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ANTIMITOTIC AND CYTOTOXIC FLAVONOLS FROM *ZIERIDIUM PSEUDOBTUSIFOLIUM* AND *ACRONYCHIA PORTERI*[1]

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ABSTRACT.—Bioassay-guided fractionation of the extracts of *Zieridium pseudobtusifolium* and *Acronychia porteri* led to the isolation of 5,3′-dihydroxy-3,6,7,8,4′-pentamethoxyflavone [**1**], which showed activity against (KB) human nasopharyngeal carcinoma cells (IC_{50} 0.04 μg/ml) and inhibited tubulin assembly into microtubules (IC_{50} 12 μM). Two other known flavonols, digicitrin [**2**] and 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone [**5**], were also isolated together with three new ones, 3-*O*-demethyldigicitrin [**3**], 3,5,3′-trihydroxy-6,7,8,4′-tetramethoxyflavone [**4**], and 3,5-dihydroxy-6,7,8,3′,4′-pentamethoxyflavone [**6**]. All of these flavonols showed cytotoxic activity against KB cells.

During the course of a testing program devoted to the search for new antimitotic drugs, the CH_2Cl_2 extract of *Zieridium pseudobtusifolium* (Guillaum.) Guillaumin (Rutaceae) and the EtOH extract of *Acronychia porteri* Hook. f. (Rutaceae), which were collected in New Caledonia and Malaysia, respectively, revealed cytotoxic activity against (KB) human nasopharyngeal carcinoma cells. Bioassay-guided fractionation led to the isolation of flavonols **1**–**4** from *Zieridium pseudobtusifolium* and **1**, **5**, and **6** from *Acronychia porteri*. Compound **1**, 5,3′-dihydroxy-3,6,7,8,4′-pentamethoxyflavone, was the most active of those compounds against KB cells and also showed inhibition of tubulin assembly into microtubules. Flavonols **1**, **2**, and **5** are known compounds and were identified by direct comparison with literature data. Compound **1** has been isolated previously from *Calycadenia* sp. (1), *Guttierrezia microcephala* (2), *G. sarothrae* (3), and *Polanisia trachysperma* (4). Digicitrin [**2**] was obtained before from *Digitalis purpurea* (5), *Polygonum orientale* (6), and *Guttierrezia microcephala* (2). 5-Hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone [**5**], was isolated previously from *Polanisia trachysperma* (4). All flavonols were purified from the crude extracts using successive cc on Si gel and reversed-phase hplc.

In the case of compound **1**, the reported ^1H and ^{13}C-nmr data (2,4) did not allow exact assignment of the substitution pattern at positions 3′ and 4′ (3′-OH, 4′-OMe). The only significant difference between **1** and its isomer **7** (4′-OH, 3′-OMe) (7,8) lay in the C-2′ and C-5′ resonances. Thus, the signal of the carbon ortho- to the C-OH group (C-2′ at δ 115.0 in **1** and C-5′ at δ 115.8 in **7**) was shifted downfield about 4 ppm in comparison with the signal of the carbon ortho- to the C-OMe group (C-6′ at δ 110.9 in **1** and C-2′ at δ 111.7 in **7**) (7,9). However, unambiguous assignments of C-2′ and C-5′ could be deduced from a ^1H-^{13}C COSY nmr experiment, which

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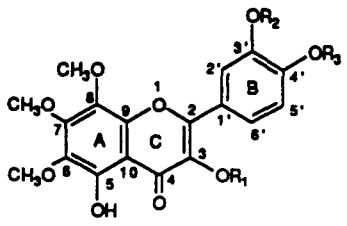
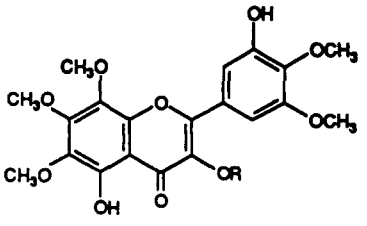
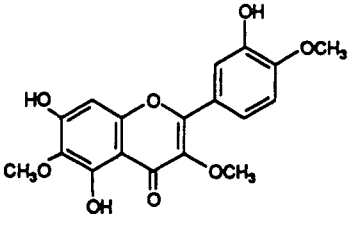


	R_1	R_2	R_3
1	CH_3	H	CH_3
4	H	H	CH_3
5	CH_3	CH_3	CH_3
6	H	CH_3	CH_3
7	CH_3	CH_3	H
8	CH_3	H	H



2 R=CH_3
3 R=H



9

was performed in the present study. Finally, the substitution pattern of ring B was confirmed by the uv spectrum and a NOESY nmr experiment. Precise uv data have not been reported previously for compound **1**, which exhibited a significant decrease in the intensity of the uv band I after addition of NaOMe indicating the absence of an OH group at C-4′ (7,10). The NOESY spectrum showed an intense cross-peak H-5′/4′-OMe (δ 3.99). Weak correlations were also observed between the OMe groups attached at C-3 and C-8 (δ 3.87 and δ 3.95) and the protons H-2′ and H-6′ (δ 7.78). These results were in accordance with molecular modeling studies, which indicated in the low-energy conformer a distance of 3.6 Å between 3-OMe and H-6′ and between 8-OMe and H-2′, whereas the distance between 4′-OMe and H-5′ was 2.58 Å.

Compound **3** revealed uv maxima at 259, 275, 339, and 378 nm, typical of a flavonoid. The hreims exhibited a molecular ion at *m/z* 420.1046 (calcd 420.1056) corresponding to the molecular formula $C_{20}H_{20}O_{10}$. Comparison of the spectral data with those of the known digicitrin [**2**] showed that the substitution pattern in ring B of the two compounds was identical. The C-2 and C-3 resonances, which were shifted upfield from those of **1** (Table 1) and **2** (5), indicated that **3** possessed an OH at C-3 instead of the OMe in **1** and thus was 3-*O*-demethyldigicitrin. This was confirmed by the HMBC nmr spectrum which disclosed correlations between 3-OH and C-2, C-3, and C-4.

Similarly, compound **4** showed a uv spectrum typical of a flavonoid (λ max 259, 288, 350, 378 nm). The hreims revealed a molecular ion at *m/z* 390.0958 (calcd 390.0951), which corresponded to the molecular formula $C_{19}H_{18}O_9$. This result, together with the lack of an OMe group in the nmr spectra, indicated that **4** possesses an OH group in place of one of the OMe groups of **1**. The OH was located at C-3 as shown by the similarity of the C-2 and C-3 resonances to those of

TABLE 1. ^{13}C-Nmr Data for Compounds **3**, **4**, and **6**.[a]

Carbon	Compound		
	3	**4**	**6**
2	145.4	145.9	146.1
3	136.3	136.0	136.1
4	175.9	175.9	176.8
5	147.9[b]	148.0	148.0
6	135.9[b]	136.0	135.8
7	153.4	153.4	153.3
8	133.2	133.5	133.3
9	145.1	145.3	145.1
10	105.3[b]	105.6	105.5
1′	126.4	124.3	123.6
2′	108.1	114.0	111.3
3′	152.2	146.0	149.1
4′	137.5	148.7	151.2
5′	149.4	110.8	110.7
6′	104.3	121.2	121.2
3-OMe	61.3[c]	61.4[c]	62.1[c]
7-OMe	62.1[c]	61.8[c]	62.2[c]
8-OMe	61.8[c]	61.9[c]	61.8[c]
3′-OMe			56.0
4′-OMe	61.2[c]	56.3	56.1
5′-OMe	56.0		

[a]The spectra were recorded at 62.5 MHz in $CDCl_3$.

[b]Assignments supported by the HMBC correlations 5-OH/C-5, 5-OH/C-6 and 5-OH/C-10.

[c]In each column, assignments may be reversed.

3 (Table 1). The substitution pattern of ring B was identical to that of **1** as deduced from the 1D nmr spectra. This structure was further confirmed by the ^1H-^{13}C COSY experiment and by the decrease in intensity of band I in the uv spectrum after addition of NaOMe. Thus, compound **4** is 3,5,3′-trihydroxy-6,7,8,4′-tetramethoxyflavone.

For compound **6**, the uv data also suggested a flavone skeleton. The hreims exhibited a molecular ion at *m/z* 404.1117 (calcd 404.1116) which corresponded to a molecular formula of $C_{20}H_{20}O_9$. Thus, **6** was an isomer of **1**. The two compounds differed from each other by the position of the OH group located at C-3 in **6** and at C-3′ in **1**. The pattern of the methine proton signals in the ^1H-nmr spectra of **1** and **6** was similar. Moreover, the C-2, C-3 resonances were almost identical to those of 3-*O*-demethyldigicitrin [**3**] and 3,5,3′-trihydroxy-6,7,8,4′-tetramethoxyflavone [**4**]. Finally, the C-2′ and C-5′ resonances at δ 111.3 and δ 110.7, respectively (Table 1), indicated that both carbons were ortho- to C-OMe groups. In the same way, C-2′ and C-5′ in the known flavonol **5** appeared at δ 111.8 and 110.9 (4). On the basis of these data, compound **6** is 3,5-dihydroxy-6,7,8,3′,4′-pentamethoxyflavone.

Compounds **1** and **6** exhibited significant in vitro cytotoxicity against KB cells (IC_{50}=0.04 and 0.1 μg/ml). The other flavonols **2**, **3**, **4**, and **5** were less active with IC_{50} values of 2, 2, 6, and 6 μg/ml respectively. These cytotoxic properties have not previously been found for compounds **1**, **2**, and **5**. However, cytotoxic activity has been reported for the closely related flavonols **7** and **8** (IC_{50}=0.20 and 0.44 μg/ml) (7,11). Compound **1** was inactive when evaluated in vivo against early-stage subcutaneous pancreatic ductal adenocarcinoma O3 in B6D2F1 female mice.

Compound **1** inhibited tubulin assembly into microtubules with an IC_{50} value of 12 μM. In the same experimental conditions (12), vinblastine displayed an IC_{50} value of 4 μM. Compound **1** is the second example of a flavonol interacting with tubulin, as data for such a tubulin inhibitory activity (IC_{50}=3 μM) have recently been published for centaureidin [**9**] (13). Compound **9** was also found to be cytotoxic (IC_{50}=0.27 μg/ml) (13).

Flavonols are well known in Rutaceae species. The fact that *Zieridium pseudobtusifolium* and *Acronychia porteri*, collected in New Caledonia and in Malaysia, respectively, contain the same flavonol **1**, which interacts with tubulin, is coincidental.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Mps (uncorrected) were determined on a micro hot-stage apparatus. Spectra were recorded as follows:

uv, Shimadzu UV-161 uv-visible spectrophotometer; ir, Nicolet 205 Ft-ir spectrometer; eims (70 eV), Kratos MS 50; nmr, Bruker AC 250 (^{1}H- and ^{13}C-nmr spectra), AM 400 (2D spectra). Cc was performed using Si gel Merck H60, prep. hplc on a Waters Delta prep 3000 Apparatus [Delta-pak C-18, (100 Å, 15 mm), 47×300, flow rate 50 ml/min, uv detection] and semi-prep. hplc on a Waters RCM Apparatus [Delta-pak C-18 (100 Å, 15 mm), 10×250, flow rate 10 ml/min, uv detection]. Molecular modeling studies were carried out on a Silicon Graphics 4D/35 Workstation using Macromodel with the included version of the MM3 force field. Experiments were performed *in vacuo*.

PLANT MATERIAL.—Leaves of *Zieridium pseudobtusifolium* (Guillaum.) Guillaumin were collected at Mont Mou, New Caledonia in June 1991, and leaves of *Acronychia porteri* Hook. f. in Mersing, Malaysia, in November 1991. Identifications were made by A. de Gouvello and F. Remy, respectively. Voucher specimens of *Z. pseudobtusifolium* (Prie-Cosson 341) and *A. porteri* (KL 4091) have been deposited at the Herbarium of ORSTOM, Nouméa, New Caledonia (*Z. pseudobtusifolium*) and at the Herbarium of the Department of Chemistry, University of Malaya, Kuala Lumpur, Malaysia (*A. porteri*).

EXTRACTION AND ISOLATION.—The dried ground leaves of *Z. pseudobtusifolium* (140 g) and *A. porteri* (200 g) were extracted with CH_2Cl_2 and EtOH, respectively. The extract of *Z. pseudobtusifolium* (14 g, yield 10%) underwent extensive cc on Si gel with CH_2Cl_2/MeOH and then cyclohexane/EtOAc. The fraction interacting with tubulin (cyclohexane-EtOAc, 70:30; 0.35 g) was further purified by prep. (MeOH-H_2O, 7:3) and then semi-prep. hplc (MeOH-H_2O-AcOH, 7:3; 2%) yielding **1** (40 mg) and **2** (10 mg). The more polar fraction from the cc (EtOAc, 0.10 g) was purified by semi-prep. hplc to give the flavonols **3** (40 mg) and **4** (10 mg).

The EtOH-soluble portion of *A. porteri* (12 g, yield 6%) was chromatographed on Si gel (CH_2Cl_2/MeOH). The fractions active in the KB test (CH_2Cl_2-MeOH, 9.5:0.5; 75% inhibition at 1 μg/ml; 0.7 g) were further purified by prep. hplc (MeOH-H_2O, 6:4 to 8:2) to give compounds **1** (22 mg), **6** (8 mg), and **5** (22 mg).

5,3′-Dihydroxy-3,6,7,8,4′-pentamethoxyflavone [**1**].—Yellow crystals, mp 170° (MeOH), [lit. (4) 169–170°]; uv λ max (MeOH) (rel. abs.) 258 (1.00), 277 (1.00) and 346 (0.89) nm; +NaOMe 277 (1.00) and 402 (0.34) nm; ^{1}H nmr ($CDCl_3$) δ 12.4 (1H, s, OH-5), 7.78 (2H, m, H-2′ and H-6′), 7.00 (1H, d, J=9 Hz, H-5′), 5.90 (1H, br s, OH-3), 3.87 (3H, s, OMe-3 or OMe-8), 3.95, 3.96 (2×3H, 2s, OMe-3 or OMe-8, OMe-7 or OMe-6), 3.99 (3H, s, OMe-4′), 4.11 (3H, s, OMe-7 or OMe-6); ^{13}C nmr ($CDCl_3$) δ 156.3 (C-2), 139.3 (C-3), 179.8 (C-4), 149.4 (C-5), 136.5 (C-6), 153.3 (C-7), 133.3 (C-8), 145.3 (C-9), 107.9 (C-10), 124.4 (C-1′), 115.0 (C-2′), 146.0 (C-3′), 149.5 (C-4′), 110.9 (C-5′), 122.0 (C-6′), 60.5, 61.5, 62.5, 62.1 (OMe-3, OMe-6, OMe-7, OMe-8), 56.5 (OMe-3′). Lit. (7) for compound **7**: ^{1}H nmr (Me_2CO-d_6) δ 12.56 (1H, s, OH-5), 7.84 (1H, d, J=2 Hz, H-2′), 7.80 (1H, dd, J=8.5 and 2 Hz, H-6′), 7.05 (1H, d, J=8.5 Hz, H-5′), 3.89 (3H, s), 3.92 (3H, s), 3.97 (3H, s), 3.98 (3H, s), 4.08 (3H, s) (5×OMe); ^{13}C nmr (DMSO-d_6) δ 155.9 (C-2), 137.7 (C-3), 178.5 (C-4), 148.1 (C-5), 135.4 (C-6), 152.3 (C-7), 132.4 (C-8), 144.3 (C-9), 106.7 (C-10), 120.6 (C-1′), 111.7 (C-2′), 147.5 (C-3′), 150.1 (C-4′), 115.8 (C-5′), 122.3 (C-6′), 59.6 (3-OMe), 60.5 (OMe-6), 61.4 (OMe-8), 55.5 (OMe-3′). ^{1}H-^{13}C COSY ($CDCl_3$) cross-peaks: H-5′ (δ 7.00)/C-5′ (δ 110.9), H-2′ (δ 7.75)/C-2′ (δ 115.0), H-6′ (δ 7.75)/C-6′ (δ 122.0).

3-O-Demethyldigicitrin [**3**].—Yellow gum. Uv λ max (MeOH) 259, 275, 339, and 378 nm; +NaOMe 263, 350 (sh) and 428 nm; +$AlCl_3$ 271, 380, 435 nm; +$AlCl_3$/HCl 269, 372, 436 nm; ir ν max ($CHCl_3$) 3580, 1650, 1640, and 1600 cm^{-1}; eims m/z 420 $[M]^+$ (100), 405 (90), 377 (23), 181 (8); ^{1}H nmr ($CDCl_3$) δ 10.25 (1H, s, OH-5), 7.55, 7.50 (2H each, 2s, H-2′ and H-6′), 6.72 (1H, br s, OH-3), 5.90 (1H, br s, OH-3′), 3.93, 3.95, 3.98, 4.00, 4.12 (each 3H, s, 5×OMe); ^{13}C nmr, see Table 1.

3,5,3′-Trihydroxy-6,7,8,4′-tetramethoxyflavone [**4**].—Yellow gum. Uv λ max (MeOH) (rel. abs.) 259 (1.00), 288 (sh), 350 (0.73) and 378 (0.80) nm; +NaOMe 262 (1.00) and 426 (0.60) nm; +$AlCl_3$ 270, 385, 436 nm; +$AlCl_3$/HCl 270, 385, 436 nm; ir ν max ($CHCl_3$) 3580, 1650, 1640, and 1600 cm^{-1}; eims m/z 390 $[M]^+$ (100), 375 (100), 347 (20), 151 (30); ^{1}H nmr ($CDCl_3$) δ 10.10 (1H, s, OH-5), 7.86 (1H, d, J=9 Hz, H-6′), 7.85 (1H, s, H-2′), 7.00 (1H, d, J=9 Hz, H-5′), 6.90 (1H, br s, OH-3), 5.75 (1H, br s, OH-3′), 3.95 (3H, s), 3.99 (6H, s), 4.12 (3H, s) (4×OMe); ^{13}C nmr, see Table 1. ^{1}H-^{13}C COSY ($CDCl_3$) cross-peaks: H-5′ (δ 7.00)/C-5′ (δ 110.8), H-2′ (δ 7.85)/C-2′ (δ 114.0), H-6′ (δ 7.86)/C-6′ (δ 121.2).

3,5-Dihydroxy-6,7,8,3′,4′-pentamethoxyflavone [**6**].—Yellow crystals, mp 145–147° (MeOH); uv λ max (MeOH) 259, 276 (sh), 349, 376 nm; +NaOMe 262, 368, 431 nm; +$AlCl_3$ 270, 380, 435 nm; +$AlCl_3$/HCl 268, 374, 439 nm; ir ν max ($CHCl_3$) 3690, 1650, 1630, and 1600 cm^{-1}; eims m/z 404 $[M]^+$ (100), 389 (95), 373 (20), 361 (20), 344 (20), 263 (30), 226 (81), 181 (82), 149 (63); ^{1}H nmr ($CDCl_3$) δ 10.10 (1H, s, OH-5), 7.93 (1H, dd, J=9 and 2 Hz, H-6′), 7.88 (1H, d, J=2 Hz, H-2′), 7.03 (1H, d, J=9 Hz, H-5′), 6.72 (1H, br s, OH-3), 3.96 (3H, s), 3.98 (3H, s), 3.99 (6H, s), 4.12 (3H, s) (5×OMe); ^{13}C nmr, see Table 1.

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