Minor Isoflavones from the Roots of *Pueraria mirifica*

John L. Ingham*, Satoshi Tahara**, and Stanley Z. Dziedzic*

* Department of Food Science, Food Studies Building, University of Reading, Whiteknights, P.O. Box 226, Reading RG6 2AP, England

** Department of Agricultural Chemistry, Faculty of Agriculture, Hokkaido University, Kita-ku, Sapporo 060, Japan

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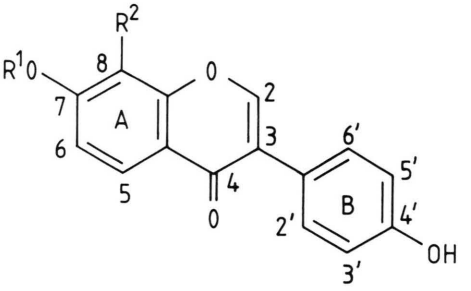
The isoflavone aglycone kwakhurin hydrate, and the glycosides genistin (genistein-7-O-glucoside) and puerarin-6″-monoacetate have been isolated from a methanolic extract of *Pueraria mirifica* roots.

Introduction

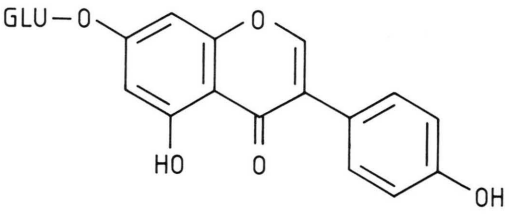
In previous papers we described the isolation of three isoflavone aglycones (daidzein, genistein and kwakhurin) [1, 2], three isoflavone glycosides (daidzin **1**, puerarin **2**, and mirificin) [1, 3], and four coumestans (coumestrol, mirificoumestan, mirificoumestan hydrate and mirificoumestan glycol) [4] from roots of the Thai forest legume *Pueraria mirifica* Airy Shaw & Suvatabandhu. In this final paper of the series we report the isolation of three further isoflavones, genistin **3** (genistein-7-O-glucoside), puerarin-6″-monoacetate **4**, and kwakhurin hydrate **5**, from *P. mirifica* roots. Isoflavones **4** and **5** are recognized for the first time as natural products.



1: R^1 = GLU; R^2 = H
2: R^1 = H ; R^2 = GLU
4: R^1 = H ; R^2 = GLU-6″-OAc

Results and Discussion

A methanolic extract of dried *P. mirifica* root was fractionated by Si gel TLC (Merck, F-254, layer thickness 0.5 mm) in $CHCl_3$–MeOH (CM, 20:1) as described earlier [1] to afford numerous fluorescence-quenching bands, many of which exhibited a blue or light blue fluorescence under long wavelength (365 nm) UV light. Apart from various known isoflavonoid aglycones [1, 2, 4], a minor component at approx. *Rf* 0.08 was identified as kwakhurin hydrate (**5**) by UV, MS and Si gel TLC comparison with authentic material previously obtained by treatment of kwakhurin with HCOOH [2].



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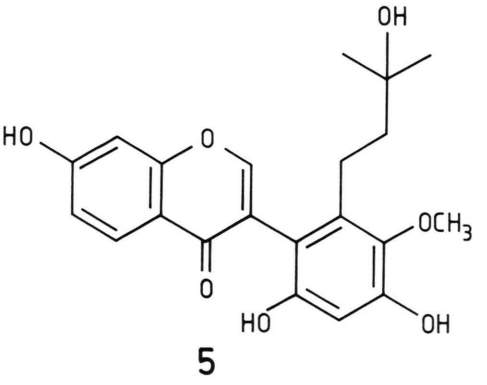
As expected, elution (MeOH) and further Si gel TLC ($CHCl_3$–MeOH–H_2O, CMH, 20:10:1) of the origin zone (*Rf* 0.00–0.05) from CM chromatograms yielded daidzin (**1**, daidzein-7-O-glucoside), puerarin



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Reprint requests to Dr. J. L. Ingham.

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(**2**, daidzein-8-C-glucoside) and mirificin (puerarin-6″-apioside) [1, 3]. However, upon TLC in benzene–MeOH (BM, 20:3, ×3) the 'daidzin' material gradually separated into two compounds, pure daidzin **1** (major component, lower zone) and genistin **3** (genistein-7-O-glucoside; minor component, upper zone). The identification of genistin was based on a comparison (UV, TLC) with synthetic material. Genistin has previously been isolated from the flowers of *Pueraria thunbergiana* (= *P. lobata*) [5].

In addition to the four glycosides already mentioned, the CMH chromatogram revealed another compound running (*Rf* 0.69) above the daidzin/genistin band (*Rf* 0.63). This compound was immediately recognized as a 5-deoxyisoflavone from its typical long wavelength (365 nm) UV fluorescence on Si gel chromatograms (light blue, intensifying when fumed with NH_3 vapour [6]), and its UV spectrum in MeOH which resembled that of puerarin (**2**). A C-7 OH group was evident from the NaOAc-induced bathochromic shift of the methanolic UV maximum at 250 nm [6]. Treatment of the glycoside in MeOH with aqueous NH_3 caused the rapid formation of puerarin, a result consistent with acetylation of an aromatic (C-4′) and/or sugar OH group. Monoacetylation was favoured from the detection of an intense FD-MS ion at *m/z* 458 (M^+, base; *cf.* puerarin, M^+ 416).

A ^{1}H NMR study indicated that ring B of puerarin and the new glycoside were identical (4′-OH) since virtually co-incident chemical shift values were in each case obtained for H-2′/6′ and H-3′/5′ (Table I). Similarly, the sugar H-1″ was evident as a doublet at δ 5.09 in the ^{1}H NMR spectrum of both compounds, whilst the 2″–5″ protons appeared as a complex series of signals between δ 3.40 and 4.19. In contrast, the 6″ protons (–$C\underline{H}_2$OH) appeared at δ 3.75dd and 3.88dd in the spectrum of puerarin, shifting by 0.47 and 0.58 ppm to δ 4.22dd and 4.46dd respectively in that of the acetylated compound (OAc signal at δ 2.03s). These shifts to significantly lower field confirm that the new glycoside is acetylated on the sugar unit at C-6″. In view of its similarity to puerarin, we suggest that the compound should be named puerarin-6″-monoacetate (**4**).

In a previous study, Bhutani *et al.* [7] isolated puerarin-4′,6″-di-O-acetate from the roots of *Pueraria tuberosa*. However, no evidence was obtained to suggest that the latter compound was present in *P. mirifica* root extracts. Attempts to detect daidzein-7,4′-di-O-glucoside, earlier found in *P. thunbergiana (P. lobata)* [8], were similarly unsuccessful.

Table I. Comparative ^{1}H NMR data (δ values) for puerarin (**2**) and puerarin-6″-monoacetate (**4**)[a].

Proton	Puerarin (**2**)	Puerarin-6″-monoacetate (**4**)
H-2	8.14s	8.13s
H-5	8.01d (*J* = 8.8 Hz)	8.01d (*J* = 8.8 Hz)
H-6	6.94d (*J* = 8.8 Hz)	6.94d (*J* = 8.8 Hz)
H-2′/H-6′ [2H]	7.36d (*J* = 8.8 Hz)	7.35d (*J* = 8.8 Hz)
H-3′/H-5′ [2H]	6.84d (*J* = 8.8 Hz)	6.83d (*J* = 8.8 Hz)
H-1″	5.09d (*J* = 9.9 Hz)	5.09d (*J* = 9.9 Hz)
H-2″		
H-3″	3.40–3.60m [3H][b]	3.46–3.57m [2H][c]
H-4″		3.62–3.75m[c]
H-5″	4.17m[b]	4.19br t[d] (*J* = *ca.* 10 Hz)
H-6″ α, β	3.75dd (*J* = 12.1, 4.8 Hz)	4.22dd (*J* = 12.1, 5.9 Hz)
	3.88dd (*J* = 12.1, 1.8 Hz)	4.46dd (*J* = 12.1, 1.8 Hz)
OAc [3H]	–	2.03s

[a] Spectra were determined in MeOH-d_4 at 270 MHz (TMS reference). Except where indicated by [2H] or [3H] the signals integrated for 1 proton. Coupling constants *(J)* are given in parentheses.

[b] 3H and 1H signals probably H-3″/H-4″/H-5″, and H-2″ respectively.

[c] 2H and 1H signals probably H-3″/H-4″, and H-5″ respectively. The shift of the H-5″ signal to lower field (Δδ ~0.2 ppm) in **4** when compared with **2** is consistent with the influence of a 6″-O-acetyl substituent, *cf.* ^{1}H NMR data in MeOH-d_4 for mosesin-2 with a β-galactoside residue (sugar H-5 at δ 3.49, and H-6 at δ 3.67 and 3.72), and its 6-acetyl derivative mosesin-1 (sugar H-5 at δ 3.71 [Δδ 0.22 ppm], and H-6 at δ 4.14 [Δδ 0.47 ppm] and 4.35 [Δδ 0.63 ppm]) [11].

[d] 1H signal, probably H-2″, virtually unaffected by acetylation.

Experimental

Si gel TLC separations were carried out on Merck pre-coated plates (F-254, layer thickness 0.5 mm) using the following solvent systems: BM = benzene–MeOH, 5:2 or 20:3; CM = $CHCl_3$–MeOH, 20:1; CMW = $CHCl_3$–MeOH–H_2O, 20:10:1;

CMH = $CHCl_3$–MeOH–hexane (60–80 °C fraction from petroleum), 5:2:3; PEAM = *n*-pentane–diethyl ether–glacial acetic acid–MeOH, 75:25:6:20. MeOH was used as the eluting solvent.

Air-dried roots of *Pueraria mirifica* Airy Shaw & Suvatabandhu were extracted with aqueous MeOH as previously reported [1]. The extract was reduced to dryness *in vacuo* (35–40 °C) and the residue was then chromatographed (Si gel TLC) in CM to give various isoflavonoids which have been the subject of earlier papers [1, 2, 4]. In addition to these known compounds, the CM chromatogram also yielded kwakhurin hydrate (**5**, approx. *Rf* 0.08) which was eluted and further purified by Si gel TLC in PEAM (*Rf* 0.42). Pure **5** was finally obtained by TLC of the eluate from PEAM plates in BM (5:2, *Rf* 0.41). Elution of the near origin zone (*Rf* 0.00–0.05) from CM chromatograms gave a mixture of isoflavone glycosides which were separated by TLC in CMW [1]. In this solvent system puerarin-6″-monoacetate (**4**, *Rf* 0.69) was located immediately above the daidzin/genistin band (*Rf* 0.63) which in turn ran above puerarin (**2**, *Rf* 0.53). Si gel TLC of the daidzin + genistin eluate in BM (20:3, ×3) gave pure genistin (**3**, upper zone) just separated from daidzin (**1**, lower zone). Puerarin-6″-monoacetate from the CMW chromatogram was finally purified by TLC in CMH (*Rf* 0.35). In the same solvent system puerarin was located at *Rf* 0.15. In addition to their isolation from *P. mirifica* root material, further quantities of isoflavones **1**–**5** were also obtained by TLC of isoflavonoid-rich fractions remaining from an earlier investigation of the oestrogenic constituents of this plant [9, 10]. Kwakhurin hydrate (**5**) was isolated from these fractions by initial Si gel TLC in CM, 5:1 (*Rf* 0.46; *cf.* kwakhurin, *Rf* 0.62 [2]) followed by purification in PEAM and BM as described above.

*Genistin **3** (genistein-7-O-glucoside)*

UV data as lit. [6]. Hydrolysis in boiling MeOH with 2 N HCl [6] afforded genistein (5,7,4′-trihydroxyisoflavone) identical (UV, TLC) with an authentic sample.

*Kwakhurin hydrate (**5**)*

UV and MS data as lit. [2]. Long wavelength (365 nm) UV fluorescence on Si gel TLC plates, faint blue intensifying upon fuming with NH_3 vapour [6].

*Puerarin-6″-monoacetate (**4**)*

UV: λ_{max}, nm: MeOH 208, 243sh, 250, 262sh, 309sh; MeOH + NaOAc 260, 338 (addition of solid H_3BO_3 regenerated the MeOH spectrum). FD-MS: *m/z*, (%): 459 ($M^+ + 1$; 50), 458 (M^+; 100), 440 (28), 416 ($M^+ - 42$; 15), 398 (21), 362 (20), 326 (29). Comparative FD-MS data for puerarin (**2**) were as follows: *m/z* 417 ($M^+ + 1$; 39), 416 (M^+; 100), 398 (15), 327 (12), 326 (28). ^1H NMR data, see Table I. *Conversion of **4** to **2**:* Conc. aqueous NH_3 (approx. 0.5 ml) was added to a solution of **4** (5 mg) in MeOH. After standing at room temperature (approx. 18 °C) for 2 h, the solution was diluted with MeOH (20 ml) and reduced to dryness *in vacuo* (40 °C). Si gel TLC of the residue afforded a product indistinguishable (UV, TLC) from authentic puerarin (**2**).

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