Fungitoxic Isoflavones from *Lupinus albus* and other *Lupinus* Species

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The constitutive isoflavones genistein, 2′-hydroxygenistein, wighteone and luteone have been isolated in varying amounts from methanolic leaf washings of eight species belonging to the legume genus *Lupinus*. These four compounds likewise occur in the flower buds, stems, roots and immature pods of *L. albus*, and in stems and roots of *L. angustifolius* and *L. mutabilis*. A minor isoflavone present in *L. albus* and *L. luteus* leaf washings has been identified by chemical and spectroscopic procedures as 5,7,4′-trihydroxy-3′-methoxy-6-(3,3-dimethylallyl)isoflavone (lupisoflavone). Apart from genistein, 2′-hydroxygenistein, wighteone and luteone, the roots of *L. albus* also contain alpinumisoflavone, licoisoflavone B and 6,3′-di-(3,3-dimethylallyl)genistein (lupalbigenin).

Introduction

A number of constitutive 5-hydroxylated isoflavones are known to occur in the roots and aboveground parts of several species belonging to the genus *Lupinus* (Leguminosae-Papilionoideae; tribe Genisteae; subtribe Lupininae) [1, 2]. As well as genistein (5,7,4′-trihydroxyisoflavone, **1**) and 5-*O*-methylgenistein, both of which are commonly found elsewhere in the Genisteae [1, 3], these compounds include various other aglycones, e.g. luteone (5,7,2′,4′-tetrahydroxy-6-(3,3-dimethylallyl)isoflavone, **2**), and at least six *O*- and *C*-glycosides [1, 2]. Luteone, first isolated from young fruits of *L. luteus* [4], is particularly notable because of its presence on the surface of lupin leaves [5] where, in conjunction with related compounds such as 5,7,4′-trihydroxy-6-(3,3-dimethylallyl)isoflavone (wighteone, **3**), it may inhibit the spore germination and/or germ tube growth of many potentially pathogenic fungi [5]. In *Lupinus* leaves, constitutive fungitoxic isoflavones appear to replace the post-infectionally formed (induced) isoflavonoid phytoalexins which occur characteristically as antimicrobial defence compounds in the tissues of a great many papilionate legumes [1, 6]. An earlier study [5] revealed that in addition to luteone and wighteone (the latter substance is denoted "LA-1" in ref. [5]), the methanolic washings of *L. albus* leaves contained a number of other phenolic isoflavones. These compounds have now been identified as genistein (**1**), 2′-hydroxygenistein (5,7,2′,4′-tetrahydroxyisoflavone, **4**) and the previously unreported 5,7,4′-trihydroxy-3′-methoxy-6-(3,3-dimethylallyl)isoflavone (**5**) for which we propose the trivial name lupisoflavone. Isoflavones **1**–**5** have also been variously detected in washings from leaves of several additional *Lupinus* species.

Results and Discussion

Lupinus albus leaves were briefly immersed (1 min) in MeOH to remove the bulk of surface isoflavones. The resulting solution was then filtered, reduced to near dryness *in vacuo*, and the residual brown syrup chromatographed (see Experimental for details) to yield small amounts of genistein (**1**) and 2′-hydroxygenistein (**4**) together with somewhat larger quantities of luteone (**2**) and wighteone (**3**) (Table I). All four compounds, but particularly **2** and **3**, proved to be fungitoxic when tested (25 and 50 μg) against *Cladosporium herbarum* using the TLC plate bioassay procedure described by Homans and Fuchs [7, 8]. Isoflavones **1**–**4** were firmly identified by UV, MS and TLC comparison with authentic material previously obtained from *Neonotonia (Glycine) wightii* (isoflavones **1**, **3** and **4** [9]) and *L. luteus* (isoflavone **2** [4]).

In addition to the above mentioned compounds, MeOH washings of *L. albus* leaves also contained traces of a new isoflavone (lupisoflavone, **5**) which,

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Table I. Isoflavones associated with the leaf surface and other parts of 8 *Lupinus* spp.

Species and cultivar	Plant part(s) examined	Isoflavone				
		Genistein (**1**)	2'-Hydroxy-genistein (**4**)	Wighteone (**3**)	Luteone (**2**)	Lupisoflavone (**5**)
L. albus cv. Kievskij Mutant	FB[a]	–	+[b]	+	++	(+) ?
	IP	< 0.5[c]	< 0.5	1	< 0.5	–
	L	< 0.5	< 0.5	4	3	< 1
	R[d]	+	+	+	+	–
	S	–	+	+	++	(+) ?
L. angustifolius cv. Beliak	L	2	1	26	7	Tr?
	R	+	+	+	(+)	–
	S	+	+	++	+	–
L. arboreus	L	< 0.1	–	< 0.1	–	–
L. arcticus	L	+++	+	(+)	++	–
L. elegans	L	< 0.1	Tr	< 0.5	< 0.5	–
L. luteus						
cv. Barpine	L	1	0.5	3	4	–
cv. Refusa	L	< 0.5	< 0.5	2	1	–
cv. Topaz	L	2	2	15	31	< 0.5
L. mutabilis	L	1	< 0.5	Tr	< 0.5	–
	R	+	+	(+)	+	–
	S	++	+	(+)	+	–
L. nanus	L	Tr	Tr	< 0.5	< 0.5	–

[a] FB = Flower bud; IP = Immature pod (30 – 50 mm in length); L = Leaf; R = Root; S = Stem.

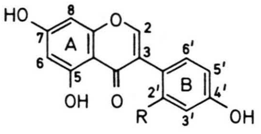
[b] Isoflavone levels assessed qualitatively using the scale, +++ = large quantity (probably > 10 µg/g fresh tissue) to (+) = trace amount (probably < 1 µg/g fresh tissue). ? = Provisional identification.

[c] Numerical values refer to isoflavone concentrations expressed as µg/g fresh tissue. These were determined spectrophotometrically using log $\varepsilon = 4.45$ at 266 nm for luteone [4] (compounds **2**, **3** and **5**) or log $\varepsilon = 4.63$ at 262 nm for genistein [28] (compounds **1** and **4**). Tr = Trace (compound detectable by Si gel TLC, but obtained in amounts insufficient for UV quantification). – = Not detected.

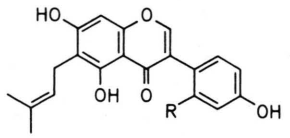
[d] Alpinumisoflavone (**11**), licoisoflavone B (**12**) and lupalbigenin (**13**) were also obtained from *L. albus* roots (see text).

like other 5-hydroxy derivatives including its co-constituents **1**–**4**, exhibited a purple-black fluorescence on TLC plates viewed under long wavelength UV light. A TLC bioassay (test organism, *C. herbarum*) indicated that lupisoflavone (25 µg) possessed antifungal properties although the resulting inhibition zone was only slight when compared with that given by comparable quantities of luteone and wighteone.

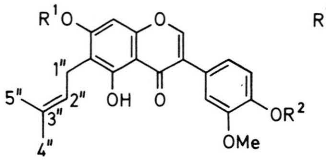
Apart from signals attributable to the methylene (H-1''), olefinic (H-2'') and non-equivalent terminal methyl (H-4'' and H-5'') protons of a 3,3-dimethylallyl side-chain, the ^{1}H NMR spectrum ($CDCl_3$) of lupisoflavone ($[M]^+$ 368) also revealed an OMe substituent (δ 3.94), a hydrogen-bonded OH group (δ 13.26) and the sharp low-field (δ 7.85) singlet characteristic of an isoflavone derivative. The MS of **5** revealed major fragments at *m/z* 325 (M^+-43) and *m/z* 313 (M^+-55) thereby providing further support for a 3,3-dimethylallyl attachment, whilst the aromatic ring substituents (*A-ring*, 3,3-dimethylallyl unit and two OH groups; *B-ring*, OH and OMe groups) were deduced from low intensity ions apparent at *m/z* 165 (A-ring derived; cf. MS data for luteone and wighteone [4, 9]) and *m/z* 148 (B-ring derived; cf. MS data for calycosin and 3'-*O*-methylorobol [10, 11]). As the MeOH UV (269 nm) maximum of lupisoflavone shifts bathochromically by 5–6 nm upon addition of $AlCl_3$ or NaOAc, the two A-ring OH substituents can only be sited at C-5 and C-7 [12]. The associated 3,3-dimethylallyl group must therefore be at either C-8 or, more probably in view of the co-occurrence of lupisoflavone with **2** and **3**, at C-6.



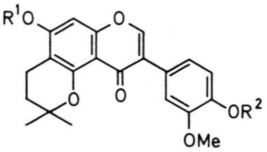
1: R = H
4: R = OH



2: R = OH
3: R = H



5: $R^1 = R^2 = H$
8: $R^1 = R^2 = Me$



6: $R^1 = R^2 = H$
9: $R^1 = R^2 = Et$

When lupisoflavone was heated with *p*-toluenesulphonic acid [13], two chromatographically distinct chroman derivatives (α-cyclolupisoflavone **6**, and β-cyclolupisoflavone **7**) were obtained in approx. equal quantities. Significantly, the α-isomer (λ_{max}, MeOH: 265 nm) gave a distinct 6 nm bathochromic UV shift with NaOAc whereas the β-isomer did not (see Experimental for full spectroscopic details). Moreover, the MeOH UV maximum of *β*-cyclolupisoflavone shifted bathochromically by 4 nm upon addition of $AlCl_3$, whilst that of α-cyclolupisoflavone was completely unaffected. These data indicate that the 3,3-dimethylallyl group of **5** occurs at C-6 rather than at the alternative C-8 position as, for example, in the recently reported *Phaseolus* isoflavone, 2,3-dehydrokievitone [14]. Lupisoflavone dimethyl ether **8** ($[M]^+$ 396; the H-bonded C-5 OH remains unmethylated) gave a blue colour on TLC plates sprayed with Gibbs reagent/aqueous Na_2CO_3 [8], an observation which also provides support for assignment of the 3,3-dimethylallyl substituent to C-6. In the ^1H NMR spectrum of lupisoflavone, the singlet resonance at δ 6.39 ($CDCl_3$) or δ 6.52 (acetone-d_6) can be attributed to the C-8 proton.

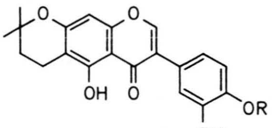
^1H NMR signals (acetone-d_6 spectrum) due to the three remaining (B-ring) aromatic protons (δ 7.25 *d*, 7.06 *dd* and 6.89 *d*) clearly suggested that **5** was oxygenated at either C-2′/C-4′, C-3′/C-4′ or C-2′/C-5′. The latter arrangement was quickly discounted, however, because all known legume isoflavones with B-ring dioxygenation are substituted at C-4′ [1]. Although a firm decision between the 2′/4′ and 3′/4′ oxygenation patterns could not be made from the spectroscopic data alone, the 3′/4′ disposition was favoured for two reasons. First, the B-ring proton signals of **5** were in very close accord with those reported for comparable compounds such as 3′-*O*-methylorobol (δ 7.26, H-2′; 7.10, H-6′; 6.86, H-5′: acetone-d_6 [11]), petalostetin (δ 7.07, 6.99 and 6.83 respectively: $CDCl_3$ [15]) and durmillone (δ 7.12, 6.97 and 6.85 respectively: $CDCl_3$ [16]). Secondly, the H-5′ resonance of 2′/4′-disubstituted isoflavones often appears between 6.30 and 6.60 ppm [14, 17–19] whereas for lupisoflavone, lupalbigenin (**13**) and other 3′/4′-disubstituted compounds [11, 15, 16] the corresponding signal is evident at about 6.90 ppm in both acetone-d_6 and $CDCl_3$.

As noted earlier, the lupisoflavone B-ring contains both an OH and an OMe substituent, and the exact location of these (3′-OMe and 4′-OH) was eventually established by chemical degradation. A mixture of α- and *β*-cyclolupisoflavone (**6** and **7**) was first ethylated (diethyl sulphate [20]) and the products (**9** and **10**) separated by Si gel TLC. After determination of their UV maxima (see Experimental), compounds **9** and **10** (both of which have identically substituted B-rings) were again combined and then degraded with H_2O_2 under alkaline conditions to afford a substance identical (UV, MS, TLC) with authentic vanillic acid ethyl ether. The degradation product could be distinguished chromatographically not only from isovanillic acid ethyl ether (synthetic or derived via alkaline peroxide treatment of ethylated calycosin [10]) but also from 2-ethoxy-4-methoxybenzoic acid and its 2-methoxy-4-ethoxy isomer; one or other of the latter acids would have been obtained if the starting isoflavone possessed oxygenation (OH/OMe) at C-2′ and C-4′. Together with information on acidic cyclisation of the 3,3-dimethylallyl group (see above), the formation of vanillic acid ethyl ether allows lupisoflavone to be correctly formulated as 5,7,4′-trihydroxy-3′-methoxy-6-(3,3-dimethylallyl)isoflavone (**5**).

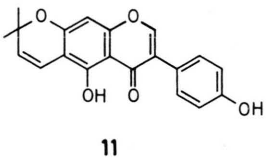
As shown in Table I, isoflavones **1–4** were generally present throughout *L. albus*, occurring in small but readily detectable quantities on leaf surfaces and in roots, pods, flower buds (apart from **1**) and stems (not **1**). Except for 2′-hydroxygenistein and luteone, which could not be isolated from leaf washings of *L. arboreus*, the same compounds were also associated with leaves and various other parts of a further seven Old and New World *Lupinus* species. The leaves of *L. angustifolius* and *L. luteus* cv. Topaz proved to be particularly rich sources of luteone and wighteone. Lupisoflavone was firmly identified only as a constituent of *L. albus* and *L. luteus* leaf washings, although some evidence was obtained to suggest that traces of this compound might similarly occur in stems and flower buds of *L. albus*, and on leaves of *L. angustifolius*.

In addition to compounds **1–4**, examination of *L. albus* roots also revealed the presence of alpinumisoflavone (**11**; < 1 μg/g fresh tissue), licoisoflavone B (**12**; 5–10 μg/g fresh tissue), and a substance (lupalbigenin) later found to be a diprenylated derivative of genistein (1–5 μg/g fresh tissue). All three isoflavones proved to be antifungal when tested (TLC plate bioassay) against *C. herbarum* at an applied level of about 25 μg. Alpinumisoflavone and licoisoflavone B were readily identified by chromatographic and spectroscopic comparison with authentic material. Licoisoflavone B has previously been found to occur only in the roots of Sinkiang licorice (*Glycyrrhiza* sp.; Leguminosae-Papilionoideae; tribe Galegeae) [21] and its appearance in *L. albus* is entirely unexpected. In contrast, however, the isolation of alpinumisoflavone is somewhat less surprising as this complex isoflavone was first discovered in *Laburnum* (Leguminosae-Papilionoideae; tribe Genisteae; subtribe Genistinae), a genus very closely allied to *Lupinus* [22]. Several other legumes also contain alpinumisoflavone [1], and in *Erythrina variegata* (as in *L. albus*) this isoflavone co-occurs with its presumed biosynthetic precursor wighteone **3** (= erythrinin B) [1, 23].

The *Lupinus* isoflavone termed lupalbigenin (**13**; M^+ 406) was hydroxylated at C-5 and C-7 (bathochromic UV shifts were obtained with $AlCl_3$ and NaOAc respectively [12]), and from its ^1H NMR spectrum clearly contained two 3,3-dimethylallyl substituents, one of these being assigned to C-6 by analogy with compounds **2**, **3**, **5** and **11**. Assuming oxygenation (here OH) at C-4′, the second 3,3-dimethylallyl group can be readily placed at C-3′ (*cf.* licoisoflavone B, **12**) from coupling observed between the aromatic B-ring protons (H-2′, *meta*-coupled; H-5′, *ortho*-coupled; H-6′, *ortho*- and *meta*-coupled). Lupalbigenin would thus appear to be 6,3′-di-(3,3-dimethylallyl)genistein (**13**), a compound also found in the aerial parts of *Millettia pachycarpa* (Leguminosae-Papilionoideae; tribe Tephrosieae) [24]. This provisional identification has recently been confirmed by direct UV, MS and TLC comparison with the *Millettia* isoflavone. The 2′-hydroxy analogue of **13** is already known to occur in roots of *L. angustifolius* where it is thought to deter feeding by certain insect larvae [25].



7: R = H
10: R = Et



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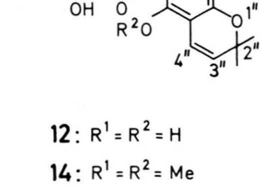
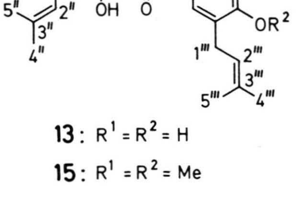


12: $R^1 = R^2 = H$
14: $R^1 = R^2 = Me$



13: $R^1 = R^2 = H$
15: $R^1 = R^2 = Me$

Experimental

Plant material. Seeds of *Lupinus albus* L. cv. Kievskij Mutant, *L. angustifolius* L. cv. Beliak, *L. mutabilis* Sweet (all supplied by Prof. W. Williams, Dept. of Agricultural Botany, Univ. of Reading), *L. luteus* L. cvs. Barpine, Refusa and Topaz (imported from The Netherlands, West Germany and Poland respectively), *L. arboreus* Sims, *L. elegans* H.B.K., *L. nanus* Dougl. (all from the Chelsea Physic Garden, London), and *L. arcticus* S. Wats. (from the Univ. of Oslo Botanic Garden) were sown in John Innes No. 1 compost and the resulting plants then grown in a glasshouse (without supplementary lighting) at approx. 20°. Leaves and other parts for chemical analysis were normally collected when the plants were 4 weeks old (*L. luteus* cv. Topaz), 13 weeks old (*L. arcticus*), or between 8 and 10 weeks old (all other species).

Isolation and purification of Lupinus isoflavones. Freshly harvested *Lupinus* leaves (sample sizes varied from about 5 g to more than 300 g) were placed in a beaker, covered with MeOH and gently stirred for 30 sec. The MeOH was then decanted and the procedure repeated for periods of 20 sec and 10 sec respectively. The combined MeOH washings for each species were suction filtered, reduced to near dryness (*in vacuo*, 40°) and the brown oily residue applied in EtOAc to one or more Si gel TLC plates (Merck, F-254, layer thickness 0.5 mm) which were then immediately developed in $CHCl_3$-MeOH (20 : 1). Bands of Si gel at R_F 0.58 (lupisoflavone, **5**), 0.27 (wighteone, **3**), 0.16 (genistein, **1** + luteone, **2**) and 0.09 (2′-hydroxygenistein, **4**) were removed, eluted with EtOAc and the material thus obtained further purified by TLC (Si gel, layer thickness 0.25 mm) in $CHCl_3$-Me_2CO-aqueous (35%) NH_3 (70 : 60 : 1). This system separated **1** (R_F 0.11) from **2** (R_F 0.22). All the *Lupinus* leaf isoflavones ran as single spots when checked for homogeneity by Si gel TLC in several additional solvent systems. Occasionally, leaf extracts were fractionated by column chromatography prior to preparative and/or analytical TLC. In a typical experiment, the washings from approx. 200 g leaf material were dissolved in EtOAc (about 0.5 ml) and the resulting solution applied to a column (1 × 8 cm) of dry Si gel (3 g). After moistening the entire column with *n*-hexane, the various leaf surface components were eluted with 20 ml portions of *n*-hexane – EtOAc as follows:

i) 100 : 5, *fraction 1* (lipid material) ii) 100 : 40, *fraction 2* (principally isoflavones **1**–**3** and **5**), and iii) 100 : 60, *fraction 3* (principally isoflavone **4**).

Stems (100 – 300 g), flower buds (20 g; *L. albus* only), immature pods (12 g; *L. albus* only) and roots (100 – 200 g) of three *Lupinus* spp. (*albus*, *angustifolius* and *mutabilis*) were cut into small pieces, placed in a beaker and covered with MeOH. After standing (with intermittent stirring) for 2 days at 20°, the MeOH extracts were collected and processed by Si gel TLC as outlined above. In $CHCl_3$-MeOH (20 : 1), the major *L. albus* root isoflavone (licoisoflavone B, **12**) was located (approx. R_F 0.60) immediately above the lupisoflavone marker. Elution (EtOAc) and additional TLC purification ($CHCl_3$-Me_2CO-aqueous NH_3, 70 : 60 : 1, ×2) of this material gave pure licoisoflavone B (lower zone) well separated from an upper fluorescence-quenching band consisting predominantly of lupalbigenin (**13**) admixed with small quantities of alpinumisoflavone (**11**). These two isoflavones were eventually resolved by Si gel TLC in *n*-pentane-Et_2O-glacial HOAc (75 : 25 : 6) (**11**, R_F 0.70; **13**; R_F 0.64). Lupisoflavone was not detected in extracts of *L. albus* roots.

5,7,4′-Trihydroxyisoflavone (**1**) *(genistein)*. UV and MS data as lit. [26].

5,7,4′-Trihydroxy-6-(3,3-dimethylallyl)isoflavone (**3**) *(wighteone)*. UV data as lit. [9]. MS: $[M]^+$ 338 (40), *m/z* 337 (4), 323 (M^+–15; 15), 309 (4), 295 (M^+–43; 100), 294 (4), 283 (M^+–55; 93), 282 (5), 270 (4), 165 (4), 123 (4), 121 (4), 118 (8).

5,7,2′,4′-Tetrahydroxyisoflavone (**4**) *(2′-hydroxygenistein)*. UV data as lit. [26]. MS: $[M]^+$ 286 (100), *m/z* 285 (13), 269 (M^+–17; 21), 229 (7), 217 (6), 161 (5), 154 (5), 153 (99), 152 (10), 135 (6), 134 (60), 124 (13), 123 (7).

5,7,2′,4′-Tetrahydroxy-6-(3,3-dimethylallyl)isoflavone (**2**) *(luteone)*. UV data as lit. [4]. MS: $[M]^+$ 354 (66), *m/z* 353 (3), 339 (M^+–15; 14), 337 (3), 312 (17), 311 (M^+–43; 100), 300 (12), 299 (M^+–55; 71), 298 (12), 281 (4), 269 (11), 241 (8), 219 (4), 205 (9), 177 (10), 165 (33), 161 (5), 149 (4), 135 (8), 134 (19), 124 (7), 123 (11).

5,7,4′-Trihydroxy-3′-methoxy-6-(3,3-dimethylallyl)isoflavone (**5**) *(lupisoflavone)*. Diazotised *p*-nitroaniline, orange; Gibbs reagent, blue (colour is initially faint but intensifies upon warming with a hair-dryer). On TLC (Si gel) plates viewed under long wavelength UV light, lupisoflavone exhibits a purple-black fluorescence unaffected by NH_3 vapour. UV: λ_{max}, nm, MeOH 269, 292 sh; +NaOH 281, 330 sh; +NaOAc 275, 342 (addition of solid boric acid regenerated the MeOH spectrum); +$AlCl_3$ 274, 327 sh. MS: $[M]^+$ 368 (42), *m/z* 367 (4), 353 (M^+–15; 11), 325 (M^+–43; 100), 314 (13), 313 (M^+–55; 70), 310 (5), 298 (7), 165 (4), 148 (4), 135 (6), 133 (8), 105 (9). ^1H NMR (400 MHz; TMS; acetone-d_6; J in Hz): δ 1.65 (*d*, $J_{5'',2''}$ = 0.97, 3H, H-5″), 1.78 (*s*, 3H, H-4″), 3.36 (*d*, $J_{1'',2''}$ = 7.32, 2H, H-1″), 3.89 (*s*, 3H, OMe), 5.27 (*br t*, 1H, H-2″), 6.52 (*s*, 1H, H-8), 6.89 (*d*, $J_{5',6'}$ = 8.30, 1H, H-5′), 7.06 (*dd*, $J_{6',5'}$ = 8.30 and $J_{6',2'}$ = 2.44, 1H, H-6′), 7.25 (*d*, $J_{2',6'}$ = 2.44, 1H, H-2′), 8.19 (*s*, 1H, H-2), approx. 13.3 (*s*, 1H, C-5 $O\underline{H}$). ^1H NMR ($CDCl_3$) δ 1.78, 1.85 (both *s*, each 3H, H-4″ and H-5″), 3.48 (*d*, $J_{1'',2''}$ = 6.59, 2H, H-1″), 3.94 (*s*, 3H, OMe), 5.29 (*br t*, 1H, H-2″), 5.70 (*br s*, 1H, OH?), 6.18 (*br s*, 1H, $O\underline{H}$?), 6.39 (*s*, 1H, H-8), 6.95 (*dd*, $J_{6',5'}$ = 8.05 and $J_{6',2'}$ = 1.47, 1H, H-6′), 6.98 (*d*, $J_{5',6'}$ = 8.05, 1H, H-5′), 7.12 (*d*, $J_{2',6'}$ = 1.47, 1H, H-2′), 7.85 (*s*, 1H, H-2), 13.26 (*s*, 1H, C-5 $O\underline{H}$). *Dimethyl ether* (**8**) (CH_2N_2; R_F 0.15 in CCl_4-$CHCl_3$, 4 : 1). Diazotised *p*-nitroaniline, pale orange; Gibbs reagent, blue. UV: λ_{max}, nm, MeOH 268, 292 sh; +NaOH 274; +$AlCl_3$ 273. The MeOH spectrum of **8** was unaffected by NaOAc. MS: $[M]^+$ 396 (32), *m/z* 395 (4), 382 (7), 381 (M^+ – 15; 11), 367 (6), 354 (15), 353 (M^+ – 43; 100), 342 (13), 341 (M^+ – 55; 70), 339 (9), 337 (6), 328 (6), 327 (9).

Alpinumisoflavone (**11**). Diazotised *p*-nitroaniline, orange, Gibbs reagent, blue (colour initially weak). Long wavelength UV fluorescence as given for **5**. UV: λ_{max}, nm, MeOH 283; +NaOH 242 sh, 291, 342 sh; +$AlCl_3$ 295, 355. The MeOH spectrum of **11** was unaffected by NaOAc. MS: $[M]^+$ 336 (20), *m/z* 335 (2), 323 (4), 322 (22), 321 (M^+ – 15; 100), 320 (3), 203 (5), 177 (5), 152 (5), 150 (4), 149 (30), 145 (5), 137 (9), 135 (16), 133 (5), 123 (8), 121 (6), 119 (8), 118 (10).

Licoisoflavone B (**12**). Diazotised *p*-nitroaniline, orange; Gibbs reagent, deep blue. Long wavelength UV fluorescence as given for **5**. UV: λ_{max}, nm, MeOH 264; +NaOH 245, 268, 342; +NaOAc 276, 326 (addition of solid boric acid regenerated the MeOH spectrum); +$AlCl_3$ 270, 308, 366. MS: $[M]^+$ 352 (7), *m/z* 338 (8), 337 (M^+ – 15; 100), 295 (2), 185 (4), 168 (3), 153 (2). ^1H NMR (400 MHz; TMS; acetone-d_6; J in Hz) δ 1.42 (*s*, 6H, 2 × Me), 5.70 (*d*, $J_{3'',4''}$ = 9.76, 1H, H-3″), 6.35 (*d*, $J_{6,8}$ = 1.95, 1H, H-6), 6.40 (*d*, $J_{5',6'}$ = approx. 8.3, 1H, H-5′), 6.50 (*d*, $J_{8,6}$ = 1.95, 1H, H-8), 6.78 (*d*, $J_{4'',3''}$ = 9.76, 1H, H-4″), 7.04 (*d*, $J_{6',5'}$ = approx. 8.3, 1H, H-6′), 8.25 (*s*, 1H, H-2), 12.52 (*s*, 1H, C-5

OH). *Dimethyl ether* (**14**) (CH_2N_2; R_F 0.76 in $CHCl_3$). Diazotised *p*-nitroaniline, orange; Gibbs reagent, blue. UV: λ_{max}, nm, MeOH 261; +NaOH 240 sh, 266; +$AlCl_3$ 271, 307, 366. The MeOH spectrum of **14** was unaffected by NaOAc. MS: $[M]^+$ 380 (21), *m/z* 379 (3), 367 (4), 366 (23), 365 ($M^+ - 15$; 100), 350 (7).

6,3′-di-(3,3-dimethylallyl)genistein (**13**) *(lupalbigenin).* Diazotised *p*-nitroaniline, orange; Gibbs reagent, blue (colour initially weak). Long wavelength UV fluorescence as given for **5**. UV: λ_{max}, nm, MeOH 268; +NaOH 279, 335; +NaOAc 273, 336 (addition of solid boric acid regenerated the MeOH spectrum); +$AlCl_3$ 274, 325 sh, 362. MS: $[M]^+$ 406 (75), *m/z* 391 ($M^+ - 15$; 6), 364 (12), 363 ($M^+ - 43$; 78), 352 (14), 351 ($M^+ - 55$; 100), 350 (6). ^{1}H NMR (400 MHz; TMS; acetone-d_6; J in Hz) δ 1.65, 1.71, 1.73, 1.78 (all *s*, each 3H, H-4″, H-5″, H-4‴, H-5‴), 3.36 (*d*, $J_{1'',2''} = J_{1''',2'''} = 7.81$, 2 × 2H, H-1″, H-1‴), 5.28, 5.38 (both *br t*, each 1H, H-2″, H-2‴), 6.51 (*s*, 1H, H-8), 6.90 (*d*, $J_{5',6'} = 7.82$, 1H, H-5′), 7.26 (*dd*, $J_{6',5'} = 7.82$ and $J_{6',2'} = 1.95$, 1H, H-6′), 7.33 (*d*, $J_{2',6'} = 1.95$, 1H, H-2′), 8.08 (*s*, 1H, H-2), 13.34 (*s*, 1H, C-5 O$\underline{\text{H}}$). *Dimethyl ether* (**15**) (CH_2N_2; R_F 0.75 in $CHCl_3$-CCl_4, 1 : 1). Diazotised *p*-nitroaniline, pale orange; Gibbs reagent, blue. UV: λ_{max}, nm, MeOH 269; +NaOH 273; +$AlCl_3$ 274. The MeOH spectrum of **15** was unaffected by NaOAc. MS: $[M]^+$ 434 (28), *m/z* 419 ($M^+ - 15$; 34), 392 (23), 391 ($M^+ - 43$; 65), 380 (28), 379 ($M^+ - 55$; 100), 378 (9).

Acidic cyclisation of lupisoflavone. A mixture of **5** (1 mg), *p*-toluenesulphonic acid (1.5 mg) and C_6H_6 (1 ml) was heated (75 – 80°) in a sealed tube for 60 min [13]. EtOAc (15 – 20 ml) was then added, and the solution shaken (× 2) with aqueous $NaHCO_3$ (0.5%; about 10 ml). The aqueous layer was discarded and the EtOAc fraction allowed to stand (12 h) over Na_2SO_4 before being reduced to dryness *in vacuo* (40°). Si gel TLC ($CHCl_3$-MeOH, 20 : 1) of the products gave α-cyclolupisoflavone **6** (approx. 300 µg; R_F 0.46) well separated from the β-isomer **7** (approx. 250 µg; R_F 0.83). A marker of uncyclised lupisoflavone was located at R_F 0.65. UV maxima recorded for isoflavones **6** and **7** were as follows: i) *α-cyclolupisoflavone* (**6**). λ_{max}, nm, MeOH 265, 290 sh; +NaOH 275, 314 sh; +NaOAc 271, 326 (addition of solid boric acid regenerated the MeOH spectrum). The MeOH spectrum of **6** was unaffected by $AlCl_3$. ii) *β-cyclolupisoflavone* (**7**). λ_{max}, nm, MeOH 270, 290 sh; + NaOH 282, 300 sh; + $AlCl_3$ 274, 320 sh. The MeOH spectrum of **7** was unaffected by anhydrous NaOAc.

Ethylation of α- and β-cyclolupisoflavone. A mixture of **6** and **7** (500 µg) was dissolved in dry Me_2CO (4 ml) and then refluxed with stirring in the presence of anhydrous K_2CO_3 (0.2 g) and diethyl sulphate (10 µl) for approx. 2 h. After removal of K_2CO_3 and Me_2CO [20], the residue was chromatographed (Si gel TLC; $CHCl_3$-MeOH, 50 : 1) to afford **9** (approx. 200 µg) and **10** (approx. 180 µg) at R_F 0.44 and 0.76 respectively. UV maxima recorded for isoflavones **9** and **10** were as follows: i) *7,4′-diethoxy-α-cyclolupisoflavone* (**9**). λ_{max}, nm, MeOH 264, 290 sh, 324 sh. The MeOH spectrum of **9** was unaffected by NaOAc or $AlCl_3$. ii) *4′-ethoxy-β-cyclolupisoflavone* (**10**). λ_{max}, nm, MeOH 270, 288 sh; +$AlCl_3$ 273, 320 sh. The MeOH spectrum of **10** was unaffected by NaOAc.

Degradation of ethylated isoflavones **9** *and* **10**. H_2O_2 (30%) was added dropwise in portions (0.1 ml; 0.05 ml; 0.05 ml; 0.1 ml) over a 4 h period to a stirred solution of **9** + **10** (combined amount, 350 µg) in EtOH (1 ml) and aqueous KOH (15%; 0.25 ml) at approx. 20°. After standing for a further 12 h, the mixture was acidified (pH 2; 2N HCl) and then extracted (×3) with EtOAc. The combined organic fractions were dehydrated (Na_2SO_4; 12 h), reduced to dryness *in vacuo* (40°), and the residue chromatographed (Si gel TLC; $CHCl_3$-MeOH, 30 : 1) to furnish a single, fluorescence-quenching product (R_F 0.22) indistinguishable (UV, MS, TLC) from synthetic vanillic acid ethyl ether (3-methoxy-4-ethoxybenzoic acid). In the same TLC system, isovanillic acid ethyl ether (3-ethoxy-4-methoxybenzoic acid; both synthetic and derived by H_2O_2 oxidation of ethylated calycosin) was located at R_F 0.24, whilst 2-ethoxy-4-methoxybenzoic acid and 2-methoxy-4-ethoxybenzoic acid ran to R_F 0.74 and 0.56 respectively. Corresponding R_F values in toluene-*p*-dioxane (4 : 1) were: vanillic acid ethyl ether (synthetic, and from isoflavones **9** and **10**), R_F 0.41; isovanillic acid ethyl ether (synthetic, and from ethylated calycosin), R_F 0.44; 2-ethoxy-4-methoxybenzoic acid, R_F 0.47; 2-methoxy-4-ethoxybenzoic acid, R_F 0.35. Vanillic acid ethyl ether and isovanillic acid ethyl ether were indistinguishable by Si gel TLC in a variety of other solvent systems.

Preparation of vanillic acid ethyl ether. A mixture of vanillic acid (50 mg), dry Me_2CO (10 ml), anhydrous K_2CO_3 (0.5 g) and diethyl sulphate

(0.06 ml) was refluxed with stirring for 2 h. After addition of H_2O (5 ml), the reaction mixture was concentrated *in vacuo* (40 °) to about 3 ml, and then shaken with an approx. equal volume of Et_2O. The ether layer was collected, and the solvent removed under reduced pressure to yield 3-methoxy-4-ethoxyvanillic acid ethyl ester (18 mg). Examination of the remaining aqueous phase (acidification with 2N HCl, followed by extraction with Et_2O and eventual TLC purification of the ether extract) revealed only unchanged vanillic acid. The preceding ethyl ester, without further purification, was refluxed (2 – 3 h) with EtOH (1 ml) and aqueous NaOH (2 N; 1 ml), and the cooled mixture then acidified (pH 2; 2 N HCl) prior to extraction with Et_2O. Si gel TLC ($CHCl_3$-MeOH, 10 : 1) of the Et_2O extract gave vanillic acid ethyl ether (approx. 5 mg) at R_F 0.63. UV: λ_{max}, nm, MeOH 254, 289; +NaOH 251, 285, 290 sh. MS: $[M]^+$ 196 (63), m/z 168 (M^+ – 28; 100), 153 (84), 151 (17), 125 (16), 97 (17), 79 (17). Identical UV and MS data were obtained for the product resulting from H_2O_2 oxidation of ethylated isoflavones **9** and **10**.

Preparation of isovanillic acid ethyl ether. Isovanillic acid (100 mg) in dry Me_2CO (10 ml) was refluxed with anhydrous K_2CO_3 (0.25 g) and diethyl sulphate (0.2 ml) for 5 h, and the reaction mixture then treated as described for preparation of vanillic acid ethyl ether to yield 3-ethoxy-4-methoxyisovanillic acid ethyl ester (20 mg). In addition, an Et_2O extract of the acidified aqueous phase afforded two substances upon Si gel TLC in $CHCl_3$-MeOH (10 : 1). One of these (R_F 0.31) proved to be unchanged isovanillic acid, whilst the other (R_F 0.63) was identified (MS) as the desired isovanillic acid ethyl ether (30 mg). UV: λ_{max}, nm, MeOH 254, 289; +NaOH 250, 286, 290 sh. MS: $[M]^+$ 196 (62), m/z 168 (M^+ – 28; 100), 153 (76), 151 (16), 125 (16), 97 (16), 79 (19). As expected, H_2O_2 oxidation of ethylated calycosin gave a single substance indistinguishable (UV, MS, TLC) from synthetic isovanillic acid ethyl ether.

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