

ISOFLAVONE GLUCOSIDES EXIST AS THEIR 6"-O-MALONYL ESTERS IN *PUERARIA LOBATA* AND ITS CELL SUSPENSION CULTURES

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Methanol extracts prepared by mild and careful procedure from cultured cells of *P. lobata* were analyzed by reverse phase high performance liquid chromatography (HPLC) to reveal that most of the isoflavone 7-O-glucosides daidzin(1) and genistin(2), and 8-C-glucoside puerarin(3), exist as their 6"-O-malonyl esters. The presence of malonyl esters of 1, 2 and 3 was also detected in the fresh roots and stems of *P. lobata*.

KEYWORDS *Pueraria lobata*; isoflavone; glucoside; malonyl ester; Leguminosae

The roots of *Pueraria lobata* Ohwi (Leguminosae) have been used as the main ingredient of a traditional Chinese prescription, Gegen Tao (Kakkon To in Japanese), for treatment of early symptoms of the common cold. The main isoflavonoid components, such as daidzin(1), puerarin(3) and daidzein(4), have been proved to stimulate cerebral and coronary blood circulation.¹⁾ Isolation of some other isoflavonoids,^{2,3)} as well as novel phenolic glycosides, have been reported.⁴⁾ Cell suspension cultures induced from the stem of this plant produce most of the isoflavonoid components found in the mother plant⁵⁾ and have been employed for extensive biosynthetic studies.⁶⁾ Recent reports on the identification of 6"-O-malonyl esters of isoflavone-7-O-glucoside in some Leguminous plants⁷⁾ and their possible role as precursors of phytoalexins^{8,9)} in plant-pathogen interactions led us to re-examine isoflavonoid constituents of cultured cells of this plant. For extraction, sonication in methanol at room temperature (Method A) was employed instead of refluxing in methanol (Method B) since prolonged storage of the extracts at elevated temperature resulted in complete hydrolysis of malonyl esters.⁷⁾ After being passed

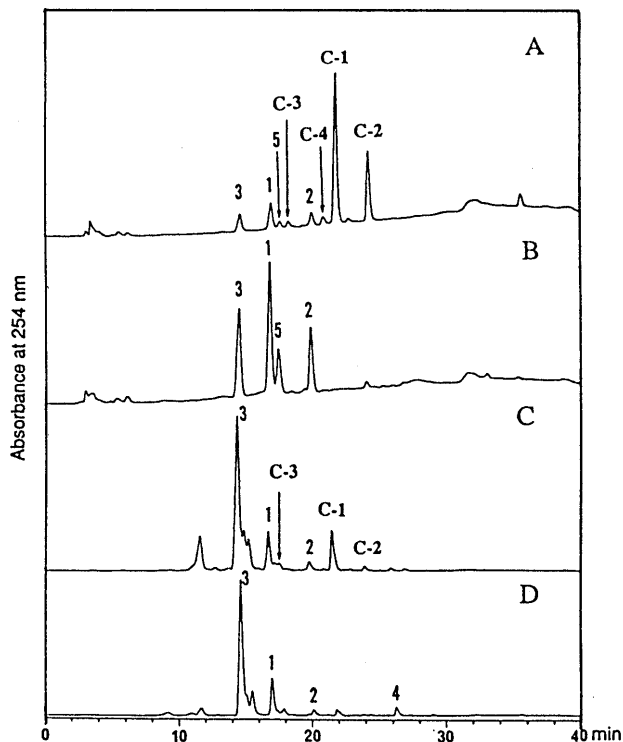
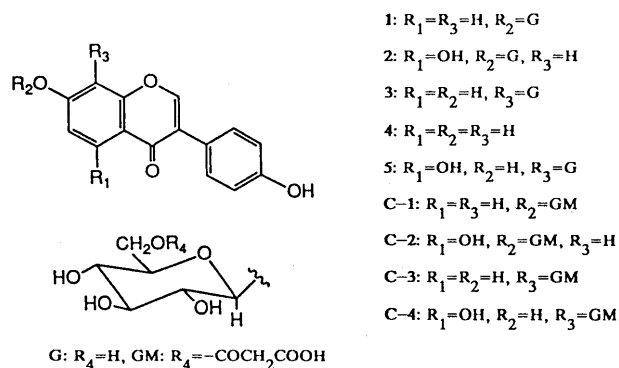


Fig. 1. HPLC of *P. lobata* MeOH Extracts
 A, B: cultured fresh cells. C: fresh roots. D: crude drug. Extracts prepared by Method A for A, C and D, by Method B for B (see Text).

through TOYOPAK-ODS (Tosoh) as methanol solution, thus obtained extracts were directly analysed by reverse phase HPLC (Tosoh ODS-80TM, 4.6 mm x 150 mm) using a linear gradient of methanol (20-80%/30 min, 0.6 ml/min, UV₂₅₄) in 1% acetic acid. As shown in Fig. 1, the chromatogram of the methanol extract of cultured fresh cells prepared by Method A was quite different from that prepared by Method B. When the former extract was kept for 48 h at r.t., the chromatogram changed into the one that is almost superimposable on that of the latter extract. Among the major peaks found in these chromatograms, 1, 2, 3 and 5 were readily identified as daidzin, genistin, puerarin and

genistein-8- β -glucoside, respectively, by their retention times. None of the four peaks, tentatively designated as C-1 to C-4, corresponded to any isoflavonoid constituent so far known in this plant. Separation of these metabolites was achieved using Lobar RP-18 (Merck, 25 mm x 310 mm) and ODS-80TM (Tosoh, 21.5 mm x 300 mm) columns by isocratic elution with 45-58% methanol in 1% acetic acid depending upon the polarity of individual components. C-1 and C-2 gave 1 and 2, respectively, by alkaline hydrolysis (1N-NaOH at r.t.) or when being kept at r.t. as methanol solution. ¹³C-NMR spectra (Table I) of C-1 and C-2 indicated the presence of malonyl group in their molecules, and they were identified as 6"-O-malonyl esters of 1 and 2 by comparison of their chemical and spectroscopic data to those reported.^{7,9,10,11)}

C-3 was obtained as colorless powder¹³⁾ and showed UV absorption maxima at 247 and 303 nm characteristic of 4',7-dihydroxyisoflavone. Either acidic (1N-HCl reflux) or alkaline (1N-NaOH at r.t.) hydrolysis afforded 3. FAB-MS determined its MW as 502 (Found m/z; 503.1219 [M+H]⁺, Calcd. for C₂₄H₂₃O₁₂; 503.1190), which is 86 mass units (C₃H₂O₃) larger than 3, suggesting the presence of a malonyl group in its molecule. In the ¹³C-NMR spectrum (Table I) of C-3, malonyl signals appeared at δ 41.38(t), 166.86(s) and 167.79(s) in addition to those corresponding to 3. Low field shift of glucose C-6 resonance at δ 64.95(t) established the ester linkage at 6" of glucose residue. C-4 afforded 5 under the

Table I. ¹³C-NMR Data for C-1, C-2 and C-3

Carbon No	C-1	C-2	C-3
2	153.15(d)	156.06(d)	152.50(d)
3	123.65(s)	124.19(s)	123.07(s)
4	174.68(s)	181.95(s)	174.80(s)
5	127.01(d)	163.27(s)	126.23(d)
6	115.24(d)	101.10(d)*	114.90(d)
7	161.07(s)	164.24(s)	160.93(s)
8	103.55(d)	96.13(d)	112.28(s)
9	157.19(s)	158.80(s)**	156.09(s)
10	118.55(s)	107.82(s)	116.81(s)
1'	122.26(s)	122.64(s)	122.49(s)
2',6'	129.95(d)	131.73(d)	129.93(d)
3',5'	114.91(d)	116.66(d)	114.90(d)
4'	156.89(s)	159.08(s)**	157.06(s)
1"	99.78(d)	101.16(d)*	73.32(d)
2"	72.97(d)	74.58(d)	70.47(d)*
3"	76.16(d)	77.73(d)	78.11(d)**
4"	69.63(d)	71.23(d)	70.08(d)*
5"	73.80(d)	75.41(d)	78.36(d)**
6"	64.01(t)	65.38(t)	64.95(t)
-CO-	166.70(s)	169.57(s)	166.86(s)
-CH ₂ -	41.37(t) ¹²⁾	42.03(t)	41.38(t)
-COOH	167.73(s)	173.57(s)	167.79(s)

*,**) may be reversed within each column.
(125 MHz, in DMSO-d₆, 35°C.)

Table II. Isoflavonoid Contents^{a)} in Roots, Stems and Cultured Cells of *P. lobata*

	Daidzin(1)	C-1	Genistin(2)	C-2	Puerarin(3)	C-3
Roots ^{b)}	8,380	8,550	1,120	630	37,050	1,625
Stems	35	103	21	27	147	n.d. ^{d)}
Cultured Cells						
5 Days Old	31	319	26	156	40	40
10 Days Old ^{c)}	119	470	53	194	86	32

a) nmol/g fr.wt. b) Fig. 1C. c) Fig. 1A. d) not detected.

hydrolytic condition described for C-3. Full spectroscopic characterization of C-4 was hampered because of its low yield and instability; however, similar chemical and chromatographic behavior of C-4 suggested that it was 6"-O-malonyl ester of 5. 6"-O-Malonyl ester of 1 was reported in the leaves, hypocotyls¹⁰⁾ and cotyledons⁹⁾ of the soybean (*Glycine max*), and that of 2 in the roots and stems of *Trifolium pratense*⁷⁾ and in the roots of *Baptisia australis*,⁷⁾ *Ononis spinosa*⁷⁾ and *Lupinus albus*.¹¹⁾ C-3 is the first example of 6"-O-malonyl ester of isoflavone-8-C-glucoside as a natural product.

Fresh roots (Fig. 1C) and stems of *P. lobata*¹⁴⁾ were extracted and analysed as described above to show the presence of C-1 to C-3, though the proportion of malonyl esters to normal glucosides was relatively low compared to that found in cultured cells, where malonyl esters dominated over normal isoflavone glucosides, especially in younger cells (Table II). The physiological significance of these malonyl esters in Leguminous plants is not clear at the moment; however, one possible explanation is that they are a storage form of biosynthetic precursors for pterocarpin phytoalexins, as has been suggested in the chickpea (*Cicer arietinum*)⁸⁾ and soybean.⁹⁾ Elicitor treatment of *P. lobata* cell suspension cultures is now in progress to substantiate this possibility.¹⁵⁾ Since no such malonyl esters were detected in the commercial crude drug (Fig. 1D),¹⁶⁾ future evaluation and preparation of this crude drug should take the above facts into considerations.

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- 12) Reported at δ 20.59 in reference 10).
- 13) mp 208°C; ¹H-NMR (500 MHz, DMSO-d₆, J in Hz); δ : 8.31 (1H, s, H-2), 7.94 (1H, d, J=8.5, H-5), 7.40 (2H, d, J=8.5, H-2',6'), 6.99 (1H, d, J=8.5, H-6), 6.80 (2H, d, J=8.5, H-3',5'), 4.84 (1H, d, J=9.5, H-1").
- 14) Collected in July 1991 on the campus of this University.
- 15) Pterocarpin tuberosin, a main phytoalexin of *P. lobata*, can be induced with a glyco-protein elicitor prepared from *Phytophthora megasperma* f. sp. *glycinea*.^{6f)}
- 16) Purchased on the Tokyo market.

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