Cytotoxic Anthraquinones from the Stems of *Rubia wallichiana* DECNE

Tian-Shung WU,*[,a] Dau-Min LIN,[a] Li-Shian SHI,[a] Amooru Gangaiah DAMU,[a] Ping-Chung KUO,[a] and Yao-Hau KUO[b]

[a] *Department of Chemistry, National Cheng Kung University; Tainan, Taiwan: and* [b] *National Research Institute of Chinese Medicine; Taipei, Taiwan.* Received March 10, 2003; accepted May 12, 2003

From the stems of *Rubia wallichiana* DECNE, thirty-four structurally related compounds were isolated and identified. Three of them, namely rubiawallin-A (1), -B (2), and -C (3), constitute the first report of their occurrence from the natural source. Their structures were determined by comprehensive analyses of their 1D and 2D NMR, and electron impact (EI) mass spectral data. Furthermore, an *in vitro* screening of cytotoxicity of the isolated compounds was also evaluated. Among the testing compounds, 1-hydroxy-2-hydroxymethyl-3-methoxyanthraquinone (4) demonstrated most effective cytotoxicity towards Hepa-3B and Colo-205 cells.

Key words rubiawallin; traditional Chinese medicine; tumor cell lines; antiproliferative

Anthraquinones are a class of natural products encompassing several hundreds of compounds, differing in the nature and positions of the substituents.[1—3] They are found in a large number of plant families. They are particularly widespread in the subclass Asteridae, comprising among others the plant families Rubiaceae, Gesneriaceae, and Scrophulariaceae. Anthraquinones are not only common constituents of plants of the Rubiaceae, but also of their tissue and cell cultures.[4—7]

In the course of phytochemical studies of medicinal plants from the Chinese traditional medicine, we have investigated *Rubia wallichiana* DECNE (Rubiaceae), a plant distributed widely in the Himalayan region and northeast India. In clinical biological experiments, plants of the genus *Rubia* showed antibacteria, anticancer, anticough and antiplatelet aggregation activities. They have also been used to enhance the number of leukocytes, and in the therapy of myocardial infraction.[8] Phytochemical studies previously performed on the genus, *Rubia* have led to the isolation of anthraquinones, naphthaquinones, naphthahydroquinones, cyclic hexapeptides, flavonoids, coumarins, iridoids, lignans, triterpenoids, and benzenoids. The present paper deals with the isolation, structural elucidation of three anthraquinones, besides thirty-one known compounds. The *in vitro* cytotoxicity of the quinonoids isolated was also assessed, taking into account that anthraquinones have been reported to exhibit strong antiproliferative properties on mammalian cells.

Results and Discussion

Dried and powdered stems of *R. wallichiana* were extracted with methanol. The residue obtained after evaporation of the solvent was fractionated with chloroform and water, followed by conventional purification procedures resulted in the isolation of thirty-four constituents, including three new anthraquinones.

Rubiawallin-A (**1**) was obtained as yellow needles, mp >280 °C. The molecular ion peak at *m/z* 284.0688 in its high resolution electron impact (HR-EI)-MS was in accordance with the molecular formula $C_{16}H_{12}O_5$, deduced also by ^{13}C-NMR and distortionless enhancement by polarization transfer (DEPT) analyses. The UV spectrum of **1** exhibited absorption maxima at 225, 270, 292 (sh) and 418 nm, suggested an anthraquinone as the basic structure,[9] with a single *peri*-hydroxyl group. It was further supported by a D_2O exchangeable singlet at δ 13.20 in ^{1}H-NMR spectrum and an IR absorption at 3524 cm^{-1}. The IR absorption bands at 1640 and 1600 cm^{-1} also indicated the presence of free carbonyl and chelated carbonyl groups, respectively. Two carbonyl groups of an anthraquinone skeleton were also found in ^{13}C-NMR at δ 181.6 and 188.7 for the nonchelated one and chelated one, respectively. In ^{1}H-NMR spectrum, a set of ABX protons at δ 7.25 (1H, dd, J=8.8, 2.4 Hz), 7.73 (1H, d, J=2.4 Hz), and 8.26 (1H, d, J=8.8 Hz), were assigned to H-7, H-5, and H-8 of monosubstituted A-ring, respectively. Two *ortho* coupled protons at δ 7.15 (1H, d, J=8.4 Hz, H-3) and 7.86 (1H, d, J=8.4 Hz, H-4), and two methoxyl groups at δ 4.00 (3H, s, 6-OCH$_3$) and 4.02 (3H, s, 2-OCH$_3$) were also observed. The heteronuclear multiple bond coherence (HMBC) spectrum confirmed the attachment of two methoxyl groups to C-2 and C-6 by displaying correlations of the methoxyls to C-6 (δ 165.0) and C-2 (δ 154.3), respectively. In final confirmation, nuclear Overhauser effect spectroscopy (NOESY) spectrum exhibited correlations of the methoxyl at δ 4.00 with H-7, and the methoxyl at δ 4.02 with H-3. The identification of **1** was also accomplished by the comparison of spectral data with those of reported synthetic sample.[10] Thus the structure **1** was established for rubiawallin-A.

Rubiawallin-B (**2**) was isolated as yellow needles, mp 136—137 °C. Its molecular formula $C_{16}H_{12}O_4$ was determined on the basis of HR-EI-MS data. The IR spectrum showed bands at 3400 cm^{-1} due to chelated hydroxyl function and bands of a free carbonyl at 1630 cm^{-1} and a chelated one at 1600 cm^{-1}. In the ^{1}H-NMR spectrum, a downfield singlet at δ 13.11, exchangeable with D_2O, was attributed to chelated C-1 hydroxyl group. It also exhibited signals of a set of ABX protons at δ 7.27 (1H, dd, J=8.7, 2.5 Hz), 7.72 (1H, d, J=2.5 Hz), and 8.24 (1H, d, J=8.7 Hz), a set of two mutually coupled protons at δ 7.51 (1H, d, J=7.3 Hz) and 7.75 (1H, d, J=7.3 Hz), a methyl group at δ 2.38 (s) and a methoxyl group at δ 3.99 (s). In the NOESY spectrum, the methoxyl at δ 3.99 (3H, s) had connectivities with a doublet of doublet at δ 7.27 and a *meta*-coupled doublet at δ 7.72 and methyl at δ 2.38 (3H, s, 2-CH$_3$) with H-3 inferred its attachment to C-7 or C-6 and C-2, respectively. The point of attachment of methoxyl group at C-6 was excluded by comparison of spectral data with those of 1-hydroxy-6-methoxy-2-methyl anthraquinone,[11] since they differed in chemical

* To whom correspondence should be addressed. e-mail: tswu@mail.ncku.edu.tw

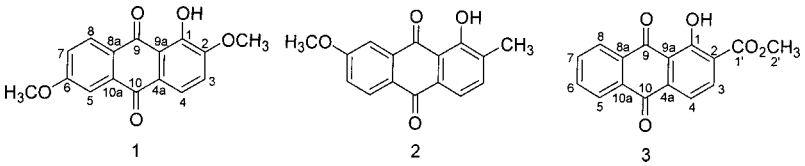

Fig. 1. Structures of Rubiawallin A—C (**1**—**3**)

shift values of methoxyl and protons of A-ring. Thus, the structure **2** was assigned as rubiawallin-B.

Rubiawallin-C (**3**) was obtained as yellow needles, mp 136—137 °C. Its HR-EI-MS spectrum with a molecular ion peak at *m/z* 282.0525 established the molecular formula $C_{16}H_{10}O_5$. The 9,10-anthraquinone chromophore with a peri-hydroxyl group was evident from its UV absorption maxima at 252, 282 (sh), 327, 402 nm. IR spectrum displayed bands for chelated hydroxyl (3400 cm^{-1}), chelated carbonyl (1630 cm^{-1}), a nonchelated carbonyl (1680 cm^{-1}), in addition to an ester carbonyl (1598 cm^{-1}). A chelated hydroxyl at δ 13.60 was located at the peri-position to the C-9 carbonyl group. In the aromatic region, the ^{1}H-NMR spectrum showed two multiplets for four symmetrical AA′ and BB′ type of aromatic protons at δ 8.31 and 7.85, and a pair of doublets at δ 8.26 (J=7.4 Hz) and 7.85 (J=7.4 Hz) indicated that anthraquinone possessing an unsubstituted A-ring and a disubstituted C-ring, respectively. A methoxyl singlet at δ 3.99 combined with an IR absorption at 1598 cm^{-1} suggested the presence of –$COOCH_3$ group in **3**. It was confirmed by the fragment ion $[M-COOCH_3]^+$ at *m/z* 222 in its EI-MS spectrum. The attachment of –$COOCH_3$ group to C-2 was determined by the downfield shift of H-3 to δ 8.26 due to anisotropic effect of carboxyl group. All these assignments were strongly supported by the NOEs: H-5/H-6, H-6/H-7, H-7/H-8, H-4/H-3, and H-3/2′-CH_3 in NOESY spectrum. The above spectral analysis strongly supported the proposed structure **3** for rubiawallin-C.

In addition, 1-hydroxy-2-hydroxymethyl-3-methoxyanthraquinone (**4**),[12] alizarin-2-methylether (**5**),[13] lucidin (**6**),[14] lucidin primeveroside (**7**),[14] munjistin methyl ether (**8**),[15] nordamnacanthal (**9**),[16] purpurin (**10**),[16] rubiadin (**11**),[15] rubiadin primeveroside (**12**),[17] rubischumin-A (**13**),[18] xanthopurpurin (**14**),[15] 1-hydroxy-3-methoxy-2-methylanthraquinone (**15**),[19] 1,3-dimethoxy-2-hydroxyanthraquinone (**16**),[20] 1-hydroxy-2-methylanthraquinone (**17**),[21] 1-hydroxy-2-hydroxymethylanthraquinone (**18**),[20] 2-methyl-1,3,6-trihydroxyanthraquinone (**19**),[21] 2-methyl-1,3,6-trihydroxyanthraquinone 3-*O*-α-L-rhamnosyl-(1→2)-β-D-glucoside (**20**),[21] 2-methyl-1,3,6-trihydroxyanthraquinone 3-*O*-(6′-*O*-acetyl)-α-L-rhamnosyl-(1→2)-β-D-glucoside (**21**),[21] 1-hydroxy-5-methoxy-2-methylanthraquinone (**22**),[22] 2-hydroxy-1-methoxyanthraquinone (**23**),[20] 1-hydroxy-3-methoxyanthraquinone (**24**),[13] 1,8-dihydroxy-2-methylanthraquinone (**25**),[13] 1,3-dihydroxy-2-methoxymethylanthraquinone (**26**),[15] scopoletin (**27**),[23] 6,7-dimethoxycoumarin (**28**),[24] 7-hydroxy-6-methoxy-8-(3-methylbut-2-enyl)coumarin (**29**),[25] (+)-medioresinol (**30**),[26] ursolic acid (**31**),[27] the mixture of β-sitosterol (**32**) and stigmasterol (**33**),[28] and docosanoic acid (**34**)[29] were also isolated from the stems of *R. wallchiana*. They were identified by the comparison of their spectral data with those in literature.

Furthermore, the compounds isolated from *R. wallchiana* were screened for their *in vitro* cytotoxicity against human nasal pharynegeal carcinoma (KB), human hepatoma (Hepa-3B), human cervix epithelioid carcinoma (Hela) and human colon carcinoma (Colo-205) tumor cell lines, and doxorubicin was used as reference compound. The results (ED_{50} values) of the cytotoxicity assay were summarized in Table 1. All the tested anthraquinones showed different degrees of activity with, **4** being the most potent one against Hepa-3B and Colo-205 cell lines. On the other hand, **14** and **19** also exhibited marked cytotoxicity against Hepa-3B and Colo-205 cell lines. **10** and **14**; and **13** and **18** are only moderately active against KB and Hepa-3B cell lines, respectively.

Table 1. Cytotoxicity ED_{50} (μg/ml) of Compounds Isolated from the Stems of *R. wallichiana* on Tumor Cell Lines

Compounds	Cell lines			
	KB	Hepa-3B	Hela	Colo-205
Doxorubicin	0.12	0.14	0.11	0.10
4	—	0.6	9.15	0.58
5	—	—	—	—
6	—	—	—	—
10	3.1	—	—	—
11	—	—	—	—
13	—	3.58	—	—
14	6.57	1.7	—	1.9
15	—	—	24.5	—
17	—	—	—	—
18	—	3.85	24.5	—
19	—	1.7	12.3	1.16
21	—	—	—	—
24	—	—	—	—
25	—	—	—	—
27	—	—	—	—
28	—	7.05	—	—
31	—	—	—	—

—: ED_{50}>25 μg/ml.

Experimental

General Experimental Methods Melting points were uncorrected. ^{1}H- and ^{13}C-NMR spectra were obtained on the Bruker AC-200, AMX-400 and Varian-400 Unity Plus NMR spectrometers, with tetramethylsilane (TMS) as internal standard. IR spectra were determined as KBr discs, and UV spectra were recorded in MeOH. EI and HR-EI-MS were measured with a 70 eV direct inlet system on a VG70-250S spectrometer. Optical rotations were recorded on a Jasco DIP-370 digital polarimeter.

Plant Material The stems of *R. wallichiana* used in this study were collected in Tibet, China Mainland, on July, 1996, and authenticated by Prof. C. S. Kuoh. A voucher specimen (TSWu 1996020) has been deposited at the Herbarium of National Cheng Kung University, Tainan, Taiwan.

Extraction and Separation The air-dried stems (4.2 kg) were cut into small pieces, extracted with MeOH (61×10), and concentrated to give a deep brown syrup (750 g). The crude extract was partitioned with H_2O and $CHCl_3$, successively. The $CHCl_3$ layer was concentrated *in vacuo* to leave a brown syrup. The residue was chromatographed on silica gel column and eluted with a gradient of C_6H_6 and $(CH_3)_2CO$ to give ten fractions. Fraction 3 was rechromatographed with silica gel column using $CHCl_3$–hexane (1 : 5)

as an eluent to obtain **25** (4.4 mg), and **34** (2.6 mg). Fraction 4 was repeatedly column chromatographed over silica gel and eluted with $CHCl_3$: hexane (1 : 1) to give **2** (1.2 mg), **10** (34.6 mg), **15** (10.3 mg) and **22** (0.3 mg), successively. Fraction 6 was rechromatographed on a silica gel column and eluted with a gradient of $CHCl_3$ and $(CH_3)_2CO$ to obtain **3** (1.2 mg), **13** (43.0 mg) and **24** (1.0 mg). Fraction 7 was rechromatographed on a silica gel column and eluted with $CHCl_3$: $(CH_3)_2CO$ (10 : 1) to give **5** (7.9 mg), **6** (74.5 mg), **11** (758.0 mg), **13** (1.1 mg) and **15** (15.4 mg). Fraction 7 was rechromatographed on a silica gel column and eluted with $CHCl_3$: $(CH_3)_2CO$ (5 : 1) to afford **28** (6.8 mg) and **31** (1.3 mg). Fraction 9 was also rechromatographed on silica gel column and eluted with $CHCl_3$: $(CH_3)_2CO$ (3 : 1) to give **8** (2.0 mg) and **27** (71.5 mg). Fraction 10 was chromatographed on silica gel column and eluted with $CHCl_3$: $(CH_3)_2CO$ (1 : 1) to obtain **14** (15.0 mg), **18** (1.8 mg), **19** (2.5 mg), **23** (2.1 mg), **29** (2.3 mg), and mixture of **32** and **33** (7.2 mg), successively. Fraction 13 was rechromatographed on a silica gel column and eluted with $CHCl_3$: $(CH_3)_2CO$ (1 : 2) to obtain **1** (2.5 mg), **4** (3.0 mg) and **16** (16.0 mg), respectively. Fraction 14 and 15 was rechromatographed on a silica gel column and eluted with $CHCl_3$: $(CH_3)_2CO$ (1 : 5) to afford **30** (7.8 mg).

The water layer was directly chromatographed on Diaion HP-20 column and eluted with a gradient of H_2O and MeOH to give 9 fractions. Fraction 6 was rechromatographed on RP-18 column and eluted with a gradient of H_2O and MeOH to give **7** (146.0 mg), **12** (73.7 mg) and **26** (1.7 mg). Fraction 7 was rechromatographed on a silica gel column and eluted with $CHCl_3$: MeOH (1 : 20) to obtain **20** (1.0 mg). Fraction 8 and 9 was treated in the similar method as fraction 7 to afford **21** (32.0 mg).

Rubiawallin-A (**1**): Red needles (acetone); mp>280 °C. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 4.00 (3H, s, 6-OCH_3), 4.02 (3H, s, 2-OCH_3), 7.15 (1H, d, J=8.4 Hz, H-3), 7.25 (1H, dd, J=8.8, 2.4 Hz, H-7), 7.73 (1H, d, J=2.4 Hz, H-5), 7.86 (1H, d, J=8.4 Hz, H-4), 8.26 (1H, d, J=8.8 Hz, H-8), 13.20 (1H, s, D_2O exchangeable, OH). ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 55.6 (6-OCH_3), 57.1 (5-OCH_3), 109.8 (C-5), 115.0 (C-3), 115.5 (C-9a), 120.3 (C-7), 121.0 (C-4), 125.5 (C-4a), 126.8 (C-8a), 128.9 (C-8), 136.4 (C-10a), 152.7 (C-1), 154.3 (C-2), 165.0 (C-6), 181.6 (C-10), 188.7 (C-9). IR (KBr) cm^{-1}: 1640, 1550. UV λ_{max} (MeOH) nm (log ε): 418 (5.12), 292 (4.37), 270 (4.33), 225 (4.21). HR-EI-MS m/z: 284.0688 (Calcd for $C_{16}H_{12}O_5$: 284.0684). EI-MS m/z: 284 (M^+), 255, 241.

Rubiawallin-B (**2**): Yellow needles (acetone); mp 136—137 °C. ^{1}H-NMR ($CDCl_3$, 200 MHz) δ: 2.38 (3H, s, 2-CH_3), 3.99 (3H, s, 7-OCH_3), 7.27 (1H, dd, J=8.7, 2.3 Hz, H-6), 7.51 (1H, d, J=7.3 Hz, H-3), 7.72 (1H, d, J=2.3 Hz, H-8), 7.75 (1H, d, J=7.3 Hz, H-4), 8.25 (1H, d, J=8.7 Hz, H-5), 13.11 (1H, s, D_2O exchangeable, OH). IR (KBr) cm^{-1}: 3400, 1630, 1600. UV λ_{max} (MeOH) nm (log ε): 411 (5.12), 334 (4.98), 292 (4.21), 261 (4.18). HR-EI-MS m/z: 268.0733 (Calcd for $C_{16}H_{12}O_4$: 268.0736). EI-MS m/z: 268 (M^+), 253, 251, 239, 225, 222, 181, 165, 152, 139, 115.

Rubiawallin-C (**3**): Yellow needles (acetone); mp 136—137 °C. ^{1}H-NMR ($CDCl_3$, 200 MHz) δ: 3.99 (3H, 2′-CH_3), 7.85 (1H, d, J=7.4 Hz, H-3), 7.85 (2H, m, H-6, 7), 8.26 (1H, d, J=7.4 Hz, H-4), 8.31 (2H, m, H-5, 8), 13.60 (1H, s, D_2O exchangeable, OH). IR (KBr) cm^{-1} 1680, 1630, 1510. UV λ_{max} (MeOH) nm (log ε): 402 (4.15), 327 (4.06), 282 (3.95, sh), 252 (3.90). HR-EI-MS m/z: 282.0525 (Calcd for $C_{16}H_{10}O_5$: 282.0528). EI-MS m/z: 282 (M^+), 250, 222.

Biological Assay The *in vitro* cytotoxicity assay against KB (nasal pharnegeal carcinoma), Hepa-3B (hepatoma), Hela (cervix carcinoma), and COLO-205 (colon carcinoma) tumor cells by the methylene blue dying method was based on reported procedures.[30—33] The cells for bioassay were cultured in RPMI-1640 medium supplemented with a 5% CO_2 incubator at 37 °C. In summary, the assay depends on binding the methylene blue to the fixed monolayer at pH 8.5 and, after washing the monolayer, releasing dye by lowering the pH. Entries and control standard agents were prepared at concentrations of 1, 10, 40, and 100 μg/ml. The detailed procedures of this experiment are in previous report.[33] Finally, the 96-well tray was dipped into a 0.01 M borated-buff solution four times for the removing the dye. Then, 100 μl/well ethanol–0.1 M HCl (1/1 v/v) was measured on a microtiter plate reader (Dynatech, MR 7000) at wavelength of 650 nm. As of the ED_{50} value was defined during a comparison with the untreated cells at the concentration of test sample resulting in 50% reduction of absorbance.

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