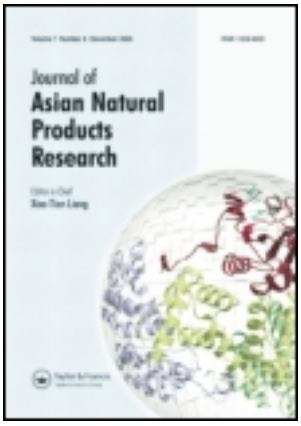

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Anthraquinones from the roots of *Knoxia valerianoides*

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Five new 9,10-anthraquinones (**1**–**5**) were isolated from an ethanol extract of the roots of *Knoxia valerianoides*. Their structures including absolute configuration of **1** were determined by spectroscopic analysis. Compounds **4** and **5** showed moderate activity against nitrogen oxide production in macrophages induced by lipopolysaccharide, at 10^{-5} M, with inhibition ratios of 50.4 ± 3.6 and 41.7 ± 2.1%, respectively.

Keywords: *Knoxia valerianoides*; Rubiaceae; anthraquinone; NO production inhibition

1. Introduction

Knoxia valerianoides Thorel ex Pitard (Rubiaceae) is a perennial plant widely distributed in the southern Chinese mainland. The dried roots are famous in traditional Chinese medicine as a purgative and anti-ulcer medicine. Previous investigation indicated that 9,10-anthraquinones were the major components of this herbal medicine, and some showed inhibitory activities against the formation of advanced glycation end products and rat lens aldose reductase [1–5]. As part of a program to assess chemical and biological diversities of traditional Chinese medicines [6], an ethanol extract of the roots of *K. valerianoides* has been investigated. This paper reports the isolation, structural elucidation, and *in vitro* bioassays of five new anthraquinones (**1**–**5**, Figure 1) from the herbal medicine. Compound **1** is a rare dihydrofurano-9, 10-anthraquinone.

2. Results and discussion

Compound **1**, an orange amorphous solid, showed IR absorptions for hydroxy (3406 cm^{-1}), conjugated carbonyl (1658 cm^{-1}), and aromatic ring (1623, 1575, and 1485 cm^{-1}) functional groups. The molecular formula of **1** ($C_{19}H_{14}O_5$) was indicated by HR-ESI-MS combined with the NMR spectral data. The ^{1}H NMR spectrum of **1** displayed signals (Table 1) attributed to a pair of *ortho*-coupled aromatic protons at δ 7.22 (d, $J = 9.0$ Hz, H-7) and 8.18 (d, $J = 9.0$ Hz, H-8), a pair of *meta*-coupled aromatic protons at δ 6.65 (d, $J = 1.5$ Hz, H-2) and 7.22 (d, $J = 1.5$ Hz, H-4), and a hydrogen-bonded phenolic hydroxy proton at δ 13.09 (s, O*H*-1). In addition, it showed signals assignable to a pair of geminal protons of an olefinic methylene at δ 5.14 (brs, H-4′a) and 4.96 (brs, H-4′b), a group of typical ABX-coupled protons of an oxymethine at δ 5.50 (dd, $J = 7.5$, 9.0 Hz, H-2′), a

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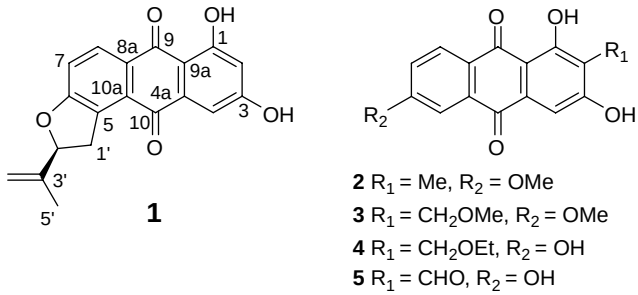

Figure 1. Structures of compounds **1**—**5**.

methylene at δ 3.91 (dd, $J = 18.0$, 9.0 Hz, H-1′a) and 3.46 (dd, $J = 18.0$, 7.5 Hz, H-1′b), and an olefinic methyl at δ 1.79 (s, CH_3-5′). The ^{13}C NMR spectrum of **1** showed five carbon resonances due to a 2′-oxygen-bearing isopent-3′-en-1′-yl unit in addition to 14 sp^2 carbon resonances including two typical carbonyl resonances at δ 186.7 (C-9) and 184.0 (C-10) (Table 2) for the 9,10-anthraquinone nucleus [7–10]. These spectroscopic data suggested that compound **1** was a tetra-substituted 9,10-anthraquinone analog containing an isopentenyl-derived unit. In the gHMBC spectrum of **1**, correlations of H-2/C-4 and C-9a, H-4/C-2, C-9a, and C-10, H-7/C-5 and C-8a, and H-8/C-6, C-9, and C-10a (Figure 2), together with their shifts, further confirmed a 1,3,5,6-tetra-substituted 9,10-anthraquinone nucleus. HMBC correlations of CH_2-1′/C-2′, C-3′, C-6, and C-10a, H-2′/C-1′, C-3′, C-4′, C-5′, and C-6, in combination with shifts of these protons and carbons, demonstrated a connection between C-1′ and C-5 and an oxygen-bridged connection between C-2′ and C-6. In addition, HMBC correlations of O*H*-1/C-1, C-2, and C-9a, in combination with the splitting patterns of H-2 and H-4 and the formula, located two hydroxy groups at C-1 and C-3, respectively. Compound **1** exhibited a positive specific rotation ($[\alpha]_D^{20} + 54$; *c* 0.7, MeOH) and positive Cotton effects at 220 and 285 nm in the CD spectrum ("Experimental" section). These data were opposite to those of compounds with a similar isopropenyldihydrofuran system, such as the *R*-(−)-3′,4′-deoxypsorospermin derivatives and *R*-(−)-tubaic acid [11–12], suggesting 2′*S* configuration for **1**. Therefore, **1** was characterized as (2*S*)-7,9-dihydroxy-2-(prop-1-en-2-yl)-1,2-dihydroanthra[2,1-b]furan-6,11-dione.

Compound **2** was obtained as orange needles (acetone) with m.p. 232°C. Its molecular formula $C_{16}H_{12}O_5$ was indicated by HR-ESI-MS at *m/z* 283.0600 $[M - H]^-$ (calculated for $C_{16}H_{11}O_5$, 283.0612) and NMR spectroscopic data (Tables 1 and 2). The UV and IR spectra showed characteristic absorptions ("Experimental" section) for 9,10-anthraquinones [1]. The NMR spectral data suggested that it was the 5-deoxy derivative of the co-occurring knoxiadin [1,2]. The substitution pattern was confirmed by the gHMBC spectrum that showed correlations from both H-4 and H-5 to C-10, from H-8 to C-9, from CH_3-11 to C-1, C-2, and C-3, and from O*Me* to C-6. Therefore, compound **2** was determined as 1,3-dihydroxy-6-methoxy-2-methyl-9,10-anthraquinone.

Compound **3**, a yellowish amorphous solid, had the molecular formula $C_{17}H_{14}O_6$ as indicated by HR-ESI-MS at *m/z* 313.0713 $[M - H]^-$ (calculated for $C_{17}H_{13}O_6$, 313.0718) and NMR spectroscopic data (Tables 1 and 2). The UV, IR, and NMR spectroscopic data of **3** were similar to those of **2**, except that the NMR

Table 1. ^{1}H NMR spectral data of compounds **1**–**5**.[a]

Position	**1**[b]	**2**	**3**	**4**[c]	**5**
1					
2	6.65 d (1.5)				
4	7.22 d (1.5)	7.33 s	7.25 s	7.25 s	7.12 s
5		7.62 d (2.5)	7.56 d (1.5)	7.61 d (2.4)	7.45 d (2.5)
7	7.22 d (9.0)	7.39 dd (8.5, 2.5)	7.44 dd (8.5, 1.5)	7.32 dd (8.4, 2.4)	7.25 dd (8.5, 2.5)
8	8.18 d (9.0)	8.22 d (8.5)	8.16 d (8.5)	8.19 d (8.4)	8.09 d (8.5)
11		2.17 s	4.44 s	4.78 s	10.37 s
1-OH	13.09 s	13.32 s	13.39 s	13.53 s	14.14 s
11-OMe			3.26 s		
3-OH		9.76 s		9.82 brs	11.20 brs
6-OH				9.82 brs	12.36 brs
6-OMe		4.02 s	3.95 s		

[a] Data were measured in acetone-d_6 at 500 MHz for 1 and 2 and at 600 MHz for 4; in DMSO-d_6 at 500 MHz for 3 and 5. The assignments were based on HMBC experiments.
[b] Data for the dihydrofuran unit of 1: δ 3.91 (dd, J = 18.0, 9.0 Hz, H-1′a), 3.46 (dd, J = 18.0, 7.5 Hz, H-1′b), 5.50 (dd, J = 7.5, 9.0 Hz, H-2′), 5.14 (brs, H-4′a), 4.96 (brs, H-4′b), and 1.79 (s, H3-5′).
[c] Data for OEt of 4: δ 3.66 (q, J = 7.2 Hz) and 1.22 (t, J = 7.2 Hz).

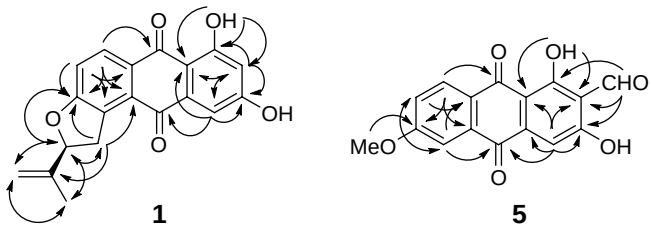

Figure 2. Selected key HMBC correlations of compounds **1** and **5**.

resonances for the C-2 methyl group in **2** were replaced by those for a methoxymethyl unit in **3** (Tables 1 and 2, "Experimental" section). This indicated that compound **3** was 1,3-dihydroxy-6-methoxy-2-methoxymethyl-9,10-anthraquinone. HMBC correlations from CH_2-11 to C-1, C-2, C-3, and O*Me* (δ 58.2) verified the methoxymethyl group at C-2 in **3**.

The spectroscopic data of compound **4** (Tables 1 and 2, "Experimental" section) indicated that it was an isomer of **3**. Comparison of the NMR spectral data of **4** with those of **3** indicated that the resonances of the two methoxy groups in **3** were replaced by those of an ethoxy group in **4** [δ_H 3.66 (q, $J = 7.2$ Hz) and 1.22 (t, $J = 7.2$ Hz), δ_C 67.0 and 15.4]. This led to an assignment of **4** as 1,3,6-trihydroxy-2-ethoxymethyl-9,10-anthraquinone, which was confirmed by the gHMBC data.

Compound **5**, an orange amorphous solid, had the molecular formula $C_{15}H_8O_6$ as indicated by HR-ESI-MS at *m/z* 283.0221 $[M - H]^-$ (calculated for $C_{15}H_7O_6$, 283.0243) and NMR spectroscopic data (Tables 1 and 2). Comparison

Table 2. ^{13}C NMR spectral data of compounds **1**–**5**.[a]

Position	**1**[b]	**2**	**3**	**4**[c]	**5**
1	166.3	163.9	164.2	163.9	166.1
2	108.7	119.0	117.4	117.7	112.7
3	165.6	162.9	164.6	164.2	166.3
4	108.6	108.0	108.3	108.6	107.3
4a	136.7	133.3	134.6	135.3	138.1
5	127.6	111.4	111.2	113.6	112.4
6	166.9	165.4	164.6	164.0	163.2
7	114.6	121.1	121.3	122.1	121.5
8	130.2	130.0	129.7	130.4	129.3
8a	130.4	127.5	126.8	126.6	124.1
9	186.7	187.2	186.1	187.2	185.3
9a	110.7	110.3	109.1	110.3	108.3
10	184.0	182.7	182.4	182.6	180.9
10a	131.2	136.4	135.6	136.6	134.7
11		8.2	61.7	63.0	190.5
11-OMe			58.2		
6-OMe		56.5	56.7		

[a] Data were measured in acetone-d_6 at 125 MHz for **1**, **2**, and **4**; in DMSO-d_6 at 125 MHz for **3** and **5**. The assignments were based on HMBC experiments.
[b] Data for the dihydrofuran unit of 1: δ 36.3 (C-1′), 88.7 (C-2′), 144.7 (C-3′), 112.6 (C-4′), and 17.1 (C-5′).
[c] Data for OEt of 4: δ 67.0 and 15.4.

of the NMR spectral data of **5** with those of **4** indicated that a formyl group in **5** (δ_H 10.37 and δ_C 190.5) replaced the C-2 ethoxymethyl group in **4**. In addition, due to hydrogen bond formation and conjugation effects of the formyl group, O*H*-1 and C-1, C-3, and C-4a in **5** were deshielded significantly, as compared with those in **4**, whereas C-2, C-8a, C-9, C-9a, C-10, and C-10a were shielded significantly. This suggested that **5** was 1,3,6-trihydroxy-2-formyl-9,10-anthraquinone. The suggestion was confirmed by gHMBC correlations from both O*H*-1 and H-11 to C-1 and C-2.

In the *in vitro* bioassay, at 10 μM, compounds **4** and **5** inhibited lipopolysaccharide (LPS)-induced nitrogen oxide (NO) production in macrophages, with inhibition ratios of 50.4 ± 3.6 and 41.7 ± 2.1%, respectively, and the positive control dexamethasone (DEX) gave a 77 ± 2.9% inhibition.

3. Experimental

3.1 General experimental procedures

Melting points were determined on a Boetius micro melting point apparatus and are uncorrected. Specific rotations were measured on a Rudolph Research Autopol III automatic polarimeter. CD spectra were recorded on a JASCO J-815 CD spectrometer. IR spectra were recorded as KBr pellets on a Nicolet 5700 FT-IR spectrophotometer. UV spectra were measured on a Cary 300 spectrophotometer. NMR spectra were obtained at 500 or 600 MHz for ^{1}H and 125 MHz for ^{13}C, respectively, on Inova 500 and 600 MHz spectrometers in DMSO-d_6 or Me_2CO-d_6 with solvent peaks being used as references. ESI-MS data were measured with a Q-Trap LC/MS (Turbo Ionspray source) spectrometer. HR-ESI-MS data were, respectively, measured using a Micromass Autospec-Ultima ETOF and an AccuToFCS JMS-T100CS spectrometers. Column chromatography (CC) was carried out with silica gel (100–200 or 200–300 mesh, Qingdao Marine Chemical Inc., Qingdao, China) and Sephadex LH-20 (Pharmacia Biotech AB, Uppsala, Sweden). HPLC separation was carried out on an instrument consisting of a Waters 600 controller, a Waters 600 pump, and a Waters 2487 dual λ absorbance detector with an Alltima (250 × 10 mm i.d.) column packed with C_{18} (5 μm). Thin layer chromatography (TLC) was carried out with glass precoated silica gel GF_{254} plates (Yantai Jiangyou Silica Gel Technology Development Co. Ltd., Yantai, China). Spots were visualized under UV light or by spraying with 10% H_2SO_4 in EtOH followed by heating.

3.2 Plant material

Roots of *K. valerianoides* were purchased at Anguo medicinal herb market of Hebei Province, which were collected in Dali, Yunnan Province, in 2006. Plant identification was done by Associate Prof. Lin Ma (Institute of Materia Medica). A voucher specimen (No. HDJ070413) has been deposited at the Herbarium of the Department of Medicinal Plants, Institute of Materia Medica, Beijing 100050, China.

3.3 Extraction and isolation

The dried roots of *K. valerianoides* (20 kg) were powdered and extracted with 10.0 l of 95% EtOH at room temperature for 3 × 48 h. The EtOH extract was evaporated under reduced pressure to yield a dark brown residue (3.9 kg). The residue was suspended in H_2O (3000 ml) and then partitioned with EtOAc (3000 ml × 3). The EtOAc layer (400 g) was chromatographed over silica gel eluting with a gradient of increasing acetone in petroleum ether (100:0–0:100, V/V) to afford nine fractions (Fr.1–Fr.9) based on TLC. Fr.4 (15 g) was chromatographed over silica gel eluting with a gradient of increasing ethyl acetate in petroleum ether (5–100%, V/V)

to afford Fr.4-1–Fr.4-46. Fr.4-14 (0.36 g) was chromatographed over Sephadex LH-20 [petroleum ether–$CHCl_3$–CH_3OH (5:5:1, V/V)] to yield 26 subfractions. Separation of Fr.4-14-16 (71 mg) by preparative TLC [petroleum ether–acetone (5:1, V/V)] afforded **2** (10.2 mg). Fr.4-14-26 (48 mg) was repeatedly recrystallized in $CHCl_3$–CH_3OH (1:1, V/V) to obtain **5** (8.4 mg). Fr.4-18 (0.29 g) was separated on a Sephadex LH-20 column [petroleum ether–$CHCl_3$–CH_3OH (5:5:1, V/V)] to give 15 subfractions, of which Fr.4-18-8 (19 mg) was separated by reversed-phase semipreparative HPLC [CH_3OH–H_2O (90:10, V/V)] to afford **1** (7.3 mg). Fr.5 (24 g) was chromatographed over silica gel, eluting with a gradient of increasing acetone in petroleum ether (10–100%, V/V), to yield 60 subfractions. Fr.5-60 (0.33 g) was separated into 11 subfractions by CC over Sephadex LH-20 [$CHCl_3$–CH_3OH (1:1, V/V)], of which Fr.5-60-1 (27 mg) was purified by reversed-phase semipreparative HPLC [CH_3OH–H_2O (75:25, V/V)] to obtain **4** (8.5 mg). Fr.6 (30 g) was subjected to CC over silica gel eluting with a gradient of increasing CH_3OH in $CHCl_3$ (1–100%, V/V) to afford Fr.6-1–Fr.6-101, of which Fr.6-17 (16 mg) was separated into six subfractions by chromatography over Sephadex LH-20 [$CHCl_3$–CH_3OH (1:1, V/V)], and then Fr.6-17-2 (5 mg) was purified by preparative TLC [$CHCl_3$–CH_3OH (25:1, V/V)] to give **3** (2.6 mg).

*3.3.1 (2S)-7,9-Dihydroxy-2-(prop-1-en-2-yl)-1,2-dihydroanthra[2,1-b]furan-6,11-dione (**1**)*

An orange amorphous solid, $[\alpha]_D^{20} + 54$ ($c = 0.7$, MeOH); UV (MeOH) λ_{max} (log ε) 221 (4.18), 285 (4.11), 352 (2.66), 434 (2.93) nm; CD ($c = 0.007$, MeOH) $\Delta\varepsilon_{220\,nm} + 1.88$, $\Delta\varepsilon_{244\,nm} + 0.01$, $\Delta\varepsilon_{285\,nm} + 1.04$, and $\Delta\varepsilon_{347\,nm} - 0.17$; IR (KBr) ν_{max} 3406, 3083, 2976, 2926, 2856, 1733, 1658, 1623, 1575, 1485, 1457, 1414, 1384, 1313, 1247, 1160, 1139, 1111, and 839 cm^{-1}; ^1H NMR (acetone-d_6, 500 MHz) spectral data, see Table 1; ^{13}C NMR (acetone-d_6, 125 MHz) spectral data, see Table 2; (−)-ESI-MS *m/z* 321 $[M - H]^-$; HR-ESI-MS *m/z* 321.0764 $[M - H]^-$ (calculated for $C_{19}H_{13}O_5$, 321.0768).

*3.3.2 1,3-Dihydroxy-6-methoxy-2-methyl-9,10-anthraquinone (**2**)*

Orange needles (acetone), m.p. 232°C; UV (MeOH) λ_{max} (log ε) 210 (3.98), 275 (4.31), 339 (2.72), and 421 (2.75) nm; IR (KBr) ν_{max} 3409, 2987, 2950, 2840, 1659, 1620, 1588, 1494, 1440, 1368, 1329, 1230, 1123, 1093, 1063, 1017, 881, 836, 789, 758, 729, 682, and 588 cm^{-1}; ^1H NMR (acetone-d_6, 500 MHz) spectral data, see Table 1; ^{13}C NMR (acetone-d_6, 125 MHz) spectral data, see Table 2; (−)-ESI-MS *m/z* 283 $[M - H]^-$; (−)-HR-ESI-MS *m/z* 283.0600 $[M - H]^-$ (calculated for $C_{16}H_{11}O_5$, 283.0612).

*3.3.3 1,3-Dihydroxy-6-methoxy-2-methoxymethyl-9,10-anthraquinone (**3**)*

A yellowish amorphous solid; UV (MeOH) λ_{max} (log ε) 214 (4.14), 271 (4.12), 434 (2.90) nm; IR (KBr) ν_{max} 3300, 2922, 2851, 1670, 1621, 1596, 1439, 1396, 1374, 1289, 1231, 1139, 1092, 1061, 1022, and 873 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) spectral data, see Table 1; ^{13}C NMR (DMSO-d_6, 125 MHz) spectral data, see Table 2; (−)-ESI-MS *m/z* 313 $[M - H]^-$; (−)-HR-ESI-MS *m/z* 313.0713 $[M - H]^-$ (calculated for $C_{17}H_{13}O_6$, 313.0718).

*3.3.4 1,3,6-Trihydroxy-2-ethoxymethyl-9,10-anthraquinone (**4**)*

A yellowish amorphous solid; UV (MeOH) λ_{max} (log ε) 216 (4.44), 281 (4.64), 340 (3.16), and 432 (3.18) nm; IR (KBr) ν_{max} 3411, 3201, 2985, 2921, 2852,

1735, 1660, 1626, 1593, 1581, 1479, 1456, 1401, 1380, 1323, 1280, 1235, 1217, 1191, 1167, 1131, 1098, 1063, 980, 890, and 850 cm^{-1}; ^1H NMR (acetone-d_6, 600 MHz) spectral data, see Table 1; ^{13}C NMR (acetone-d_6, 125 MHz) spectral data, see Table 2; (−)-ESI-MS *m/z* 313 $[M - H]^-$; (−)-HR-ESI-MS *m/z* 313.0711 $[M - H]^-$ (calculated for $C_{17}H_{13}O_6$, 313.0718).

*3.3.5 1,3,6-Trihydroxy-2-formyl-9,10-anthraquinone (**5**)*

An orange amorphous solid; UV (MeOH) λ_{max} (log ε) 208 (3.87), 275 (4.29), 302 (sh, 3.68), 340 (3.08), and 433 (2.60) nm; IR (KBr) ν_{max} 3066, 2923, 2616, 1648, 1623, 1577, 1467, 1431, 1412, 1378, 1316, 1298, 1279, 1233, 1196, 1173, 1137, 1038, 1021, 991, 906, 826, 798, 733, and 610 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) spectral data, see Table 1; ^{13}C NMR (DMSO-d_6, 125 MHz) spectral data, see Table 2; (−)-HR-ESI-MS *m/z* 283.0221 $[M - H]^-$ (calculated for $C_{15}H_7O_6$, 283.0243).

3.4 Inhibition assay of NO production in macrophage

Mouse peritoneal macrophages (PEM™) were treated with carrier control (DMSO only), LPS, or test compounds (LPS + compounds of 1×10^{-6} M, final concentration), or DEX (1×10^{-6} M, final concentration) [13]. After cells (5×10^5 cells) were incubated at 37°C, 5% CO_2 for 24 h, NO was monitored by measuring nitrite levels in culture media using the Griess reagent. Absorbance was measured at 540 nm after incubating culture media with Griess reagent for 10 min. Treated cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (2 μg/ml) for 4 h. Media were then removed and absorbance was assayed at 540 nm. Analysis of variance was used for all statistical analyses with independent experiments and data are represented as means ± standard error of the mean. Individual values were compared by Student's *t*-test and a *P*-value of <0.05 is considered as significant.

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