

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.

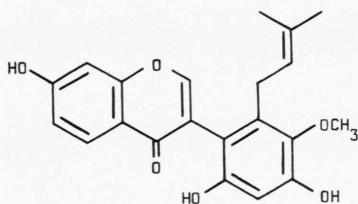
Introduction

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

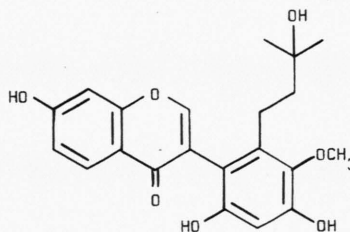
P. mirifica roots [2] suggested that PM-1 might possibly be the corresponding coumestan analogue (**3**). In this paper we present chemical and spectroscopic evidence which confirms structure **3** for PM-1, and which additionally allows coumestans PM-4 and PM-6 to be formulated as **4** and **5**, respectively. 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



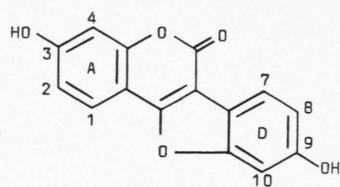
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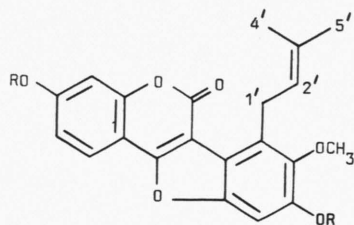
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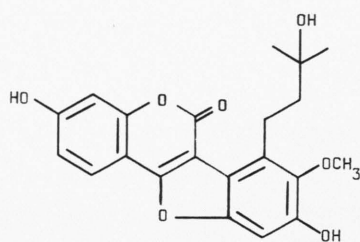
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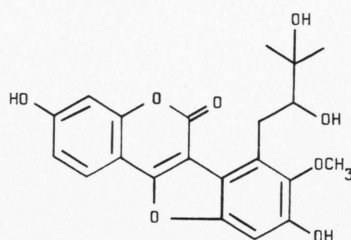
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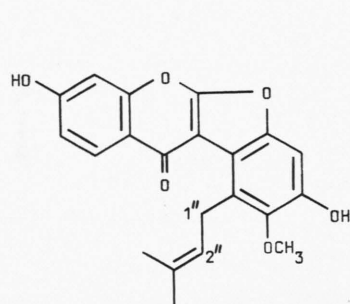
3 (PM-1) : R = H

6 : R = CH₃

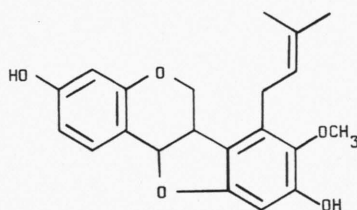
4 (PM-4)



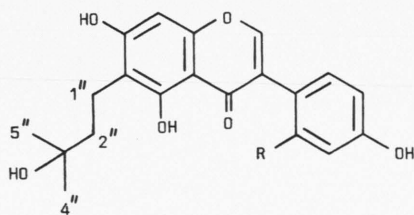
5 (PM-6)



7



8



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 $1'\text{-CH}_2$ signal at very low field (δ 4.15) when compared with kwakhurin (**1**, CH_2 signal at approx. δ 3.20 [2]) was attributed to the strong deshielding effect of the carbonyl group as observed earlier in the ^1H NMR spectrum of coumaronochromone **7** (CH_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. ^1H 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
OCH_3	3.82 s (3H)	3.88 s (3H)	3.89 s (3H)
H-1' } H-2' } c	4.15 br d (2H) (J = 6.8 Hz)	3.35–3.60 m (2H)	3.50–3.70 br s (3H)
	5.30 br t (J = ca. 6.8 Hz)	1.70–1.90 m (2H)	
H-4'5' ^c	$\left\{ \begin{array}{l} 1.63 \text{ d (3H)} \\ (J = 0.98 \text{ Hz}) \\ 1.84 \text{ d (3H)} \\ (J = 0.98 \text{ Hz}) \end{array} \right.$	1.29 s (6H)	$\left\{ \begin{array}{l} 1.30 \text{ s (3H)} \\ 1.32 \text{ s (3H)} \end{array} \right.$

^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 ^1H 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.

¹H 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'-CH₂ and 2'-CH of **3** were replaced in the ¹H NMR spectrum of PM-4 by two multiplets (each integrating for 2H) at δ 1.70–1.90 (centre at δ 1.81, 2'-CH₂) and δ 3.35–3.60 (centre at δ 3.47, 1'-CH₂), 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'-CH₂, these signals are compatible with a sidechain having tertiary hydroxylation as in the isoflavone luteone hydrate (**9**, δ 2.78m = 1''-CH₂; δ 1.71m = 2''-CH₂; δ 1.26s = 4''- and 5''-CH₃ [6]). Major MS fragments at *M*⁺ – H₂O (*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 wightone **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 ¹H 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*⁺ – H₂O), 364 (*M*⁺ – 2 × H₂O), 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 ¹H NMR spectrum (acetone-d₆) which contained signals at δ 1.30 and 1.32 (both s, each 3H, 4'- and 5'-CH₃ 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'-CH₂ and 2'-CHOH). In a mixture of acetone-d₆ and methanol-d₄, the same signals were evident as a 6H singlet (δ 1.30, 4'- and 5'-CH₃) 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'-CH₂ signals of PM-6 appear at lower field when compared, for example, with those of luteone glycol (1''-CH₂; δ 2.62 and 3.25, two dd; 2''-CHOH: δ 3.65dd)

[6]. Confirmation of PM-6 as mirificoumestan glycol (**5**) was provided by treatment of **3** with OsO₄ (see Experimental) followed by hydrolysis of the resulting osmate ester. The reaction product, isolated in good yield, was identical (UV, MS, ¹H 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 CHCl₃–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 NH₃ vapour. UV: λ_{max}, 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*⁺ – CH₃; 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). ¹H NMR data, see Table I. Dimethyl ether (**6**). A mixture of **3** (approx. 2 mg), K₂CO₃ (200 mg), dimethyl sulphate (100 μl) and acetone (7 ml) was refluxed for 2 h, and then diluted with H₂O (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: λ_{max}, 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). ¹H NMR (100 MHz, acetone-d₆, TMS reference): δ 1.64, 1.83 (both s, each 3H, 4'- and 5'-CH₃), 3.80s (3H, 8-OCH₃), 3.98s (6H, 3- and 9-OCH₃), 4.14br d (2H, *J* = 6.8 Hz, 1'-CH₂), 5.28br t (1H, *J* = 6.8 Hz, 2'-CH), 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: λ_{max}, 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⁺ - H₂O; 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). ¹H 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: λ_{max}, 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⁺ - H₂O; 30), 364 (M⁺ - 2 × H₂O; 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). ¹H NMR data, see Table I.

Conversion of kwakhurin (1) to mirificoumestan (3)

The pterocarpan derivative **8** was first prepared by treatment of kwakhurin with NaBH₄ as reported earlier [2]. Purification of **8** was by Si gel TLC in CHCl₃-MeOH (20:1; *R_F* 0.58) followed by elution and further TLC in *n*-hexane-Et₂O-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 stand-

ing for a further 90 min, the reaction mixture was chromatographed (Si gel TLC) in CHCl₃-acetone-EtOAc-MeOH (60:20:20:1) to afford impure **3** at *R_F* 0.66. Elution and additional Si gel TLC (*n*-pentane-Et₂O-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 CHCl₃-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)

OsO₄ (2.3 mg) in CH₂Cl₂ (0.25 ml) was slowly added to a solution of mirificoumestan (**3**; 3 mg) in CH₂Cl₂ (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 Na₂SO₃ (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 NaHCO₃ and brine, dried over Na₂SO₄, 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, ¹H NMR, Si gel TLC) with the *Pueraria* compound.

Acknowledgements

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