JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Isoflavonoid Composition of a Callus Culture of the Relict Tree *Maackia amurensis* Rupr. et Maxim

Sergey A. Fedoreyev,[†] Victor P. Bulgakov,*[,§] Olga V. Grishchenko,[§] Marina V. Veselova,[†] Olga E. Krivoschekova,[†] Nadezda I. Kulesh,[†] Vladimir A. Denisenko,[†] Galina K. Tchernoded,[§] and Yury N. Zhuravlev[§]

Pacific Institute of Bioorganic Chemistry and Institute of Biology and Soil Science, Far East Branch of Russian Academy of Sciences, Vladivostok, Russian Federation

Isoflavonoids, an interesting and restricted group of secondary metabolites of legumes, exhibit estrogenic, antiangiogenic, and anticancer activities and are now popular as dietary supplements. Plant cell cultures that possess an increased ability to synthesize these metabolites were examined. During the investigation, cell cultures of the Far Eastern relict tree *Maackia amurensis* (Leguminosae) were established. A selection of seed-derived cell aggregates yielded the callus line designated A-18. This culture produces 20 isoflavonoids, namely, the isoflavones genistein, daidzein, formononetin, calycosin, derrone, and pseudobaptigenin and their glycosylated conjugates genistin, 6″-*O*-malonylgenistin, ononin, 6″-*O*-malonylononin, daidzin, 3′-methoxydaidzin, 4′-*O*-*β*-D-glucopyranosyldaidzin, 4′-*O*-*β*-D-glucopyranosylgenistin, and 7-*O*-*β*-D-glucopyranosylcalycosin; the pterocarpans maackiain and medicarpin and their glycosylated conjugates 6′-*O*-malonyl-3-*O*-*β*-D-glucopyranosylmaackiain and 6′-*O*-malonyl-3-*O*-*β*-D-glucopyranosylmedicarpin; and the new pterocarpan glucoside 6′-*O*-malonyl-3-*O*-*β*-D-glucopyranosyl-6,6a-dehydromaackiain. These isoflavonoids, possessing a hepatoprotective activity, were stably produced by the A-18 calli for prolonged periods of observation.

KEYWORDS: Callus culture; hepatoprotective activity; isoflavonoids; isoflavones; *Maackia amurensis*; pterocarpans

INTRODUCTION

Isoflavones are compounds with estrogenic activity (phytoestrogens) that are widely distributed in many plant species. Because of their structural similarity to *β*-estradiol, the health benefits of isoflavones have been evaluated in age-related and hormone-dependent diseases (*1, 2*). The presence of isoflavonoids is almost entirely restricted to the family Leguminosae, but they are also occasionally found in some other angiosperm families (*3*). Isoflavones are usually present in plants as free compounds and 7-*O*-*β*-D-glucoside derivatives. The 6″-*O*-malonyl-7-*O*-*β*-D-glucoside derivatives are the predominant forms of glucosylated isoflavones, and these forms are precursors of 6″-*O*-acetyl-7-*O*-*β*-D-glucosides (*4*). The biological activities of isoflavonoids have been well-studied. For example, genistein possesses inhibitory activity against topoisomerase II and tyrosine kinase and inhibits cancer cell proliferation (*5, 6*). Many of the health-promoting benefits of isoflavonoids are linked to the ability of these substances to serve as antioxidants (*7*). As phytoestrogens, they are believed to block estrogen activity by competitive inhibition of estrogen receptors. They may reduce the risk of some hormone-dependent cancers including prostate and breast cancers (*1, 8*). Inclusion of isoflavonoid-rich soybeans in the diet protected patients against coronary heart diseases by reducing blood lipids, oxidized LDL, homocysteine, and blood pressure (*1, 9*).

Maackia amurensis Rupr. et Maxim. is the only woody plant representative of the Leguminosae family in the flora of the Russian Far East. This species is a relict of the tertiary flora (*10*). The polyphenolic complex from the heartwood (PHW) of *M. amurensis*, named the maksar preparation, was registered in the Russian Federation as a hepatoprotective drug in 2004 (*11*). The specific activity of the maksar preparation was found to be higher than that of currently used commercial hepatoprotective drugs (*10–12*). HPLC and NMR analyses of PHW yielded piceatannol and resveratrol (stilbenes), formononetin, genistein, retuzin, afromozin, daidzein, orobol, tectorigenine, 3-hydroxivestiton (isoflavones), maakiain and medicarpin (pterocarpans), maackiasin (isoflavonostilben), scirpusin A, scirpusin B, maackin (dimeric stilbenes), and maackolin (stilbenolignan) (*10*). Callus cultures of *M. amurensis* derived from leaf petioles, inflorescences, leaves, and apical meristems have been examined for biosynthetic activity. Analyses have shown

* Address correspondence to this author at the Institute of Biology and Soil Science, 159 Stoletija Str., Vladivostok 690022, Russia (fax +7-4232-310193; telephone +7-4232-375279; e-mail bulgakov@ibss.dvo.ru).

[†] Pacific Institute of Bioorganic Chemistry.

[§] Institute of Biology and Soil Science.

10.1021/jf801227q CCC: $40.75
Published on Web 08/01/2008

the presence of the isoflavones daidzein, retuzin, genistein, and formononetin and the pterocarpans maakiain and medicarpin, although these cultures did not contain stilbenes (*13*). Polyphenolic compounds extracted from both core wood and callus cultures of *M. amurensis* decreased the acute toxicity of tetratrachloromethane on liver with similar efficacy, showing a prominent favorable effect on animal survival (*14*). This effect resulted from the suppression of dystrophy and hepatocyte necrosis, normalization of aminotransferase and γ-glutamyltransferase activities in the blood, and stimulation of bilirubin conjugation (*14*). Thus, the possibility of creating a renewable source of *Maackia* isoflavonoids has been demonstrated. However, these calli possessed a slow growth. The aim of the present investigation was to establish actively growing callus culture producing isoflavonoids at high yields and to provide detailed chemical analysis of isoflavonoids isolated from the calli. The analysis revealed the presence in the calli of numerous glycosylated isoflavones and pterocarpans, in addition to previously isolated isoflavones.

MATERIALS AND METHODS

Plant Material and Callus Culture. Seeds of *M. amurensis* Rupr. et Maxim. were collected in the southern regions of the Primorsky Territory (Russian Far East). Plants and seeds of *M. amurensis* were identified in the Botany Department of the Institute of Biology and Soil Science. The A-18 callus culture was established from seeds of *M. amurensis* using the procedure described previously (*13*). The seeds were surface sterilized and germinated in vitro, and then 3−5 mm explants were cut from the seedlings and placed on $W_{B/A}$ medium (*15*) supplemented with the following components (mg/L): thiamin−HCl (0.2), nicotinic acid (0.5), pyridoxine−HCl (0.5), meso-inositol (100), peptone (100), sucrose (25000), agar (6000), 6-benzylaminopurine (0.5), and α-naphthaleneacetic acid (2.0). Calli emerging from the explants were transferred to fresh $W_{B/A}$ medium and cultivated at 25 °C in the dark at 30 day subculture intervals. Further callus tissues were selected for growth and isoflavonoid content. The selected actively growing culture was designated the A-18 line, which was further cultivated in 100 mL Erlenmeyer flasks containing 30 mL of the $W_{B/A}$ agarized medium supplemented with 100 μM DL-phenylalanine. The inoculum mass was 1 g of fresh callus weight in all experiments. The growth index was calculated as $W_f - W_i/W_i$, where W_f is final biomass and W_i represents inoculum biomass. For evaluation of growth dynamics, 10 flasks were selected weekly.

General Apparatus and Chemicals. Melting points (uncorrected) were determined on a Boetius apparatus. Optical rotations $[\alpha]_{576}^{20}$ were measured with a Perkin-Elmer 343 polarimeter. CD spectra were recorded on a JASCO-J 500A spectropolarimeter. UV spectra were obtained on a Cecil CE 7250 spectrophotometer. IR spectra were recorded on a Vector 22 IR-FT spectrophotometer. ^{1}H and ^{13}C NMR spectra were determined on a NMR Bruker AVANCE DRX-500 instrument. Samples were run in DMSO-d_6, with tetramethylsilane as the internal standard. The chemical shift values are reported in parts per million (δ), and the coupling constants (*J*) are given in hertz. MALDI-MS spectra were measured using a BIFLEX III (Bruker) spectrometer (direct sample inlet, ionizing energy = 10 eV). Reagents for the preparation of nutrient media and dihydroquercetin (DHQ) were obtained from Sigma. The standards of polyphenols, such as daidzein (**9**), calycosin (**10**), genistein (**12**), formononetin (**13**), (6a*R*,11a*R*)-maackiain (**19**), and (6a*R*,11a*R*)-medicarpin (**20**) were previously isolated from the heartwood (*10, 11*) as well as callus cultures of *M. amurensis* during the course of the present series of investigations and identified by ^{1}H and ^{13}C NMR. The purity of the seven reference compounds was >98% by the peak area normalization method using HPLC UV (detection at 280 nm).

Analytical and Preparative HPLC. The dried and powdered callus culture sample (100 mg) was extracted with 96% EtOH (3 mL) for 2 h at 40−45 °C. An ethanolic solution of dihydroquercetin (0.050 mL, 1 mg/mL) was added to 0.450 mL of the extract as an internal standard. The sample solution was membrane filtered (0.45 μm, Agilent), and 5 μL aliquots were used for analysis. The analytical HPLC was carried out using an Agilent Technologies 1100 series HPLC system equipped with a VWD detector (λ = 280 nm). Extracts and fractions were analyzed using a Hypersil BDS-C-18 column (5 μm, 250 × 5 mm) thermostated at 30 °C. The mobile phase consisted of 1% aqueous acetic acid (A) and acetonitrile containing 1% acetic acid (B). For the analyses, the following seven gradient steps were programmed: 0−5 min, 5−10% B; 5−10 min, 10−20% B; 10−30 min, 20−30% B; 30−35 min, 30−40% B; 40−45 min, 50−90% B; 45−50 min, 90−50% B; 50−60 min, 5% B. The flow rate was 1 mL/min. The data were analyzed with the ChemStation program var. 09 (Agilent Technologies, Waldbronn, Germany). The preparative HPLC was carried out using an Agilent Technologies 1100 series HPLC system equipped with a VWD detector (λ = 280 nm) and a Zorbax Eclipce XDB-C-8 column (5 μm, 150 × 4.6 mm) thermostated at 30 °C. The mobile phase consisted of 1% aqueous acetic acid (A) and acetonitrile containing 1% acetic acid (B). For the preparative HPLC, the following five gradient steps were programmed: 0−5 min, 5−10% B; 5−10 min, 10−25% B; 10−20 min, 25−50% B; 20−25 min, 50−55% B; 25−35 min, 55−5% B. The flow rate was 1 mL/min. Correlation coefficients (*K*) as well as retention times (t_R) were measured in triplicate for all pure standard compounds relative to DHQ and used for analysis of isoflavonoids.

Extraction and Isolation. Cells or tissue samples of *M. amurensis* (46.85 g) dehydrated under hot air flow (50 °C) were extracted three times with a mixture of $CHCl_3$ and EtOH (3:1) for 2 days at 50 °C. The combined extract was evaporated under reduced pressure to 20 mL and applied to a Toyopearl HW-50F column (3 × 40 cm). The column was eluted with H_2O−EtOH (containing 0.04% HCOOH), with gradually increasing amounts of EtOH. Fractions of 10, 20, 30, 40, 50, and 60% EtOH eluates were evaporated to obtain fraction A (80 mg), fraction B (398 mg), and fraction C (469 mg). Fractions A−C were subsequently chromatographed using a column of Toyopearl HW-40F (2 × 40 cm). The column was eluted with H_2O−EtOH (containing 0.04% HCOOH), and the EtOH concentration was gradually increased (20−50%) to obtain 13 subfractions: 1A (12 mg), 1B (19 mg), 1C (15 mg), 1D (23 mg), 2A (34 mg), 2B (32 mg), 2C (38 mg), 2D (57 mg), 2E (19 mg), 3A (48 mg), 3B (57 mg), 3C (137 mg), and 3D (24 mg). Subfractions 1A, 1C, 2B, 2D, and 3C were subsequently subjected to a column (2 × 40 cm) of Toyopearl HW-40F chromatography under the same conditions to yield compounds **1** (5 mg), **6** (13 mg), **9** (25 mg), **11** (25 mg), **19** (35 mg), and **20** (40 mg). Subfractions 1B, 1D, 2A, 2C, 2E, and 3A were then chromatographed by preparative HPLC to yield compounds **2** (5 mg), **3** (9 mg), **4** (4 mg), **5** (8 mg), **7** (14 mg), **8** (20 mg), **10** (12 mg), **16** (12 mg), **12** (8 mg), **17** (6 mg), **18** (7 mg), and **14** (28 mg). Subfraction 3B was subjected to silica gel column (1 × 10 cm, 230 mesh, 9 g) chromatography with hexane/acetone (8:1 → 2:1) to yield compound **17** (48 mg). Subfraction 3D was repeatedly chromatographed on a Sephadex LH-20 column (1.0 × 50 cm) in $CHCl_3$/EtOH (12:1) to yield compound **15** (8 mg).

Acid Hydrolysis of *M. amurensis* Cell Culture Extract. The combined extract of *M. amurensis* (56 mg) in MeOH (3 mL) and aqueous 4 M HCl (20 mL) was refluxed under nitrogen for 5 h at 100 °C. After hydrolysis, the mixture was extracted three times with EtOAc (30 mL). The combined EtOAc extract was concentrated in vacuo to yield the isoflavone mixture (35 mg). The isoflavone mixture was dissolved in EtOH (8 mg/mL), membrane filtered (0.45 μm, Agilent), and analyzed by analytical HPLC.

*4′-O-β-D-Glucopyranosyldaidzin (**1**):* white powder; mp 140−145 °C; $[\alpha]_{576}^{20}$ −32° (*c* 0.97, C_2H_5OH); MALDI, *m/z* 601.4 ($[M + Na]^+$); IR (KBr) ν_{max} 3380, 2924, 2170, 1715, 1630, 1511 cm^{-1}; UV λ_{max} nm 205, 260 (C_2H_5OH); lit. mp and spectral data (*16*); ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz), see **Tables 1** and **2**.

*4′-O-β-D-Glucopyranosylgenistin (**2**):* white powder; mp 188−190 °C; $[\alpha]_{576}^{20}$ −38° (*c* 0.8, C_2H_5OH); MALDI, *m/z* (relative intensity) 617.7 (10) $[M + Na]^+$, 455 (8), 271.2 (70), 269.2 (70); IR (KBr) ν_{max} 2930, 1631, 1512 cm^{-1}; UV λ_{max} nm 203, 264 (C_2H_5OH); lit. mp and spectral data (*17*); ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz), see **Tables 1** and **2**.

*Daidzin (**3**):* white powder; mp 246−248 °C; $[\alpha]_{576}^{20}$ −70° (*c* 0.57, C_2H_5OH); MALDI, *m/z* 439.74 $[M + Na]^+$; IR (KBr) ν_{max} 3407, 2170,

Table 1. ^{1}H NMR Spectral Data (*J* in Hertz) for Compounds **1**−**8** and **11** at (500 MHz) at 30 °C, Recorded in DMSO-d_6

site	1	2	3	4	5	6	7	8	11
2	8.45, s	8.49, s	8.31, s	8.42, s	8.39, s	8.42, s	8.40, s	8.43, s	8.37, s
5	8.06, d (8.8)		8.05, d (8.5)	8.06, d (9.0)	8.05, d (8.85)			8.06, d (8.8)	8.08, d (8.8)
6	7.15, dd (8.8, 2.5)	6.47, d (1.8)	7.14, dd (2.7, 8.5)	7.15, dd (2.28, 9.0)	7.14, dd (2.3, 8.85)	6.72, d (2.2)	6.70, d (2.0)	7.15, dd (8.8, 2.2)	7.15, dd (2.26, 8.8)
8	7.25, d (2.5)	6.74, d (1.8)	7.23, d (2.7)	7.24, d (2.28)	7.23, d (2.3)	6.47, d (2.2)	6.47, d (2.0)	7.24, d (2.2)	7.22, d (2.26)
2′	7.53, d (8.8)	7.52, m,[a] (8.7)	7.41, d (8.7)	7.19, d (2.10)	7.06, s	7.40, d (8.6)	7.40, d (8.3)	7.53, d (9.0)	7.53, d (9.04)
3′	7.09, d (8.8)	7.15, m,[a] (8.7)	6.82, m,[a] (8.7)			6.83, d (8.6)	6.83, d (8.8)	7.00, d (9.0)	6.99, d (9.04)
5′	7.09, d (8.8)	7.15, m,[a] (8.7)	6.82, m,[a] (8.7)	6.82, d (8.24)	6.96, br s	6.83, d (8.6)	6.83, d (8.8)	7.00, d (9.0)	6.99, d (9.04)
6′	7.53, d (8.8)	7.52, m,[a] (8.7)	7.41, d (8.7)	7.01, dd (2.10, 8.24)	6.96, br s	7.40, d (8.6)	7.40, d (8.3)	7.53, d (9.0)	7.53, d (9.04)
1″	5.11, d (7.6)	5.07, d (7.6)	5.10, m (7.58)	5.10, d (7.33)	5.10, d (7.33)	5.05, d (7.48)	5.11, d (7.54)	5.11, d (7.2)	5.12, m (7.35)
2″	3.29−3.33, m	3.26, m	3.46, m	3.25−3.38, m	3.30, m	3.26, m	3.31, m	3.31, m	3.35, m
3″		3.28, m	3.46, m		3.32, m	3.30, m	3.33, m	3.32, m	3.35, m
4″	3.18, m	3.16, m	3.19, m	3.17, m	3.19, m	3.17, m	3.21, m	3.19, m	3.22, m
5″	3.47, m	3.34, m	3.46, m	3.48, m	3.46, m	3.45, m	3.75, m	3.46, m	3.75, m
6″	3.47, m	3.46, m	3.71, m	3.73, m	3.48, m	3.47, m	4.13, m	3.47, m	4.13, d (7.06, 11.87)
	3.70, m	3.70, m	3.46, m	3.48, m	3.72. m	3.71, m	4.39, m	3.72, m	4.40, dd (2.0, 11.87)
1‴	4.91, d (7.4)	4.92, d (7.1)							
2‴	3.29−3.33, m	3.26, m							
3‴		3.28, m							
4‴	3.18, m	3.16, m							
5‴	3.34, m	3.45, m							
6‴	3.47, m	3.46, m							
	3.70, m	3.70, m							
OCH_3				3.79, s	3.79, s			3.79, s	3.79, s
CH_2(mal)							3.37, d, (15.7)		3.41, d, (15.82)
							3.33, d, (15.7)		3.37, d (15.82)
OH		12.88, s, OH-5				9.64, br s, OH-4′ 12.94, br s, OH-5	9.63, br s, OH-4′ 12.95, br s, OH-5		

[a] Doublet-like multiplets of AA′XX′ spin system.

Table 2. ^{13}C NMR Spectral Data for Compounds **1**−**8** and **11** at (500 MHz) at 30 °C, Recorded in DMSO-d_6

site	1	2	3	4	5	6	7	8	11
2	153.8	155.2	153.3	153.4	153.5	154.6	154.6	153.7	159.0
3	123.4	124.1	122.3	123.7	123.5	121.0	122.7	123.4	126.7
4	174.7	180.9	174.8	174.6	174.6	180.5	180.6	174.7	174.4
5	127.0	161.7	127.0	129.9	126.9	163.1	161.7	126.9	126.9
6	115.7	99.8	115.6	115.5	115.5	94.6	99.7	115.7	118.5
7	161.6	163.2	161.5	161.4	161.4	161.7	162.7	161.5	166.3
8	103.6	94.5	103.5	103.4	103.4	99.6	94.6	103.5	103.6
9	157.1	157.3	157.1	156.9	156.9	157.6	157.3	157.1	153.0
10	118.6	106.2	118.5	118.4	118.4	106.1	106.3	118.5	123.3
1′	125.4	122.3	123.8	125.9	124.4	122.6	121.1	124.1	123.8
2′	130.0	130.2	130.1	115.2	119.6	130.2	130.2	131.1	129.7
3′	116.1	116.2	115.0	146.5	153.5	115.1	115.2	113.7	113.5
4′	157.3	157.4	157.3	147.1	147.6	157.2	157.6	159.1	161.0
5′	116.1	116.2	115.0	113.3	111.9	115.1	115.2	113.7	113.5
6′	130.0	130.2	130.1	121.5	116.4	130.2	130.2	131.1	129.7
1″	100.2	99.9	100.1	99.9	99.9	99.9	99.7	100.1	99.9
2″	73.2	73.2	73.2	73.1	73.1	73.0	73.1	73.8	72.9
3″	76.6	76.5	76.5	76.4	76.4	76.5	76.2	76.2	76.1
4″	69.8	69.8	69.7	69.6	69.6	69.7	69.7	69.7	69.6
5″	77.1	77.2	77.3	77.2	77.2	77.2	73.9	77.3	73.9
6″	60.8	60.7	60.7	60.6	60.6	60.7	64.0	60.7	63.8
1‴	100.5	100.4							
2‴	73.3	73.3							
3‴	76.8	76.7							
4‴	69.9	69.7							
5‴	77.3	77.3							
6‴	60.7	60.8							
OCH_3					55.7	55.6		55.2	54.9
CO							168.0		169.7
							167.1		167.1
CH_2 (mal)							41.8		41.1

1623, 1514 cm^{-1}; UV λ_{max} nm 235, 260.4 (C_2H_5OH); lit. mp and spectral data (*18*); ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz), see **Tables 1** and **2**.

*3′-Methoxydaidzin (**4**):* white powder; mp 188−190 °C (dec); $[\alpha]_{576}^{20}$ ± 0° (*c* 0.7, C_2H_5OH); MALDI, *m/z* 468.85 $[M + Na]^+$; IR (KBr) ν_{max} 3378, 2920, 2851, 2170, 1625, 1514 cm^{-1}; UV λ_{max} nm 211, 249, 264 (C_2H_5OH); lit. mp and spectral data (*19*); ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz), see **Tables 1** and **2**.

*7-O-β-D-Glucopyranosylcalycosin (**5**):* white powder; mp 248−255 C; $[\alpha]_{576}^{20}$ ± 0° (*c* 0.6, C_2H_5OH); MALDI, *m/z* (relative intensity) 469.08 (30), $[M + Na]^+$, 285.13 (100) $[M]^+$; IR (KBr) ν_{max} 3379, 2922, 2853, 2170, 1728, 1624, 1512 cm^{-1}; UV λ_{max} nm 216, 250, 262, 286

Table 3. ^{1}H and ^{13}C NMR Spectral Data (*J* in Hertz) for Compounds **16**−**18**, Recorded in DMSO-d_6 at 30 °C (500 MHz for ^{1}H and 125 MHz for ^{13}C)

	16					**17**		**18**	
site	δ_H	δ_C	^{1}H−^{1}H 2D COSY correlated H for **16**, H	^{13}C−^{1}H 2D HSQC correlated C for **16**, C	^{13}C−^{1}H 2D HMBC correlated C for **16**	δ_H	δ_C	δ_H	δ_C
1	7.37, d (8.6)	131.9	H2 (6.72)	131.9	C11a, C3, C4a	7.39, d (8.5)	132.1	7.41, d (8.6)	132.1
2	6.72, dd (2.4, 8.6)	110.4	H1 (7.37)	110.4	C4, C11b	6.69, dd (2.6, 8.5)	110.2	6.70, dd (8.6), (2.5)	110.3
3		158.5					158.3		158.3
4	6.57, d (2.4)	104.1		104.1	C2, C11b	6.54, d (2.6)	104.3	6.54, d (2.5)	104.2
4a		156.2					156.4		156.2
6	8.31, s	153.9		153.9	C11a, C7a, C4a	3.61, m	65.9	3.59−3.67, m	66.0
						4.27, m		4.28, dd (4.3, 10.7)	
6a		125.6				3.61, m	39.9	3.59−3.67, m	38.9
6b		118.3					118.3		119.3
7	6.99, s	105.3		105.3	C6a, C9, C10a	6.98, s	105.5	7.25, d (8.8)	125.3
8		141.2					141.2	6.44, dd (2.2, 8.8)	106.2
9		147.5					147.6		160.6
10	6.52, s	93.2		93.2	C7a, C8	6.53, s	93.4	6.44, s	96.4
10a		153.6					153.7		160.3
11a	6.06, s	101.1		101.1	C1, C4a, C6, C7a	5.55, d (6.8)	77.7	5.58, d (6.9)	77.8
11b		114.2					110.3		114.2
1′	4.84, d (7.55)	100.4	H2′ (3.22)	100.4	C3	4.86, d (7.7)	102.1	4.87, d (7.6)	100.1
2′	3.22, m	73.2	H3′ (3.28)	73.2		3.25, dd (7.7, 8.8)	73.2	3.25, m	73.1
3′	3.28, m	76.6	H4′ (3.15)	76.6		3.29, t, (8.8)	76.4	3.29, t (9.0)	76.4
4′	3.15, m	69.7	H5′ (3.69)	69.7		3.17, dd (8.8, 9.5)	69.9	3.16, t (9.0)	69.9
5′	3.69, m	73.9	H6′ (4.28)	73.9		3.61, m	73.8	3.59, m (9.0, 11.9)	73.7
6′	3.69, m	64.1		64.1		4.09, dd (7.12, 11.7)	64.1	4.36, dd (2.0, 11.9)	64.2
	4.28, m					4.36, dd (2.0, 11.7)		4.08, dd (7.2, 11.9)	
7′, 9′		167.8					168.1		167.9
		166.7					167.2		166.9
OCH_3								3.69, s	55.3
O-CH_2−O	5.91, d (0.69)	101.1		101.1	C8, C9	5.91, d (0.8)	101.5		
	5.95, d (0.69)			101.1		5.95, d (0.8)			
CH_2(mal)	3.41, m	41.4		41.4	C7′, C9′	3.33, d (15.9)	41.9	3.35, d (16)	41.4
						3.36, d (15.9)		3.38, d (16)	

(C_2H_5OH); lit. mp and spectral data (*20, 21*); ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz), see **Tables 1** and **2**.

*Genistin (**6**):* white powder; mp 252−254 °C; $[\alpha]_{576}^{20} \pm 0°$; (*c* 0.74, C_2H_5OH); MALDI, *m/z* (relative intensity) 455 (45) $[M + Na]^+$, 271 (70); IR (KBr) ν_{max} 3441, 2554, 2402, 2170, 1643, 1620, 1581, 1553, 1517 cm^{-1}; UV λ_{max} nm 202, 262, 327 (C_2H_5OH); lit. mp and spectral data (*17*); ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz), see **Tables 1** and **2**.

*6″-O-Malonylgenistin (**7**):* white powder; mp 175−179 °C; $[\alpha]_{576}^{20}$ −43° (*c* 0.64, C_2H_5OH); MALDI, *m/z* 541 $[M + Na]^+$; IR (KBr) ν_{max} 3317, 1655, 1615 cm^{-1}; UV λ_{max} nm 203, 262 (C_2H_5OH); lit. mp and spectral data (*22*); ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz), see **Tables 1** and **2**.

*Ononin (**8**):* white powder; mp 203−205 °C; $[\alpha]_{576}^{20}$ −48° (*c* 0.86, C_2H_5OH); MALDI, *m/z* (relative intensity) 453.5 (10) $[M + Na]^+$, 269 (20); IR (KBr) ν_{max} 3402, 2924, 2517, 2170, 1625, 1513 cm^{-1}; UV λ_{max} nm 208, 259 (C_2H_5OH); lit. mp and spectral data (*20, 21, 23*); ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz), see **Tables 1** and **2**.

*6″-O-Malonylononin (**11**):* colorless needles; mp 153−155 °C; $[\alpha]_{576}^{20}$ +67° (*c* 0.75, C_2H_5OH); MALDI, *m/z* (relative intensity) 539.3 (10) $[M + Na]^+$, 517.2 (5), 269.2 (100); IR (KBr) ν_{max} 3422, 2923, 2170, 1733, 1631, 1515, 1446 cm^{-1}; UV λ_{max} nm 260 (C_2H_5OH); lit. mp and spectral data (*23*); ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz), see **Tables 1** and **2**.

*Pseudobaptigenin (**14**):* colorless needles; mp 293−295 °C; MALDI, *m/z* 305.21 $[M + Na]^+$; IR (KBr) 3410, 1730, 1620, 1572, 1500, cm^{-1}; UV, λ_{max} nm 220 (sh), 249, 262 (sh), 294 (C_2H_5OH); lit. mp and spectral data (*27, 28*).

*Derrone (**15**):* white powder; mp 216−218 °C; $[\alpha]_{576}^{20}$ −14°(*c* 0.79, C_2H_5OH); MALDI, *m/z* 359.13 $[M + Na]^+$; IR (KBr) ν_{max} 3400, 3035, 1647, 1616, 1579, 1515, 1482 cm^{-1}; UV λ_{max} nm 211, 268, 304 (sh) (C_2H_5OH); lit. mp and spectral data (*29, 30*).

*6′-O-Malonyl-3-O-β-D-glucopyranosyl-6,6a-dehydromaackiain (**16**):* white powder; mp 155−158 °C (dec); $[\alpha]_{576}^{20}$ −100° (*c* 1.0, C_2H_5OH); MALDI, HR *m/z* 553.4337 $[M + Na]^+$; IR (KBr) ν_{max} 3381, 2922, 2854, 2360, 2258, 2170, 2128, 1738, 1620, 1503 cm^{-1}; UV λ_{max} nm 206, 267, 285, 302 (C_2H_5OH); CD $[\theta]_{nm}$ (*c* 1.0, MeOH) $[\theta]_{208}$ −29841, $[\theta]_{285}$ +2275, $[\theta]_{300}$ +1481, $[\theta]_{326}$ −385; lit. mass spectral data (*24*); ^{1}H NMR (500 MHz) and^{13}C NMR (125 MHz), see **Table 3**.

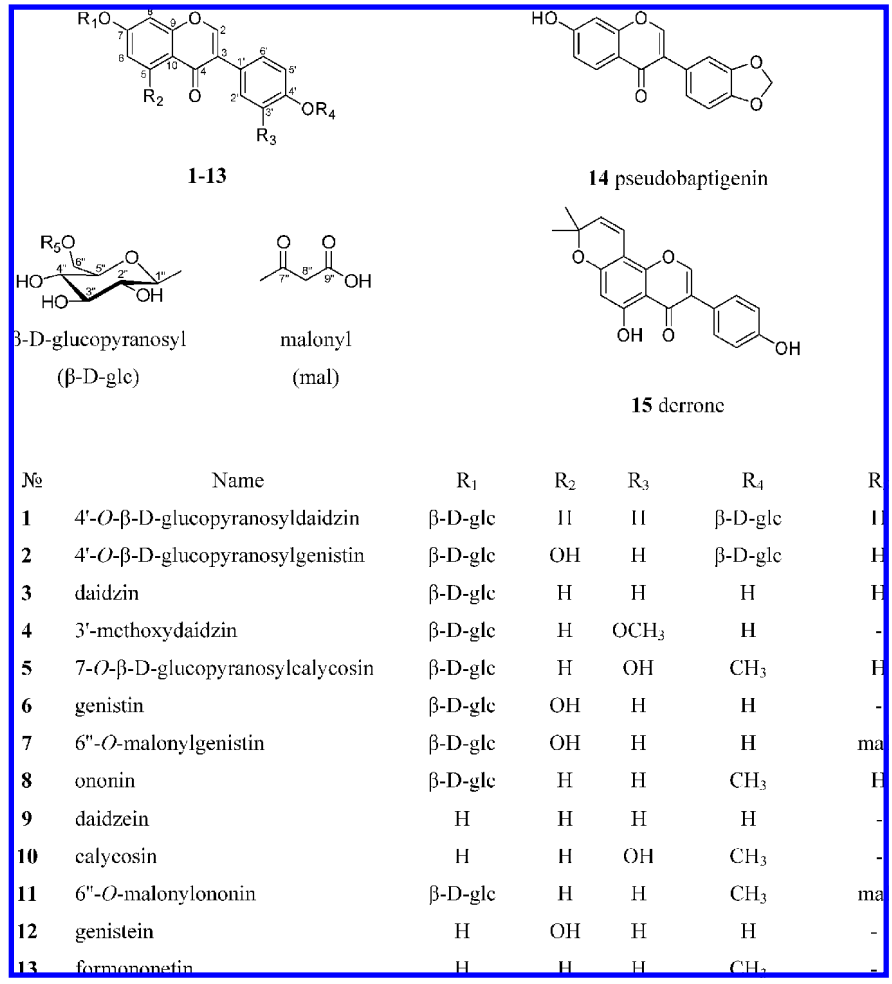
*(6aR,11aR)-6′-O-Malonyl-3-O-β-D-glucopyranosylmaackiain (**17**):* colorless plates; mp 125−127 °C; $[\alpha]_{576}^{20}$ +125° (*c* 0.8, C_2H_5OH); MALDI, *m/z* (relative intensity) 555.3 (50) $[M + Na]^+$, 284.1 (100); IR (KBr) ν_{max} 3428, 2898, 2169, 1746, 1620, 1588, 1503, 1475 cm^{-1}; UV, λ_{max} nm 283, 308 (C_2H_5OH); lit. mp and spectral data (*24, 25*); ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz), see **Table 3**.

*(6aR,11aR)-6′-O-Malonyl-3-O-β-D-glucopyranosylmedicarpin (**18**):* colorless plates; mp 185−190 °C; $[\alpha]_{576}^{20}$ −108° (*c* 1.0, C_2H_5OH); MALDI, *m/z* 541.2 $[M + Na]^+$; IR (KBr) ν_{max} 3410, 2924, 2170, 1745, 1621, 1597, 1498 cm^{-1}; UV, λ_{max} nm 278, 284, 316 (C_2H_5OH); lit. $[\alpha]_D$, mp, and spectral data (*26*); ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz), see **Table 3**.

Statistical Aalysis. For statistical evaluation, Student's *t* test was used for comparison between two independent groups. For comparison among multiple data, analysis of variance (ANOVA) followed by a multiple-comparison procedure was employed. Tukey's HSD post hoc test was employed for intergroup comparison. A difference of $P < 0.05$ was considered to be significant.

RESULTS

Establishing the A-18 Callus Line. The peculiarity of the relict Leguminosae tree *M. amurensis* is that it is difficult to regenerate callus tissue from explants of the tree. These primary calli grew slowly as brown tissues. In a previous work (*13*), we reported the establishment of callus cultures derived from leaves, leaf petioles, inflorescences, and apical meristems of the plant. However, the growth index of these calli was no higher than 8 (*13*). The seedling-derived primary calli consisted of pale, light brown, and dark brown cell aggregates with different



№	Name	R_1	R_2	R_3	R_4	R
1	4′-*O*-β-D-glucopyranosyldaidzin	β-D-glc	H	H	β-D-glc	H
2	4′-*O*-β-D-glucopyranosylgenistin	β-D-glc	OH	H	β-D-glc	H
3	daidzin	β-D-glc	H	H	H	H
4	3′-methoxydaidzin	β-D-glc	H	OCH_3	H	-
5	7-*O*-β-D-glucopyranosylcalycosin	β-D-glc	H	OH	CH_3	H
6	genistin	β-D-glc	OH	H	H	-
7	6″-*O*-malonylgenistin	β-D-glc	OH	H	H	ma
8	ononin	β-D-glc	H	H	CH_3	H
9	daidzein	H	H	H	H	-
10	calycosin	H	H	OH	CH_3	-
11	6″-*O*-malonylononin	β-D-glc	H	H	CH_3	ma
12	genistein	H	OH	H	H	-
13	formononetin	H	H	H	CH_3	-

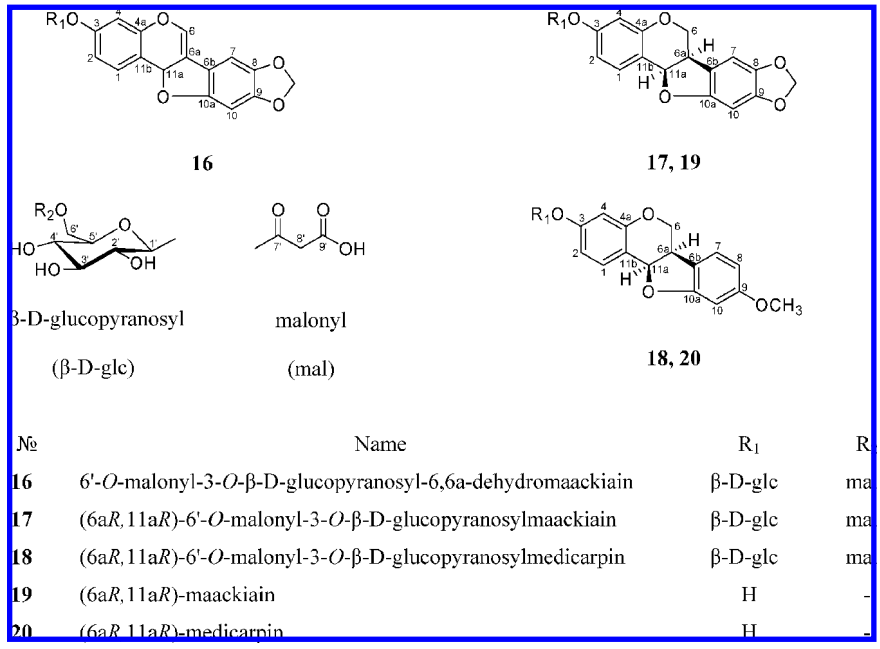
Figure 1. Chemical structures of isoflavones and their β-glucosides and malonylglucosides in *M. amurensis* A-18 callus culture. glc, glucoside; mal, malonate.

growth intensities. More deeply pigmented aggregates showed slower growth but a higher ability to produce isoflavonoids. Therefore, we selected vigorously growing light brown cell aggregates. A 3-year period was necessary to obtain homogeneous calli (A-18 callus culture) with a growth index of 16.7−24.2. After this period, we started chemical investigation of the culture.

Isoflavonoid Composition of *M. amurensis* A-18 Calli. Repeated column chromatography using Toyopearl HW-50F and Toyopearl HW-40F columns and preparative HPLC chromatography of the cell culture extract followed by recrystallization yielded 20 isoflavonoids, 6 of which were identified as free isoflavones (**9**,**10**, and **12**−**15**); 7 mono- and di-β-D-glucoside derivatives (**1**−**6** and **8**); 2 6″-*O*-malonyl-7-*O*-β-D-glucoside derivatives (**7** and **11**); 2 free pterocarpans (**19** and **20**); 2 6′-*O*-malonyl-3-*O*-β-D-glucoside prerocarpans (**17** and **18**); and a new pterocarpan, **16** (**Figures 1** and **2**). The physical, spectroscopic, and ^{1}H and ^{13}C NMR data for the isolated compounds showed identity with the following previously identified isoflavones: daidzein (**9**), calycosin (**10**), genistein (**12**), formononetin (**13**), pseudobaptigenin (**14**), and derrone (**15**); their β-glucosides and malonylglucosides, 4′-*O*-β-D-glucopyranosyldaidzin (**1**), 4′-*O*-β-D-glucopyranosylgenistin (**2**), daidzin (**3**), 3′-methoxydaidzin (**4**), 7-*O*-β-D-glucopyranosylcalycosin (**5**), genistin (**6**), 6″-*O*-malonylgenistin (**7**), ononin (**8**), 6″-*O*-malonylononin (**11**); and pterocarpans and their malonyl glucosides, (6a*R*,11a*R*)-maackiain (**19**), (6a*R*,11a*R*)-medicarpin (**20**), (6a*R*,11a*R*)-6′-*O*-malonyl-3-*O*-β-D-glucopyranosylmaackiain (**17**), (6a*R*,11a*R*)-6′-*O*-malonyl-3-*O*-β-D-glucopyranosylmedicarpin (**18**). Only a few authors have provided full NMR spectral assignments for the glucosidic component of the conjugated forms of isoflavonoids. Therefore, we summarized these data in **Tables 1**–**3**.

The results show that isoflavones are predominantly present as glucoside and malonyl-glucoside conjugated forms in the *M. amurensis* A-18 callus culture (**Figure 3**). It is known that the absence of acetyl glycosides in plant extracts is an indication that aggressive processing or inadequate preparation conditions were not used during the chemical analysis of isoflavonoids (*4, 31*). Because we have not detected such glycosides, it can be assumed that our isolation/purification procedure was adequate for the A-18 calli. This proposition was confirmed by acid hydrolysis of the isoflavonoid complex, yielding aglycones**9**, **10**, **12**−**14**, **19**, and **20** from the corresponding glycoconjugates.

Analysis of Compound 16. The high-resolution MALDI-MS $[M + Na]^+ = 553.4337$ (requires 553.4344) for **16** was in agreement with the molecular formula $C_{25}H_{22}NaO_{13}$. The molecular ion peak at *m*/*z* $[M + Na]^+$, which is smaller than that of (6a*R*,11a*R*)-6′-*O*-malonyl-3-*O*-β-D-glucopyranosylmaackiain by 2 mass units (**17**), suggests the presence of a double bond in the molecular structure of **16**. The ^{13}C and ^{13}C DEPT NMR spectra showed 25 signals (**Table 3**), 6 of which were assigned to the saccharide portion, 3 to a malonat portion, and 16 to a pterocarpan moiety. From the mass spectroscopy and ^{13}C and ^{13}C DEPT NMR data, the molecular formula of **16** was deduced to be $C_{25}H_{22}O_{13}$. The aromatic side of the ^{1}H NMR spectrum of **16** (**Table 3**) was similar to that of **17** and showed



№	Name	R_1	R_2
16	6'-*O*-malonyl-3-*O*-β-D-glucopyranosyl-6,6a-dehydromaackiain	β-D-glc	mal
17	(6a*R*,11a*R*)-6'-*O*-malonyl-3-*O*-β-D-glucopyranosylmaackiain	β-D-glc	mal
18	(6a*R*,11a*R*)-6'-*O*-malonyl-3-*O*-β-D-glucopyranosylmedicarpin	β-D-glc	mal
19	(6a*R*,11a*R*)-maackiain	H	-
20	(6a*R*,11a*R*)-medicarpin	H	-

Figure 2. Chemical structures of pterocarpans and their malonyl glucosides in *M. amurensis* A-18 callus culture. glc, glucoside; mal, malonate.

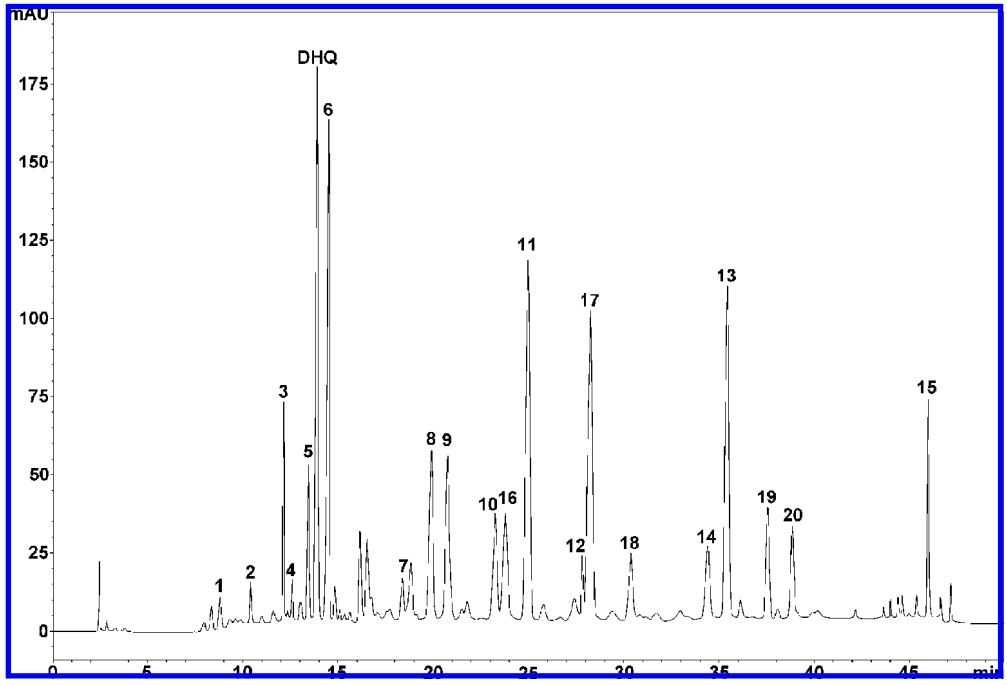


Figure 3. Reversed-phase HPLC analysis of isoflavonoids from the *M. amurensis* A-18 callus culture. $\lambda = 280$ nm. Peaks: 1, 4′-*O*-β-D-glucopyranosyldaidzin; 2, 4′-*O*-β-D-glucopyranosylgenistin; 3, daidzin; 4, 3′-methoxydaidzin; 5, 7-*O*-β-D-glucopyranosylcalycosin; 6, genistin; 7, 6″-*O*-malonylgenistin; 8, ononin; 9, daidzein; 10, calycosin; 16, 6′-*O*-malonyl-3-*O*-β-D-glucopyranosyl-6,6a-dehydromaackiain; 11, 6″-*O*-malonylononin; 12, genistein; 17, (6a*R*,11a*R*)-6′-*O*-malonyl-3-*O*-β-D-glucopyranosylmaackiain; 18, (6a*R*,11a*R*)-6′-*O*-malonyl-3-*O*-β-D-glucopyranosylmedicarpin; 14, pseudobaptigenin; 13, formononetin; 19, (6a*R*,11a*R*)-maackiain; 20, (6a*R*,11a*R*)-medicarpin; 15, derrone; DHQ, dihydroquercetin. The experimental conditions are given under Materials and Methods.

the presence of an ABX-type aromatic proton system appearing as a pattern of meta-coupled protons of ring A at δ 7.37 (d, J = 8.6 Hz, H-1), 6.72 (dd, J = 8.6, 2.4 Hz, H-2), and 6.57 (d, J = 2.4 Hz H-4). Signals at δ 6.99 (s, H-7) and 6.52 (s, H-10) belong to the aromatic protons of ring B located in a para arrangement. Signals at δ 5.95 (d, J = 0.69 Hz, 1H) and 5.91 (d, J = 0.69, 1H) in the ^{1}H NMR spectrum of **16** specify the presence of a methylenedioxy fragment (O−CH_2−O) in **16**, located in ring B. Moreover, the ^{1}H NMR spectrum of **16** shows the signal of an anomeric proton at δ 4.84 (d, J = 7.55, H-1′).

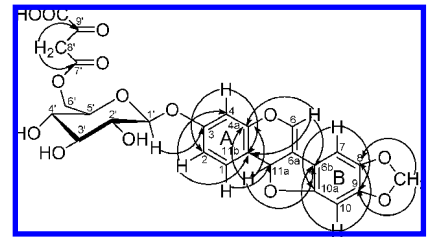


Figure 4. HMBC correlations of compound **16**.

Table 4. Important Chromatographic Constants of Isoflavonoids **1**−**20** and Their Content (% DW) in *M. amurensis* A-18 Calli

	retention time (t_R), correlation coefficients (K), and molecular weight (MW) of standard compds			harvesting time[a]						
compd	t_R	K	MW	March 2006	May 2006	Dec 2006	Feb 2007	Aug 2007	Oct 2007	mean ± SEM
Isoflavones, Their β-Glucosides, and Malonylglucosides										
1	8.8	0.17	578	0.022	0.066	nd[b]	0.005	nd	tr[c]	0.016 ± 0.011
2	10.1	0.41	594	0.140	0.100	0.007	0.012	0.052	0.049	0.060 ± 0.021
3	12.2	0.33	416	0.047	0.083	0.051	0.067	0.076	0.053	0.063 ± 0.006
4	12.6	0.46	446	0.013	0.016	0.007	0.011	tr	0.007	0.009 ± 0.002
5	13.4	0.25	446	0.035	0.056	0.077	0.089	0.027	0.027	0.052 ± 0.011
6	14.6	0.53	432	0.132	0.194	0.095	0.129	0.152	0.097	0.133 ± 0.015
7	18.2	0.35	518	0.035	0.095	0.022	0.028	0.094	0.105	0.063 ± 0.016
8	20.2	0.43	430	0.051	0.102	0.089	0.098	0.038	0.075	0.076 ± 0.011
9	21.2	0.85	254	0.004	0.021	0.044	0.049	tr	tr	0.020 ± 0.009
10	23.2	0.73	284	0.029	0.038	0.034	0.037	0.014	0.024	0.029 ± 0.004
11	25.3	0.46	516	0.258	0.381	0.165	0.192	0.085	0.234	0.219 ± 0.041
12	28.2	0.90	270	0.008	0.015	0.008	0.009	0.007	0.020	0.011 ± 0.002
13	35.5	0.92	268	0.037	0.066	0.074	0.084	tr	0.055	0.053 ± 0.012
14	34.2	0.44	282	0.016	0.030	0.038	0.043	nd	0.041	0.028 ± 0.007
15	46.0	1.10	336	0.058	0.064	0.027	0.029	0.013	0.028	0.037 ± 0.008
Pterocarpans and Their Malonylglucosides										
16	23.6	0.22	530	0.056	0.174	0.128	0.139	0.041	0.156	0.116 ± 0.022
17	28.9	0.13	532	0.121	0.231	0.487	0.619	0.101	0.130	0.282 ± 0.089
18	30.0	0.20	518	0.147	0.125	0.070	0.093	0.073	0.068	0.096 ± 0.013
19	37.6	0.24	284	0.030	0.040	0.072	0.090	tr	tr	0.039 ± 0.015
20	38.8	0.41	270	0.054	0.056	0.040	0.046	tr	0.016	0.035 ± 0.009
total isoflavonoids				1.293	1.953	1.535	1.888	0.773	1.233	1.437 ± 0.178

[a] Calli in 10 cultural vessels were cultivated for 45 days, combined and analyzed by HPLC as described under Materials and Methods. [b] Not detectable by HPLC. [c] Trace amounts (a substance was detectable by HPLC, but its quantity was below the level of determination, approximately <0.003% DW). Summary of all effects shows homogeneity of these probes (General MANOVA, α-level = 0.05, Wilks' lambda = 0.828, Rao's $R = 1.03, P > 0.05$).

Table 5. Main Biotechnological Parameters of the A-18 Line[a]

fresh biomass (g/L)	dry biomass (g/l)	% of dry biomass	isoflavonoid content (% DW)	isoflavonoid production (mg/L)
234 ± 21	13.5 ± 1.2	5.8	1.44 ±0.18	194 ± 17

[a] Calli were cultivated for 45 days. The data (mean ± SE) were obtained from 10 independent experiments with 10 replicates each.

The positions of the β-glucopyranosyloxy group at C-3 and methylenedioxy fragment (O−CH_2−O) at C-8 and C-9 in **16** were determined from the HMBC spectrum as shown in **Table 3** and **Figure 4**. Signals at δ_H 8.31 (s, H-6),$_C$ 153.9 (C-6), and 125.6 (C-6a) in the ^{1}H and ^{13}C NMR spectra of **16** specify the position of a double bond between C-6 and C-6a carbons at a pyran cycle of **16**. The signals at δ_C 167.8, 166.7, and 41.4 in the ^{13}C NMR, which suggest the presence of a malonyl group located at C-6′ of a glucosyl moiety, were confirmed by the presence of an additional methylene 2H multiplet resonance at δ_H 3.40 (m, H-8′) in the corresponding ^{1}H NMR spectrum of **16**. Proton and carbon signals were further assigned as shown in **Figure 4** by analysis of the COSY, HMQC, and HMBC spectra of **16** (**Table 3**). Thus, the structure of compound **16** was determined to be *6′-O-malonyl-3-O-β-D-glucopyranosyl-6,6a-dehydromaackiain*. Previously, compound **16** was proposed to be a minor component of *Oudneya africana* of the Brassicaceae family, but this compound has not been isolated as an individual compound, and identification of its molecular structure was not performed by NMR spectroscopy (*24*). Therefore, we consider that compound **16** was described in the present work for the first time. Compound **16** is one of the main isoflavonoids of the A-18 *M. amurensis* cell culture (**Table 4**). It was unstable and subject to decomposition during several NMR experiments in DMSO-d_6 at 30 °C. Degradation of this substance was not observed if the NMR experiments were performed for 4 h or less.

Isoflavonoid Production by A-18 Calli. The A-18 callus line has been analyzed by HPLC for two years (2006−2007) (**Table 4**). During this time, the calli produced total amounts of isoflavonoids equal to 1.437 ± 0.178% DW, that is comparable to levels of monomeric polyphenols (stilbenes and isoflavonoids) in *M. amurensis* heartwood [1.57−1.83% DW (*10*)]. Analysis showed that the A-18 callus culture produced free and glycosylated isoflavones at a ratio of ∼1:7 and free and glycosylated pterocarpans at a ratio of ∼1:5. The homogeneity test (General MANOVA) revealed no significant differences in total isoflavonoid content in the calli cultivated from 2006−2007 (**Table 4**). There were also no significant difference between the groups of isoflavonoids and pterocarpans and between the groups of aglycones and their conjugated forms (ANOVA followed by Tukey HSD test, $P > 0.05$). This result indicates stable production of isoflavonoids during prolonged periods of cultivation. The kinetic of growth of the A-18 culture was presented by the typical sigmoid curve with a lag phase (0−7 days), the phase of exponential growth (8−21 days), linear growth (22−35 days), and the phase of stationary growth (36−50 days). The dynamics of isoflavonoid accumulation during subculture were characterized by continuously increasing levels of these substances up to days 45−50 (stationary phase of growth); after that, their levels declined (data not shown). The main biotechnological parameters of the A-18 calli are presented in **Table 5**.

Our results show that the chemical compositions of PHW and PCC of *M. amurensis* are considerably different. Monomeric and dimeric stilbenes identified as main components of the polyphenolic complex of heartwood (*10*) were not found in substantial quantities in the A-18 callus culture.

Interestingly, the biosynthetic route of isoflavonoid production in the *M. amurensis* calli is orchestrated so that genistein (**12**), daidzein (**9**), formononetin (**13**), and both pterocarpans (**19** and **20**) can be rapidly metabolized to their conjugated forms: **1**−**3**,

6−**8**, **11**, **17**, and **18**. In contrast, calycosin (**10**), pseudobaptigenin (**14**), and derrone (**15**) seem to have a decreased ability to form conjugates because no glycosides of **14** and **15** were found, and only a small amount of the calycosin derivative **5** was identified (**Table 4**). Maackiain (**19**) and maackiain-derived isoflavonoids were predominant in the calli (0.437% DW); other polyphenols ranged as follows (aglycone and its derivatives, % DW): formononetin (**13**), 0.348; genistein (**12**), 0.267; daidzein (**9**), 0.151; medicarpin (**20**), 0.131; calycosin (**10**), 0.038; derrone (**15**), 0.037; and pseudobaptigenin (**14**), 0.028.

DISCUSSION

Our results indicate that isoflavonoids could be readily obtained from *M. amurensis* callus cultures without special elicitor treatments commonly used to induce secondary metabolism in cultured plant cells. During the time of investigation (two years), the A-18 calli produced total amounts of isoflavonoids equal to 1.44 ± 0.18% DW, that is comparable to levels of monomeric polyphenols in *M. amurensis* heartwood (1.57−1.83% DW of a sum of stilbenes and isoflavonoids) (*10*). Isoflavonoid content in A-18 calli was stable during long-term cultivation. Both types of the main Leguminosae isoflavones, liquiritigenin-derived daidzein and naringenin-derived genistein, as well as other types of isoflavones and pterocarpans and their derivates were produced by the calli. Despite such complex composition, the proportions of isoflavonoid types were quite stable, demonstrating high conservation of the isoflavonoid biosynthetic machinery in the A-18 callus line. This result is in agreement with numerous observations indicating stable production of these secondary metabolites in different plant cell cultures. For example, Federici et al. (*32*) reported that 25-year-old *Glycine max* cell cultures maintained the ability to produce high levels of isoflavones. These secondary metabolites have been shown to function as constitutive phytoalexins, and their preformed pools serve to defend plants against microbial pathogens (*33*).

Interestingly, despite the ability of *M. amurensis* plants to produce large amounts of stilbenes (as mentioned in the Introduction), we could not detect these substances in our callus cultures. This resembles the situation for *Vitis* spp. calli, for which numerous attempts to obtain large amounts of resveratrol by plant cell culture failed. The inability of plant cells to produce high levels of resveratrol and the low efficiency of standard biotechnological methods pose major problems to resveratrol production in vitro. It was recently suggested that a component of the signaling network that controls stilbene biosynthesis in plant cells is constitutively repressed by Tyr phosphorylation (*34*). Therefore, the activation of stilbene production requires special manipulations such as integration of a transgene encoding a protein tyrosine phosphatase (*34*). It is likely that stilbene and isoflavonoid production in *M. amurensis* cells are controlled by signaling pathways that use different regulatory mechanisms.

Pharmacological and cytological investigations as well as magnetic resonance imaging data are under investigation to confirm the interest of such compounds for the treatment of acute and chronic liver diseases in animal models.

ABBREVIATIONS USED

DW, dry weight; FW, fresh weight; PHW, polyphenolic complex from the heartwood of *M. amurensis*; PCC, polyphenolic complex from the cell culture of *M. amurensis*.

LITERATURE CITED

(1) Veitch, N. C. Isoflavonoids of the Leguminosae. *Nat. Prod. Rep.* **2007**, *24*, 417–464.

(2) McCue, P.; Shetty, K. Health benefits of soy isoflavonoids and strategies for enhancement: a review. *Crit. Rev. Food Sci. Nutr.* **2004**, *44*, 361–367.

(3) Reynaud, J.; Guilet, D.; Terreux, R.; Lussignol, M.; Walchshofer, N. Isoflavonoids in non-leguminous families: an update. *Nat. Prod. Rep.* **2005**, *22*, 504–515.

(4) Lin, L.-Z.; He, X.-G.; Lindenmaier, M.; Yang, J.; Cleary, M.; Qiu, S.-X.; Cordell, G. A. LC-ESI-MS study of the flavonoid glycoside malonates of red clover (*Trifolium pratense*). *J. Agric. Food Chem.* **2000**, *48*, 354–365.

(5) Akiyama, T.; Ishida, J.; Nakagawa, S.; Ogawara, H.; Watanabe, S.; Itoh, N.; Shibuya, M.; Fukami, Y. Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J. Biol. Chem.* **1987**, *262*, 5592–5595.

(6) Wang, S. Y.; Yang, K. W.; Hsu, Y. T.; Chang, C. L.; Yang, Y. C. The differential inhibitory effects of genistein on the growth of cervical cancer cells in vitro. *Neoplasma* **2001**, *48*, 227–233.

(7) Cos, P.; Calomme, M.; Sindambiwe, J. B.; De Bruyne, T.; Cimanga, K.; Pieters, L.; Vlietinck, A. J.; Vanden Berghe, D. Cytotoxicity and lipid peroxidation-inhibiting activity of flavonoids. *Planta Med.* **2001**, *67*, 515–519.

(8) Merken, H. M.; Beecher, G. R. Measurement of food flavonoids by high-performance liquid chromatography: a review. *J. Agric. Food Chem.* **2000**, *48*, 577–599.

(9) Cornwell, T.; Cohick, W.; Raskin, I. Dietary phytoestrogens and health. *Phytochemistry* **2004**, *65*, 995–1016.

(10) Fedoreyev, S. A.; Kulesh, N. I.; Glebko, L. I.; Pokushalova, T. V.; Veselova, M. V.; Saratikov, A. S.; Vengerovskii, A. I.; Chuchalin, V. S. Maksar: a preparation based on Amur Maackia. *Pharm. Chem. J.* **2004**, *38*, 605–610.

(11) Maksimov, O. B.; Kulesh, N. I.; Fedoreyev, S. A.; Glebko, L. I.; Pokushalova, T. V.; Stepanenko, L. S.; Krivoschekova, O. E.; Gorovoy, P. G.; Saratikov, A. S.; Chuchalin,V. S.; Vlasova, T. V. RF Patent 2104027. Hepatoprotective means. *Byull. Izobret.* **1998**, No. 4.

(12) Vengerovskii, A. I.; Baturina, N. O.; Chuchalin, V. S.; Saratikov, A. S. Effect of polyphenol-containing hepatoprotectors on the experimental chronic hepatitis pattern. *Pharm. Chem. J.* **1996**, *30*, 69–71.

(13) Fedoreyev, S. A.; Pokushalova, T. V.; Veselova, M. V.; Glebko, L. I.; Kulesh, N. I.; Muzarok, T. I.; Seletskaya, L. D.; Bulgakov, V. P.; Zhuravlev, Yu. N. Isoflavonoid production by callus cultures of *Maackia amurensis*. *Fitoterapia* **2000**, *71*, 365–372.

(14) Saratikov, A. S.; Chuchalin, V. S.; Rat'kin, A. V.; Rat'kin, E. V.; Fedoreyev, S. A.; Bulgakov, V. P. Hepatoprotive properties of polyphenolic complexes from the wood and cell culture of *M. amurensis*. *Eksp. Klin. Farmakol.* **2005**, *68*, 51–54.

(15) Bulgakov, V. P.; Tchernoded, G. K.; Mischenko, N. P.; Khodakovskaya, M. V.; Glazunov, V. P.; Radchenko, S. V.; Zvereva, E. V.; Fedoreyev, S. A.; Zhuravlev, Yu. N. Effect of salicylic acid, methyl jasmonate, ethephon and cantharidin on anthraquinone production by *Rubia cordifolia* callus cultures transformed with the rolB and rolC genes. *J. Biotechnol.* **2002**, *97*, 213–221.

(16) Kobayashi, M.; Ohta, Y. Induction of stress metabolite formation in suspension cultures of *Vigna angularis*. *Phytochemistry* **1983**, *22*, 1257–1261.

(17) Watanabe, K.; Kinjo, J.; Nohara, T. Three new isoflavonoid glycosides from *Lupinus luteus* and *L. polyphyllus*× *arboreus*. *Chem. Pharm. Bull.* **1993**, *41*, 394–396.

(18) Yuldashev, M. P.; Batirov, E.; Kh., Malikov, V. M.; Yuldasheva, N. P. Flavonoids of *Psoralea drupaceae* and *Reseda luteola*. *Chem. Nat. Compd.* **1996**, *32*, 923–924.

(19) Yahara, S.; Ogata, T.; Saijo, R.; Konishi, R.; Yamahara, J.; Miyahara, K.; Nohara, T. Isoflavan and related compounds from *Dalbergia odorifera*. *Chem. Pharm. Bull.* **1989**, *37*, 979–987.

(20) Ma, X.; Tu, P.; Chen, Y.; Zhang, T.; Wei, Y.; Ito, Y. Preparative isolation and purification of two isoflavones from *Astragalus membranaceus* Bge. var. *mongholicus* (Bge.) Hsiao by high-speed counter-current chromatography. *J. Chromatogr., A* **2003**, *992*, 193–197.

(21) Lee, H.-J.; Lee, O.-K.; Kwon, Y.-H.; Choi, D.-H.; Kang, H.-Y.; Lee, H.-Y.; Paik, K.-H.; Lee, H.-J. Isoflavone glycosides from the bark of *Amorpha fruticosa*. *Chem. Nat. Compd.* **2006**, *42*, 415–418.

(22) Park, H.-H.; Hakamatsuka, T.; Noguchi, H.; Sanakawa, U.; Ebizuka, Y. Isoflavone glucosides exist as their 6″-*O*-malonyl esters in *Pueraria lobata* and its cell suspension cultures. *Chem. Pharm. Bull* **1992**, *40*, 1978–1980.

(23) Coronado, C.; Zuanazzi, J. A. S.; Sallaud, C.; Quirion, J.-C.; Esnault, R.; Husson, H.-P.; Kondorosi, A.; Ratet, P. Alfalfa root flavonoid production is nitrogen regulated. *Plant Physiol.* **1995**, *108*, 533–542.

(24) Stocker, P.; Yousfi, M.; Salmi, C.; Perrier, J.; Brunel, J. M.; Moulin, A. Maackiain 3-*O*-(6′-*O*-malonyl-*β*-D-glucopyranoside) from *Oudneya africana*, a powerful inhibitor of porcine kidney acylase I. *Biochimie* **2005**, *87*, 507–512.

(25) Yamamoto, H.; Ichimura, M.; Tanaka, T.; Iinuma, M.; Mizuno, M. A trifolirhizin malonate from *Sophora flavescens* var. *angustifolia* and its stability. *Phytochemistry* **1991**, *30*, 1732–1733.

(26) Weidemann, C.; Tenhaken, R.; Hohl, U.; Barz, W. Medicarpin and maackiain 3-*O*-glucoside-6′-*O*-malonate conjugates are constitutive compounds in chickpea (*Cicer arietinum* L.) cell cultures. *Plant Cell Rep.* **1991**, *10*, 371–374.

(27) Ohashy, H.; Goto, M.; Imamura, H. Flavonoids from the wood *Cladrastis platicarpa*. *Phytochemistry* **1976**, *15*, 354–355.

(28) Yoon, J. S.; Sung, S. H.; Park, J. H.; Kim, Y. C. Flavonoids from *Spatholobus suberectus*. *Arch. Pharm. Res.* **2004**, *27*, 589–592.

(29) Chibber, S. S.; Sharma, R. P. Derrone, a new pyranoisoflavone from *Derris robusta* seeds. *Phytochemistry* **1980**, *19*, 1857–1858.

(30) Rao, M. K. S. R.; Rukmani, C. S.; Iyer, P. R. Synthesis of alpinum isoflavone, derrone and related pyranoisoflavones. *Tetrahedron* **1987**, *43*, 30153019.

(31) Wiseman, H.; Casey, K.; Clarke, D. B.; Barnes, K. A.; Bowey, E. Isoflavone aglycon and glucoconjugate content of high- and low-soy U.K. foods used in nutritional studies. *J. Agric. Food Chem.* **2002**, *50*, 1404–1410.

(32) Federici, E.; Touche, A.; Choquart, S.; Avanti, O.; Fay, L.; Offord, E.; Courtois, D. High isoflavone content and estrogenic activity of 25 year-old *Glycine max* tissue cultures. *Phytochemistry* **2003**, *64*, 717–724.

(33) Subramanian, S.; Graham, M. Y.; Yu, O.; Graham, T. L. RNA interference of soybean isoflavone synthase genes leads to silencing in tissues distal to the transformation site and to enhanced susceptibility to *Phytophthora sojae*. *Plant Physiol.* **2005**, *137*, 1345–1353.

(34) Kiselev, K. V.; Dubrovina, A. S.; Veselova, M. V.; Bulgakov, V. P.; Fedoreyev, S. A.; Zhuravlev, Yu. N. The *rolB* gene-induced overproduction of resveratrol in *Vitis amurensis* transformed cells. *J. Biotechnol.* **2007**, *128*, 681–692.

Received for review April 17, 2008. Revised manuscript received June 23, 2008. Accepted June 25, 2008. This work was supported by grants from the Russian Foundation for Basic Research, the Grant Program in "Molecular and Cell Biology" and "Fundamental sciences for medicine" of the Russian Academy of Sciences, a FEB RAS grant, and a grant "Leading Schools of Thought" from the President of the Russian Federation.

JF801227Q