reaction. They exhibited absorptions assignable to hydroxyl, conjugated carbonyl and aromatic functions in the infrared (IR) spectra and maximal absorptions at 270—277 and 306—317 nm in the ultraviolet (UV) spectra. From these data M1—5 were deduced to have a flavone skeleton.

Acidic hydrolysis of M1—5 with dilute HCl gave glucuronic acid, which was identified by gas liquid chromatography (GLC),[5)] suggesting glucuronide-conjugated structure for M1—5. Furthermore, enzymatic hydrolysis of M1, M3 and M5 with β-glucuronidase as well as that of M4 with crude arylsulfatase (containing β-glucuronidase activity) gave the aglycones, which were all assumed to be baicalein from their HPLC behavior. On the other hand, the hydrolysis of M2 with β-glucuronidase gave the aglycone, which was assumed to be oroxylin A from the HPLC behavior. UV spectral shifts[6)] on addition of $AlCl_3$ suggested the presence of free C5-hydroxyls in M1—5.

The appearance of OH proton signals at low field positions (12.70—13.04 ppm) in the proton nuclear magnetic resonance (^{1}H-NMR) spectra suggested the presence

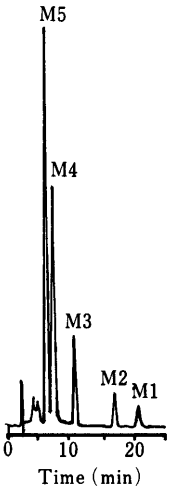


Fig. 1. HPLC Chromatogram of Bile Excreted during 6—8 h after Oral Administration of Baicalin (100 mg/kg) to Rats

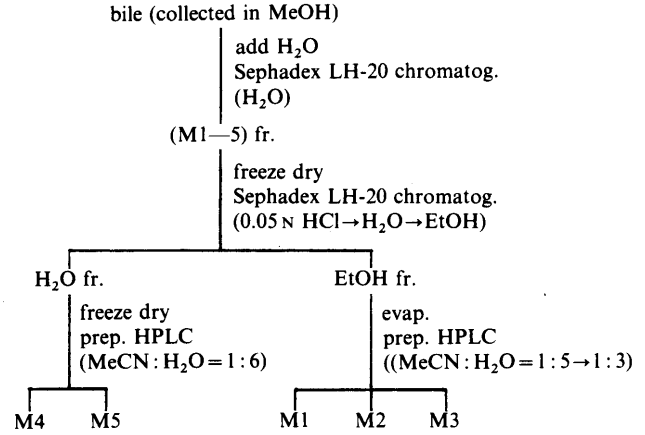
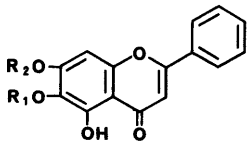


Chart 1. Isolation of the Metabolites Excreted in Bile



	R_1	R_2
M1:	GlcUA	H
M2:	CH_3	GlcUA
M3:	H	GlcUA
M4:	GlcUA	SO_3^-
M5:	GlcUA	GlcUA

GlcUA: β-glucopyranuronosyl

Chart 2. Structures of the Metabolites

of free C5-hydroxyl groups in M1, M2, M3 and M5. The ^{1}H-NMR spectra showed one doublet attributable to one β-anomeric proton for each of M1, M2 and M3 and two doublets attributable to two β-anomeric protons for M5. These NMR spectral data indicated that the presence of one glycosyl moiety each in M1—3 and two glycosyl moieties in M5.

Based on the above findings, structure elucidation of five metabolites was performed, taking account of additional chemical and/or spectral data, as follows (Chart 2).

M1 showed in the fast atom bombardment mass spectrum (FAB-MS) a molecular ion peak at *m/z* 447 $(M+H)^+$, corresponding to baicalein monoglucuronide. A comparison of the carbon-13 (^{13}C-) NMR spectrum of M1 with that of baicalein showed that the C6 signal of M1 was shifted 1.4 ppm upfield, accompanied with downfield shifts of C5 (5.9 ppm) and C7 (3.8 ppm). These shifts suggested the presence of the glucuronide group at C6. Thus, M1 was identified as baicalein 6-*O*-β-glucopyranuronoside.[7]

M2 showed in the FAB-MS a molecular ion peak at *m/z* 461 $(M+H)^+$ and was identified as 6-*O*-methyl-baicalein 7-*O*-β-glucopyranuronoside (oroxylin A 7-*O*-β-glucuronide) by comparison of its spectral data with those described in the literature.[8]

M3 was identified as baicalin by direct comparison with the reference sample.

The intense absorption at 1041 cm^{-1} in the IR spectrum and SO_4^{2-} formation on carbonization suggested the sulfate-conjugated structure for M4. The negative FAB-MS of M4 exhibited ion peaks corresponding to $(M-H+Na)^-$, $(M-H)^-$ and $(M-SO_3H)^-$ at *m/z* 547, 525 and 445, respectively, thereby indicating the presence of one sulfate group in M4. Partial hydrolysis of M4 gave a product which was identified as M1 by comparison of the spectral data. A comparison of the ^{13}C-NMR spectrum of M4 with those of M1 showed that the C7 signal of M4 was shifted 5.7 ppm upfield accompanied with the downfield shifts of C6 (1.8 ppm) and C8 (4.5 ppm). From these shift values and the foregoing bathochromic shifts of the UV absorption band I with $AlCl_3$, the sulfate group in M4 should be attached to C7-OH of M1. Thus, M4 was identified as 6-*O*-β-glucopyranuronosyl-baicalein 7-*O*-sulfate.

M5 showed in the FAB-MS a molecular ion peak at *m/z* 623 $(M+H)^+$, corresponding to baicalein diglucuronide. Based on this datum and the foregoing chemical and spectral evidence, M5 was identified as baicalein 6,7-di-*O*-β-glucopyranuronoside.

The remaining ^{1}H- and ^{13}C-NMR data given in Table I and the experimental section also supported the structures proposed above.

Table I. ^{13}C-NMR Data for Baicalein and the Metabolites (δ in DMSO-d_6)

Carbon No.	M1	M2	M3	M4	M5	Baicalein
C 2	163.2	163.7	163.9	163.8	163.8	162.9
C 3	104.1	104.9	104.1	104.8	105.1	104.4[b]
C 4	182.2	182.4	182.4	182.4	182.5	182.1
C 5	152.9[a]	152.5[a]	146.5	152.3[a]	152.9[a]	147.0[a]
C 6	127.9	132.6	130.1[a]	129.7	129.1	129.3[c]
C 7	157.4	156.2	150.9	151.7[a]	155.6	153.6
C 8	94.4	94.1	93.7	98.9	94.8	94.0
C 9	152.5[a]	152.2[a]	149.3	151.8[a]	152.6[a]	149.8[a]
C 10	104.6	106.1	106.0	106.4	106.3	104.3[b]
C 1′	130.6	130.6	130.3[a]	130.5	130.5	130.9[c]
C 2′, 6′	126.3	126.4	126.1	126.5	126.4	126.2
C 3′, 5′	129.1	129.1	129.1	129.1	129.1	129.0
C 4′	131.9	132.1	132.1	132.1	132.1	131.7
6-*O*-Me		60.2				
6-*O*-GlcUA Cl″	103.5			103.5	103.3	
C 2″	73.5			73.6	72.9	
C 3″	75.9[b]			76.0	75.6[b]	
C 4″	71.3			71.5	71.3	
C 5″	75.5[b]			76.0	75.2[b]	
C 6″	169.9			171.1	169.9	
7-*O*-GlcUA Cl‴		99.4	99.8		100.5	
C 2‴		72.8	72.7		73.8	
C 3‴		75.8[b]	75.3[b]		75.8[b]	
C 4‴		71.1	71.4		71.3	
C 5‴		75.4[b]	75.1[b]		75.5[b]	
C 6‴		169.9	170.7		169.8	

a—*c*) These assignments may be interchanged in each column.

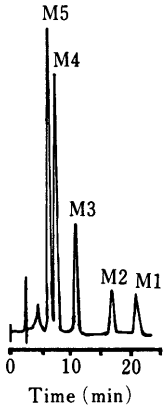


Fig. 2. HPLC Chromatogram of Bile Excreted during 1—2 h after Oral Administration of Baicalein (60 mg/kg) to Rats

Many flavonoid glycosides undergo microbial hydrolysis in the gastrointestinal tract.[9a,c] Further, in the foregoing study the bile of rats given baicalin orally was demonstrated to contain metabolites having free or sulfated hydroxyls at the C7-OH position, suggesting that at least some portion of baicalin was hydrolyzed to the aglycone prior to absorption.

These findings prompted us to investigate the biliary excretion patterns of metabolites following administration of baicalein, the aglycone of baicalin.

On HPLC analysis, the bile of rats given baicalein was shown to contain the same biliary metabolites as those obtained with baicalin, as can be seen in a typical chromatogram (Fig. 2).

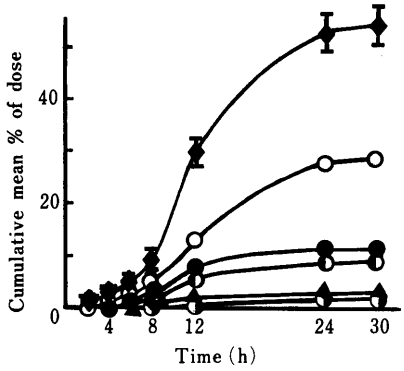


Fig. 3. Cumulative Excretion of Metabolites as a Function of Time Following Oral Administration of Baicalin (100 mg/kg)

(◑), M1; (▲), M2; (◐), M3; (●), M4; (○), M5; (◆), total. Each point represents the mean ± S.E. of five rats.

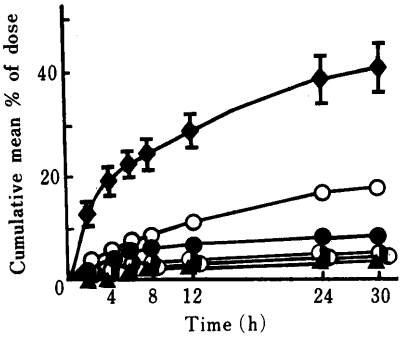


Fig. 4. Cumulative Excretion of Metabolites as a Function of Time Following Oral Administration of Baicalein (60 mg/kg)

(◑), M1; (▲), M2; (◐), M3; (●), M4; (○), M5; (◆), total. Each point represents the mean ± S.E. of five rats.

Biliary excretion profiles of the metabolites after administering 100 mg/kg of baicalin and 60 mg/kg of baicalein are shown in Figs. 3 and 4.

The total cumulative amounts of the five metabolites excreted during the period of 30 h corresponded to 54 ± 4% (mean ± S.E., $n=5$) of the dose for baicalin administration and 40 ± 5% (mean ± S.E., $n=5$) for baicalin administration. During the first 4 h period after administration, 19% of the dose was excreted in the bile as conjugates in the baicalein group compared with 3% in the baicalin group. The delayed excretion of the metabolites after administration of baicalin indicated that baicalin was absorbed as the aglycone after hydrolysis.

The high biliary excretion levels of these flavonoids observed in the present investigation were consistent with previous findings involving other flavonoids.[9b,c] Further, the biliary metabolites of baicalin and those of baicalein were demonstrated to be mainly composed of M5 and M4 with high polarity and large molecular weight, both of which are important factors facilitating excretion of flavonoids in bile.[9c] Additionally, there were no significant differences between the two groups in the percentages of respective metabolites excreted during a 30 h period.

Examination of fecal and urinary excretions and plasma concentrations of the metabolites and related compounds is in progress in order to elucidate fully the biological fate, including enterohepatic cycling, of the flavonoids.

Experimental

Melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. Specific rotations were measured with JASCO DIP-181, IR spectra with a Nicolet FT-IR 60SX diffuse reflectance spectrometer and UV spectra with a Shimadzu UV-250 spectrometer. NMR spectra were recorded on a JEOL JNM-GX 400 (400 MHz for ^1H and 100 MHz for ^{13}C) spectrometer. Chemical shifts are given in δ values (ppm) downfield from tetramethylsilane (s, singlet; d, doublet; m, multiplet; br, broad). FAB-MS were measured with a JEOL JMS-DX 300 mass spectrometer. HPLC was carried out on a Waters liquid chromatograph equipped with a model 510 pump and a model 481 UV detector. GLC was carried out on a Shimadzu GC-6A unit.

Materials Baicalin and baicalein were isolated from Scutellariae Radix according to the method of Takido *et al.*[10]

Animal Male Wistar (Japan SLC, Inc.) rats weighing 210—250 g were used. The rats were deprived of food but given free access to water for 16 h prior to the experiments.

Animal Experiments Under light anesthesia with ether bile duct cannulations were performed with polyethylene tubing on all rats. In the experiments for isolation of the metabolites, seventy rats were orally administered 300 mg/kg of finely ground baicalin suspended in 0.5% carboxymethyl cellulose (CMC)–Na solution and the biles were collected in 10 ml of MeOH with cooling for each animal for 30 h. For identification and determination of the metabolites, five rats were orally administered 100 mg/kg of baicalin or 60 mg/kg of baicalein suspended in 0.5% CMC–Na solution and bile samples were taken at 2, 4, 6, 8, 12, 24 and 30 h, each in 2 ml of MeOH–0.5 M NaH_2PO_4 (10 : 1) with cooling. All samples were stored below −20 °C until used.

HPLC Conditions For identification and quantitation of the metabolites a 5 μM octadecyl silica (ODS) column (Nucleosil $5C_{18}$, M. Nagel, 4.6 mm i.d. × 200 mm), and a mobile phase of MeOH–0.3 M phosphate buffer pH 2.1 (31 : 40) were used. The flow rate was 1 ml/min and the column eluate was monitored at 315 nm. For isolation of the metabolites a 15 μm ODS column (YMC-Pack S-343, Yamamura Chem. Lab., 20 mm i.d. × 250 mm) was used with a mobile phase of CH_3CN–H_2O (1 : 6 → 1 : 3). The flow rate was 5 ml/min and the column eluate was monitored at 210 nm.

GLC Conditions A glass column (3 mm i.d. × 1 m) packed with 2% OV-1 on Chromosorb W (AW-DMCS), 80—100 mesh was used. The column oven temperature was kept at 170 °C. Nitrogen carrier gas flow rate was 50 ml/min.

Isolation of Metabolites Bile samples taken from rats were combined and diluted with the equivalent volume of water and subjected to Sephadex LH-20 column chromatography (H_2O as the eluent) to furnish the metabolites (M1—5) fraction. Further fractionation and purification of the fraction by Sephadex LH-20 column chromatography and subsequent preparative HPLC afforded M1 (5 mg), M2 (4 mg), M3 (4 mg), M4 (15 mg) and M5 (60 mg) (Chart 1).

Acidic Hydrolysis of Metabolites M1, M2, M3, M4 and M5 (each 1 mg) were each refluxed in 2 ml of 2 N HCl–50% aqueous dioxane (1 : 1) for 3 h. The reaction mixture was filtered and the filtrate was evaporated to dryness *in vacuo*. The residue was trimethylsilylated by the usual method and subjected to GLC analysis[5] to identify glucuronolactone (main) and glucuronic acid (trace).

Enzymatic Hydrolyses of Metabolites M1, M2, M3 and M5 (each 1 mg) were incubated with β-glucuronidase (0.1 ml, Sigma, Type H-2) in 0.1 M citrate buffer (pH 5.2) for 2 h at 37 °C. M4 (1 mg) was treated with crude arylsulfatase (0.1 ml, Sigma, Type H-1) in the same way as described above. Each reaction mixture was extracted with ether, then the organic layer was washed with 1 N HCl and H_2O, and evaporated *in vacuo* to yield the aglycone.

Detection of Sulfate Function in M4 M4 (1 mg) was carbonized in a Pt crucible, and extracted with 0.3 ml of H_2O. After centrifugation the supernatant was treated with 0.2% $BaCl_2$ to afford a white precipitate ($BaSO_4$).

Partial Hydrolysis of M4 M4 (4 mg) was incubated with glacial acetic acid (10 ml) for 10 min at room temperature. The reaction mixture was diluted with H_2O and subjected to preparative HPLC under the same conditions as for metabolite M1 to afford M1 (2 mg).

Quantitation of Biliary Metabolites Bile samples were diluted with MeOH–0.5 M NaH_2PO_4 (10 : 1) to make 10 ml. An aliquot of the sample was taken, and 3 ml of methanol containing 3 μg of α-naphthol was added. A 10 μl aliquot of the solution was subjected to HPLC. Quantitation of the metabolites was performed by measuring their peak heights relative to that of the internal standard. Calibration plots of the peak height ratios of each

metabolite relative to α-naphthol plotted against the metabolite concentrations were linear over the ranges of 0.6—6.0 μg/ml for M1 and M2, 0.6—10.0 μg/ml for M3 and M4, and 0.6—50.0 μg/ml for M5. The recoveries of each metabolite in bile were confirmed to range from 97 to 103% by determining standard samples added to drug-free bile.

M1: A light yellow powder. mp 195—197 °C. Mg–HCl(+). FAB-MS *m/z*: 447 $(M+H)^+$. IR (KBr) cm^{-1}: 3370 (OH), 1740 (COOH), 1657 (conjugated C=O), 1625 (arom. C=C). UV λ_{max}^{MeOH} nm (log ε): 270 (4.33), 317 (4.00); $\lambda_{max}^{MeOH+NaOAc}$ nm (log ε): 268 (4.36), 361 (3.97); $\lambda_{max}^{MeOH+AlCl_3}$ nm (log ε): 254 sh (3.97), 282 (4.30), 334 (4.36). ^{1}H-NMR (DMSO-d_6) δ: 5.00 (1H, d, *J*=7.5, anomeric H of C7-glucuronic acid), 6.66 (1H, s, C8-H), 6.98 (1H, s, C3-H), *ca.* 7.6 (3H, m, C3′, 4′,5′-H), *ca.* 8.0 (2H, m, C2′, 6′-H), 10.63 (1H, s, C7-OH), 13.04 (1H, s, C5-OH). ^{13}C-NMR: as given in Table I.

M2: A light yellow powder. mp 192—194 °C. Mg–HCl(+). FAB-MS *m/z*: 461 $(M+H)^+$, 285 $(MH-glcUA)^+$. IR (KBr) cm^{-1}: 3456 (OH), 1735 (COOH), 1658 (conjugated C=O), 1612 (arom. C=C). UV λ_{max}^{MeOH} nm (log ε): 245 sh (4.03), 272 (4.42), 309 (4.16); no shifts were observed with NaOAc; $\lambda_{max}^{MeOH+AlCl_3}$ nm (log ε): 250 sh (4.01), 284 (4.40), 333 (4.24). ^{1}H-NMR (DMSO-d_6) δ: 3.78 (3H, s, C6-OCH_3), 5.34 (1H, d, *J*=7.0, anomeric H of C6-glucuronic acid), 7.06 (1H, s, C3-H), 7.13 (1H, s, C8-H), *ca.* 7.6 (3H, m, C3′, 4′,5′-H), *ca.* 8.0 (2H, m, C2′, 6′-H), 12.82 (1H, s, C5-OH). ^{13}C-NMR: as given in Table I.

M3: A light yellow powder. mp 220—222 °C. Mg–HCl(+). FAB-MS *m/z*: 447 $(M+H)^+$. IR (KBr) cm^{-1}: 3390 (OH), 1729 (COOH), 1662 (conjugated C=O), 1612 (arom. C=C). UV λ_{max}^{MeOH} nm (log ε): 245 (3.96), 277 (4.41), 313 (4.15); no shifts were observed with NaOAc; $\lambda_{max}^{MeOH+AlCl_3}$ nm (log ε): 289 (4.35), 340 (4.23). ^{1}H-NMR (DMSO-d_6) δ: 5.24 (1H, d, *J*=7.1, anomeric H of C7-glucuronic acid), 6.98 (1H, s, C3-H), 7.09 (1H, s, C8-H), *ca.* 7.6 (3H, m, C3′, 4′,5′-H), *ca.* 8.0 (2H, m, C2′, 6′-H), 12.70 (1H, s, C5-OH). ^{13}C-NMR: as given in Table I.

M4: A light yellow powder. mp 191—193 °C. Mg–HCl(+). Negative FAB-MS *m/z*: 547 $(M+Na-H)^-$, 525 $(M-H)^-$, 445 $(M-SO_3H)^-$. IR (KBr) cm^{-1}: 3449 (OH), 1718 (COOH), 1653 (conjugated C=O), 1621 (arom. C=C), 1041 (–O–SO_3^-). UV λ_{max}^{MeOH} nm (log ε): 275 (4.37), 306 sh (4.07); no shifts were observed with NaOAc; $\lambda_{max}^{MeOH+AlCl_3}$ nm (log ε): 247 sh (3.88), 287 (4.35), 329 (4.16). ^{1}H-NMR (DMSO-d_6) δ: 5.08 (1H, br s,[11a,b]) anomeric H of C6-glucuronic acid), 7.04 (1H, s, C3-H), 7.33 (1H, s, C8-H), *ca.* 7.6 (3H, m, C3′, 4′, 5′-H), *ca.* 8.0 (2H, m, C2′, 6′-H). ^{13}C-NMR: as given in Table I.

M5: A light yellow powder. mp >300 °C (dec.). Mg–HCl(+). $[\alpha]_D^{22}$ −96.1° (*c*=0.5, H_2O). FAB-MS *m/z*: 623 $(M+H)^+$, 447 $(MH-glucuronic\ acid)^+$. IR (KBr) cm^{-1}: 3431 (OH), 1749 (COOH), 1658 (conjugated C=O), 1625 (arom. C=C). UV λ_{max}^{MeOH} nm (log ε): 244 sh (3.93), 273 (4.38), 306 (4.10); no shifts were observed with NaOAc; $\lambda_{max}^{MeOH+AlCl_3}$ nm (log ε): 280 (5.34), 327 (4.08). ^{1}H-NMR (DMSO-d_6) δ: 5.00 (1H, d, *J*=7.5, anomeric H of C6-glucuronic acid), 5.29 (1H, d, *J*=7.6, anomeric H of C7-glucuronic acid), 7.06 (1H, s, C3-H), 7.12 (1H, s, C8-H), *ca.* 7.6 (3H, m, C3′, 4′, 5′-H), *ca.* 8.0 (2H, m, C2′, 6′-H), 12.89 (1H, s, C5-OH), ^{13}C-NMR: as given in Table I.

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11) *a*) The fact that the signal ascribable to the anomeric proton in M4 was observed as "br s" for M4 is assumed to be correlated with the sulfate-conjugation at C7, since the signal was observed as a doublet (*J*=7.5 Hz) for the desulfated derivative of M4; *b*) The C5-hydroxyl proton signal was not clearly observed in the ^{1}H-NMR spectrum of M4, which might be explained by the replacement of the hydrogen with a metal ion.