Bioactive Isoflavonols and Other Components from *Trifolium subterraneum*

Shaofang Wang,[†] Emilio L. Ghisalberti,*[,†] and James Ridsdill-Smith[‡]

Centre for Legumes in Mediterranean Agriculture, Department of Chemistry, University of Western Australia, Nedlands, Western Australia 6907, Australia, and CSIRO Entomology, Private Bag, Wembley, Western Australia 6014, Australia

Received November 14, 1997

Two new isoflavonols (**1**, **2**) with insect antifeedant activity have been isolated from the trifoliates of *Trifolium subterraneum* (Leguminosae). The structures of the compounds were determined by spectroscopic analyses. In addition, oct-1-en-3-yl arabinopyranosyl-(1→6)-β-glucopyranoside (**3**), a new metabolite, and phaselic acid (**4**), previously obtained from *Trifolium repens*, were isolated and characterized.

The redlegged earth mite, *Halotydeus destructor* (Acarina; Penthaleidae), is a major pest of pastures, crops, and vegetables in Australia, New Zealand, and South Africa. Considerable effort has been invested in developing resistant plant varieties of subclover (*Trifolium subterraneum* L.), medics (*Medicago* sp.), and canola (*Brassica napus*) to reduce the economic losses caused by the mite.[1] In continuation of our work[2] aimed at determining the chemical basis of resistance of certain subclover varieties toward the redlegged earth mite, we have investigated the metabolites of subclover trifoliates for antifeedant activity. Subclover is known to produce significant quantities of the isoflavones formononetin, biochanin A, and genistein, which have been isolated in the free and "bound" form (glycosylated).[3] The presence of these isoflavones has created considerable interest because they are responsible for reproductive abnormalities in stock feeding on the plant.[4] We now report the isolation and structure elucidation of four unusual metabolites from a resistant variety of subclover; the two isoflavonols (**1** and **2**), the alkylated dissaccharide (**3**), and phaselic acid (**4**). To our knowledge, only **4** has been isolated before.

Trifoliates of subclover, *T. subterraneum* L. (Leguminosae), were extracted by two methods. The leaf surface components, including exudate of the trichomes, were obtained by dipping the plant material in MeOH for 10 s. This method provided an extract free from chlorophyll, carotenes, waxes, and lipids.[5] The residual plant material was powdered and extracted with MeOH to provide the second extract.

Chromatographic separation of the acidic portion of the second extract was carried out utilizing the bioassay for mite feeding deterrence described previously.[6] This led to the isolation of two active compounds, (**1**) (29 mg, 0.005%) and (**2**) (14 mg, 0.0023%). HRMS measurements of the highest mass peaks showed the composition to be $C_{16}H_{12}O_5$ and $C_{15}H_{10}O_5$, respectively. The proton NMR spectra showed that both compounds contained resonances in the aromatic region that were characteristic of genistein (**7**) and biochanin A, two isoflavones found in extracts of *T. subterraneum*;[3] however, the vinylic proton expected at δ_H 8.4 (H-2) was absent. In fact, the NMR spectra of **1** showed that this proton was replaced by an AB spin system at δ_H 5.81 and 3.83 (J = 3.2 Hz) in which the proton at δ_H 5.81 was attached to a hemiacetal carbon (δ_C 99.1; HMQC). Moreover, two series of signals in the ratio of 3:1 were observed, indicating the presence of two anomers. Thus, compound **1** is a mixture of diastereoisomers of 2,5,7-trihydroxy-4′-methoxyisoflavanone. The complete assignment of NMR signals was made with the aid of HMQC and HMBC measurements, and the results are listed in Table 1. The salient correlations in the HMBC were as follows: the hemiacetal proton (δ_H 5.81 and 5.80) correlated with the carbonyl group (δ_C 196.7 and 197.1), and the benzylic methine (δ_H 3.83 and 4.30) correlated with the carbonyl group, the hemiacetal carbon (δ_C 99.1 and 98.4), and C2′, C6′ (δ_C 130.4 and 132.7). MM2 energy minimization procedures[7] revealed that, on a structure with the 3*S*-configuration (see below), the α-anomer is considerably more stable than the β-anomer.

The more polar compound was similarly deduced to be a 3:1 mixture of the two anomers of 2,5,7,4′-tetrahydroxyisoflavanone (**2**). In this case, the coupling between H-2 and H-3 was not evident (Table 1).

2-Hydroxyisoflavanones have been implicated in the biosynthesis of isoflavones but have not been isolated previously as natural products. Using a microsomal preparation from elicitor-treated soy bean cell-suspension cultures, it was shown that 2*S*-naringenin (**6**) is converted to genistein (**7**) via an intermediate, which was suggested to be 2,5,7,4′-tetrahydroxyisoflavanone on the basis of its mass spectral properties.[8] The isoflavonol **2** showed an identical MS to that reported, thus the NMR characterization of **2** provides confirmation of the structure proposed for the intermediate. The intermediacy of 2-hydroxyisoflavones in the formation of isoflavones has been supported by the isolation of 2,7,4′-trihydroxyisoflavanone (**8**) on incubation of liquiritigenin (**9**) with microsomes from cell cultures of elicitor-challenged *Pueraria lobata*.[9] Evidence for the involvement of cytochrome P-450 in the rearrangement has been obtained.[10] The mechanism suggested by these authors, and adapted here for the formation of **1** and **2** (Scheme 1), assumes that migration of the C-ring occurs with retention of stereochemistry leading to the

* To whom correspondence should be addressed. Tel.: (08) 9380-3174; Fax: (08) 9380-1005. E-mail: elg@chem.uwa.edu.au.
[†] University of Western Australia.
[‡] CSIRO Entomology.

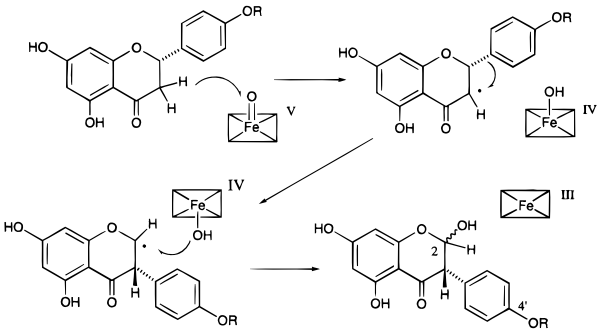
S0163-3864(97)00503-X CCC: $15.00
Published on Web 03/21/1998

Table 1. ^{1}H and ^{13}C NMR Spectral Data for the Isoflavonols **1** and **2**[a]

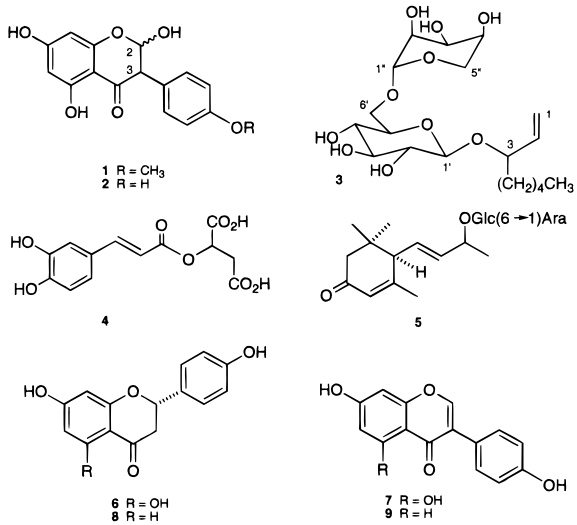
	compound									
	1[b]				**1**[c]		**2**[c]			
	major	minor			major	minor	major	minor		
position	δ_H (mult, *J* Hz)	δ_H (mult, *J* Hz)	major δ_C	minor δ_C	δ_H (mult, *J* Hz)	δ_H (mult, *J* Hz)	δ_H (mult, *J* Hz)	δ_H (mult, *J* Hz)	major δ_C	minor δ_C
2	5.81 (d, 3.2)	5.80 (d, 3.2)	99.1	98.4	5.64 (d, 3.4)	5.67 (d, 3.4)	5.62 (s)	5.65 (s)	99.6	100.1
3	3.83 (d, 3.2)	4.30 (d, 3.2)	57.4	57.3	3.73 (d, 3.4)	4.18 (d, 3.4)	3.35 (s)	3.35 (s)	49.9	49.3
4			196.7	197.1					197.4	198.4
5			165.4	164.9					165.6	165.0
6	5.96 (app. s.)	5.95 (app. s.)	97.0	96.8	5.90 (app. s.)	5.92 (app. s.)	5.92 (app. s.)	5.93 (app. s.)	97.3	97.1
7			167.8	167.2					168.8	168.2
8	5.96 (app. s.)	5.95 (app. s.)	96.6	96.5	5.90 (app. s.)	5.92 (app. s.)	5.92 (app. s.)	5.93 (app. s.)	97.0	96.9
9			160.1	160.0					161.2	162.0
10			102.7	103.2					102.9	103.5
1′			128.1	126.3					127.3	125.5
2′, 6′	7.25 (AA′BB′)	7.30 (AA′BB′)	130.4	132.7	7.17 (AA′BB′)	7.24 (AA′BB′)	7.08 (AA′BB′)	7.14 (AA′BB′)	130.6	132.8
3′, 5′	6.85 (AA′BB′)	6.85 (AA′BB′)	114.9	114.2	6.87 (AA′BB′)	6.85 (AA′BB′)	6.72 (AA′BB′)	6.74 (AA′BB′)	116.6	115.9
4′			160.6	161.1					158.2	158.0
OMe	3.75 (s)	3.78 (s)	55.5	55.4	3.75 (s)	3.77 (s)				
OH	12.11 (s)	12.07 (s)								

[a] ^{1}H NMR at 500.1 MHz; ^{13}C NMR at 125.8 MHz. [b] Me_2CO-d_6. [c] MeOH-d_4.

Scheme 1. Steps in the Cytochrome P-450 Catalyzed Aryl Migration in Isoflavonol Biosynthesis



isoflavonol with the 3*S*-configuration. The timing of the methylation of the 4′-hydroxyl is not known. The mechanism suggested in Scheme 1 allows for methylation to occur either pre- or post-aryl migration.



Reversed-phase chromatographic separation of the leaf surface components (first extract) yielded two compounds. The NMR spectral properties of the more polar compound (**3**) indicated the presence of a β-glucopyranosyl, an α-arabinopyranosyl, and an aglycon moiety with eight carbon atoms. The nature of the aglycon was established from HMQC and HMBC measurements to be oct-1-en-3-ol (Table 2). Evidence for the arabinopyranosyl-(1→6)-β-glucopyranoside entity came from interpretation of the NMR spectral parameters and by the close correspondence of these with those described for a model compound (**5**), which contains this disaccharide unit.[11] The presence of a glycosylated oct-1-en-3-yl metabolite is interesting. In previous work, we have shown that damage of cotyledons by redlegged earth mites results in release of oct-1-en-3-ol and the corresponding ketone.[12] These compounds are known to be derived from linoleic acid by lipoxidase-induced reactions. It was also found that the amount of ketone produced was correlated with cotyledon resistance to the mites. The less polar component was identified as phaselic acid (**4**), a compound origi-

Table 2. ^{1}H and ^{13}C NMR Data of Compound **3** and Model Compound **5**[a]

	3			**5**
position	δ_H (mult, *J* Hz)	δ_C	HMBC	δ_C
1a	5.19 (dd, 0.8, 17.3)			
		116.3	H3	
1b	5.08 (dd, 0.8, 10.4)			
2	5.83 (ddd, 7.1, 10.4, 17.3)	140.5	H1a, H1b	
3	4.08 (app. q., 7.1)	82.9	H1a, H1b, H1′	
4	1.66, 1.52 (m)	35.5		
5	1.32 (m)	25.4		
6	1.28 (m)	32.8		
7	1.29 (m)	23.4	H8	
8	0.87 (t, 6.7)	14.3		
1′	4.29 (d, 8.0)	103.0	H2′	102.6
2′	3.19 (m)	74.8	H3′	75.2
3′	3.34 (m)	77.8		77.9
4′	3.34 (m)	71.2	H6	71.5
5′	3.34 (m)	76.3	H6	76.8
6a′	4.02 (dd, 3.0, 11.5)			
		68.9	H1″, H5″	69.5
6b′	3.70 (dd, 5.0, 11.5)			
1″	4.31 (d, 6.7)	104.4	H6′, H5″, H2″, H3″	105.2
2″	3.52 (m)	72.0		72.4
3″	3.58 (m)	73.7		74.3
4″	3.85 (m)	68.9		69.4
5a″	3.85 (dd, 3.5, 12.2)			
		66.5	H1″	66.8
5b″	3.50 (dd, 1.6, 11.5)			

[a] MeOH-d_4.

nally isolated from the kidney bean (*Phaseolus vulgaris*) and also found in red clover (*T. pratense*).[13,14]

Experimental Section

General Experimental Procedures. ^{1}H and ^{13}C NMR spectra were obtained for MeOH-d_4, Me_2CO-d_6, or D_2O solutions using a Bruker ARX-500 instrument operating at 500.1 and 125.8 MHz. MS was carried out using a VG Autospec mass spectrometer (70 eV). Flash column chromatography was performed using 40−60 μm Si gel (BDH) and Si gel 100 C18-reversed-phase (Merck) as the stationary phase. For TLC, precoated AL SIL G/UV (Whatman) plates were used, and the compounds were detected by spraying with 3% ceric sulfate in 65% H_2SO_4. Optical rotations were measured using a Perkin-Elmer 141 polarimeter with a microcell of 1-dm path length.

Plant Material. The seeds of the variety of subclover (*T. subterraneum* L.; noncommercial resistant variety 8E014) were from a cross-bred line developed and provided by Mr. P. G. H. Nichols, Plant Breeder, Subterranean Clover Project, Department of Agriculture, Western Australia. Seeds were sown in pots with standard soil mix.[15] The pots were moved to a glasshouse after seed germination. Leaves were harvested 4−5 weeks after seed sowing.

Extraction and Isolation. Fresh leaves (600 g) were washed with MeOH for 10 s, and the MeOH extract was concentrated in vacuo to give pale yellow residue (600 mg). A portion (300 mg) was subjected to column chromatography over C18 reversed-phase support using a mobile-phase gradient of MeOH in H_2O to give seven fractions (A1 to A7). Fraction A2 was purified by flash column chromatography reversed-phase C-18 with an eluent of MeOH−H_2O to afford compound **3** (1 mg). Similarly, fraction A4 was separated by chromatography on reversed-phase C-18 to yield compound **4** (2.2 mg, 0.0009%).

The residual leaves were frozen in liquid nitrogen, powdered and extracted four times with MeOH. The combined MeOH solutions were concentrated under reduced pressure to yield an extract (28 g). A portion (10 g) was partitioned between CH_2Cl_2, EtOAc, *n*-BuOH, and H_2O to yield four fractions, CH_2Cl_2 (37%), EtOAc (8%), *n*-BuOH (22%), and H_2O (33%). The CH_2Cl_2 fraction (3.7 g), dissolved in $CHCl_3$, was extracted with 1% NaOH. The combined basic solution was neutralized with 2.5 M HCl to pH 5, extracted with EtOAc, and dried with anhydrous Na_2SO_4. The acidic fraction, obtained as a green oil (1 g), was subjected to flash column chromatography. Gradient elution with hexane and increasing amounts of EtOAc gave seven fractions (C1−C7). Further separation of fractions C5 and C6 yielded **1** (29 mg, 0.005%) and **2** (14 mg, 0.0023%).

2,5,7-Trihydroxy-4′-methoxyisoflavonol:[1] solid; $[\alpha]_D$ −70°(*c*, 0.12, MeOH); ^{1}H and ^{13}C NMR, see Table 1; HREIMS, *m/z* ($M^+ - H_2O$), calcd for $C_{16}H_{12}O_5$, 284.0685; found 284.0674.

2,5,7,4′-Tetrahydroxyisoflavonol:[2] solid; $[\alpha]_D$ −41°-(*c*, 0.11, MeOH); ^{1}H and ^{13}C NMR, see Table 1; HREIMS, *m/z* ($M^+ - H_2O$), calcd for $C_{15}H_{10}O_5$, 270.0528; found 270.0520.

Oct-1-en-3-yl arabinopyranosyl-(1→6)-*β*-glucopyranoside:[3] solid; ^{1}H and ^{13}C NMR, see Table 2.

Phaselic acid (*trans*-caffeoyl-malic acid):[4] solid; ^{1}H NMR (D_2O, 500.1 MHz) δ 6.90 (1H, d, J = 8.2 Hz, H-3), 7.10 (1H, br d, J = 8.2 Hz, H-4), 7.17 (1H, br s, H-6), 7.62 (1H, d, J = 16.0 Hz, H-7), 6.38 (1H, d, J = 16.0 Hz, H-8), 5.12 (1H, dd, J = 2.7, 11.3 Hz, H-2′), 2.77 (1H, dd, J = 2.8, 15.4 Hz, H-3′), 2.61 (1H, dd, J = 11.4, 15.4 Hz, H-3′); ^{13}C NMR (D_2O, 125.8 MHz) δ 178.4 (s, C-1′), 177.5 (s, C-4′), 147.2 (s, C-1), 146.3 (d, C-7), 144.4 (s, C-2), 127.3 (s, C-5), 122.9 (d, C-4), 116.4 (d, C-8), 115.3 (d, C-3), 114.7 (d, C-6), 73.9 (d, C-2′), 39.7 (t, C-3′).

Acknowledgment. We thank J. A. Reidy-Crofts for technical assistance with the bioassays.

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NP970503R