Cytotoxic Principles from *Ventilago leiocarpa*

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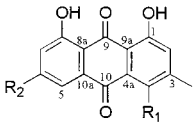
Three new anthraquinones, islandicin 4-methyl ether (**1**), 1,2,6-trihydroxy-7,8-dimethoxy-3-methylanthraquinone (**2**), and 2-hydroxyemodin 1-methyl ether (**3**) as well as two known triterpenoids [taraxerol (**4**), lupeol (**5**)], six anthraquinones [chrysophanol (**6**), islandicin (**8**), parietin (**9**), emodin (**10**), catenarin (**11**), skyrin (**15**)], a 2,3-dihydroflavonol [(+)-aromadendrin (**12**)], two benzisochromanquinones [ventiloquinone K (**13**) and ventiloquinone I (**14**)], and stigmasterol (**7**) were isolated from *Ventilago leiocarpa*. The cytotoxicity of these compounds to various tumor cell lines was evaluated, and compound **15** significantly suppressed growth of HeLa, Vero, K562, Raji, Wish, and Calu-1 tumor cell lines. With the exception of K562 cells, the proliferation of other tumor cell lines was inhibited by compounds **3** and **10**.

Ventilago leiocarpa Benth. belongs to the Rhamnaceae family, which is a scandent glabrous shrub growing throughout the thickets at low and medium altitudes in Taiwan.[1] It is used in Chinese folk medicine as an analgesic and for the treatment of rheumatism.[2] Previous chemical studies[3−7] of the genus *Ventilago* have shown the presence of a variety of anthraquinones, naphthoquinones, quinones, and benzisochromanquinones. In the course of our search for biologically active substances in nature, we found that the crude extract from the stems of *V. leiocarpa* possessed in vitro cytotoxicity to cancer cells. Since the bioactivity and chemical constituents of *V. leiocarpa* have not been studied, we investigated the chemical constituents of dried stems of this plant.

The ethanolic extract of the stems of *V. leiocarpa* was fractionated by solvent partition and separated by column chromatography to yield anthraquinones, benzisochromanquinones, 2,3-dihydroflavonol, and phytosterol. The structures of **4**−**15** have been established as taraxerol (**4**),[8] leupol (**5**),[9] chrysophanol (**6**),[10] stigmasterol (**7**), islandicin (**8**),[11] parietin (**9**),[12] emodin (**10**),[13] catenarin (**11**), (+)-aromadendrin (**12**),[14,15] ventiloquinone K (**13**)[3], ventiloquinone I (**14**),[3] and skyrin (**15**),[16] on the basis of spectral analyses and by comparison with reported data.



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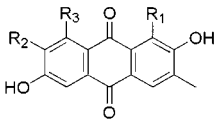

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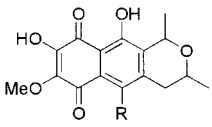

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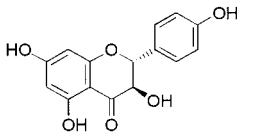

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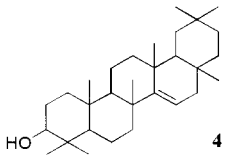

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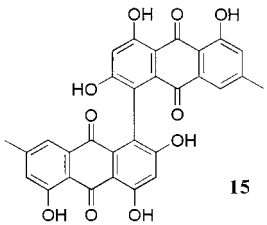

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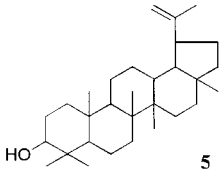

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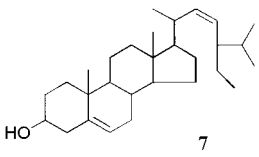

7

Compound **1** has the molecular formula $C_{16}H_{12}O_5$, which was confirmed by HREIMS. The IR spectrum shows absorption bands 1667 and 1620 cm^{-1}, and its UV spectrum (λ_{max} 441 nm) suggested a 1,8-dihydroxyanthraquinone structure. The ^{1}H NMR spectrum shows the presence of an aromatic methyl (δ 2.41), two *peri*-hydroxyl protons (δ 12.06, 12.51), four aromatic protons (one singlet at δ 7.18, and an ABC system at δ 7.27, 7.68, 7.81), and a methoxyl group at δ 3.89. Acetylation of **1** with acetic anhydride/pyridine afforded a diacetate (**1a**). The ^{1}H NMR data of **1** showed similarities to those of islandicin,[6] except for the presence of a methoxyl group. In the HMBC spectrum, the C-4 signal at δ 153.8 was correlated with resonances at δ 3.89 (OCH_3-4), 7.18 (H-2), and 2.41 (CH_3-3), and the signal at δ 126.9 (C-2) correlated with the 1-OH signal (δ 12.51) and CH_3-3 (δ 2.41), indicating that C-4 was methoxylated. Therefore, compound **1** was identified as the new compound, islandicin 4-methyl ether.

Compound **2** has the molecular formula $C_{17}H_{14}O_7$, which was confirmed by HREIMS. The ^{1}H NMR spectrum of **2** shows signals for an aromatic methyl (δ 2.35), two methoxyl groups (δ 3.98, 3.99), two isolated aromatic protons (δ 7.55 and 7.57), and three hydroxyl protons (one *peri*-OH at δ 13.32, and two free-OH at δ 8.92, 9.63), which were confirmed by formation of a triacetate derivative (**2a**). The HMBC spectrum revealed the three-bond coupling of C-9a (δ 116.3) to H-4 (δ 7.55) and OH-1 (δ 13.32), C-2 (δ 150.8) to CH_3-3 (δ 2.35) and H-4 (δ 7.55), C-10 (δ 181.0) to H-4 (δ 7.55) and H-5 (δ 7.57), and C-7 (δ 147.3) to OCH_3-7 (δ 3.98) and H-5 (δ 7.57), indicating compound **2** possessed a 1,2-dihydroxy-3-methylanthraquinone component. In the NOESY spectrum, no correlations were observed between

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Table 1. Inhibitory Activity (%) of Compounds **3**, **10**, and **15** on Various Tumor Cells Growth[a]

		cell line					
compound	dose (μM)	Calu-1	HeLa	K562	Raji	Vero	Wish
3	100	93.8 ± 7.8	100 ± 8.8	34.1 ± 7.8	100 ± 6.8	85.9 ± 2.5	79.3 ± 5.6
	50	91.7 ± 5.4	49.9 ± 7.2	N.D.	100 ± 7.2	71.1 ± 3.1	56.0 ± 3.8
	25	71.0 ± 3.3	21.4 ± 2.9	N.D.	72.4 ± 6.3.	42.6 ± 2.8	−0.30 ± 3.2
	12.5	60.5 ± 6.8	1.3 ± 4.2	N.D.	71.7 ± 5.0	13.8 ± 3.2	−5.5 ± 2.9
	6.25	−4.8 ± 3.3	−22.3 ± 3.6	N.D.	63.7 ± 4.9	9.9 ± 4.0	−9.5 ± 5.1
IC_{50} (μM)		21.3 ± 5.0	50.0 ± 6.2	>100	<6.25	32.5 ± 4.5	55.0 ± 6.4
10	100	100 ± 7.5	100 ± 5.3	29.5 ± 6.5	100 ± 6.5	69.1 ± 2.5	80.7 ± 4.5
	50	100 ± 6.8	100 ± 6.1	N.D.	47.2 ± 4.9	64.4 ± 4.1	63.3 ± 6.3
	25	100 ± 5.3	78.3 ± 3.8	N.D.	45.8 ± 6.1.	26.7 ± 3.8	61.9 ± 4.7
	12.5	60.6 ± 4.8	41.9 ± 3.5	N.D.	41.2 ± 3.7	13.7 ± 2.2	22.0 ± 5.5
	6.25	49.4 ± 3.3	24.7 ± 2.0	N.D.	0.30 ± 7.2	3.3 ± 1.5	8.7 ± 2.6
IC_{50} (μM)		6.25 ± 2.9	15.6 ± 4.2	>100	43.8 ± 7.3	40.0 ± 1.7	28.8 ± 1.9
15	50	100 ± 7.0	100 ± 5.6	100 ± 8.5	100 ± 6.7	100 ± 6.5	100 ± 6.8
	25	100 ± 5.3	100 ± 7.2	50.6 ± 4.7	82.1 ± 4.9	70.5 ± 7.3	60.0 ± 7.2
	12.5	24.9 ± 4.7	55.0 ± 1.2	33.5 ± 3.0	48.1 ± 8.1	34.8 ± 3.9	37.8 ± 4.5
	6.25	20.6 ± 3.0	27.0 ± 3.2	22.2 ± 5.5	36.8 ± 3.2	23.1 ± 4.3	23.4 ± 6.3
	3.125	15.5 ± 2.1	5.8 ± 1.8	29.7 ± 6.3	33.8 ± 5.0	19.9 ± 2.9	24.7 ± 2.9
IC_{50} (μM)		14.3 ± 2.5	11.3 ± 3.5	27.3 ± 5.0	12.3 ± 4.1	18.3 ± 2.6	21.3 ± 3.2

[a] The method is described in the Experimental Section. Each datum represents the mean of three independent experiments. N.D.: not determined.

the two methoxyl signals and the two aromatic protons, indicating that the methoxyl groups are located at C-7 and C-8. Therefore, compound **2** was characterized as 1,2,6-trihydroxy-7,8-dimethoxy-3-methylanthraquinone.

Compound **3** has the molecular formula $C_{16}H_{12}O_6$, confirmed by HREIMS. The ^{1}H NMR spectrum of **3** shows signals for a methyl group (δ 2.29), a methoxyl group (δ 3.80), and a *peri*-hydroxy group (δ 13.12), an isolated aromatic proton (δ 7.79), and two meta-coupled protons (δ 6.56 and 7.06). On acetylation, **3** also yielded a triacetate. In the HMBC spectrum, the C-10 signal at δ 181.6 was correlated with resonances at δ 7.79 (H-4) and 7.06 (H-5), the signal at δ 156.5 (C-2) correlated with the CH_3-3 (δ 2.29) and H-4 (δ 7.79) signals, and the resonance at δ 147.9 (C-1) correlated with the OCH_3-1 (δ 3.80) signal. These data suggested the presence of three hydroxyl groups located at C-2, C-6, and C-8 and the methoxyl group located at C-1. Therefore, compound **3** was identified as 2-hydroxyemodin 1-methyl ether.

All of the isolated compounds were tested against a panel of cancer cell lines according to established protocols.[17] As shown in Table 1, compound **15** displayed cytotoxicity against all six tumor cell lines, while compounds **3** and **10** significantly suppressed the growth of Vero, Wish, Calu-1, Raji, and HeLa tumor cells. All other test compounds had IC_{50} values higher than 100 μM against all cell lines. These results suggest that the 1,3,8-trihydroxy for the anthraquinone plays a significant role in the cytotoxic activity.

Experimental Section

General Experimental Procedures. Melting points were determined with a Yanagimoto micro-melting point apparatus and are uncorrected. IR spectra were obtained as KBr pellets on a Nicolet Avatar 320 IR spectrometer. UV spectra were obtained on a Hitachi U-3200 spectrophotometer in MeOH. ^{1}H, ^{13}C, and 2D NMR spectra were measured with a Varian Inova-500 spectrometer with deuterated solvent as internal standard. EIMS, HREIMS, and APCIMS were recorded on a Finnigan MAT 95S and Finnigan LCQ spectrometer, respectively.

Plant Material. The stems of *Ventilago leiocarpa* were collected at Shihting, Taipei, Taiwan, in October 1999. A voucher specimen (No. 195898) has been deposited in the herbarium of the Department of Botany of the National Taiwan University.

Extraction and Isolation. The stems of *V. leiocarpa* (8.5 kg) were extracted with 95% EtOH (50 L × 3). The ethanolic extracts were combined and concentrated under vacuum to a volume of 1.5 L. The concentrated ethanolic extract was then partitioned successively between H_2O and EtOAc, followed by *n*-BuOH (each 1 L × 3). The EtOAc extract (110 g) was subjected to silica gel column chromatography with a gradient of EtOAc in *n*-hexane, and 11 fractions were collected. Fraction 2 (2.6 g) was rechromatographed over silica gel (*n*-hexane) and further purified by preparative TLC (25% benzene/*n*-hexane) to give compounds **1**, **4**, **5**, and **6**. Fractions 4 and 5 were combined (1.7 g) and purified by Sephadex LH-20 (EtOAc) and silica gel column chromatography (12−26 μm, performed at pressure ~10 bar, 2% EtOAc/*n*-hexane) to give **7**, **8**, and **9**. A precipitate from fraction 7 was recrystallized with EtOAc/*n*-hexane to give **10**. The filtrate from fraction 7 was purified on a silica gel column (10% EtOAc/*n*-hexane) and Sephadex LH-20 (MeOH) to yield **10** and **11**. Fraction 10 (6.9 g) gave **2**, **12**, **13**, **14**, and **15** after repeated Sephadex LH-20 (MeOH, acetone) and silica gel column chromatography. Fraction 8 was further purified by silica gel column chromatography (40−63 μm, performed at pressure ~10 bar, 10% EtOAc/benzene) to yield **3**.

Islandicin 4-methyl ether (1): red needles (EtOAc/*n*-hexane); mp184−186 °C; UV (MeOH) λ_{max} (log ϵ) 441 (3.85), 287 (3.80), 251 (4.12), 227 (4.42) nm; IR (KBr) ν_{max} 3400 (OH), 1667, 1620 (C═O) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 2.41 (3H, s, CH_3-3), 3.89, (3H, s, OCH_3-4), 7.18 (1H, s, H-2), 7.27 (1H, d, J = 7.5 Hz, H-7), 7.68 (1H, t, J = 7.5 Hz, H-6), 7.81 (1H, d, J = 7.5 Hz, H-5), 12.06 (OH-8), 12.51 (OH-1); ^{13}C NMR ($CDCl_3$) δ 17.2 (3-CH_3), 61.0 (4-OCH_3), 114.0 (C-9a), 115.5 (C-8a), 120.0 (C-5), 123.6 (C-7, 4a), 126.9 (C-2), 134.8 (C-10a), 137.1 (C-6), 146.6 (C-3), 153.8 (C-4), 159.6 (C-1), 162.0 (C-8), 181.4 (C-10), 192.3 (C-9); HMBC correlations, C-1 → OH-1, H-2; C-2 → OH-1, 3-CH_3; C-4 → 4-OCH_3, H-2, 3-CH_3;C-7 → OH-8, H-5; C-8 → OH-8, H-6; C-8a → OH-8, H-5, H-7; C-9a → OH-1, H-2; C10 → H-5; EIMS m/z 284 [M]$^+$; HREIMS m/z 284.0696 [M]$^+$ (calcd 284.0685 for $C_{16}H_{12}O_5$).

Diacetate 1a. Acetylation of compound **1** (AC_2O/pyridine) gave **1a**: ^{1}H NMR ($CDCl_3$) δ 2.41 (3H, s, CH_3-3), 2.42, 2.43 (each 3H, s, OAc × 2), 3.93, (3H, s, OCH_3-4), 7.23 (1H, s, H-2), 7.35 (1H, d, J = 8.0 Hz, H-7), 7.73 (1H, t, J = 8.0 Hz, H-6), 8.13 (1H, d, J = 8.0 Hz, H-5); EIMS m/z 368 [M]$^+$, 326 [M − 42]$^+$, 284 [M − 42 × 2]$^+$.

1,2,6-Trihydroxy-7,8-dimethoxy-3-methylanthraquinone (2): yellow needles (EtOAc/*n*-hexane); mp 271−273 °C; UV (MeOH) λ_{max} (log ϵ) 410 (3.86), 313 (4.14), 283 (4.57), 317 (4.13), 301 (4.17), 225 (4.24) nm; IR (KBr) ν_{max} 3453 (OH), 1630 (C═O), 1561, 1456, 1299, 1272 cm^{-1}; ^{1}H NMR (acetone-d_6) δ 2.35 (3H, s, CH_3-3), 3.98, 3.99 (each 3H, s, OCH_3 × 2), 7.55

(1H, s, H-4), 7.57 (1H, s, H-5), 8.92, 9.64 (OH × 2), 13.33 (OH-1); ^{13}C NMR (acetone-d_6) δ 16.3 (3-CH_3), 61.5, 61.7 (OCH_3 × 2), 111.9 (C-5), 116.3 (C-9a), 120.2 (C-8a), 122.4 (C-4), 124.3 (C-10a), 131.3 (C-3), 132.9 (C-4a), 147.3 (C-7), 150.4 (C-1), 150.8 (C-2), 156.6 (C-8), 157.4 (C-6), 181.0 (C-10), 188.8 (C-9); HMBC correlations, C-1 → OH-1; C-2 → 3-CH_3, H-4; C-3 → 3-CH_3, OH-2; C-4 → 3-CH_3; C-6 → H-5; C-7 → 7-OCH_3, H-5; C-8 → 8-OCH_3; C-8a → H-5; C-9a → OH-1, H-4; C10 → H-4, H-5; EIMS *m/z* 330 $[M]^+$; HREIMS *m/z* 330.0736 $[M]^+$ (calcd 330.0740 for $C_{17}H_{14}O_7$).

Triacetate 2a. Acetylation of compound **2** (AC_2O/pyridine) gave **2a**: ^{1}H NMR ($CDCl_3$) δ 2.35 (3H, s, CH_3-3), 2.38, 2.39, 2.48 (each 3H, s, OAc × 3), 3.94, 4.01, (each 3H, s, OCH_3 × 2), 7.80 (1H, s, H-5), 8.08 (1H, s, H-4); EIMS *m/z* 456 $[M]^+$, 414 $[M - 42]^+$, 372 $[M - 42 \times 2]^+$, 330 $[M - 42 \times 3]^+$.

2-Hydroxyemodin 1-methyl ether (3): yellow needles (EtOAc/*n*-hexane); mp 292−294 °C; UV (MeOH) λ_{max} (log ϵ) 393 (3.92), 311 (4.08), 284 (4.42), 228 (4.36) nm; IR (KBr) ν_{max} 3422 (OH), 1629 (C═O), 1582, 1455, 1377, 1314, 1267 cm^{-1}; ^{1}H (DMSO-d_6) δ 2.29 (3H, s, CH_3-3), 3.80, (3H, s, OCH_3-1), 6.56 (1H, br s, H-7), 7.06 (1H, br s, H-5), 7.79 (1H, s, H-4), 10.30 (OH-2), 11.12 (OH-6), 13.12 (OH-8); ^{13}C (DMSO-d_6) δ 17.2 (3-CH_3), 61.9 (1-OCH_3), 107.8 (C-5), 108.1 (C-7), 111.0 (C-8a), 124.4 (C-9a), 125.6 (C-4a), 126.7 (C-4), 132.6 (C-3), 135.3 (C-10a), 147.9 (C-1), 156.5 (C-2), 164.9 (C-6), 165.3 (C-8), 181.6 (C-10), 187.2 (C-9); HMBC correlations, C-1 → 1-OCH_3; C-2 → 3-CH_3, H-4; C-4 → 3-CH_3; C-5 → H-7; C-8 → OH-8, H-7; C-8a → H-5, H-7, OH-8; C10 → H-4, H-5; APCIMS *m/z* 301 $[M + H]^+$; HREIMS *m/z* 300.0652 $[M]^+$ (calcd 300.0634 for $C_{16}H_{12}O_6$).

Triacetate 3a. Acetylation of compound **3** (AC_2O/pyridine) gave **3a**: ^{1}H NMR ($CDCl_3$) δ 2.34 (3H, s, CH_3-3), 2.37, 2.41, 2.47 (each 3H, s, OAc × 3), 3.88 (3H, s, OCH_3 × 2), 7.98 (1H, s, H-4), 7.94 (1H, br s, H-5), 7.25 (1H, br s, H-7); EIMS *m/z* 426 $[M]^+$, 384 $[M - 42]^+$, 342 $[M - 42 \times 2]^+$, 300 $[M - 42 \times 3]^+$.

Cell Lines. The K562, Raji, Vero, Calu-1, HeLa, and Wish cell lines were utilized as target cells in the cytotoxic assay. K562 and Raji cells are erythroleukemia and EBV-transformed B cell lines, respectively (American Type Culture Collection, ATCC, Rockville, MD). They were cultured in RPMI-1640 medium (Hyclone, Logan, UT) containing 10% fetal calf serum (FCS, Gibco, Grand Island, NY), 100 u/mL penicillin, and 100 μg/mL streptomycin. The Vero cell is a green monkey kidney tumor cell line (ATCC, Rockville, MD). The Wish cell is a transformed epithelial cell line, and the Calu-1 cell is a human lung carcinoma cell line (ATCC, Rockville, MD). The HeLa cell is a human cervical carcinoma cell line (ATCC, Rockville, MD). The Vero, Wish, Calu-1, and HeLa cell lines were cultured in MEM medium containing 10% FCS, 100 μg/mL streptomycin, and 100 u/mL penicillin. These cell lines were cultured at 37 °C in an atmosphere of humidified 5% CO_2.

Growth Inhibition Assay. Growth inhibition was assessed as described previously.[17] Each tumor cell line was cultured with or without various concentrations of compound **3**, **10**, or **15** for 3 days, after which [^{3}H]-thymidine was added and incubation continued for 16 h before harvest. Radioactivity was determined by a scintillation counter, and inhibitory activity was calculated as the IC_{50} (inhibition of 50% cell proliferation).

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