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Two New Cytotoxic Naphthoquinones from *Mendoncia cowanii* from the Rainforest of Madagascar

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Abstract

Bioassay-guided fractionation of the root and stem extracts of *Mendoncia cowanii* led to the isolation of two new and two known naphthoquinones. The structures of the new compounds, avicequinones D and E (**1** and **2**), were determined using 1D and 2D NMR spectroscopy and by chemical conversion of compound **1** to **2**. The new compounds were active in the A2780 human ovarian cancer cell line with IC₅₀ values of 7.4 - 50 μ M, respectively, and **1**, **3**, and **4** were subsequently found to be weakly active in an assay for inhibition of the kinase Akt.

In our continuing search for bioactive molecules from the rainforests of Madagascar as part of an International Cooperative Biodiversity Group (ICBG) program [1], we obtained extracts of *Mendoncia cowanii* (S. Moore) Benoist roots and stems. *M. cowanii* is known locally as vahimpianaomby, where it is used against syphilis [2]. These extracts were selected for bioassay-guided fractionation based on their cytotoxic activities in the A2780 human ovarian cancer cell line, with IC₅₀ values of 17 μ g/mL for the root extract and 21 μ g/mL for the stem extract, and also because no previous chemical studies have been reported for this genus. After solvent partitioning, reversed-phase flash chromatography, and reversed-phased HPLC the crude extract yielded the two new naphthoquinones, avicequinone D and E (**1** and **2**), and the two known naphthoquinones, avicequinone C (**3**) and stenocarpoquinone-B (**4**) [3,4].

1 R = OH
3 R = H

2 R = OH
4 R = H

Avicequinone D (**1**) was isolated as a yellow powder. Its UV spectrum, with absorptions at 249 and 295 nm, indicated that it was a naphthoquinone derivative [5]. Its HR-FAB-MS was consistent with a molecular formula of $C_{15}H_{12}O_5$ (m/z 273.0786 $[M + H]^+$). Its 1H -NMR data (Table 1) indicated the presence of two equivalent methyl groups that resonated at δ_H 1.67 (s), four sp^2 methines that resonated at δ_H 6.81 (s), δ_H 7.25 (dd, $J = 1.2, 8.3$ Hz), δ_H 7.58 (dd, $J = 7.5, 8.3$ Hz), and δ_H 7.70 (dd, $J = 1.2, 7.5$), and one chelated hydroxyl proton that resonated at δ_H 12.04 (s). The ^{13}C -NMR spectrum (Table 1) contained a methyl resonance of double intensity, an sp^3 quaternary carbon resonance at δ_C 69.5, four sp^2 methine resonances, six sp^2 quaternary carbon resonances, and two carbonyl resonances. These 1H - and ^{13}C -NMR spectra indicated that **1** was a naphthoquinone.

HMBC correlations (Fig. 1) and 1H NMR coupling constants established two fragments. Based on the HMBC experiment the hydroxyl group (δ_H 12.04) was attached to the carbon that resonated at δ_C 162.6. Adjacent to it were two sp^2 carbons, one was a quaternary carbon that resonated at δ_C 114.8, and the other was a methine that resonated at δ_C 125.2 (δ_H 7.25). Based on coupling constants this methine was clearly one of three methines [δ_H 7.25 (δ_C 125.2), δ_H 7.58 (δ_C 136.2), and δ_H 7.70 (δ_C 120.1)] in an ABC spin system with the methine that resonated at δ_H 7.58 (dd, $J = 7.5, 8.3$ Hz) in the B position. The methine that resonated at δ_H 7.58 (δ_C 136.2) produced HMBC correlations to the phenolic carbon (δ_C 162.6) and to an sp^2 quaternary carbon (δ_C 133.3). The third methine of the ABC spin system (δ_H 7.70/ δ_C 120.1) was found to produce HMBC correlations to the sp^2 quaternary carbon that resonated at δ_C 114.8 as well as the carbonyl carbon that resonated at δ_C 179.9. The second fragment consisted of a substituted furan ring. The methine that resonated at δ_H 6.81 (s) (δ_C 103.0) showed correlations to the sp^2 quaternary carbons that resonated at δ_C 132.1, δ_C 151.0, and δ_C 168.6. The methyl resonances (δ_H 1.67 (s), δ_C 28.8) were found to show HMBC correlations to the quaternary carbon that resonated at δ_C 69.5 and the sp^2 quaternary carbon that resonated at δ_C 168.6. Unfortunately, there were no HMBC correlations revealing the orientation of the two fragments to each other, and two structures were therefore possible. HMBC experiments were carried out with the long range J_{CH} set at 5, 8 (the original value), 12, and 15 Hz, in order to determine all relevant correlations, since an HMBC correlation from the H-1' methine at δ_H 6.81 (s) (δ_C 103.0) to one of the carbonyl resonances was vital to the structural determination. In the event, an HMBC correlation from this methine to the carbonyl carbon at δ_C 179.9 was only observed at the 5 Hz setting, thus providing the connectivity of the two fragments and establishing the structure as **1**. In keeping with the nomenclature previously established we propose the trivial name avicequinone D [3].

Avicequinone E (**2**) was isolated in low yield as a yellow powder. Its UV spectrum was similar to that of **1**, showing that it was also a naphthoquinone derivative. Attempts at obtaining HR-FAB-MS data were not successful, but a weak molecular ion was observed at m/z 275 $[M + H]^+$ in its LR-ESI-MS; this value was consistent with the composition $C_{15}H_{14}O_5$. The composition was supported by resonances in the 1H -NMR spectrum (Table 1) at δ_H 3.14 (d, 10.1) for the protons of a methylene group and at δ_H 4.83 (t, 10.1) for the proton of a methine group, as well as resonances for two nonequivalent methyls (δ_H 1.24 (s) and δ_H 1.39 (s)). These data indicated that **2** was a dihydrofuran analog of **1**. Due to the small amount of compound isolated it was only possible to obtain a partial ^{13}C -NMR spectrum. The structure of **2** was confirmed by hydrogenating a small amount of **1** (1.2 mg) over Pd on carbon in THF at 35 psi [12]. The major product was racemic **2** contaminated with about 35% of the corresponding hydroquinone; pure semisynthetic **2** was obtained by HPLC and had the same retention time as the natural product. The 1H NMR spectrum of semisynthetic **2** was identical to that of the natural product. The HMBC spectrum of semisynthetic **2** (Fig. 1) was in agreement with the partial ^{13}C -NMR spectrum of the natural product, and it also provided all but one of the carbon chemical shifts (Table 1). Compounds

3 and **4** were identified as avicequinone C and stenocarpoquinone-B, respectively, by comparison of their spectroscopic data with literature data [3,4].

Compounds **1-3** were cytotoxic against the A2780 human ovarian cancer cell line with IC₅₀ values of 7.4, 8.8, and 9.8 μM, respectively; compound **4** was much less active with an IC₅₀ value of 50 μM. Compounds **1, 3**, and **4** were subsequently found to be weakly active in an Akt kinase inhibition assay with IC₅₀ values of 110, 100, and 70 μM, respectively. Akt has been shown to prevent apoptosis at a postmitochondrial stage through the phosphorylation and inactivation of a number of cellular apoptosis inducers. Glycogen synthase kinase-3, BAD, caspase-9, and several transcription factors such as FKHRL1 have been identified as targets [6-10]. Over expression of Akt has also been implicated in oncogenesis [7], and Akt is thus a promising target in the search for anticancer agents.

Materials and Methods

General experimental methods were as previously described [11].

The roots and stems of *Mendoncia cowanii* (S. Moore) Benoist (Acanthaceae) were collected at 17°39'46"S, 48°59'5"E near the village of d'Ambarifotsy in Zahamena, Madagascar, by L. J. Razafitsalama *et al.* on May 29, 2003. A voucher specimen (Razafitsalama 473) was deposited at the Missouri Botanical Garden, and was identified by L. J. Razafitsalama, 2003. The specimen was a liana with green fruit growing in a dense humid forest at an elevation of 560 m.

The roots and stems were extracted separately with EtOH to give the crude extracts MG 1918 (IC₅₀ = 17 μg/mL) and MG 1919 (IC₅₀ = 21 μg/mL), respectively. MG 1918 (345 mg) was partitioned with EtOAc/25% aq. MeOH. The EtOAc fraction (269 mg) was found to be active (IC₅₀ = 11 μg/mL) while the aq. MeOH fraction was not active. The EtOAc fraction was eluted on a Supelco Discovery C-18 SPE cartridge (1 g) with 100% MeOH to remove nonpolar material, and then subjected to preparative HPLC on a C18 Varian Dynamax column (8 μm, 250 × 21.4 mm), eluting with 70% aq. MeOH to produce compounds **4** (t_R 9.8 min) (1.3 mg), **2** (t_R 10.6 min) (0.6 mg), **3** (t_R 13.7 min) (4.8 mg), and **1** (t_R 17.6 min) (3.5 mg). MG 1919 was fractionated using similar conditions to yield the same compounds.

Avicequinone D (**1**)

Yellow amorphous solid; UV (MeOH): λ_{max} (log ε) = 249 (2.93), 295 (3.48) nm; IR (film): ν_{max} = 3412 (br), 2925, 2854, 1727, 1686, 1646 cm⁻¹; ¹H- and ¹³C-NMR, see Table 1; HR-FAB-MS: *m/z* 273.0786 [M + H]⁺ (calcd for C₁₅H₁₃O₅ 273.0763).

Avicequinone E (**2**)

Yellow amorphous solid; [α]_D²⁵ = +11.0° (c 0.15, CHCl₃); UV (MeOH): λ_{max} (log ε) = 250 (sh), 277 (3.19) nm; IR (film): ν_{max} = 3416 (br), 2925, 2855, 1711, 1644, 1617 cm⁻¹; ¹H- and ¹³C-NMR, see Table 1; ESI-MS: *m/z* 275 [M + H]⁺.

Hydrogenation of **1**

Compound **1** (1.2 mg) was hydrogenated with Pd on C in THF at 35 psi. The reaction mixture was filtered to remove the Pd on carbon to yield 1.2 mg of a mixture of **2** and its hydroquinone derivative in a 2:1 ratio as verified by ¹H-NMR [12].

Cytotoxicity assay

The A2780 human ovarian cancer cell line assay was performed at Virginia Polytechnic Institute and State University as previously reported [13]. Actinomycin D at 2 µg/mL was used as a positive control its IC₅₀ value was consistently in the range 8 - 24 × 10⁻⁴ µM [11].

Akt inhibition assay

A 96-well microtiter-based (IMAP™ Akt Assay Kit from Molecular Devices) fluorescence polarization assay was performed to measure the Akt inhibitory effects of **1-4**. Assays were performed according to the manufacturer's recommendations. Briefly, 100, 10, 1, and 0.1 µg/mL of compound **1-4** in IMAP reaction buffer were incubated with 0.2 µg/mL Akt, 100 nM fluorescently labeled Akt substrate peptide, 5 µM ATP in a total volume of 20 µL for 60 minutes at room temperature. IMAP binding solution (60 µL) was added to each well and incubated at room temperature for 30 minutes. Samples with no drug or enzyme were run in parallel. Data were collected on an Analyst GT (Molecular Devices) and analyzed using SoftMaxPro software. The positive control staurosporine was obtained from Sigma (IC₅₀ = 2 - 6 × 10⁻³ µM).

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Fig. 1.
HMBC correlations for **1** and **2**.

Table 1¹H and ¹³C NMR data for **1** and **2** (CDCl₃)

	Avicequinone D (1)		Avicequinone E (2)	
	¹ H NMR	¹³ C NMR	¹ H NMR	¹³ C NMR
1		178.5		<i>a</i>
2		151.0		159.7 ^{<i>b</i>}
3		132.1		126.1 ^{<i>b</i>}
4		179.9		180.6 ^{<i>b</i>}
4a		133.3		133.4 ^{<i>b</i>}
5	7.70 dd (1.2, 7.5)	120.1	7.59 dd (1.9, 7.4)	119.2
6	7.58 dd (7.5, 8.3)	136.2	7.57 dd (7.4, 7.6)	136.9
7	7.25 dd (1.2, 8.3)	125.2	7.19 dd (1.9, 7.6)	124.2
8		162.6		162.0
8a		114.8		114.3 ^{<i>b</i>}
1'	6.81 s	103.0	3.14 d (10.1)	28.4
2'		168.6	4.83 t (10.1)	92.4
3'		69.5		71.6
3' Me	1.67 s	28.8	1.24 s	24.2
3' Me	1.67 s	28.8	1.39 s	25.9
8-OH	12.04 s		11.64 s	

^{*a*}No signal observed in the ¹³C NMR and no HMBC correlation observed.^{*b*}Chemical shift determined by HMBC.