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# PLANT ANTIMUTAGENIC AGENTS, 5. ISOLATION AND STRUCTURE OF TWO NEW ISOFLAVONES, FREMONTIN AND FREMONTONE FROM *PSOROTHAMNUS FREMONTII*[1]

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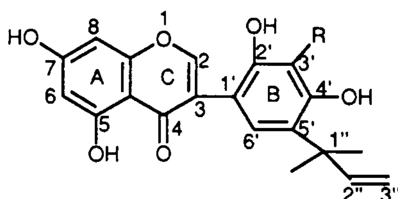
ABSTRACT.—Two new isoflavones, fremontin [**1**] and fremontone [**2**], were isolated from roots of *Psorothamnus fremontii* (Fabaceae), a desert plant. Compound **1** has the structure 5′-(α,α-dimethylallyl)-5,7,2′,4′-tetrahydroxyisoflavone; compound **2** is similar but also contains the 3′-(γ,γ-dimethylallyl) substituent. The α,α-dimethylallyl substituent is rarely observed, and the combination of the α,α- and γ,γ-dimethylallyl substituents is unprecedented. Both **1** and **2** were nontoxic toward *Salmonella typhimurium* and were both highly active in the inhibition of mutagenicity of ethyl methanesulfonate (EMS) at all concentrations tested. Compound **2** was more active than **1** in the inhibition of mutagenicity of 2-aminoanthracene (2AN) and acetylaminofluorene (AAF) toward *S. typhimurium*.

In connection with our screening of the plant kingdom for antimutagenic agents, we report the bioassay-directed isolation of two new isoflavones from the roots of *Psorothamnus fremontii* (Torr.) Barneby (Fabaceae). This plant is a desert shrub found in arid areas of Nevada and California and may have had medicinal uses by local Indians. These compounds, which we have named fremontin [**1**] and fremontone [**2**], respectively, have an unusual substitution pattern in ring B. The structure elucidation and antimutagenicity of these new compounds are described in this paper.




**1** R=H

**2** $R=\overset{1'''}{CH_2}-\overset{2'''}{CH}=\overset{3'''}{C}(Me)_2$

## EXPERIMENTAL

MUTAGENIC INHIBITION.—Procedures for determining the inhibition of the mutagenic activity of 2-aminoanthracene (2AN) toward *Salmonella typhimurium* (T-98) by crude and purified plant extracts have been described in previous papers (1,2). Similar procedures for determining the inhibition of the mutagenic activity of other mutagens such as acetylaminofluorene (AAF), benzo[*a*]pyrene (B[*a*]P), and ethyl methanesulfonate (EMS) by pure compounds have also been described (1,2). All of these mutagens with the exception of EMS require metabolic activation by the Ames S-9 preparation (3) as described previously (1). In the case of EMS the T-100 strain of *S. typhimurium* was used (4). Toxicity of extracts and pure compounds toward *S. typhimurium* was determined in the absence of mutagen but in the presence of histidine as described previously (1,2).

GENERAL ISOLATION AND CHARACTERIZATION PROCEDURES.—Melting points were determined on a Kofler hot stage microscope and are uncorrected. $^{1}H$- and $^{13}C$-nmr spectra were obtained with a Bruker WM250 spectrometer using TMS as internal standard. High resolution mass spectra (hrms) were obtained with an AEI MS-902 instrument. Uv spectra were obtained in MeOH with a Varian 2290-UV-

[1]For Part 4 in this series, see M.E. Wall, M.C. Wani, K. Gaetano, G. Manikumar, H. Taylor, and R. McGivney, *J. Nat. Prod.*, **51**, 1226 (1988).

VIS spectrometer and ir spectra with a Perkin-Elmer 467 Grating spectrometer. Standard chromatography was carried out on Si gel E. Merck 230–40 mesh, or Baker Flash chromatography Si gel, using in general $CH_2Cl_2$ as eluent with a gradient of 0.5–10.0% MeOH, collecting 15-ml fractions with an automatic fraction collector. For tlc determinations, precoated Si gel plates were utilized; normal phase, EM precoated Si gel 60, F254, usual solvent 10% MeOH in $CH_2Cl_2$; reversed-phase, Baker precoated Si gel $C_{18}$-F plates, usual developer 5–10% $H_2O$ in MeOH. Exposure of plates to $I_2$ vapor was used as a general detection agent; alternatively, spraying with phosphomolybdate reagent followed by heating was utilized. Preparative hplc was conducted utilizing a Waters Model Prep-3000 instrument. In most cases, a Dynamax reversed-phase $C_{18}$ column (21.5 mm × 25 cm) was utilized, with 10–50% $H_2O$ in MeOH as solvent; for highly nonpolar compounds a similar Dynamax 10μ Si column was used.

PLANT MATERIAL.—Roots of *P. fremontii* collected in Nevada in May 1980 were supplied through the auspices of the Drug Research and Development Branch, National Cancer Institute by the Medicinal Plant Resources Laboratory, Plant Genetics and Germplasm Institute, Agricultural Research Service, USDA, Beltsville, Maryland. An herbarium specimen documenting this collection is deposited in the Herbarium of the National Arboretum, Agricultural Research Service, USDA, Washington, D.C.

EXTRACTION AND ISOLATION.—Pulverized, dried roots of *P. fremontii* (300 g) were extracted with hot 95% EtOH, the extract concentrated in vacuo and partitioned between $CHCl_3$-$H_2O$ (1:1). The $CHCl_3$ fraction (8.0 g) exhibited 94% 2AN inhibition at 0.6 mg per plate and was nontoxic at 0.6 and 0.3 mg. An aliquot of the crude extract (4.0 g) was flash chromatographed on 50 g Baker Si gel. Elution of the column was carried out initially with 0.5% MeOH in $CH_2Cl_2$ (1725 ml), followed by 2% MeOH in $CH_2Cl_2$ (960 ml) and finally 4% MeOH in $CH_2Cl_2$ (4260 ml). Most of the total material chromatographed (93%) was recovered. The various fractions eluted were concentrated and analyzed by reversed-phase tlc. Fractions with similar tlc patterns were combined, weighed, and assayed for 2AN inhibition. Active fractions, combined weight, and % inhibition activities were 63–80, 1.6 g, (98); 96–115, 3.2 g, (62); 131–180, 0.5 g, (47); and 321–400, 0.4 g, (81). Fraction 96–115 (2.2 g) crystallized from $C_6H_{14}$/$CH_2Cl_2$ to yield 0.65 g of off-white crystals, mp 244–247°. This compound, designated fremontin [**1**], [5′-(α,α-dimethylallyl)-5,7,2′,4′-tetrahydroxyisoflavone] showed 2AN inhibition of 92% at 0.6 and 0.3 mg/plate and was shown to be toxic at 0.6 and nontoxic at 0.3 mg/plate in the toxicity test (1).

Attempts to crystallize fraction 63–80 were unsuccessful. The entire sample (1.64 g) was subjected to preparative hplc in eight equal portions on a Dynamax C18 column (21.5 mm × 25 cm) using MeOH-$H_2O$ (7:3) as an eluent; detector λ max 230 nm; flow rate 10 ml/min. Fractions were collected at 2 min intervals. A minor peak was collected between 14 and 16 min and a large, major peak was collected between 16 and 22 min. The major fraction, after removal of solvent, weighed 1.3 g. After crystallization from EtOAc/$C_6H_{14}$, 0.45 g of light-yellow crystals, mp 124–126°, was obtained. This compound was designated fremontone [**2**] {3′-(γ,γ-dimethylallyl)-5′-(α,α-dimethylallyl)-5,7,2′,4′-tetrahydroxyisoflavone}. Fremontone was nontoxic at concentrations of 0.6 and 0.3 mg/plate. The results of the assays of **1** and **2** for the inhibition of 2AN, AAF, B[*a*]P and 2NF or EMS are shown in Table 1.

TABLE 1. Percentage Inhibition of Various Mutagens by Fremontin [**1**] and Fremontone [**2**].

| Compound | Mutagen[a] and Dose (μg/plate) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2AN | | | | AAF | | | | EMS | | | |
| | 300 | 150 | 75 | 37.5 | 300 | 150 | 75 | 37.5 | 300 | 150 | 75 | 37.5 |
| **1** . . . . . . . . | 93 | 24 | 0 | 65 | 0 | 0 | 0 | 0 | 87 | 81 | 90 | 58 |
| **2** . . . . . . . . | 93 | 17 | 78 | 89 | 70 | 27 | 3 | 0 | 94 | 86 | 81 | 63 |

[a]2AN = 2-aminoanthracene; AAF = acetylaminofluorene; EMS = ethyl methanesulfonate.

FREMONTIN [**1**].—Mp 244–247°; λ max (MeOH) 256 (log ε 4.36), 292 (3.97), λ max (MeOH + NaOH) 270 (4.49), 325 (4.13); ir ν max (KBr) 3300, 1650, 1610, 1570, 1500, 1440, 1360, 1275, 1045 $cm^{-1}$; hrms *m/z* (rel. int. %) $[M]^+$ 354.1107 ($C_{20}H_{18}O_6$ = 354.1103) (31), 339 (5), 285 (100), 153 (38); $^1H$ nmr ($Me_2CO$-$d_6$) δ 13.01 (1H, s, 5-OH), 9.59 (1H, s, 2′-OH), 7.68 (1H, s, 7-OH), 7.84 (1H, s, 4′-OH), 7.79 (1H, s, H-2), 7.01 (1H, s, H-6′), 6.54 (1H, s, H-3′), 6.38 (1H, d, *J* = 2 Hz, H-8), 6.25 (1H, d, *J* = 2 Hz, H-6), 6.0 (1H, dd, *J* = 10 Hz, H-2″), 4.7 (2H, dd, *J* = 10 and 18 Hz, H-3″), 1.32 (3H, s, 1″-Me), 1.28 (3H, s, 1″-Me); $^1H$-nmr (DMSO-$d_6$) δ 12.95 (1H, s, 5-OH), 8.96 (2H, broad s, 2 × OH), 7.95 (1H, s, H-2), 6.90 (1H, s, H-6′), 6.40 (1H, s, H-3′), 6.37 (1H, d, *J* = 2 Hz, H-8), 6.22 (1H, d, *J* = 2 Hz, H-6), 5.92 (1H, dd, *J* = 10 Hz, H-2″), 4.64 (2H, dd, *J* = 10 and 18 Hz, H-

3″), 1.27 (3H, s, 1″-Me), 1.21 (3H, s, 1″-Me); $^{13}$C-nmr (DMSO-$d_6$) δ 181.7 (C-4), 164.2 (C-7), 161.9 (C-5), 157.7 (C-9), 154.5 (C-2), 148.9 (C-2″), 144.5 (C-4′), 142.8 (C-2′), 138.3 (C-5′), 125.3 (C-1′), 120.4 (C-3′), 120.3 (C-3), 114.8 (C-6′), 108.9 (C-3″), 104.3 (C-10), 98.8 (C-6), 93.6 (C-8), 41.1 (C-1″), 29.4, 28.8 (2 × 1″-Me).

FREMONTIN [**1**] ACETATE.—Compound **1** (30 mg) was acetylated with $Ac_2O$ (1.0 ml) and pyridine (1.0 ml) by stirring at room temperature for 24 h. The product was purified by recrystallization from EtOAc to give a crystalline tetraacetate (22 mg): mp 195°, λ max (MeOH) 306 (log ε 3.77), 236 (4.40), 214 sh (4.52); ir ν max ($CHCl_3$) 1770, 1650, 1625, 1370, 1265, 1175 $cm^{-1}$; hrms *m/z* (rel. int. %) $[M]^+$ 522.1529 ($C_{28}H_{26}O_{10}$ = 522.1526) (30), 493 (100), 480 (30), 438 (37), 411 (77), 396 (48), 369 (67), 354 (17), 285 (50); $^1$H nmr (DMSO-$d_6$) δ 8.20 (1H, s, H-2), 7.47 (1H, d, *J* = 2 Hz, H-8), 7.36 (1H, s, H-6′), 7.07 (1H, d, *J* = 2 Hz, H-6), 6.94 (1H, s, H-3′), 5.90 (1H, dd, *J* = 10 Hz, H-2″), 4.67 (2H, dd, *J* = 10 and 17 Hz, H-3″), 2.29, 2.28, 2.27, 2.23 (12H, 4s, 4 × $COCH_3$), 1.30, 1.27 (6H, 2s, 2 × 1″-Me).

FREMONTONE [**2**].—Mp 124–126°; λ max (MeOH) 256 (log ε 4.35), 290 (3.96), 330 sh (3.61), λ max (MeOH + NaOH) 270 (4.48), 325 (4.11); ir ($CHCl_3$) ν max 3540, 3300, 2970, 2900, 1650, 1615, 1585, 1500, 1480, 1440, 1365, 1285, 1190, 830 $cm^{-1}$; hrms *m/z* (rel. int. %) $[M]^+$ 422.1729 ($C_{25}H_{26}O_6$ = 422.1729) (30), 353 (60), 270 (100), 153 (48); $^1$H-nmr (DMSO-$d_6$) δ 13.05 (1H, s, 5-OH), 10.84 (1H, s, 2′-OH), 9.33 (1H, s, 7-OH), 8.19 (1H, s, 4′-OH), 7.75 (1H, s, H-2), 6.88 (1H, s, H-6′), 6.38 (1H, d, *J* = 2 Hz, H-8), 6.23 (1H, d, *J* = 2 Hz, H-6), 5.93 (1H, dd, *J* = 10 Hz, H-2″), 4.91 (1H, t, *J* = 7 Hz, H-2‴), 4.54 (2H, dd, *J* = 10, 17 Hz, H-3″), 3.24–2.73 (2H, m, H-1‴), 1.51, 1.27, 1.23, 1.21 (12H, 4s, 1″ and 3‴-2 × Me); $^{13}$C nmr (DMSO-$d_6$) δ 182.06 (C-4), 163.8 (C-7), 161.7 (C-5), 157.3 (C-9), 154.8 (C-2), 148.7 (C-2″), 144.3 (C-4′), 140.9 (C-2′), 137.9 (C-5′), 129.0 (C-3′), 128.9 (C-6″), 123.3 (C-5″), 122.07 (C-1″), 120.3 (C-3), 111.5 (C-6′), 108.0 (C-3″), 104.0 (C-10), 98.4 (C-6), 93.1 (C-8), 40.9 (C-1″), 29.4, 29.3 (2 × 1″-Me), 26.4 (C-1‴), 25.2, 16.7 (2 × 3‴-Me).

FREMONTONE [**2**] ACETATE.—Compound **2** (30 mg) was acetylated with $Ac_2O$ (1.0 ml) and pyridine (1.0 ml) by stirring at room temperature for 24 h. The product was recrystallized from EtOAc to give 20.0 mg of powdery fremontone tetraacetate: mp 96–98°; λ max (MeOH) 307 nm (log ε 3.64), 239 (4.25); ir ν max ($CHCl_3$) 3000, 1770, 1645, 1620, 1425, 1365, 1265, 1230, 1180, 1115, 1045, 1015, 900 $cm^{-1}$; hrms *m/z* (rel. int. %) 590 $[M]^+$ ($C_{33}H_{34}O_{10}$) (3), 548 (6), 521 (13), 479 (10), 437 (12), 395 (10), 354 (45), 312 (100), 270 (60); $^1$H nmr ($CDCl_3$) δ 7.37 (1H, s, H-2), 7.25 (1H, s, H-6′), 7.22 (1H, d, *J* = 2 Hz, H-8), 6.84 (1H, d, *J* = 2 Hz, H-6), 5.94 (1H, dd, *J* = 10 Hz, H-2″), 4.81–4.65 (3H, m, H-2‴, H-3″), 3.25–2.75 (2H, m, H-1‴), 2.37, 2.34, 2.27, 2.25 (12H, 4s, 4 × Ac), 1.55, 1.35 (6H, 2s, 3‴-Me), 1.30 (6H, s, 2 × 1″-Me).

## RESULTS AND DISCUSSION

STRUCTURE OF FREMONTIN [**1**].—The ir spectrum of **1** showed a strong carbonyl band at 1650 $cm^{-1}$, and the uv λ max in MeOH was at 256 nm (log ε 4.36) shifting to 270 nm (4.49) after treatment with NaOH; the above data provisionally indicated that **1** was possibly an isoflavone with a 5,7-dihydroxy moiety in ring A. The hrms spectrum showed a molecular ion $[M]^+$ at *m/z* 354.1107 giving a molecular formula of $C_{20}H_{18}O_6$, consistent with a flavonoid with four hydroxyl groups; this was confirmed by acetylation yielding a tetraacetate with a molecular formula of $C_{28}H_{26}O_{10}$. A fragment found at *m/z* 153 is in accord with the ms spectrum of isoflavones with a 2′-hydroxy substituent and two hydroxyl groups in ring A (5). The ms spectrum also showed the presence of a dimethylallyl moiety, a strong ion at *m/z* 285 $[M - C_5H_9]^+$ being the base peak.

All remaining structural features were established by $^1$H- and $^{13}$C-nmr spectroscopy. The $^1$H-nmr spectrum in DMSO-$d_6$ (see Experimental) revealed the presence of two meta-coupled aromatic protons at δ 6.37 (*J* = 2.0 Hz) and 6.22 (*J* = 2.0 Hz) in accord with a 5,7-dihydroxy moiety in ring A. Three isolated aromatic singlets at δ 7.95, 6.90, and 6.40 were also observed. The signal at δ 7.95 was attributed to H-2 in ring C, confirming that **1** is an isoflavone. The $^1$H-nmr spectrum of **1** in $(CD_3)_2CO$ showed all aromatic hydroxyl protons as singlets at δ 13.01, 9.59, 7.86, and 7.84, of which the farthest downfield is due to the 5-OH which is strongly hydrogen-bonded to the 4-carbonyl group; the signal at δ 9.59 is due to the partially hydrogen-bonded 2′-

OH group; the remaining two signals are attributable to the proven 7-OH and probable 4′-OH.

The singlets at δ 6.90 and 6.40 were assigned to the H-6′ and H-3′ respectively. Characteristic olefinic methine and methylene shifts were observed at δ 5.92 and 4.64, respectively, along with two nonequivalent methyl singlets δ 1.27 and 1.23. This data in conjunction with the mass spectral results show that the $C_5H_9$ group at 5′ is the α,α-dimethylallyl moiety.

Assignments in ring B were confirmed by nOe experiments in $(CD_3)_2CO$. Irradiation of the methyl singlet at δ 1.2 changed the integral of the signal at δ 6.90 (H-6′) by 20%, suggesting the assignment of the α,α-dimethyllallyl group to 5′ position. Further confirmation of the assignments were made by $^{13}C$-nmr spectroscopy. The off-resonance spectrum showed 2 methyl, 1 methylene, 6 methine, and 11 quaternary carbons in agreement with the proposed structure. Assignments of the protonated carbons were made using a 2D-proton carbon correlation experiment (HETCOR). The results are shown in Table 2. The nonprotonated carbons were then assigned with a long range 2D proton-carbon correlation experiment (COLOC). The results (Table 3) confirm the structural assignments made for compound **1**.

TABLE 2. Proton-Carbon Correlation Nmr Data.

| Compound | Proton | $^1H$ Shift | $^{13}C$ Shift |
|---|---|---|---|
| Fremontin [**1**] . . . . . | H-2 | 7.95 | 154.5 |
| | H-8 | 6.36 | 93.6 |
| | H-6 | 6.25 | 98.8 |
| | H-3′ | 6.44 | 120.4 |
| | H-6′ | 6.93 | 114.8 |
| Fremontone [**2**] . . . . . | H-2 | 7.75 | 154.8 |
| | H-8 | 6.38 | 93.4 |
| | H-6 | 6.27 | 98.7 |
| | H-6′ | 6.88 | 111.8 |

STRUCTURE OF FREMONTONE [**2**].—Compound **2** was shown to be a 3′-prenyl (γ,γ-dimehylallyl) analogue of **1**. The ir and uv spectra of **2** were similar to that of **1** (see Experimental). Hrms showed that the molecular formula of **2** was $C_{25}H_{26}O_6$. The compound on acetylation formed a tetraacetate. The $^1H$-nmr spectrum of **2** was similar to that of **1** with the exception that the characteristic prenyl signals were present and the singlet at δ 6.40 (H-3′) was absent. Hence, **2** can be considered as 3′-prenylfremontin. Long range proton-carbon correlation values for **2** were almost identical with those found for **1** (Table 3).

Fremontin [**1**] and fremontone [**2**] are new compounds with a number of interesting features. All isoflavones in general have a very limited taxonomic distribution, being found usually in the Fabaceae and occasionally in a few other plant families (6). Although substitution of the prenyl group in various locations of rings A and B is common in both flavonoids and isoflavonoids, substitution of the α,α-dimethyllallyl group is rare and the combination of both substituents in ring B as in **2** is unprecedented to the best of our knowledge.

BIOLOGICAL ACTIVITY.—Compound **1** was somewhat more toxic than **2**, which was nontoxic at 0.6 mg/plate. Both **1** and **2** were nontoxic at lower concentrations, i.e., 0.3–0.075 mg/plate. Results for testing at various concentrations of **1** and **2** for activity in the inhibition of the mutagenic effects on *S. typhimurium* of 2AN, AAF, B[*a*]P, and EMS are shown in Table 1. In addition to inhibiting 2AN at concentrations as low

TABLE 3. Long Range Proton-Carbon Correlation Data for Fremontin [**1**].

| $^{13}C$ Shift | $^{1}H$ Correlation Shift | Correlation Proton | Coupling | Carbon Assignment |
|---|---|---|---|---|
| Ring A | | | | |
| 164.2 . . . . . . . . | 6.37 | H-8 | 2 bond φ | C-7 |
| | 6.22 | H-6 | 2 bond φ | |
| 161.9 . . . . . . . . | 12.97 | 5-OH | 2 bond N | C-5 |
| | 6.22 | H-6 | 2 bond φ | |
| 157.7 . . . . . . . . | 7.95 | H-2 | 3 bond φ | C-9 |
| | 6.37 | H-8 | 2 bond φ | |
| 104.3 . . . . . . . . | 12.97 | 5-OH | 3 bond N | C-10 |
| | 6.37 | H-8 | 3 bond φ | |
| | 6.22 | H-6 | 3 bond φ | |
| 98.9 . . . . . . . . | 12.97 | 5-OH | 3 bond N | C-6 |
| | 6.37 | H-8 | 3 bond φ | |
| 93.6 . . . . . . . . | 6.37 | H-8 | 1 bond φ | C-8 |
| | 6.22 | H-6 | 3 bond φ | |
| Ring C | | | | |
| 181.7 . . . . . . . . | 7.95 | H-2 | 3 bond φ | C-4 |
| 154.2 . . . . . . . . | No adjacent protons | | | C-2 |
| 120.2 . . . . . . . . | 6.90 | H-2′ | 3 bond φ | C-3 |
| Ring B | | | | |
| 144.5 . . . . . . . . | 6.90 | H-6′ | 3 bond φ | C-4′ |
| | 6.40 | H-3′ | 2 bond φ | |
| 142.8 . . . . . . . . | 6.90 | H-6′ | 3 bond φ | C-2′ |
| 138.3 . . . . . . . . | 6.40 | H-3′ | 3 bond φ | C-5′ |
| | 1.27 | 1″-$CH_3$ | 3 bond N | |
| 125.3 . . . . . . . . | 7.95 | H-2 | 3 bond φ | C-1′ |
| | 6.40 | H-3′ | 3 bond φ | |
| 120.4 . . . . . . . . | No adjacent protons | | | C-3′ |
| 114.8 . . . . . . . . | 6.90 | H-6′ | 1 bond φ | C-6′ |

as 37.5 μg/plate, compounds **1** and **2** were both strongly active in inhibition of the mutagenicity of EMS at low concentrations. There was some activity in the inhibition of AAF with higher concentrations, i.e., 300 μg/plate. The mechanisms of the mutagenic inhibition and SAR studies that would reflect the effects of the various dimethylallyl sidechains are beyond the scope of this study but would certainly be of interest. It should be noted that in contrast to 2AN, EMS does not require metabolic activation but reacts directly with the various DNA bases (7).

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