New Isoflavone Glucosides from White Lupin (*Lupinus albus* L.)

Yusuke Shibuya, Satoshi Tahara, Yutaka Kimura, and Junya Mizutani

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

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Further investigation of polar constituents in white lupin roots revealed the presence of genistein 7-O-β-glucoside (**1a**), genistein 4′-O-β-glucoside (**1b**), 2′-hydroxygenistein 7-O-β-glucoside (**2a**) and 2′-hydroxygenistein 4′-O-β-glucoside (**2b**) as simple isoflavone glucosides; luteone 7-O-β-glucoside (**3a**), licoisoflavone A 7-O-β-glucoside (**4a**) and licoisoflavone A 4′-O-β-glucoside (**4b**) as prenylated (= 3,3-dimethylallyl-substituted) isoflavone glucosides; and genistein 7-O-β-(6″-O-malonyl)glucoside (**1am**) and 2′-hydroxygenistein 7-O-β-(6″-O-malonyl)glucoside (**2am**) as malonylated isoflavone glucosides. Six compounds (**2a, 2b, 3a, 4a, 4b,** and **2am**) are new naturally occurring isoflavone glucosides.

Introduction

According to the latest reviews [1, 2], about 160 kinds of isoflavonoid glycosides have been characterized. Although 50 kinds of isoflavonoids (41 isoflavones, 7 coumaronochromones and 2 coumaranochroman-4-ones) have been isolated from white lupin [3–5], there is no precise survey of the glucosides except an earlier work [6].

Initially, the prenylated isoflavones, wighteone and luteone (**3**) in white lupin were identified as preinfectional fungitoxins (prohibitin [7]) [8, 9], whilst the amounts of those isoflavones have increased by biotic elicitation [10]. It has long been interested if the glucoside pool of simple isoflavones is available to the inductive biosynthesis of complex isoflavonoides in plants under biotic or abiotic stresses, and the conclusions are still contradictory [11–14]. For the further biochemical studies on lupin isoflavonoids, the present experiment was conducted to qualify the isoflavone glucosides in lupin roots. Although we could isolate seven isoflavone glucosides and two isoflavone malonylglucosides, some of the former ones may be derived from the corresponding malonylglucosides during extraction and isolation because of their labilities in a methanolic or an aqueous solution [15]. The structures of two more malonyl-glucosides were tentatively identified.

Reprint requests to Dr. S. Tahara.

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Results and Discussion

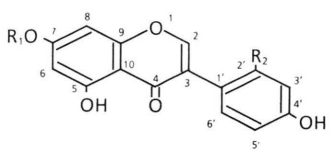
The ethyl acetate soluble constituents from the methanol extract of white lupin roots were fractionated by liquid chromatography over silica gel, TSK gel, Unisil QC-18 and Lichroprep RP-8, and preparative TLC. As shown in Table I, 11 isoflavone glucosides were isolated and fully or partially characterized.

The isoflavonoid nature of the isolated compounds (**1a, 1am, 1b, 2a, 2am, 2b, 3a, 4a, 4b**) was suggested by UV absorption maximum around 260–264 nm [16] and ^{1}H NMR detection of a sharp singlet proton around δ 8.00–8.34 which is characteristic of the isoflavone 2-H [17]. The molecular weights of isolated compounds (except **1bm** and **2bm**) were determined by FD-MS. Enzymatic (β-glucosidase) hydrolysis of **1a** and **1b,** or **2a** and **2b** yielded genistein (**1**) or 2′-hydroxygenistein (**2**), respectively. In the hydrolysate of **3a,** luteone (**3**) was isolated as the aglycone, whereas in those of **4a** and **4b,** licoisoflavone A (**4**) was identified. In every case, glucose was a sole sugar constituent in the hydrolysate of lupin glucosides. Together with the fact that **1a, 1b, 2a, 2b, 3a, 4a** and **4b** were susceptible to β-glucosidase, ^{1}H NMR coupling constants of those anomeric protons (J = 6.6–7.1 Hz) clearly indicated that the isolates were β-glucosides. Furthermore, the isolated compounds (**1a, 1b, 2a, 2b, 3a** and **4b**) were laevorotatory as was observed with authentic phenyl-β-D-glucoside [18]. Therefore, the lupin isoflavone glucosides are plausibly all β-D-glucosides.

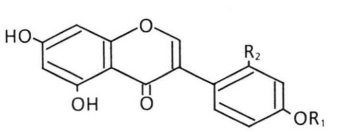
Table I. Physicochemical and structural properties of lupin isoflavone glucosides[a].

Compd.	TLC R_f CAM	TLC R_f EMWAm	Gibbs test	UV λ_{max} [nm] MeOH	+$AlCl_3$	+NaOAc	Aglycone	Substituent
1a	0.34	0.50	slow white-blue	261.5 290sh	272 290sh	261.5	genistein (**1**)	7-O-glc
1am	0.04	0.30	slow white-blue	261.5	272.5	261.5	**1**	7-O-(6″-O-malonyl)glc
1b	0.34	0.43	slow blue	261 300sh	269 315sh	272[b] 330sh	**1**	4′-O-glc
1bm[c]	0.03	0.18	slow blue				**1**	4′-O-(6″-O-malonyl)glc
2a	0.25	0.48	rapid purple-blue	260 287sh	268	260	2′-hydroxy-genistein (**2**)	7-O-glc
2am	0.03	0.24	rapid purple-blue				**2**	7-O-(6″-O-malonyl)glc
2b	0.25	0.42	rapid clear-blue	260.5 295sh	268 305sh	270[b] 330sh	**2**	4′-O-glc
2bm[c]	0.03	0.17	rapid clear-blue				**2**	4′-O-(6″-O-malonyl)glc
3a	0.38	0.60	rapid purple-blue	264 290sh	274 310sh	265	luteone (**3**)	7-O-glc
4a	0.36	0.52	rapid brownish purple-blue	262.5	270 310sh	262.5	licoiso-flavone A (**4**)	7-O-glc
4b	0.40	0.49	rapid clear-blue	262	269 310sh	275[b]	**4**	4′-O-glc

[a] All compounds isolated in the present study showed a dark purple fluorescence on TLC plates under long-wavelength UV light.
[b] The MeOH spectra were regenerated on the addition of H_3BO_3.
[c] Structures were tentatively determined.

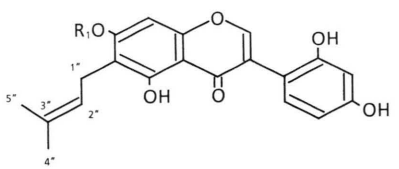


1: $R_1 = R_2 = H$, genistein
1a: R_1 = glc, $R_2 = H$
1am: R_1 = (6"-O-malonyl)glc, $R_2 = H$
2: $R_1 = H$, R_2= OH, 2'-hydroxygenistein
2a: R_1 = glc, R_2 = OH
2am: R_1 = (6"-O-malonyl)glc, R_2 = OH

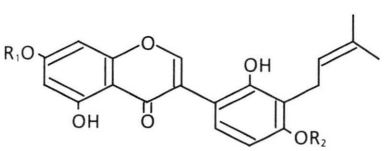


1b: R_1 = glc, $R_2 = H$
1bm: R_1 = (6"-O-malonyl)glc, $R_2 = H$ *
2b: R_1 = glc, R_2 = OH
2bm: R_1 = (6"-O-malonyl)glc, R_2 = OH *

* tentatively identified



3: $R_1 = H$, luteone
3a: R_1 = glc



4: $R_1 = R_2 = H$, licoisoflavone A
4a: R_1 =glc, $R_2 = H$
4b: $R_1 = H$, R_2 = glc

The O-glucosyl substitution in the A-ring (5 or 7) was deduced from the response of each compound to UV shift reagents (+$AlCl_3$ shifted: free 5-OH, **1a, 1b, 2a, 2b, 3a, 4a** and **4b;** +NaOAc shifted: free 7-OH, **1b, 2b** and **4b;** and +NaOAc unchanged: 7-OH substituted, **1a, 2a, 3a** and **4a**) [16]. In case of O-glucosylation in the B-ring (2′ or 4′) of the 2′-hydroxygenistein skeleton, the position was immediately differentiated from the result of Gibbs test. As shown in our earlier paper [19], the 2′-OH modified derivative of 2′-hydroxygenistein yielded a green-blue – blue pigment slowly, whilst the 4′-OH derivatized one, a clear blue pigment rapidly. Thus the B-ring glucosyl-substituted compounds **2b, 2bm** and **4b** affording a clear blue colour rapidly on Gibbs reagent spray must be 4′-O-glucosyl derivatives.

The more polar isolates **1am, 1bm, 2am** and **2bm** were gradually hydrolyzed in aqueous methanol to yield less polar compounds indistinguishable from **1a, 1b, 2a** and **2b,** respectively. Therefore, those polar compounds were supposed to be unstable derivatives of **1a, 1b, 2a** and **2b.** The FD-MS of the methyl ester of **1am** (**1am-Me**) exhibited the molecular ion at m/z 532 (88%) associated with a deacylation ion at m/z 432 ($M^+ - CH_2OCOCH_2CO$, 85%) and an acylated ion at m/z 632 ($M^+ + CH_2OCOCH_2CO$, 14%), whilst **1am** and **2am** gave m/z 474 ($M^+ - CO_2$, 31%) and 490 ($M^+ - CO_2$, 23) respectively. The former fragment m/z 474 was associated with m/z 432 ($M^+ - CO_2 - CH_2CO$, 15%), 516 ($M^+ - CO_2 + CH_2CO$, 20) and 558 ($M^+ - CO_2 + 2 \times CH_2CO$, 5), and the latter fragment m/z 490 associated with m/z 448 ($M^+ - CO_2 - CH_2CO$, 8%), 532 ($M^+ - CO_2 + CH_2CO$, 14) and 574 ($M^+ - CO_2 + 2 \times CH_2CO$, 5) consistent with the detection of ions m/z 632 and 432 in the mass spectrum of **1am-Me**. The appearance of those unexpected ions on the FD-MS spectra of **1am, 2am** and **1am-Me** was suggestive of the occurrence of disproportionation reaction in the spectrometer.

Together with molecular weights confirmed by FD-MS, the detection of malonyl methylene protons on ^1H NMR spectra of **1am** and methyl esters of **1am** and **2am** (Table II and experimental) at the expected magnetic field around δ 3.39–3.54 indicated the presence of malonyl residue as a glucose acylation group. The fact that the malonyl group was quite easily released from the present com-

Table II. ^1H NMR data for lupin glucosides in DMSO-d_6.

Compd. / Proton	**1a**	**1am**	**1b**	**2a**	**2am**-Me	**2b**	**3a**	**4a**	**4b**
[illegible]	8.34 s		8.33 s	8.25 s	8.24 s	8.17 s	8.24 s	8.23 s	8.00 s
[illegible]	6.47 d *J* = 2.2	6.47 d *J* = 2.2	6.16 br. s	6.47 d *J* = 2.2	6.47 d *J* = 2.2	6.20 d *J* = 2.2		6.47 br. s	5.93 br. s
[illegible]	6.73 d *J* = 2.2	6.68 d *J* = 2.2	6.31 br. s	6.71 d *J* = 2.2	6.69 d *J* = 2.2	6.36 d *J* = 2.2	6.78 s	6.71 br. s	6.02 br. s
[illegible]′	7.41 d (2H)	7.38 d (2H)	7.45 d (2H)						
[illegible]′	*J* = 8.3	*J* = 8.8	*J* = 8.8	6.99 d *J* = 8.2	6.99 d *J* = 8.2	7.09 d *J* = 8.2	6.98 d *J* = 8.3	6.76 d *J* = 8.2	6.92 d *J* = 8.4
[illegible]′	6.83 d (2H)	6.82 d (2H)	7.09 d (2H)	6.37 d *J* = 2.2	6.37 d *J* = 2.2	6.58 d *J* = 2.2	6.37 d *J* = 2.2		
[illegible]′	*J* = 8.3	*J* = 8.8	*J* = 8.8	6.27 dd *J* = 8.2, 2.2	6.29 dd *J* = 8.2, 2.2	6.54 dd *J* = 8.2, 2.2	6.27 dd *J* = 8.3, 2.2	6.38 d *J* = 8.2	6.65 d *J* = 8.4
Glucose [illegible]nomeric proton	5.10 d *J* = 6.4	5.07 d *J* = 7.1	4.91 d *J* = 7.1	5.06 d *J* = 7.7	5.13 d *J* = 7.1	4.82 d *J* = 7.1	5.01 d *J* = 7.0	5.06 d *J* = 7.1	4.79 d *J* = 6.6
[illegible]) Malonyl methylene and methoxyl		a) 3.39 s (2H)			a) 3.52 d-like (2H) 3.61 s (3H) COOMe		c) 3.28 br. d *J* = 7.2 (2H) 5.22 br. t *J* = 7.2 1.75 s (3H) 1.62 s (3H)	c) 3.26 br. d *J* = 6.6 (2H) 5.19 br. t *J* = 6.6 1.71 s (3H) 1.62 s (3H)	c) 3.27 br. d *J* = 6.6 (2H) 5.22 br. t *J* = 6.6 1.73 s (3H) 1.61 s (3H)
) Glucosyl 6″-H_2		b) 4.03–4.31 m (2H)			b) 4.10 m (1H) and 4.37 m (1H)				
[illegible]) Prenyl									

1a and **4a** were analyzed at 100 MHz and 500 MHz, respectively. The remainder were at 270 MHz. Coupling constants (*J*) are given in Hz.

pound suggested the substitution at C-6-OH of glucose [20]. The estimation was confirmed by ^{1}H NMR in which 6″-CH_2 was detected in the lower field at δ 4.0–4.4 due to acylation of the 6″-hydroxyl group [20].

The known isoflavone glucosides **1a** [21], **1am** [22], and **1b** [21], gave the reasonably identical spectroscopic data to those found in literature. The ^{1}H and ^{13}C NMR data for new isoflavone glucosides **2a, 2am, 2b, 3a, 4a** and **4b** (Table II and experimental) supported well those estimated structures. Although the spectroscopic evidence for **1bm** and **2bm** was incomplete, the estimated structures are reliable enough, because the free acids or methyl derivatives behaved in parallel with **1am** and **2am** or those methyl esters on thin-layer plates in addition to the former fact that **1bm** and **2bm** were easily hydrolyzed to yield **1b** and **2b,** respectively.

The first prenylated isoflavone glucoside, 5-hydroxy-4′-methoxy-3′-prenylisoflavone 7-O-β-D-(2″-O-*p*-coumaroyl)glucoside has been recently isolated from *Sopubia delphinifolia* [23]. The following isoflavone 7-O-(6″-O-malonyl)glucosides were found and characterized: biochanin A, formononetin and genistein derivatives in Leguminosae [15, 22, 24], and orobol and pratensein derivatives in a bryophyte [25].

Experimental

General procedures

Analytical and preparative TLC (PTLC) separations were carried out using Merck pre-coated silica gel 60 plates (F-254, layer thickness, 0.25 mm). The developing solvent systems were as follows: (a) CAM, $CHCl_3$–acetone–MeOH = 4:1:1 and (b) EMWAm, EtOAc–MeOH–H_2O–conc. ammonia water = 60:15:15:1. Isoflavone glucosides were eluted from TLC plates with MeOH and contaminated silica gel was separated by HPLC using a reverse phase column (Unicil QC-18). Detection of isoflavone on developed TLC plates was carried out by inspection under long (365 nm) and short (254 nm) wavelength UV lights, and by the characteristic colouration with Gibbs reagent. FD-MS and UV spectra were respectively recorded on a JEOL JMS-01 SG-2 and a Hitachi EPS-3 T. NMR spectra were determined by a JEOL JNM-FX-100, a FX-270, or a Bruker AM-500. Wako-gel C-200 (Wako Pure Chem. Ind.) and TSK-gel HW-40 F (Tosoh Co. Ltd.) were used as adsorbents for column chromatography. Low pressure liquid chromatography and HPLC were carried out respectively using a Lobar column (Lichroprep RP-8, 31 × 2.5 cm) and a Unicil QC-18 column. Eluates were monitored by a UV detector at 260 nm.

Extraction and isolation of lupin isoflavone glucosides

The white lupin (*Lupinus albus* L. *cv.* Kievskij Mutant) roots (10.2 kg) were collected at the early flowering stage, washed and air-dried overnight. The chopped roots were extracted with 90% MeOH (20 l × 4) at room temperature and combined extracts were reduced to 1.5 l *in vacuo*. After being stood at −20 °C, the liquid phase 1 l was separated and combined with 50% MeOH washings (200 ml) of the semi-solid phase. The extracts were successively washed with hexane (1 l), benzene (1.2 l × 3) and EtOAc (1.2 l × 5). The extractives (15.5 g) in the EtOAc were initially subjected to silica gel (500 ml) column chromatography with $CHCl_3$–acetone–MeOH mixture as eluting solvent, Fr-1 (56:7:7), 500 ml; Fr-2–4 (112:21:21), 150 ml each; Fr-5–7 (14:3:3), 150 ml each; and Fr-8–12 (4:1:1), 150 ml each. The major constituents (**1am,** R_f 0.30 and **4b,** R_f 0.49 in EMWAm) in Fr-5 (600 mg) were at first fractionated by TSK-gel. The earlier eluate was subjected to PTLC in EMWAm to yield 28 mg of **1am** which was also obtained from Fr-6 (*ca.* 20 mg). A constituent in the later eluate was purified by HPLC (Unisil QC 18 column; solvent, CH_3CN – 3% CH_3COOH = 3:2) to give 6.8 mg of **4b.** The constituents in Fr-6 (600 mg) were divided into three major fractions by TSK-gel column chromatography with 60% MeOH. From the earlier eluates, **1a** (5.3 mg), **2a** (2.0 mg, R_f 0.48), **1b** (1.8 mg) which was also isolated from Fr-7 (8.4 mg), and **2am** (13.1 mg, R_f 0.24) were isolated by re-chromatography over TSK-gel with 35% MeOH and PTLC in EMWAm. From the middle fraction, **3a** (3.1 mg, R_f 0.52 in EMWAm) was isolated by PTLC in EMWAm. The later eluates were further fractionated over a Lobar column to yield **4b** (2.1 mg, R_f 0.52 in EMWAm) and **4a** (1.2 mg, R_f 0.49) which was also isolated from Fr-7 (1.3 mg). The Fr-7 (140 mg) was subjected to Lobar column chromatography to elute with 60% MeOH at a rate of 2.9 ml/min and the eluates

from 37–51 min were concentrated and charged on PTLC to separate 1.2 mg of **2b,** R_f 0.42 in EMWAm. The same compound (17 mg) was also obtained from Fr-10 and Fr-11. During the isolation of major constituents, minor glucosides **1bm** (R_f 0.18 in EMWAm) and **2bm** (R_f 0.17) were respectively isolated from Fr-6, and Fr-5 and Fr-6.

Methylation of malonylglucoside

The malonylated glucoside (**1am** or **2am,** 4–10 mg) was methylated in a solution of *p*-TsOH 1 mg/4 ml MeOH standing at room temperature for 12 h. The concentrated reaction mixture was applied to PTLC in EMWAm to yield the corresponding methyl ester.

Enzymatic hydrolysis of isoflavone glucoside

The isolated glucosides (**1a, 1b, 2a, 2b, 3a, 4a** and **4b**) were respectively hydrolyzed in an aqueous solution (*ca.* 1 mg/4 ml) with 2 mg of β-glucosidase (Sigma 4.0 units/mg) at 25 °C for 18 h. The reaction products were partitioned between EtOAc and water. The former was concentrated to give an aglycone and the latter to give glucose, which were compared respectively with authentic isoflavones (genistein [26], 2′-hydroxygenistein [26], luteone [26] and licoisoflavone A [18]) and glucose by TLC in $CHCl_3$–MeOH = 50:2 or n-BuOH–CH_3COOH–H_2O = 3:1:1.

Physicochemical properties of isolated glucosides

Gibbs test colours, UV absorption maxima in MeOH and ^1H NMR data for the isolated compounds are shown in Tables I and II.

2′-Hydroxygenistein 7-O-β-glucoside (**2a**). FD-MS *m/z* (rel. int. %): 448 (M^+, 100), 286 (M^+–162, 26). ^{13}C NMR δ (DMSO-d_6, 25 MHz): 60.6 (C-6″), 69.6 (C-4″), 73.1 (C-2″), 76.4 (C-5″), 77.2 (C-3″), 94.5 (C-8), 99.4 (C-6), 99.9 (C-1″), 102.6 (C-3′), 106.1 (C-10), 106.2 (C-5′), 108.3 (C-1′), 120.7 (C-3), 132.1 (C-6′), 155.9 (C-2), 156.4 (C-2′), 157.2 (C-9), 158.6 (C-4′), 161.5 (C-5), 162,9 (C-7), 180.6 (C-4).

2′-Hydroxygenistein 4′-O-β-glucoside (**2b**). FD-MS *m/z* (rel. int. %): 448 (M^+, 100), 286 (M^+–162, 62). ^{13}C NMR δ (DMSO-d_6, 125 MHz): 60.5 (C-6″), 69.5 (C-4″), 73.1 (C-2″), 76.5 (C-5″), 76.9 (C-3″), 93.6 (C-8), 99.0 (C-6), 100.3 (C-1″), 103.8 (C-3′), 104.3 (C-10), 106.6 (C-5′), 111.5 (C-1′), 120.0 (C-3), 132.0 (C-6′), 155.3 (C-2), 156.2 (C-2′), 157.6 (C-9), 158.4 (C-4′), 161.7 (C-5), 164.3 (C-7), 180.1 (C-4).

Luteone 7-O-β-glucoside (**3a**). FD-MS *m/z* (rel. int. %): 516 (M^+, 100), 354 (M^+–162, 36). ^{13}C NMR δ (DMSO-d_6, 125 MHz): 17.7 (C-5″), 21.1 (C-1″), 25.4 (C-4″), 60.6 (C-6‴), 69.6 (C-4‴), 73.3 (C-2‴), 76.6 (C-5‴), 77.2 (C-3‴), 93.0 (C-8), 100.4 (C-1‴), 102.6 (C-3′), 105.7 (C-10), 106.0 (C-5′), 108.3 (C-1′), 112.5 (C-6), 120.6 (C-3), 121.9 (C-2″), 130.7 (C-3″), 132.0 (C-6′), 155.3 (C-2), 155.6 (C-9), 156.5 (C-2′), 157.9 (C-4′), 158.7 (C-5), 160.5 (C-7), 180.7 (C-4).

Licoisoflavone A 7-O-β- and 4′-O-β-glucosides (**4a** *and* **4b**). **4a:** FD-MS *m/z* (rel. int. %): 516 (M^+, 49), 354 (M^+–162, 100). **4b:** FD-MS *m/z* (rel. int. %): 516 (M^+, 41), 354 (M^+–162, 100).

Genistein 7-O-β-(6″-O-malonyl)glucoside (**1am**) *and its methylation product* (**1am-Me**). **1am:** FD-MS *m/z* (rel. int. %): 558 (M^+–44+2 × 42, 5), 516 (M^+–44+42, 20), 474 (M^+–44, 31), 432 (M^+–44–42, 15), 270 (aglycone, 100). **1am-Me:** R_f 0.56 in CAM. FD-MS *m/z* (rel. int. %): 632 (M^++CH_2OCOCH_2CO, 14), 574 (M^++CH_2CO, 7), 532 (M^+, 88), 474 (M^+–CH_2OCO, 26), 432 (M^+–CH_2OCOCH_2CO, 85), 270 (aglycone, 100). ^1H NMR δ (DMSO-d_6, 500 MHz): 3.50 and 3.54 (both 1H, two d, J = 16.2 Hz, malonyl-CH_2), 3.60 (3H, s, $-OCH_3$), 4.16 and 4.39 (both 1H, two dd, J = 11.8, 6.9 and 11.8, 2.0 Hz, 6″-H_2), 5.13 (1H, d, J = 7.5 Hz, 1″-H), 6.47 (1H, d, J = 2.2 Hz, 6-H), 6.71 (1H, d, J = 2.2 Hz, 8-H), 6.83 (2H, d, J = 8.8 Hz, 3′- and 5′-H), 7.41 (2H, d, J = 8.8 Hz, 2′- and 6′-H), 8.36 (1H, s, 2-H).

2′-Hydroxygenistein 7-O-β-(6″-O-malonyl)glucoside (**2am**) *and its methylation product* (**2am-Me**). **2am:** FD-MS *m/z* (rel. int. %): 574 (M^+–44+2 × 42, 5), 532 (M^+–44+42, 14), 490 (M^+–44, 23), 448 (M^+–44–42, 8), 286 (aglycone, 100). **2am-Me:** R_f 0.47 in CAM.

Genistein and 2′-hydroxygenistein 4′-O-β-(6″-O-malonyl)glucosides (**1bm** *and* **2bm**), *and their methylation products* (**1bm-Me** *and* **2bm-Me**). **1bm** and **2bm:** see Table I. **1bm-Me:** R_f 0.56 in CAM. **2bm-Me:** R_f 0.47 in CAM.

Acknowledgement

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