Coumestans from the Roots of *Pueraria mirifica*

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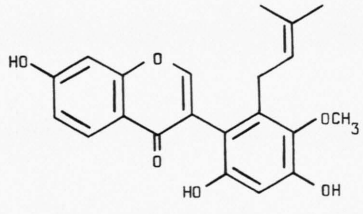
Three isoflavonoids obtained from a methanolic extract of *Pueraria mirifica* roots have been identified as 3,9-dihydroxy-8-methoxy-7-(3,3-dimethylallyl)-coumestan (mirificoumestan), 3,9-dihydroxy-8-methoxy-7-(3-hydroxy-3-methylbutyl)-coumestan (mirificoumestan hydrate), and 3,9-dihydroxy-8-methoxy-7-(2,3-dihydroxy-3-methylbutyl)-coumestan (mirificoumestan glycol). These new coumestans co-occur with coumestrol (3,9-dihydroxycoumestan), a compound already found in *P. mirifica* roots.

Introduction

Root extracts of the Thai legume *Pueraria mirifica* Airy Shaw & Suvatabandhu have previously been found to contain three isoflavone aglycones (daidzein, genistein and kwakhurin **1**), two isoflavone O-glycosides (daidzin and mirificin), an isoflavone C-glycoside (puerarin), and four coumestans (coumestrol **2**, and the partially identified compounds denoted PM-1, PM-4 and PM-6) [1–3]. Although only limited structural data were originally obtained for the three latter coumestans, it was clear from the NaOAc-induced bathochromic shift of the UV (MeOH) maximum at 347 nm [4] that each was hydroxylated at C-3 (ring A). Moreover, the mass spectrum of PM-1 (M^+ 366) afforded a prominent fragment at $M^+ - 55$ consistent with the presence of a 3,3-dimethylallyl side attachment. The recent identification of kwakhurin (**1**) as a major constituent of *P. mirifica* roots [2] suggested that PM-1 might possibly be the corresponding coumestan analogue (**3**). In this paper we present chemical and spectroscopic evidence which confirms structure **3** for PM-1, and which additionally allows coumestans PM-4 and PM-6 to be formulated as **4** and **5**, respectively. The trivial names mirificoumestan (**3**), mirificoumestan hydrate (**4**) and mirificoumestan glycol (**5**) are proposed for these new *Pueraria* coumestans.

Results and Discussion

Upon methylation with dimethyl sulphate, coumestan PM-1 readily afforded a non-phenolic dimethyl ether (M^+ 394; **6**). Apart from a methoxyl group and a 3,3-dimethylallyl (prenyl) side attachment, the ^1H NMR spectrum of PM-1 revealed signals attributable



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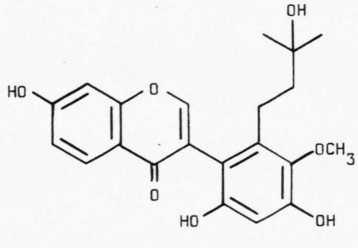

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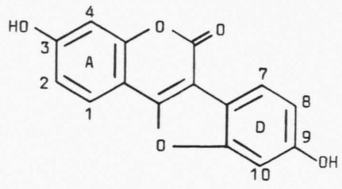

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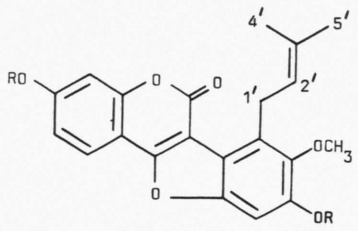
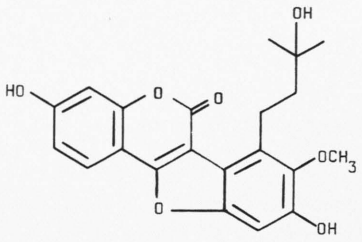
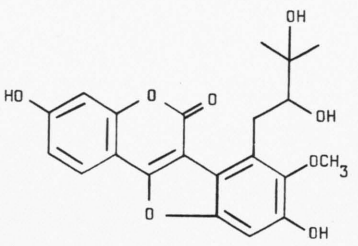


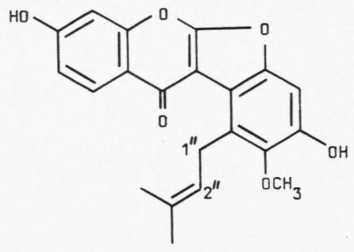
3 (PM-1) : R = H
6 : R = CH_3



4 (PM-4)



5 (PM-6)



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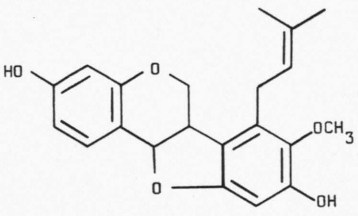

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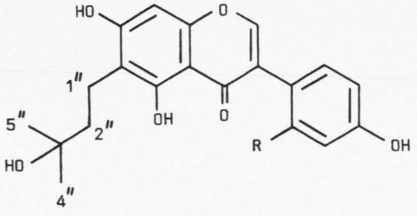

9: R = OH
10: R = H

to four aromatic protons (Table I). One of these was evident as a singlet (δ 7.08) with the others exhibiting *ortho* (δ 7.77 d, J = 8.8 Hz), *meta* (δ 6.82 d, J = 2.4 Hz) and *ortho-meta* (δ 6.91 dd, J = 8.8 & 2.4 Hz) coupling. The appearance of the sidechain 1′-$C\underline{H}_2$ signal at very low field (δ 4.15) when compared with kwakhurin (**1**, $C\underline{H}_2$ signal at approx. δ 3.20 [2]) was attributed to the strong deshielding effect of the carbonyl group as observed earlier in the ^{1}H NMR spectrum of coumaronochromone **7** ($C\underline{H}_2$ signal at δ 4.26 [2]). Such an effect can be explained if the sidechain of PM-1 is located at C-7. Assuming that PM-1 (in common with coumestrol **2** and other reported natural coumestans [5]) is oxygenated at C-9, the three *o*-, *m*- and *o*/*m*-related aromatic protons must reside on ring A which is known to be hydroxylated at C-3 [1]. The remaining aromatic proton can therefore be assigned to ring D along with the sidechain (C-7), an OH group, and the OCH_3 substituent (δ 3.82).

As *P. mirifica* roots contain the comparably substituted isoflavone kwakhurin (**1**) [2], it is logical to suggest that in addition to C-9 (OH), the D-ring of PM-1 is also oxygenated at C-8 (OCH_3). The provisional identification of PM-1 as 3,9-dihydroxy-8-methoxy-7-(3,3-dimethylallyl)-coumestan (**3**) was subsequently confirmed by chemical synthesis from kwakhurin (**1**). Treatment of **1** with $NaBH_4$ and HCl as previously described [2] afforded the pterocarpan **8** which was then oxidized with DDQ (see Experimental) to yield a product identical (UV, Si gel TLC) with PM-1 (mirificoumestan).

Table I. ^{1}H NMR data for mirificoumestan (**3**; = PM-1), mirificoumestan hydrate (**4**; = PM-4) and mirificoumestan glycol (**5**; = PM-6) from *Pueraria mirifica* roots[a,b].

Proton	Mirificoumestan (**3**)	Mirificoumestan hydrate (**4**)	Mirificoumestan glycol (**5**)
H-1	7.77 d (J = 8.8 Hz)	7.84 d (J = 8.3 Hz)	7.90 d (J = 8.6 Hz)
H-2	6.91 dd (J = 8.8 & 2.4 Hz)	6.98 dd (J = 8.3 & 2.0 Hz)	7.02 dd (J = 8.6 & 2.1 Hz)
H-4	6.82 d (J = 2.4 Hz)	6.90 d (J = 2.0 Hz)	6.94 d (J = 2.1 Hz)
H-10	7.08 s	7.09 s	7.16 s
$OC\underline{H}_3$	3.82 s (3H)	3.88 s (3H)	3.89 s (3H)
H-1′	4.15 br d (2H) (J = 6.8 Hz)	3.35–3.60 m (2H)	
H-2′	5.30 br t (J = *ca.* 6.8 Hz)	1.70–1.90 m (2H)	3.50–3.70 br s (3H)
H-4′5′[c]	1.63 d (3H) (J = 0.98 Hz); 1.84 d (3H) (J = 0.98 Hz)	1.29 s (6H)	1.30 s (3H); 1.32 s (3H)

[a] Data (δ values) are for spectra determined in acetone-d_6 (TMS reference) at 100 MHz. Except where indicated, the signals integrated for one proton.

[b] Comparative ^{1}H NMR data (acetone-d_6; 100 MHz) for authentic coumestrol (**2**; 3,9-dihydroxycoumestan) supplied by Dr. E. M. Bickoff (Western Regional Research Laboratory, US Dept. of Agriculture, Albany, California, USA) are as follows :– δ 7.87 (1H, d, J = 8.8 Hz, H-1), 7.80 (1H, d, J = 8.8 Hz, H-7), 7.11 (1H, dd, J = 8.8 & *ca.* 2.0 Hz, H-2), 6.99 (1H, dd, J = 8.4 & *ca.* 2.0 Hz, H-8) and 6.94 (2H, d, J = *ca.* 2.0 Hz, H-4 and H-10).

[c] In a mixture of acetone-d_6 and methanol-d_4 (100 MHz) the H-1′/H-2′ and H-4′/H-5′ signals of mirificoumestan glycol appeared respectively as a 3H multiplet or broad triplet-like resonance at δ 3.50–3.70, and as a 6H singlet at δ 1.30.

^{1}H NMR data obtained for coumestan PM-4 (M^+ 384) indicated that apart from sidechain differences this compound resembled mirificoumestan (Table I). Thus the broad doublet and broad triplet signals attributable respectively to the 1′-C$\underline{H}_2$ and 2′-C$\underline{H}$ of **3** were replaced in the ^{1}H NMR spectrum of PM-4 by two multiplets (each integrating for 2H) at δ 1.70–1.90 (centre at δ 1.81, 2′-C$\underline{H}_2$) and δ 3.35–3.60 (centre at δ 3.47, 1′-C$\underline{H}_2$), whilst the sidechain methyls resonated (δ 1.29) as a 6H singlet rather than two 3H doublets (J = 0.98 Hz). Allowing for a deshielding effect, particularly on the 1′-C$\underline{H}_2$, these signals are compatible with a sidechain having tertiary hydroxylation as in the isoflavone luteone hydrate (**9**, δ 2.78m = 1″-C$\underline{H}_2$; δ 1.71m = 2″-C$\underline{H}_2$; δ 1.26s = 4″- and 5″-C$\underline{H}_3$ [6]). Major MS fragments at $M^+ - H_2O$ (*m/z* 366, 70%) and $M^+ - 73$ (*m/z* 311, 100%) provided further support for a hydrated (3-hydroxy-3-methylbutyl) side attachment (*cf.* MS data for the hydrates of luteone **9** [6] and wighteone **10** [7] both of which afford comparable fragments). In an earlier study it was shown that kwakhurin (**1**) could be converted into the corresponding hydrate (**11**) by heating with 88% formic acid [2], and under similar conditions mirificoumestan (**3**) yielded a product indistinguishable (UV, MS, Si gel TLC) from PM-4. Coumestan PM-4 (mirificoumestan hydrate) must therefore have structure **4**.

Like that of **4**, the ^{1}H NMR spectrum of PM-6 (M^+ 400) suggested that, except for its side attachment, this compound was identical with mirificoumestan (Table I). Prominent MS fragments at *m/z* 382 ($M^+ - H_2O$), 364 ($M^+ - 2\times H_2O$), 341 ($M^+ - 59$), 312 ($M^+ - 88$) and 311 ($M^+ - 89$) were attributed to the 2,3-dihydroxy-3-methylbutyl residue found earlier in luteone glycol [6], the presence of such a sidechain being supported by the ^{1}H NMR spectrum (acetone-d_6) which contained signals at δ 1.30 and 1.32 (both s, each 3H, 4′- and 5′-C$\underline{H}_3$ on a carbinol carbon) with the three remaining aliphatic protons appearing as a broad singlet (δ 3.50–3.70) centred at δ 3.60 (1′-C$\underline{H}_2$ and 2′-C$\underline{H}$OH). In a mixture of acetone-d_6 and methanol-d_4, the same signals were evident as a 6H singlet (δ 1.30, 4′- and 5′-C$\underline{H}_3$) and as a 3H triplet-like resonance (δ 3.50–3.70 with centre at δ 3.60). As in both **3** and **4**, the deshielding influence of the carbonyl substituent accounts for the fact that the 1′-C$\underline{H}_2$ signals of PM-6 appear at lower field when compared, for example, with those of luteone glycol (1″-C$\underline{H}_2$: δ 2.62 and 3.25, two dd; 2″-C$\underline{H}$OH: δ 3.65dd) [6]. Confirmation of PM-6 as mirificoumestan glycol (**5**) was provided by treatment of **3** with OsO_4 (see Experimental) followed by hydrolysis of the resulting osmate ester. The reaction product, isolated in good yield, was identical (UV, MS, ^{1}H NMR and Si gel TLC) with a sample of PM-6 derived from *P. mirifica* roots.

Experimental

Air dried roots of *Pueraria mirifica* Airy Shaw & Suvatabandhu were obtained from Thailand as reported elsewhere [1]. A methanolic extract of the powdered root material was chromatographed (Si gel TLC, layer thickness 0.5 mm, F-254, Merck) in $CHCl_3$–MeOH (20:1) to afford mirificoumestan (**3**; PM-1), mirificoumestan hydrate (**4**; PM-4) and mirificoumestan glycol (**5**; PM-6) at approx. R_F 0.52, 0.27 and 0.18 respectively. Coumestrol (**2**), admixed with the isoflavone genistein, was located at R_F 0.33. After elution (MeOH), each coumestan was further purified as described in ref. [1].

*Coumestrol **2** (3,9-dihydroxycoumestan)*

UV maxima in MeOH, MeOH + NaOH, and MeOH + NaOAc as lit. [1, 4].

*Mirificoumestan **3** [PM-1; 3,9-dihydroxy-8-methoxy-7-(3,3-dimethylallyl)-coumestan]*

Fluorescence on Si gel thin-layer plates viewed under long wave-length (365 nm) UV light: deep blue becoming pinkish when fumed with NH_3 vapour. UV: λmax, nm: MeOH 208, 254, 294, 306, 347, 362sh; +NaOH 208, 268, 322, 388; +NaOAc 255, 280sh, 312, 366, 382sh (addition of boric acid regenerated the MeOH spectrum). EI-MS (rel. int.): $[M]^+$ 366 (100), *m/z* 351 ($M^+ - CH_3$; 6), 335 (11), 333 (6), 322 (6), 321 (8), 311 ($M^+ - 55$; 49), 310 ($M^+ - 56$; 24), 309 (15), 308 (8), 297 (12), 296 (15), 268 (6), 265 (7), 253 (5), 239 (9), 168 (10), 137 (5). ^{1}H NMR data, see Table I. *Dimethyl ether* (**6**). A mixture of **3** (approx. 2 mg), K_2CO_3 (200 mg), dimethyl sulphate (100 μl) and acetone (7 ml) was refluxed for 2 h, and then diluted with H_2O (10 ml). After acidification to pH 3 with HCl, the solution was extracted with EtOAc (3×30 ml). Si gel TLC of the EtOAc extract in benzene–EtOAc (5:1) afforded **6** (1.5 mg) at R_F 0.77. UV: λmax, nm: MeOH 214, 246sh, 252, 294sh, 303, 330sh, 343, 354sh. EI-MS (rel. int.):

$[M]^+$ 394 (100), *m/z* 379 ($M^+ - 15$; 9), 363 (7), 349 (6), 348 (7), 340 (17), 339 ($M^+ - 55$; 82), 337 (9), 325 (23), 324 (90), 321 (6), 309 (10), 281 (10), 279 (7), 253 (5), 182 (11), 169 (5), 162 (6). ^{1}H NMR (100 MHz, acetone-d_6, TMS reference): δ 1.64, 1.83 (both s, each 3H, 4′- and 5′-C$\underline{H}_3$), 3.80s (3H, 8-OC$\underline{H}_3$), 3.98s (6H, 3- and 9-OC$\underline{H}_3$), 4.14br d (2H, J = 6.8 Hz, 1′-C$\underline{H}_2$), 5.28br t (1H, J = 6.8 Hz, 2′-C$\underline{H}$), 7.07d/dd (2H, J = 9.2 & 2.2 Hz, H-2 and H-4), 7.32s (1H, H-10), 7.93d (1H, J = 9.2 Hz, H-1).

Mirificoumestan hydrate ***4*** *[PM-4; 3,9-dihydroxy-8-methoxy-7-(3-hydroxy-3-methylbutyl)-coumestan]*

Long wave-length UV fluorescence as given for **3**. UV: λmax, nm: MeOH 212, 254, 293sh, 305, 347, 362sh; +NaOH 210, 270, 320, 390; +NaOAc 255, 282sh, 313, 366, 383sh (addition of boric acid regenerated the MeOH spectrum). EI-MS (rel. int.): $[M]^+$ 384 (56), *m/z* 366 ($M^+ - H_2O$; 70), 326 (51), 325 (15), 311 ($M^+ - 73$; 100), 310 (82), 309 (28), 308 (14), 297 (28), 296 (43), 268 (19), 267 (22), 265 (16), 239 (19), 155 (15), 130 (41), 119 (25). ^{1}H NMR data, see Table I.

Mirificoumestan glycol ***5*** *[PM-6; 3,9-dihydroxy-8-methoxy-7-(2,3-dihydroxy-3-methylbutyl)coumestan]*

Long wave-length UV fluorescence as given for **3**. UV: λmax, nm: MeOH 212, 254, 293sh, 306, 347, 362sh; + NaOH 208, 268, 306sh, 323, 393; + NaOAc 256, 282sh, 305sh, 313, 368, 383sh (addition of boric acid regenerated the MeOH spectrum). EI-MS (rel. int.): $[M]^+$ 400 (7), *m/z* 383 (8), 382 ($M^+ - H_2O$; 30), 364 ($M^+ - 2 \times H_2O$; 6), 355 (8), 354 (19), 343 (8), 342 (41), 341 ($M^+ - 59$; 89), 333 (14), 324 (14), 313 (21), 312 ($M^+ - 88$; 100), 311 ($M^+ - 89$; 38), 309 (11), 298 (17), 297 (51), 296 (18), 283 (21), 281 (16), 268 (8), 255 (10), 239 (8), 84 (11), 71 (13), 69 (10), 66 (12), 59 (8). ^{1}H NMR data, see Table I.

*Conversion of kwakhurin (**1**) to mirificoumestan (**3**)*

The pterocarpan derivative **8** was first prepared by treatment of kwakhurin with $NaBH_4$ as reported earlier [2]. Purification of **8** was by Si gel TLC in $CHCl_3$–MeOH (20:1; R_F 0.58) followed by elution and further TLC in *n*-hexane–Et_2O–glacial HOAc–MeOH (75:25:3:8; R_F 0.22). Pterocarpan **8** (5 mg) in benzene (0.5 ml) was added dropwise over a 20 min period to a solution of 2,3-dichloro-5,6-dicyano-*p*-benzoquinone (DDQ; 10 mg) in benzene (0.5 ml) at room temp. (approx. 18 °C). After standing for a further 90 min, the reaction mixture was chromatographed (Si gel TLC) in $CHCl_3$–acetone–EtOAc–MeOH (60:20:20:1) to afford impure **3** at R_F 0.66. Elution and additional Si gel TLC (*n*-pentane–Et_2O–glacial HOAc–MeOH, 50:16:2:1) gave the pure coumestan (approx. 0.3 mg; R_F 0.27). Mirificoumestan (**3**) prepared from kwakhurin *via* pterocarpan **8** proved to be identical (UV, Si gel TLC) with the *Pueraria*-derived compound.

*Conversion of mirificoumestan (**3**) to mirificoumestan hydrate (**4**)*

A solution of mirificoumestan (2.5 mg) in EtOAc (0.5 ml) and benzene (0.5 ml) was stirred for 2 h at 50–60 °C with 88% formic acid (0.5 ml) in a stoppered tube. The mixture was then diluted with EtOAc (5 ml) and MeOH (5 ml) and reduced to dryness *in vacuo* (40 °C). Si gel TLC of the residue in $CHCl_3$–MeOH (20:1) gave mirificoumestan hydrate (**4**; approx. 0.5 mg; R_F 0.20) together with unchanged starting material (R_F 0.45). Synthetic **4** was indistinguishable from the *Pueraria* root compound by UV, MS and Si gel TLC.

*Conversion of mirificoumestan (**3**) to mirificoumestan glycol (**5**)*

OsO_4 (2.3 mg) in CH_2Cl_2 (0.25 ml) was slowly added to a solution of mirificoumestan (**3**; 3 mg) in CH_2Cl_2 (0.5 ml) and pyridine (20 µl). The mixture was allowed to stand for 20 h at room temp. before being poured into a solution of Na_2SO_3 (100 mg in 50% aqueous EtOH, 4 ml). After acidification (pH 2; 6N HCl), the solution was shaken (×3) with EtOAc. The combined EtOAc extracts were washed successively with 5% aqueous $NaHCO_3$ and brine, dried over Na_2SO_4, and concentrated to near dryness under reduced pressure (40 °C). Si gel TLC of the residue in benzene–MeOH (4:1) gave unchanged starting material (R_F 0.59) and mirificoumestan glycol (1.8 mg; R_F 0.47) identical (UV, MS, ^{1}H NMR, Si gel TLC) with the *Pueraria* compound.

Acknowledgements

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