Phytochemistry, Vol. 25, No. 7, pp. 1772–1775, 1986.
Printed in Great Britain.

0031-9422/86 $3.00 + 0.00
Pergamon Journals Ltd.

PUERARIN 6″-*O*-β-APIOFURANOSIDE, A *C*-GLYCOSYLISOFLAVONE *O*-GLYCOSIDE FROM *PUERARIA MIRIFICA*

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(*Received* 26 *November* 1985)

Key Word Index—*Pueraria mirifica*; Leguminosae–Papilionoideae; isoflavonoids; isoflavone *C*-glycosides; puerarin; puerarin 6″-*O*-β-apiofuranoside; ^{1}H and ^{13}C NMR; gated spin echo (GASPE).

Abstract—In addition to puerarin (7,4′-dihydroxyisoflavone 8-*C*-β-glucopyranoside), the air-dried tuberous roots of *Pueraria mirifica* have been found to contain a second, previously unreported, isoflavone *C*-glycoside. This new compound (mirificin), which has now been identified by chemical and spectroscopic (UV, ^{1}H NMR, ^{13}C NMR including GASPE) procedures as puerarin 6″-*O*-β-apiofuranoside is the first *O*″-glycoside of an isoflavone *C*-glycoside to be discovered in nature. Mirificin contains a rare 1 → 6 interglycosidic linkage between apiose and the glucose unit which is unique in flavonoids. It is proposed that 1 → 2 and 1 → 6 linked apioglucosides can be distinguished by ^{1}H NMR spectroscopy in the same manner as used for the equivalent rhamnoglucosides.

INTRODUCTION

Extracts of *Pueraria* roots (Leguminosae–Papilionoideae; tribe Phaseoleae) have yielded various isoflavonoids [1], including a series of novel isoflavone *C*-glucosides of which puerarin (7,4′-dihydroxyisoflavone 8-*C*-β-glucopyranoside, **1**) is probably the best known example. Puerarin was first obtained from the roots of Chinese and Japanese *Pueraria* species (*P. thunbergiana* = *P. lobata*, *P. pseudo-hirsuta* and *P. thomsonii*) [2, 3] although it has since been found in *P. tuberosa* roots [4], and in callus tissue derived from the stem of *P. thunbergiana* [5]. Four other related isoflavone *C*-glycosides, namely 3′-hydroxy and 3′-methoxypuerarin [6], the partially identified diglycoside 'puerarin xyloside' [2, 3], and 4′,6″-di-*O*-acetylpuerarin [4] have also been detected in *Pueraria* roots.

Our interest in *P. mirifica* Airy Shaw & Suvatabandhu, a woody climber indigenous to parts of northern Thailand, arises from the discovery of a potent oestrogen (miroestrol, **4**) in its tuberous roots [7, 8]. Although miroestrol does not possess the 1,2-diphenylpropane skeleton common to all isoflavonoids [1], its A- and B-rings closely resemble the corresponding A- and C-rings of daidzein (7,4′-dihydroxyisoflavone, **3**), an isoflavone exhibiting weak oestrogenic activity [9]. This suggested that *P. mirifica* might also be a source of daidzein-related isoflavones, a possibility strengthened by the claim that root extracts contain other oestrogens apart from miroestrol itself [10]. In this paper we describe the isolation of puerarin (**1**) and a novel isoflavone *C*-glycoside (mirificin), similarly based on the aglycone daidzein, from *P. mirifica* roots. Using chemical and spectroscopic procedures, mirificin has been identified as puerarin 6″-*O*-β-apiofuranoside (**2**).

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RESULTS AND DISCUSSION

Silica gel TLC ($CHCl_3$–MeOH, 20:1) of a *P. mirifica* root extract yielded numerous bands fluorescing blue under long wavelength (*ca* 365 nm) UV light. However, our attention was drawn particularly to the lowest region of the chromatogram (R_f 0.00–0.05) which fluoresced pale blue, changing to bright blue upon fuming with NH_3 vapour, a feature often associated with isoflavones lacking oxygenation at C-5 [11]. Further purification of this near-origin material in chloroform–methanol–water (20:10:1) gave two compounds (**1** at R_f 0.53 and **2** at R_f 0.37), both of which exhibited UV maxima similar to those of authentic daidzein (**3**). Upon treatment with boiling 2 N HCl [11] compound **1** was unaffected, whereas **2** readily yielded **1** and a sugar that was not glucose, galactose, rhamnose, xylose or arabinose. These data, together with its R_f values (TLC) on cellulose plates developed in TBA (0.69) and 15% acetic acid (0.72), suggested that **1** was an isoflavone *C*-glycoside, and that **2** was an *O*-glycoside of **1** with the additional sugar(s) probably being attached to the *C*-glycosyl moiety.

The ^{1}H NMR spectrum of **1** revealed a pattern of aromatic proton signals resembling that of daidzein (Table 1) except for the H-8 doublet at δ6.83 which was absent, and the lack of *meta*-coupling in H-6. Thus **1** was provisionally formulated as an 8-*C*-glycosylated derivative of daidzein, a view later confirmed by ^{13}C NMR spectroscopy (Table 2). The ^{13}C NMR data also established the *C*-linked sugar as β-glucopyranose, evidence for a β-linkage being additionally provided by the 9.3 Hz, H-1″/H-2″ coupling constant in the ^{1}H NMR

Table 1. ^{1}H NMR data (δ values) for daidzein (3), puerarin (1) and mirificin (2)*

Proton	Daidzein† (3)	Puerarin (1)	Mirificin (2)
H-2	8.22 (1H, *s*)	8.21 (1H, *s*)	8.19 (1H, *s*)
H-5	7.94 (1H, *d*, *J* = 9.0)	7.82 (1H, *d*, *J* = 8.8)	7.78 (1H, *d*, *J* = 8.8)
H-6	6.90 (1H, *dd*, *J* = 9.0 *and* 2.0)	6.85 (1H, *d*, *J* = 8.8)	6.80 (1H, *d*, *J* = 8.8)
H-8	6.83 (1H, *d*, *J* = 2.0)	—	—
H-2',6'	7.36 (2H, *d*, *J* = 9.0)	7.40 (2H, *d*, *J* = 8.5)	7.39 (2H, *d*, *J* = 8.6)
H-3',5'	6.79 (2H, *d*, *J* = 9.0)	6.80 (2H, *d*, *J* = 8.5)	6.80 (2H, *d*, *J* = 8.6)
Sugar H-1	—	4.82 (1H, *d*, *J* = 9.3)	4.76‡ (1H, *d*, *J* = 10.8) 4.79 (1H, *d*, *J* = 3.0)

*Spectra were determined in DMSO-d_6 at 80 MHz (1), 100 MHz (3) and 200 MHz (2). Coupling constants (*J* in Hz) are given in parentheses.

†Data for daidzein (3) are taken from ref. [5].

‡Upper δ value refers to H-1 of the C-linked sugar (glucose); lower δ value is for H-1 of the *O*-linked sugar (apiose).

spectrum. Isoflavone **1** is therefore defined as daidzein 8-*C*-β-glucopyranoside (puerarin). Samples of puerarin derived from *P. mirifica* root and stem callus of *P. thunbergiana* [5] proved to be inseparable when co-chromatographed (TLC) on cellulose plates in 15% acetic acid (R_f 0.72) and TBA (R_f 0.69).

Apart from an extra H-1 sugar signal, the ^{1}H NMR spectrum of isoflavone **2** appeared very similar to that of puerarin (Table 1). The *C*-linked glucose H-1 signal was evident as a doublet at δ4.76 (J = 10.8 Hz), and the second H-1 resonance at δ4.79 (J = 3 Hz) was therefore assigned to the hydrolysable *O*-linked sugar. In the ^{13}C NMR spectrum, this sugar residue gave a pattern of five signals with chemical shift values closely resembling those previously published for glycosides containing β-apiose [12, 13], an unusual branched chain pentafuranose. The presence of apiose in **2** was confirmed in two ways. First, by a gated spin echo (GASPE) [14] ^{13}C NMR experiment which defined the three signals at δ78.9, 73.4 and 63.4 as representing quaternary or methylene carbons, and the remaining signals (δ109.1 and 75.9) as methine carbons. This experiment also confirmed the assignments shown for these signals in Table 2. Secondly, apiose was identified in the hydrolysate of **2** by paper chromatographic comparison with authentic apiose derived from the flavone apiin [15]. Attachment of apiose, via an *O*-link, to the glucose residue rather than to the isoflavone nucleus at C-7 or C-4' was apparent from the UV shift observed with sodium acetate (C-7 OH [11]), and from the B-ring proton δ values (Table 1) which were essentially identical with those obtained for puerarin and daidzein. Lastly, the interglycosidic linkage in **2** was clearly defined as 1 → 6 by the ^{13}C NMR spectrum. Thus the glucose C-6 signal was shifted downfield by 7 ppm relative to that of puerarin (Table 2), whereas the C-5 signal exhibited the expected upfield shift. The chemical shift of the apiose C-1 was essentially the same as previously reported for C-1 linked apiosides [12, 13]. Compound **2** is therefore 7,4'-dihydroxy-8-*C*-β-glucopyranosylisoflavone 6''-*O*-β-apiofuranoside (puerarin 6''-*O*-β-apiofuranoside), a new natural product for which we propose the name mirificin.

To date, puerarin has only been associated with the genus *Pueraria* [1], and the present work extends its known occurrence to yet another *Pueraria* species. Both flavonoid and isoflavonoid glycosides containing apiose are rare in nature but have been isolated on two previous occasions from leguminous plants. Thus, in 1967 Malhotra *et al.* [16] extracted the isoflavone biochanin A 7-*O*-apiosyl(1 → 2) glucoside from the roots of *Dalbergia lanceolaria*, and more recently the flavanone liquiritigenin 4'-*O*-apiosyl(1 → 2)glucoside was obtained from licorice (*Glycyrrhiza uralensis*) roots [13]. However, whilst an *O*-linked 1 → 6 apioglucoside of a xanthone has already been discovered in *Polygala caudata* (Polygalaceae) [17], mirificin from *P. mirifica* is apparently the first *C*-linked apiosyl(1 → 6)glucoside of any kind to be recognised as a natural product.

The present work has also revealed an interesting analogy between the sugar H-1 chemical shift data of rhamnosyl(1 → 6)glucosides (rutinosides)/rhamnosyl (1 → 2)glucosides (neohesperidosides) and the equivalent apiosylglucosides. With the two rhamnosylglucosides, it has been observed that although the glucose H-1 shifts are similar, the rhamnose H-1 signal in neohesperidosides occurs at about 0.5 ppm downfield from its position in rutinosides. This difference is considered to be a reliable method of distinguishing between these two most common rhamnosylglucosides [11]. Significantly, the apiosylglucosides closely parallel this situation, with the

Table 2. ^{13}C NMR data (δ values) for daidzein (3), puerarin (1), mirificin (2) and apiose*

	Daidzein (3)†	Puerarin (1)	Mirificin (2)	Apiose (apiin)†
Isoflavone				
C-2	152.9	152.0	151.8	—
C-3	123.6[b]	123.1[c]	123.2[b]	—
C-4	175.0	174.8	174.8	—
C-5	127.4	125.8	125.7	—
C-6	115.3	114.3	115.1	—
C-7	162.7	166.4	167.1	—
C-8	102.2	111.9	111.8	—
C-9	157.3[a]	157.1[a]	157.2	—
C-10	116.7	117.4	117.6	—
C-1′	122.7[b]	122.9[c]	122.8[b]	—
C-2′	130.2	130.1	130.1	—
C-3′	115.1	115.1	115.1	—
C-4′	157.7[a]	157.3[a]	157.2	—
C-5′	115.1	115.1	115.1	—
C-6′	130.2	130.1	130.1	—
C-sugar				
C-1″	—	74.1	74.2	—
C-2″	—	70.8[b]	70.9	—
C-3″	—	79.2	78.9	—
C-4″	—	70.5[b]	70.9	—
C-5″	—	81.6	80.1	—
C-6″	—	61.3	68.4	—
O-sugar				
C-1‴	—	—	109.1	109.0
C-2‴	—	—	75.9	76.5
C-3‴	—	—	78.9	79.1
C-4‴	—	—	73.4	74.0
C-5‴	—	—	63.4	64.4

*Spectra were determined in DMSO-d_6 (30°) at 20 MHz. Assignments bearing the same superscript (a, b or c) in any one spectrum may be reversed.

†Assignments are based on data given in ref. [12].

apiose H-1 signal appearing at δ4.79 in the (1 → 6)-linked diglycoside (Table 1), and at δ5.38 in the (1 → 2)-linked diglycoside [13]. It is likely, therefore, that ^1H NMR data for apiosylglucosides has diagnostic value similar to that previously demonstrated for rhamnosylglucosides.

EXPERIMENTAL

Plant material. Tuberous roots of *Pueraria mirifica* Airy Shaw & Suvatabandhu [18] (collected by Dr. S. Bartlett from the Doi Suthep area near Chiangmai in northern Thailand) were sliced and air-dried before shipment to the U.K. Upon receipt, the root material was stored in sealed glass jars away from sunlight until required for extraction.

Extraction, isolation and purification of puerarin (1) *and mirificin* (2). The powdered root (870 g) was first stirred with boiling 95% MeOH (4.3 l.) for 30 min. After cooling and filtering, the residue was extracted twice more with a mixture of 95% MeOH (2 × 3.5 l.) and H_2O (2 × 870 ml). The combined filtrates (*ca* 15 l.) were taken to dryness *in vacuo* (40°) and the residue was then stirred with 900 ml of 95% MeOH. The resulting suspension was filtered to remove insoluble waxy material, and the filtrate was then concd *in vacuo* to *ca* 450 ml before being filtered again. An equal vol. of Et_2O was added to the filtrate, and after standing overnight at room temp. the clear MeOH–Et_2O soln was decanted. The gummy ppt was washed with more Et_2O (2 × *ca* 100 ml) and these washings were added to the MeOH–Et_2O soln which was immediately reduced to dryness. The residue thus obtained was dissolved in MeOH (130 ml) and gently shaken with 300 ml of Et_2O. Upon settling, the clear supernatant was decanted and the remaining solid was re-dissolved in MeOH (200 ml). A final quantity of Et_2O (300 ml) was added to this MeOH soln giving a ppt which was removed and air-dried. The resulting brownish cake was then treated as follows: (a) a small portion (*ca* 100 mg) was dissolved in boiling MeOH (*ca* 10 ml) and applied to a number of pre-coated preparative silica gel thin-layer plates (Merck, glass-backed, F-254, layer thickness 0.5 mm) which were developed in $CHCl_3$–MeOH (20:1) to afford various bands fluorescing blue under long wavelength (*ca* 365 nm) UV light. The zone of silica gel extending from the origin to *ca* R_f 0.05 was removed and its isoflavonoid components were eluted with MeOH (50 ml). Concn of the eluate *in vacuo* (40°) to *ca* 5 ml afforded a yellow–brown soln which was chromatographed (silica gel TLC, layer thickness 0.25 mm) in $CHCl_3$–MeOH–H_2O

(20:10:1) to give puerarin (1) and mirificin (2) at R_f 0.53 and 0.37 respectively. (b) A larger quantity (*ca* 3.5 g) of the finely powdered Et_2O-precipitated material was applied to a dry pack silica gel column (Merck, Type 7734, 300 g) prior to elution with $CHCl_3$–MeOH–H_2O (20:10:1). Eluant fractions (10 ml each) were monitored by silica gel TLC, with puerarin and mirificin being detected by their characteristic pale blue fluorescence under 365 nm UV light. Fractions containing mainly mirificin were combined and reduced to dryness *in vacuo* (45°). The residue was then chromatographed (silica gel TLC) in $CHCl_3$–MeOH–H_2O (20:10:1) to afford, after elution of the appropriate silica gel band with Me_2CO, *ca* 14 mg of pure mirificin. This material was principally used for the spectroscopic (^1H and ^{13}C NMR) studies.

Puerarin (*7,4'-dihydroxyisoflavone 8-C-β-glucopyranoside*, **1**). Appearance on silica gel TLC plates viewed under long wavelength UV light before and after fuming with NH_3 vapour, pale blue and bright blue respectively. Colour with diazotized *p*-nitroaniline reagent, orange. UV λ_{max}^{MeOH} nm (% intensity): 206 (100%), 243 sh (83%), 251 (88%), 263 sh (77%), 309 (30%); $\lambda_{max}^{MeOH-NaOH}$ nm: 213, 265, 292 sh, 336; $\lambda_{max}^{MeOH-NaOAc}$ nm: 260, 342 (addition of H_3BO_3 regenerated the MeOH spectrum). Comparative UV maxima recorded for synthetic daidzein (7,4'-dihydroxyisoflavone, **3**) were: λ_{max}^{MeOH} nm: 211, 242 sh, 250, 263 sh, 306; $\lambda_{max}^{MeOH+NaOH}$ nm: 215, 260, 291 sh, 331; $\lambda_{max}^{MeOH+NaOAc}$ nm: 255, 272 sh, 337 (addition of H_3BO_3 regenerated the MeOH spectrum). ^1H and ^{13}C NMR data for puerarin are shown in Tables 1 and 2 respectively.

Mirificin (*7,4'-dihydroxy-8-C-β-glucopyranosylisoflavone 6"-O-β-apiofuranoside*, **2**). Fluorescence characteristics and colour with diazotized *p*-nitroaniline reagent as for puerarin (**1**). UV λ_{max}^{MeOH} nm (% intensity): 208 (98%), 242 sh (90%), 252 (100%), 263 sh (88%), 308 (33%); $\lambda_{max}^{MeOH-NaOH}$ nm: 210, 263, 293 sh, 336; $\lambda_{max}^{MeOH-NaOAc}$ nm: 259, 340 (addition of H_3BO_3 regenerated the MeOH spectrum). For ^1H and ^{13}C NMR data see Tables 1 and 2 respectively. Mirificin was hydrolysed with boiling 2 N HCl according to standard practice [11]. Apiose was detected in the hydrolysate by PC in *n*-BuOH–C_6H_6–pyridine–H_2O (5:1:3:3). Relative R_f values were: galactose 0.27, glucose 0.32, arabinose 0.35, xylose 0.41, apiose 0.49 and rhamnose 0.53. The hydrolysate also yielded a compound inseparable from puerarin (**1**) by silica gel TLC in $CHCl_3$–MeOH–H_2O (20:10:1, R_f 0.53).

Acknowledgements—We thank Dr. H. Itokawa (Tokyo College of Pharmacy, Japan) for a sample of puerarin, and Dr. H. Wong (Chemistry Division, D.S.I.R., Petone, New Zealand) for determining the ^1H and ^{13}C NMR spectra.

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