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ISOFLAVENES FROM THE ROOTS OF *CICER JUDAICUM*

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Key Word Index—*Cicer judaicum*; Leguminosae; wild chickpeas; isoflav-3-ene; isoflav-3-ene glycosides; maackiain glycosides; chemotaxonomy.

Abstract—Three new isoflav-3-enes, 7-hydroxy-2′-methoxy-4′,5′-methylenedioxyisoflav-3-ene (judaicin), judaicin 7-*O*-glucoside and judaicin 7-*O*-(6″-*O*-malonylglucoside), have been isolated from the roots of *Cicer judaicum* together with the known pterocarpans, maackiain 3-*O*-glucoside and maackiain 3-*O*-(6′-*O*-malonylglucoside). Their structures were determined using 1D and 2D NMR techniques in conjunction with other physical methods of analysis. The malonylglucoside derivatives were found to decarboxylate in solution to give the corresponding acetylglucosides. Copyright © 1996 Published by Elsevier Science Ltd

INTRODUCTION

Cicer judaicum Boiss. is an annual herb native to the Middle East and one of *ca* 40 species in the genus *Cicer* (Leguminosae-Papilionoideae) [1]. The majority of previous phytochemical studies on this genus have been concerned with the cultivated chickpea, *C. arietinum* L., largely due to its economic value as a major source of human and domestic animal food in the semi-arid tropics [2, 3]. This species is noted particularly for its content of isoflavonoids, including the pterocarpan phytoalexins maackiain and medicarpin. Wild *Cicer* species are less well studied, although biochanin A, formononetin, medicarpin and maackiain have been identified previously in stem material from 14 species [4]. A single investigation of root material from an additional wild species, *C. mogolatvicum*, yielded seven relatively common isoflavonoids [5]. The chemistry of wild species is of current interest with respect to agriculturally beneficial characters and especially for resistance to fungal wilt caused by *Fusarium oxysporum* f.sp. *ciceri*. The pathogen occurs as different races or pathotypes and while there are sources of resistance [6, 7], no cultivar is resistant to all pathotypes [8]. This paper describes the isolation and characterization of three new isoflav-3-enes from the roots of *C. judaicum*, including the first report of glycosylated forms of this uncommon isoflavonoid class. Comparative spectroscopic data are also presented for two known pterocarpan glycosides found additionally in the roots of *C. judaicum*, and possible biogenetic relationships between these compounds noted.

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RESULTS AND DISCUSSION

The major components of a methanolic extract of *C. judaicum* roots were readily separated by analytical HPLC with gradient elution, as illustrated in Fig. 1. Compounds **1**, **2a** and **3** exhibit highly similar UV spectra, with maxima at 337 and 235 nm and a shoulder at 300 nm, although **1** and **2a** are distinguished from **3** due to their much shorter retention times and slightly more prominent shoulders at 300 nm in the corresponding UV spectra. The analytical method was scaled-up with no loss of resolution, and the major phenolic components were isolated by semi-preparative HPLC. Mass spectral data indicated that **3** must be the aglycone of **1** and **2a**, with the former giving a strong $[M]^{+\cdot}$ ion at *m/z* 298 and a second prominent ion, $[M + H + Na]^+$, at *m/z* 322. The molecular ions of **1** and **2a** occurred at *m/z* 460 and 546, respectively, in addition to a common ion at *m/z* 298. These data correspond to loss of glycosyl $[C_6H_{10}O_5]^+$ and acylated glycosyl fragments in **1** and **2a**, respectively. The molecular structure of **1** was determined unambiguously using ^1H and ^{13}C NMR spectroscopy with recourse to standard 1D and 2D experiments. Chemical shift parameters and assignments for **1**–**3** are summarized in Tables 1 and 2. The quaternary carbon atoms of **3** were assigned from long-range connectivities recorded in a COLOC experiment.

The ^1H NMR spectrum of **1** indicated the presence of an *O*-linked glycosyl unit, with a characteristic 1H proton doublet at δ 4.81 exhibiting a coupling constant

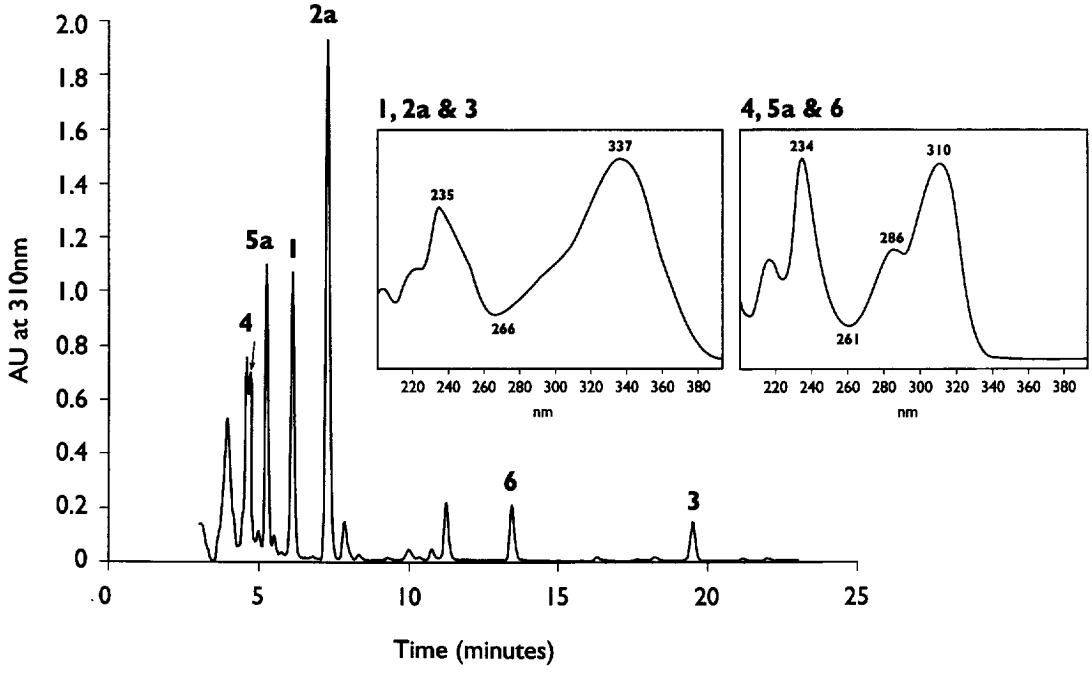


Fig. 1. Analytical HPLC separation of isoflavonoids in 10 μl of a 1 g fresh root ml^{-1} methanol extract of *Cicer judaicum*. UV spectra of compounds **3** and **6** are given as an illustration of those obtained here for isoflav-3-enes and pterocarpans, respectively. It should be noted that while the UV spectra of **1** and **2a** are almost identical to that of **3**, the shoulder at 300 nm is more prominent in the former cases. Chromatographic protocols are reported in detail in the experimental section.

of 7.0 Hz. A $^3J_{1'',2''}$ constant of this magnitude can be taken to indicate that the glycosyl moiety adopts the β-configuration [9]. The remaining glycosyl resonances in the ^{1}H NMR spectrum were assigned by sequential connectivities in a COSY experiment. Their chemical shift values, together with those of the set of six corresponding glycosyl resonances in the ^{13}C spectrum, confirmed **1** to be an *O*-linked glucoside. Analysis of the ^{1}H NMR data for the aglycone moiety of **1** indicated an isoflav-3-ene skeleton, principally from the observation of a 2H singlet at δ 4.88 and a 1H singlet at δ 6.60, typical of this isoflavonoid class. These two resonances also gave a cross-peak in the corresponding COSY spectrum due to allylic coupling in this A_2X spin system. Aromatic proton resonances at δ 7.04 (*d*, J = 8.2 Hz), 6.59 (*dd*, J = 8.2 and 2.1 Hz) and 6.51 (*d*, J = 2.1 Hz) showed the A ring to be substituted at C-7. The remaining proton resonances, two aromatic proton singlets at δ 6.90 and 6.82, a methylenedioxy 2H singlet at δ 5.99 and a methoxyl 3H singlet at δ 3.74 were assigned to the B ring. The ^{13}C spectrum of **1** consisted of 23 distinct resonances, five of which could be assigned immediately to C–O linkages, in addition to those expected for the glucosyl moiety. This enabled the empirical formula of **1** to be deduced from DEPT experiments as $C_{23}H_{24}O_{10}$ (M_r = 460.42). The molecular structure of **1**, representing the only consistent solution to the NMR data, is therefore, 7-*O*-β-D-glucopyranosyloxy-2′-methoxy-4′,5′-methylenedioxyisoflav-3-ene, a new isoflav-3-ene glycoside.

Table 1. ^{13}C NMR data for the isoflav-3-enes **1**, **2b** and **3** (δ in DMSO-d_6, 67.8 MHz, 37°)

C	1	2b	3
2	67.6	67.6	67.5
3	129.1	129.1	127.5
4	120.9	120.8	121.3
4a	117.6	117.6	115.1
5	127.3	127.2	127.4
6	109.5	109.4	108.6
7	158.0	157.6	158.2
8	103.5	103.4	102.3
8a	153.8	153.8	154.1
1′	119.2	119.1	119.5
2′	152.5	152.5	152.4
3′	95.6	95.5	95.5
4′	147.8	147.7	147.5
5′	141.0	141.0	141.0
6′	107.5	107.5	107.5
1″	100.5	100.0	
2″	73.2	73.1	
3″	76.5	76.3	
4″	69.2	69.9	
5″	77.1	73.6	
6″	60.7	63.3	
OMe	56.5	56.5	56.5
OCH_2O	101.2	101.2	101.1
OCOMe		170.1	
$OCOCH_3$		20.5	

The ^{13}C NMR spectrum of a second compound, which, according to Fig. 1, eluted just after **1**, was essentially identical to that of the latter with the exception of two additional resonances at δ 170.1 and

Table 2. ^{1}H NMR chemical shift assignments, coupling constant data and long-range connectivities for the isoflav-3-enes **1**, **2b** and **3** (δ in DMSO-d_6, 270 MHz, 37°)

H	1	2b	3	3 δ(^{13}C) long-range connectivities
2	4.88 *s*	4.88 *s*	4.83 *s*	119.5 (C-1′), 121.3 (C-4), 127.5 (C-3), 154.1 (C-8a)
4	6.60 *s*	6.60 *s*	6.54 *s*	119.5 (C-1′), 127.5 (C-3), 154.1 (C-8a)
5	7.04 *d* (8.2)	7.05 *d* (8.2)	6.92 *d* (8.2)	154.1 (C-8a), 158.2 (C-7)
6	6.59 *dd* (8.2, 2.1)	6.59 *dd* (8.2, 2.1)	6.33 *dd* (8.2, 2.1)	115.1 (C-4a)
8	6.51 *d* (2.1)	6.50 *d* (2.1)	6.24 *d* (2.1)	115.1 (C-4a)
3′	6.82 *s*	6.82 *s*	6.81 *s*	119.5 (C-1′) 147.5 (C-4′), 152.4 (C-2′)
6′	6.90 *s*	6.90 *s*	6.87 *s*	127.5 (C-3), 141.0 (C-5′), 147.5 (C-4′)
OMe	3.74 *s*	3.74 *s*	3.73 *s*	152.4 (C-2′)
OCH_2O	5.99 *s*	5.99 *s*	5.98 *s*	
1″	4.81 *d* (7.0)	4.85 *d* (7.6)		
2″	3.21 *m*	3.23 *m*		
3″	3.30 *m*	3.29 *m*		
4″	3.15 *m*	3.22 *m*		
5″	3.32 *m*	3.58 *m*		
6″	3.47 *dd* (11.9, 5.8)	4.11 *dd* (11.6, 6.7)		
	3.72 *dd* (11.9, 1.8)	4.27 *br d* (11.6)		

20.5. These indicated the presence of an acetyl group as was also confirmed by observation of an additional singlet resonance at δ 1.90 in the corresponding ^{1}H NMR spectrum. The inference of acetyl is not supported in the first instance by the mass spectral data, which indicates malonyl as the acyl group present in **2a**. This apparent discrepancy is readily resolved, however, with reference to the facile decarboxylation of malonyl to acetyl, which is known to occur in solution in some instances [10]. The site of acylation was confirmed as C-6″ from the downfield shift of +2.64 ppm observed for this carbon atom in the ^{13}C spectrum, and the downfield shifts of +0.64 and +0.55 ppm for C-6″H_2 in the ^{1}H spectrum, when compared with appropriate chemical shift data for **1**. Compound **2a** is, therefore, 7-*O*-(6″-*O*-malonyl-β-D-glucopyranosyloxy)-2′-methoxy-4′,5′-methylenedioxyisoflav-3-ene, the first acylated isoflav-3-ene glycoside to be described. In solution, compound **2b**, 7-*O*-(6″-*O*-acetyl-β-D-glucopyranosyloxy)-2′-methoxy-4′,5′-methylenedioxyisoflav-3-ene, is readily formed and it is this acylated derivative for which NMR data are presented in Tables 1 and 2.

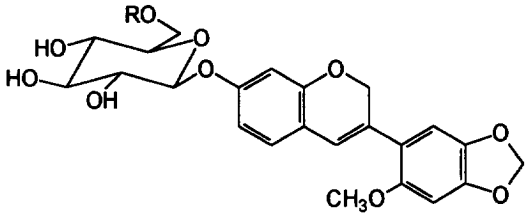
The ^{1}H and ^{13}C NMR data for **3** confirmed it to be the aglycone of **1** and **2a**, in accordance with the mass spectral and HPLC data described above and in Fig. 1. Compound **3** is, therefore, 7-hydroxy-2′-methoxy-4′,5′-methylenedioxyisoflav-3-ene, a new isoflav-3-ene for which we propose the common name 'judaicin'. The co-occurrence of an aglycone with glycoside and acyl glycoside is not uncommon for isoflavonoids, one example being that of biochanin A, biochanin A 7-*O*-glucoside and biochanin A 7-*O*-(6″-*O*-malonyl-glucoside) found previously in root material of *C. arietinum* [11]. It should be noted, however, that the present report is the first in which isoflav-3-ene glycosides are described. Figure 1, which illustrates the chromatographic properties of **1**, **2a** and **3**, also indicates their relative abundance in fresh root. Judaicin is a minor component compared to its glucoside and malonyl glucoside. Its UV spectrum is given here for reference purposes as similar data for isoflav-3-enes have not been published. The structure of **3** is also of interest as it includes a methylenedioxy group not found previously in the 12 recorded examples of the isoflav-3-ene class [12–20]. In fact, the overall substitution pattern of **3** is reproduced in three structurally related compounds, namely cuneatin (7-hydroxy-2′-methoxy-4′,5′-methylenedioxyisoflavone) from stem material of *C. cuneatum* [4], onogenin (7-hydroxy-2′-methoxy-4′,5′-methylenedioxyisoflavanone) from *Dalbergia stevensonii* [21] and astraciceran (7-hydroxy-2′-methoxy-4′,5′-methylenedioxyisoflavan) from fungus-inoculated leaflets of *Astragalus cicer* [22].

The data presented in Fig. 1 show that another class of isoflavonoid is present in the methanolic root extract of *C. judaicum* in addition to the isoflav-3-enes described in detail above. Compounds **4**, **5a** and **6** have essentially identical UV spectra, with a maximum absorbance at 310 nm and a characteristic shoulder at 286 nm. These spectral features are typical of the pterocarpan, maackiain. A reference sample of this compound subjected to the HPLC procedure described in the legend to Fig. 1 gave identical UV spectra to those of **4** and **5a**, but had the same retention time as **6**. Positive confirmation of the identity of **6** as maackiain was obtained by co-chromatography of the standard and the methanolic extract of the root material of *C. judaicum*. The shorter retention times of **4** and **5a** indicate that, as with the isoflav-3-enes, glycosidic forms are present. This premise was confirmed by analysis of the ^{1}H NMR spectrum of **4**, which was characteristic of maackiain 3-*O*-β-D-glucopyranoside and in accordance with previously published ^{1}H NMR data for trifolirhizin or (−)-maackiain 3-*O*-β-D-glucopyranoside [23]. The ^{1}H NMR spectrum of the additional maackiain derivative was very similar to that of **4**, with the exception of significant downfield shifts of +0.60 and +0.64 ppm to the 6′-CH_2 protons of the glucoside moiety (δ 4.27 and 4.09) when compared with those of **4** (δ 3.67 and 3.45). These protons were assigned by means of COSY experiments owing to the

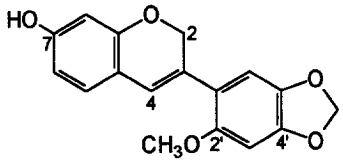
complexity of the resonances present in the spectral region between 3.00 and 4.50 ppm of both compounds. The chemical shift perturbations noted between the ^1H spectra of the two derivatives indicate that the site of acylation is at C-6′ as was also found in the case of the isoflav-3-enes.

The β-configuration of the glucopyranoside moiety of both maackiain derivatives was deduced from the magnitude of the $^3J_{1'',2''}$ coupling constant as before. Once again an additional resonance at δ 1.90 in the ^1H NMR spectrum of the acylated derivative suggested an acetyl functional group. However, mass spectral data for **5a** confirmed the presence of a malonylated maackiain derivative, with prominent mass ions of *m/z* 532 and 533 corresponding to $[M]^{+\cdot}$ and $[M + H]^+$, respectively. Major mass ions for the maackiain aglycone moiety were also recorded at *m/z* 284 and 285, corresponding to $[A]^{+\cdot}$ and $[A + H]^+$, respectively. Compound **5a** is, therefore, maackiain 3-*O*-(6′-*O*-malonyl-β-D-glucopyranoside). This has been isolated previously from roots and callus cultures of *Sophora flavescens* var. *angustifolia* [24], but is known only in *Cicer* from cell suspension cultures of a cultivar of *C. arietinum* [10]. It appears that, as with the analogous conversion of **2a**, compound **5a** undergoes decarboxylation in solution to give maackiain 3-*O*-(6′-*O*-acetyl-β-D-glucopyranoside) (**5b**). This derivative has only been reported previously in the root of *S. subprostata*, which constitutes the Chinese crude drug preparation known as Guang-Dou-Gen [25]. In this instance, the root was extracted three times in boiling methanol, a procedure likely to promote decarboxylation of malonylglucoside derivatives. The parent glucoside, trifolirhizin, in contrast to the acylated derivatives, is known from a number of sources, including the root material of *C. mogolatvicum* [5]. The relative amounts of the aglycone, glucoside and malonyl glucoside of maackiain in root material of *C. judaicum* are similar to those of judaicin and its conjugates. Note that the aglycones are present at low concentrations (Fig. 1). Enzymes which catalyse the conversion of these glycosidic conjugates into aglycones have been isolated from *C. arietinum* [26, 27]. It has been postulated, therefore, that the conjugated forms are the principal source of free aglycones produced in cell cultures in response to fungal elicitors [28]. The presence of relatively high concentrations of maackiain conjugates in *C. judaicum* roots may be an agriculturally desirable property absent from the cultivated species.

It is noteworthy that isoflav-3-enes and pterocarpans occur together in *C. judaicum* as these classes of isoflavonoid may share the biogenetic precursor, 2′-hydroxyisoflavanol [29]. In addition to biosynthetic considerations, the discovery of a class of compounds previously unknown in *Cicer* has additional implications for systematic studies in the genus. The taxonomy of *Cicer* has been described authoritatively in a monograph by Van der Maesen [1]. Although a later comparative study of the chemistry of constitutive and induced isoflavonoids in 14 wild *Cicer* species identified quantitative variation in levels of formononetin, biochanin A, medicarpin and maackiain, its findings did not oppose the conclusions of the earlier study [4]. Our preliminary analysis of a range of wild *Cicer* species from the series *Arietina* in section *Monocicer* of the subgenus *Pseudononis* indicates that the occurrence and distribution of judaicin, judaicin 7-*O*-glucoside and judaicin 7-*O*-(6″-*O*-malonylglucoside) does not support the published taxonomy. These compounds appear to be valuable chemotaxonomic markers and further de-



1 (R = H), **2a** (R = malonyl), **2b** (R = acetyl)



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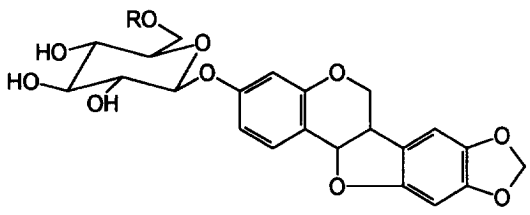
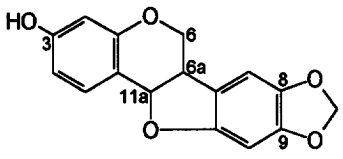


4 (R = H), **5a** (R = malonyl), **5b** (R = acetyl)



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tailed systematic studies will determine whether some revision of the genus is necessary.

EXPERIMENTAL

Plant material. Seeds of *C. judaicum* ICCW73 were obtained from the Genetic Resources Unit of the International Crops Research Institute for the Semi-Arid Tropics. These were grown under greenhouse conditions at the Royal Botanic Gardens, Kew (accession number 1995-438). Root material was taken when the plants were at the flowering stage (60 days after sowing) and freeze-dried.

General. NMR spectra were recorded at 270 and 67.8 MHz for ^{1}H and ^{13}C, respectively. Samples were dissolved in DMSO-d_6 with TMS as a primary reference. A temp. of 37° was used for all NMR experiments. FAB-MS (positive mode); 3-nitrobenzyl alcohol matrix.

Isolation procedures. Freeze-dried root material (25 g) was ground with a minimum vol. of MeOH. A further 250 ml MeOH was then added and the root material allowed to extract at room temp. for 24 hr. The resulting slurry was filtered and the filtrate evapd to dryness under red. pres. This material was redissolved in MeOH to give an extract corresponding to 1 g ml^{-1} of original plant material. The filtered extract (0.45 μm Millipore filters) was injected in 200 μl aliquots directly on to a Spherisorb 5 ODS semi-prep. column, 10 mm (i.d.) × 250 mm. A Waters HPLC system consisting of a LC600 pump and 996 photodiode array detector was used in gradient elution mode. A two-solvent sepn system was optimal, with A = 65% at t = 0 min; A = 55% at t = 20 min and A = 20% at t = 25 min, where A = 2% HOAc and B = 2% HOAc in MeCN. Six major components, compounds **1–6**, eluted as indicated in Fig. 1, and were collected manually. The extract used here for semi-prep. HPLC contained a higher concn of the aglycones **3** and **6** than the glucoside (**1** and **4**) and acylated glucoside (**2a** and **5a**) derivatives, respectively, such that the yield from a 200 μl aliquot was typically **1** (90 μg), **2a** (75 μg), **3** (200 μg), **4** (50 μg), **5a** (75 μg) and **6** (130 μg). This semi-prep. HPLC step was repeated as necessary to provide sufficient material for spectroscopic characterization. Note that the chromatogram presented in Fig. 1 shows the profile obtained with fresh root material, where the glycosylated derivatives are more prominent. This gives a more accurate representation of the relative concns of compounds occurring in the root itself. All isolated compounds were dried under a stream of N_2 followed by drying in a desiccator immediately prior to spectroscopic analysis.

Judaicin 7-O-*glucoside* (7-O-β-D-*glucopyranosyloxy*-2′-*methoxy*-4′,5′-*methylenedioxyisoflav*-3-*ene*) (**1**). UV λ_{max}^{MeCN} nm: 235, 300sh, 337. ^{1}H and ^{13}C NMR: Tables 1 and 2. FAB-MS (positive) m/z: 460 $[M]^{+\cdot}$, 298 $[A]^+$.

Judaicin 7-O-(6″-O-*malonylglucoside*) (7-O-(6″-O-*malonyl* - β - D - *glucopyranosyloxy*) - 2′ - *methoxy*-4′,5′-*methylenedioxyisoflav*-3-*ene*) (**2a**). UV λ_{max}^{MeCN} nm: 235, 300sh, 337. FAB-MS (positive) m/z: 546 $[M]^{+\cdot}$, 298 $[A]^+$.

Judaicin 7-O-(6″-O-*acetylglucoside*) (7-O-(6″-O-*acetyl* - β - D - *glucopyranosyloxy*) - 2′ - *methoxy* - 4′,5′-*methylenedioxyisoflav*-3-*ene*) (**2b**). UV λ_{max}^{MeCN} nm: 235, 300sh, 337. ^{1}H and ^{13}C NMR: Tables 1 and 2.

Judaicin (7-*hydroxy*-2′-*methoxy*-4′,5′-*methylenedioxyisoflav*-3-*ene*) (**3**). UV λ_{max}^{MeCN} nm: 235, 300sh. 337. ^{1}H and ^{13}C NMR: Tables 1 and 2. FAB-MS (positive) m/z: 298 $[M]^{+\cdot}$.

Trifolirhizin [(−)-*Maackiain* 3-O-β-D-*glucopyranoside*] (**4**). UV λ_{max}^{MeCN} nm: 286sh, 310. ^{1}H NMR (DMSO-d_6): δ 7.35 (1H, *d*, J = 8.5 Hz, H-1), 6.70 (1H, *dd*, J = 8.5, 2.0 Hz, H-2), 6.55 (1H, *d*, J = 2.0 Hz, H-4), 3.65–3.67 (3H, *m*, H-6$_\alpha$, H-6a, H-6′), 4.27 (1H, *dd*, J = 9.7, 3.2 Hz, H-6$_\beta$), 6.98 (1H, *s*, H-7), 6.52 (1H, *s*, H-10), 5.57 (1H, *d*, J = 7.0 Hz, H-11a), 5.93 (2H, *d*, J = 9.8 Hz, -OCH$_2$O-), 4.83 (1H, *d*, J = 7.3 Hz, H-1′), 3.16–3.28 (3H, *m*, H-2′, H-3′, H-4′), 3.32 (1H, *m*, H-5′), 3.45 (1H, *m*, H-6′).

Maackiain 3-O-(6′-O-*malonyl*-β-D-*glucopyranoside*) (**5a**). UV λ_{max}^{MeCN} nm: 286sh, 310. FAB-MS (positive) m/z: 533 $[M+H]^+$, 532 $[M]^{+\cdot}$, 285 $[A+H]^+$, 284 $[A]^{+\cdot}$.

Maackiain 3-O-(6′-O-*acetyl*-β-D-*glucopyranoside*) (**5b**). UV λ_{max}^{MeCN} nm: 286sh, 310. ^{1}H NMR (DMSO-d_6): δ 7.38 (1H, *d*, J = 8.5 Hz, H-1), 6.71 (1H, *dd*, J = 8.5, 2.0 Hz, H-2), 6.54 (1H, *d*, J = 2.0 Hz, H-4), 3.58–3.61 (2H, *m*, H-6$_\alpha$, H-6a), 4.27 (2H, *m*, H-6$_\beta$, H-6′), 6.97 (1H, *s*, H-7), 6.52 (1H, *s*, H-10), 5.55 (1H, *d*, J = 7.0 Hz, H-11a), 5.92 (2H, *d*, J = 9.7 Hz, -OCH$_2$O-), 4.86 (1H, *d*, J = 7.3 Hz, H-1′), 3.17–3.28 (3H, *m*, H-2′, H-3′, H-4′), 3.59 (1H, *m*, H-5′), 4.09 (1H, *dd*, J = 11.6, 6.8 Hz, H-6′), 1.90 (3H, *s*, OAc).

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