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New naphthoquinones and a new δ-lactone produced by endophytic fungi from Costa Rica

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Abstract

While searching for compounds with antimalarial activity, two new naphthoquinones, delitzchianones A (**1**) and B (**2**), were separated from *Delitzchia winteri*, an endophytic fungus from Costa Rica. The same search also led to a new 8-acetoxy pestalopyrone (**3**) and the known compound, pestalopyrone (**4**) from another Costa Rican endophytic fungus, *Phomatospora bellaminuta*. The structures of the three new compounds **1**, **2** and **3** were established with extensive NMR and MS analyses. All four compounds were tested for activity in a growth / no growth Dd2 assay, but only compound **4** had measurable activity with an IC_{50} value of 37 μM.

Keywords

endophytic fungi; anti-malaria; naphthoquinones; δ-lactones

While malaria poses a major public health burden – 300 to 500 million cases a year – to populations in tropical and subtropical areas around the world, the most affected areas are in southern Africa where the majority of the 1 to 1.5 million annual deaths occur. Four malaria parasite species affect humans, but *Plasmodium falciparum* is both the most widespread and most lethal. Four different compound classes have provided drugs that are currently useful for malaria, but the development of parasite strains resistant to all commonly used drugs has eroded our ability to combat the disease.1

In a continuing search for antimalarial natural products, endophytic fungi from Costa Rica, a biodiversity hot spot,2 have been examined. This report deals with two extracts (CR237A and CR1092F), which were obtained from the endophytic fungi *Delitzchia winteri* and *Phomatospora bellaminuta*, respectivly.3 Neither species had been examined previously, although flutimide, (Z)-5-(2-methylpropyl)-3-(2-methylpropylidene)-1-hydroxy-3H-pyrazine-2,6-dione, which has been shown to selectively inhibit cap-dependent endonuclease activity of influenza virus A, was isolated from *Delitschia cofertaspora*.4

CR237A was first fractionated over a C18 SPE column, and fraction III was further separated by C-18 prep-HPLC to yield compounds 1 and 2; Compounds 3 and 4 were

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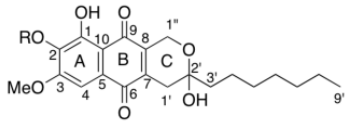
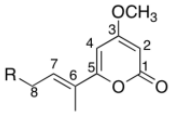
obtained from CR1092F after solvent partition and HPLC purification.5 Compound 4 was identified as pestalopyrone.6





Compound **1**7 was isolated as yellow powder. The HRMS (positive-ion mode) had an ion peak at *m/z* 387.1819, consistent with a molecular composition of $C_{22}H_{27}O_6$ ([M-H_2O+H]), calcd 387.1808). A molecular formula of $C_{22}H_{28}O_7$ requires nine double-bond equivalents. The ^{1}H NMR spectrum of **1** in $CDCl_3$ showed one chelated hydroxyl, one aromatic proton, two methoxys, eight methylenes, and one methyl group (Table 1). Its ^{13}C NMR spectrum exhibited twenty-two signals, including two carbonyls, eight sp2 carbons, one oxygenated sp3 quaternary carbon, two methoxys, and one methyl group. These were further confirmed by the HSQC. In the HMBC spectrum (Figure 1), the aromatic proton (H-4, δ_H 7.27 s) showed correlations to C-2 (δ_C 141.4), C-3 (δ_C 157.9), C-5 (δ_C 127.6), C-6 (δ_C 182.5), C-9 (δ_C 187.5, a weak ^5J correlation with a W shape), and C-10 (δ_C 110.7). On the other hand, the chelated hydoxyl proton (δ_H 12.05 s) correlated to C-1 (δ_C 155.7), C-2, and C-10, indicating that this chelated hydoxyl group (1-OH) was *para* to the aromatic proton (H-4). Also on the same ring (ring A), 2-OMe (δ_H 4.05 s) and 3-OMe (δ_H 3.99 s) exhibited HMBC correlations to C-2 and C-3, respectively. The methylene at δ_H 4.68/4.73 (d, J = 18.6 Hz, H_2-1″) showed correlations to C-7 (δ_C 141.3) and C-2′ (δ_C 96.0), and another methylene at δ 2.79/2.53 (d, J = 20.1 Hz, H_2-1′) correlated to C-2′, C-8 (δ_H 140.4) and C-6. From the above information, it could be deduced that ring C was a 2,3-dihydropyran and the oxygenated methylene (H_2-1″) was at the same side as the chelated hydroxyl group (1-OH). The low field chemical shift of the quaternary sp3 carbon C-2′ (δ 96.0) indicated that it must be a hemiacetal connected to a hydroxyl group and a heptyl, –$(CH_2)_6CH_3$. Hence, the structure of **1** was determined as shown.

Compound **2**8 was isolated as red powder, and had a molecular formula of $C_{22}H_{26}O_6$, which is 14 units less than **1**. A ^{1}H NMR spectrum in $CDCl_3$ was collected immediately after compound **2** was purified, and only one methoxy at δ_H 4.01 (s) was observed. The only difference between **1** and **2** was the substituent at the 2-position. Inspection of the 1D and 2D NMR spectra of **2** in MeOH-d_4 (Table 1) indicated that the compound consisted of two major tautomers (Figure 2), designated **2** (a *para* naphthoquinone) and **2α**a (an *ortho* naphthoquinone) in the approximate proportion of 7:6, indicating that compounds **2** was a 2-O-demethyl product of compound **1**. Hence, the structure of **2** was determined as shown.

Compound **3**9 was isolated as colorless powder. The NMR data of compound **3** were very similar to those of compound **4** except 8-position and the substituent at C-8. The chemical shifts of H-8 (4.77 d J = 6.0 Hz) and C-8 (60.8) indicated that there was an acetoxy group at 8-position (δ_H 2.04 s/δ_C 21.0 and δ_C 169.5). Hence, compound **3** was determined as 8-acetoxy pestalopyrone.

The two new naphthoquinones, **1** and **2,** and the two δ-lactones, **3** and **4**, were evaluated in *a Plasmodium falciparum* (Dd2) assay,10 but only **4** showed marginal activity against Dd2 with an IC_{50} value of 37 μM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References and notes

1. Na-Bangchang K, Karbwang J. Fundamental Clin. Pharmacol. 2009; 23:387–409.
2. a Cao S, Ross L, Tamayo G, Clardy J. Org. Lett. 2010 in press (DOI: 10.1021/ol101972g). b Kontnik R, Clardy J. Org. Lett. 2008; 10:4149–4151. [PubMed: 18698786]
3. **Sequencing and species identification.** For identification by internal transcribed spacer (ITS) sequencing, CR237A and CR1092F were cultured in potato dextrose broth for 5 days. The mycelium was then retrieved by filtration and ground to a fine powder in liquid N2. Genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega), and large subunit rDNA was amplified by PCR using primers LR5 (5′-TCCTGAGGGAAACTTCG-3′) and LROR (5′-ACCCGCTGAACTTAAGC-3′). PCR products were transformed into *E. coli* TOP10 cells using a TOPO TA Cloning Kit (Invitrogen), according to manufacturer's protocols. Transformed plasmids were isolated and sequenced at Genewiz (http://www.genewiz.com/). The following consensus sequence were used in a BLAST search against deposited sequences:
237A:CCCCTATGCCCAAATTTGACGATCGATTTGCACGTCAGAACCGCTGCGAGCCTCC
ACCAGAGTTTCCTCTGGCTTCACCCTATTCAGGCATAGTTCACCATCTTTCGGGTCCCA
ACAGCTATGCTCTTACTCAAATCCATCCGAAGACATCAGGATCGGTCGATGGTGCGCC
AGAGCTCGCGCCCTGGGTCCCACCTCCGTTCACTTTCATTCCGCGCCCGGGCTTGACAC
CCAAACACTCGCATAGATGTTAGACTCCTTGGTCCGTGTTTCAAGACGGGCCGCTTACG
ACCATTACGCCAGCATCCTAGCCGAAGCGCGGACCTCAGTCGGGGCTGGCTGCATGAC
GCCCTGGGCTATAACACTCCCCGAAGAGAGCTACATTCCCAAGGCCTTTCTCCAGCCGC
CCCAACTGATGCTGGCCTGCCTGCCGCCGAGTGCACAGGGGACGGACCCCCGATGAAC
AGCGGCAGCCAAGTCTGGTTGCAAGCGCTTCCCTTTCAACAATTTCACGTGCTGTTTGA
CTCTCTTTCCAAAGTGCTTTTCATCTTTCGATCACTCTACTTGTGCGCTATCGGTCTCTG
GCCAGTATTTAGCTTTAGAAGAAATATACCTCCCATTTAGAGCTGCATTCCCAAACAAC
TCGACTCGTCGAAGGGGGTTCACATGGCGCAGGCACCTGCCGCGTACGGGGTTCTCAC
CCTCTCTGACGTCCCGTTCCAAGGAACTTAGACAGGCGNCGTTGCCGAACCACCNTCTG
CAAAGTACAACTCGGANCCCGCAAGGAGCCAGATTTCAAATTTGAGCTGTTGCCGCTT
CACTCGCCGTTACTGAGGCAAT
CR1092F:AGAGGTTGATAGTCTTTCGCCCCCATGCTCATGTTTGACGATCGATTTGCACG
TCAGAACCGCTGCGAGCCTCCACCAGAGTTTCCTCTGGCTTCACCCTACACAAGCATAG
TTCACCATCTTTCGGGTCCAAGCGGCAAGGCTCTTACTCAAATCCATCCGAAGACTTCA
GGATCGGTCGATGGTGCGCCGAGGCTCCCACCTACGTTCACTTTCATTTCGCGTGCGGG
TTTTACACCCAAACACTCGCCCTAATGCTTGACTCCTTGGTCCGTGTTTCAAGACGGGT
CGCTGGTGACCATTACGCCAGCATCCTTGCAATGCGCGGTCCTCGGTCCCCGCGAGGGC
ATTGAGCAACGGGCTATAACACTCCCGGAGGAGCCACATTCCCGAGGCCTTTATCCCC
CCGCGAGAACCGATGCTGGCCCGAGCCCGGCGGAGTGCACCGGCGAGAACGCCGGAT
GATCCGCCGGGCGCGAGTCTGGTCACAGGCGCTTCCCTTTCAACAATTTCACGTGCTTT
TTAACTCTCTTTTCAAAGTGCTTTTCATCTTTCGATCACTCTACTTGTGCGCTATCGGTCT
CTGGCCGGTATTTAGCTTTAGAAGAAATTTACCTCCCGCTTTGAGCAGCATTCCCAAAC
TACTCGACTCGTCGAAGGAGCTTTACAGAGGCTCGGCGTCCGCCTGTACGGGGCTCTCA
CCCTCTATGGCGTCCCGTTCCAGGGAACTCGGACGGCGCCTTGCCAAAAGCATCCTCTA
CAGATTACAACTCGGGCCCTGGGGACCAGATTTCAAATCTGAGCTGTTGCCGCTTCACT
CGCCGTTACTGGGGCAATCCCTGTTGGTTTCTTTTCCTCCGCTTATTGATATGGTTAGTT
TCAANCGGGGTAA
4. Singh SB, Tomassini JE. J. Org. Chem. 2001; 66:5504–5516. [PubMed: 11485475]
5. **Culturing and extraction.** Agar plugs of CR237A and CR1092F were initially grown at 25°C on yeast malt agar plates supplemented with 30 μg/ml streptomycin and 12 μg/ml chlortetracycline. After one week, agar plugs of this plate were placed in 150 ml of rich seed media in 1 L flasks. They were grown at 25 °C and 150 rpm for 7 days. 450 ml of 0.66% (w/v) malt extract and 10 g HP-20 resin were then added to each flask, and the fungi were cultured under the same conditions for 21 days. The fungal cultures were then held at 25 °C without shaking for 5 days. Extraction of

the mycelium was accomplished by three rounds of sonication in ethanol. Rich seed media: 5 g peptone, 10 g dextrose, 3 g yeast extract, 10 g malt extract per 1 L water (pH 6.2). **Separation.** Extract CR237A was loaded on C-18 SPE and three fractions were collected. Compounds **1** (t_R: 33.0 min, 1.5 mg) and **2** (t_R: 28.5 min, 1.0 mg) were collected after a C-18 HPLC column (250 × 21.2 mm, 5 μ, 10 ml/min, 80% MeOH for 20 min then to 100% MeOH in 10 min) from fraction 3. Extract CR1092F was was suspended in aqueous MeOH (MeOH-H_2O, 9:1, 100 mL) and extracted with hexanes (3 × 100 mL portions). The aqueous layer was then diluted to 70 % MeOH with H2O and extracted with CH2Cl2 (3 × 100 mL portions). After C-18 SPE, the hexanes extract was separated using HPLC to yield compound **3** (t_R: 8 min, ~0.1 mg; C-18, Phemonenex, Luna, 250 × 10 mm, 5 μ, 4 ml/min, 45% MeOH) and compound **4** (t_R: 9.5 min, 2.0 mg; C-18, Phemonenex, Luna, 250 × 10 mm, 5 μ, 2 ml/min, 60~100% MeOH in 20 min).

6. Venkatasubbaiah P, Van Dyke CG, Chilton WS. Phytochemistry. 1991; 30:1471–1474.

7. **Delitzchianone A (1)**: yellow powder; $[\alpha]^{26}_D$ +120 (*c*, 0.06 MeOH); UV (MeOH) λ_{max} 215, 260, 295 (sh), 422 nm; ^{1}H NMR (600 MHz, CD_3OD): see Table 1; ^{13}C NMR (150 MHz, CD_3OD): see Table 1; HRMS *m/z* 387.1819 ([M-H_2O+H]), calcd for $C_{22}H_{27}O_6$, 387.1808).
8. **Delitzchianone B (2)**: red powder; $[\alpha]^{26}_D$ +161 (*c*, 0.08 MeOH); UV (MeOH) λ_{max} 222, 273, 300 (sh), 428 nm; ^{1}H NMR (600 MHz, $CDCl_3$ and CD_3OD): see Table 1; ^{13}C NMR (150 MHz, CD3OD): see Table 1; HRMS *m/z* 373.1719 ([M-H_2O+H]), calcd for $C_{21}H_{25}O_6$, 373,1651).
9. **8-Acetoxy pestalopyrone (3)**: colorless powder; UV (50% MeOH/H_2O) λ_{max} UV (50% MeOH/H_2O) λ_{max} 310 nm; ^{1}H NMR (600 MHz, DMSO-d_6): δ_H 1.88 s (H_3-9), 2.04 s (OAc), 3.82 s (OMe), 4.77 d *J* = 6.0 Hz (H_2-8), 5.65 br s (H-4), 6.31 br s (H-2), 6.35 t *J* = 6.0 Hz (H-7); ^{13}C NMR (150 MHz, DMSO-d_6): 12.7 (C-9), 21.0 (OCO$\underline{C}H_3$), 56.8 (OMe), 60.8 (C-8), 89.1 (C-4), 99.3 (C-2), 127.6 (C-6), 127.6 (C-6), 158.1 (C-5), 169.5 (O$\underline{C}$OCH_3), 170.1 (C-3), 173.8 (C-1); HRMS *m/z* 239.0925 ([M+H]), calcd for $C_{12}H_{15}O_5$, 239.0920).
10. **Antimalarial Screen.** The antimalarial screen was carried out as previously described.2 Briefly, extracts dissolved in DMSO were arrayed in dilution series in 384-well plates. The solutions were pintransferred to assay plates containing red blood cells parasitized with *Plasmodium falciparum*. Afluorescent DNA stain (DAPI) was added after a 72-hour incubation period, and the plates imaged to quantify levels of parasitic nuclei.

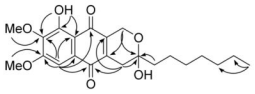

Figure 1.
HMBC Correlations of **1**

Figure 2.
Tautomerization of **2**

Table 1

^{1}H and ^{13}C NMR Data of Compounds **1** and **2**

#	**1** (^{1}H)[a]	**2** (^{1}H)[a]	**2** (^{1}H)[b]	#	**1** (^{13}C)[c]	**2** (^{13}C)[d]
1				1	155.7	154.7
2				2	141.4	142.7
3				3	157.9	154.4
4	7.27 (s)	7.27 (s)	7.25/7.27 (s)	4	104.3	107.3/107.7
5				5	127.6	129.5/130.5
6				6	182.5	182.8
7				7	141.3	140.8
8				8	140.4	140.8
9				9	187.5	189.9
10				10	110.7	111.9
1′	2.79 (d 20.1)	2.78 (d 19.8)	2.63/2.67 (d 19.8)	1′	31.7	32.3
	2.53 (d 20.1)	2.52 (d 19.8)	2.41/2.43 (d 19.8)			
2′				2′	96.0	96.8
3′	1.80 (m)	1.77 (m)	1.73 (m)	3′	42.2	42.8
4′	1.2~1.6 (m)	1.2~1.6 (m)	1.2~1.6 (m)	4′	22.6	23.7
5′	1.2~1.6 (m)	1.2~1.6 (m)	1.2~1.6 (m)	5′	30.6	31.0
6′	1.2~1.6 (m)	1.2~1.6 (m)	1.2~1.6 (m)	6′	29.7	30.8
7′	1.2~1.6 (m)	1.2~1.6 (m)	1.2~1.6 (m)	7′	29.2	30.4
8′	1.2~1.6 (m)	1.2~1.6 (m)	1.2~1.6 (m)	8′	23.0	24.5
9′	0.90 (t 6.6)	0.88 (t 6.6)	0.91 (t 6.6)	9′	14.1	14.4
1″	4.73 (d 18.6)	4.71 (d 18.6)	4.59 (m)	1″	56.5	56.5
	4.68 (d 18.6)	4.66 (d 18.6)				
1-OH	12.05	11.90 (s)				
2-OCH_3	4.05 (s)			2-OCH_3	61.0	
3-OCH_3	3.99 (s)	4.01 (s)	3.94/3.90 (s)	3-OCH_3	57.4	58.1

[a] δ (ppm) 500 MHz in $CDCl_3$; multiplicities; *J* values (Hz) in parentheses.

[b] δ (ppm) 500 MHz in CD_3OD; multiplicities; *J* values (Hz) in parentheses.

[c] δ (ppm) 150 MHz in $CDCl_3$.

[d] δ (ppm) 150 MHz in CD_3OD.