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ORIGINAL ARTICLE

Bioactive anthraquinone dimers from the leafhopper pathogenic fungus *Torrubiella* sp. BCC 28517

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Torrubiellins A (1) and B (2), two new dimeric anthraquinones were isolated from the leafhopper pathogenic fungus *Torrubiella* sp. BCC 28517. The structures of the new compounds were elucidated by analyses of the NMR spectroscopic and mass spectrometry data. Torrubiellin B (2) exhibited broad range of biological activities.
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INTRODUCTION

Torrubiella is a genus of arthropod-pathogenic fungi that attacks spiders, scale-insects and hoppers.[1] Recently, a reclassification of the genus *Torrubiella* (family Clavicipitaceae) based on a multi-gene phylogeny was reported.[1] Three scale insect pathogens *T. luteorostrata*, *T. tenuis* and *T. petcii* were assigned to the newly described genera *Conoideocrella* and *Orbiocrella*, while spider-pathogenic species (largest group) and hopper pathogens (minor group) were retained as *Torrubiella*, but they were assigned to family Cordycipitaceae. Two scale insect pathogenic species, *Conoideocrella luteorostrata* (formerly *T. luteorostrata*) and *C. tenuis* (formerly *T. tenuis*) have recently been investigated as sources of novel bioactive compounds, such as paecilodepsipeptides[2] and conoideocrellides[3] (hexadepsipeptides), luteorides[4] (prenylated tryptophan analogs), isocoumarin glucosides,[3,5] bioxanthracenes,[3] and hopane-type triterpenes.[3] In contrast, there has been only one report on bioactive compounds from the spider-pathogenic *Torrubiella*, torrubiellones A–D (pyridone and tetramic acid derivatives),[6] and no report on leafhopper pathogens. As part of our research program on novel bioactive compounds from arthropod-pathogenic fungi, we selected a leafhopper-pathogenic *Torrubiella* sp., strain BCC 28517, for large-scale fermentation and chemical studies. A mycelial extract of this strain exhibited cytotoxicity to oral cavity cancer cells (KB) and small-cell lung cancer cells (NCI-H187) with respective IC_{50} values of 1.66 and 0.79 $\mu g\,ml^{-1}$. We report here the isolation, structure elucidation and biological activities of two new anthraquinone dimers, torrubiellins A (**1**) and B (**2**), together with the known anthraquinones, chrysophanol (**3**),[7,8] aloe-emodin (**4**)[7] and emodin (**5**) (Figure 1).[7,9]

RESULTS AND DISCUSSION

Torrubiellin A (**1**) was obtained as a dark-brown solid, and the molecular formula was established as $C_{30}H_{20}O_8$, from the protonated *quasi*-molecular ion peak in the HRESIMS. The ^{13}C NMR, DEPT135 and HMQC spectroscopic data indicated the presence of 30 carbons categorized as three conjugated ketones (δ_C 192.5, 190.1 and 183.1), fourteen quaternary sp^2 carbons (δ_C 172.3–102.6), eight sp^2 methines (δ_C 142.4–119.0), an oxygenated methine (δ_C 71.9), an aliphatic quaternary carbon, a methine, a methylene and a methyl group (Table 1). In addition, the ^1H NMR spectrum showed the presence of four chelated phenolic hydroxy groups. The structure of the A-ring was deduced from the COSY correlations of three aromatic protons H-5/H-6/H-7, and the HMBC correlations from these protons and a chelated phenolic OH (δ_C 12.12, 8-OH) to carbons of the trisubstituted benzene (A-ring) (Figure 2). Intense HMBC correlation from H-5 to a conjugated ketone carbon at δ_C 183.1 (C-10) required the connection of this ketone to C-10a. The presence of a chelated OH (8-OH) suggested that C-8a was also connected to a ketone (C-9, δ_C 192.5 or 190.1). The structure of the CDEFG-ring system was established on the basis of the HMBC correlations. Thus, HMBC correlations from 1′-OH, H-2′, H-4′ and H_3-11′ to carbons of the tetrasubstituted benzene revealed the G-ring. HMBC correlations from an oxymethine H-10′ to C-4′, C-9a′, C-5′, C-8a′ and C-10a′, and the correlations from H-4′ and H-5′ to the methine carbon (C-10′) indicated that this methine linked G-ring (C-4a′) and E-ring (C-10a′). The chelated hydroxy groups, 1′-OH and 8′-OH, requested the presence of a ketone (δ_C 190.1 or 192.5, C-9′) functionality to link C-9a′ and C-8a′. The remaining pentasubstituted benzene (C-ring) was suggested based on the HMBC correlations from H-2 to C-1, C-2 and C-4, and the correlations from 1-OH to C-1, C-2 and C-9a, although no correlation to the quaternary sp^2 carbon at δ_C 129.5 (C-4a) was observed. The connection of C-4 (C-ring) and C-5′ (E-ring) was revealed by the HMBC correlations from H-5′ and H-6′ to C-4. HMBC correlations from nonequivalent methylene protons H_2-11 to C-2, C-3, C-4, C-5′, C-8a′, C-10′ and C-10a′ demonstrated that this

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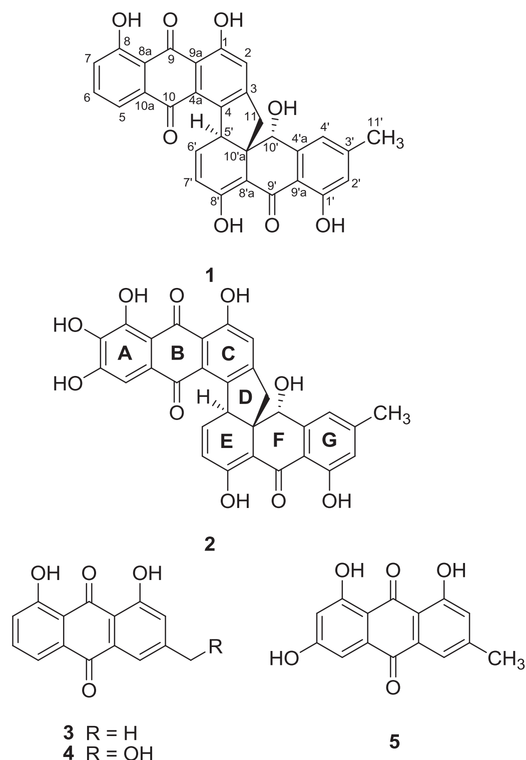

Figure 1 Structures of compounds 1–5.

methylene group linked C-3 and C-10a′ to constitute the D-ring. On the basis of these data, two fragments, A-ring (attached with two ketone carbons) and CDEFG-ring, covering all atoms required from the molecular formula. Two possible linkage patterns to constitute the ABC-ring, 1,8-dihydroxyanthraquinone and 1,5-dihydroxyanthraquinone, were proposed. The latter structure should be ruled out due to the large chemical shift differences of the two ketone carbons; δ_C 183.1 (C-9) and δ_C 192.5 or 190.1 (C-10). These carbon chemical shifts were consistent with those of monomeric 1,8-dihydroxyanthraquinone derivatives, such as **4** (C-9 δ_C 191.5, C-10 δ_C 181.4, in DMSO-d_6).[8] The relative configuration of **1** was assigned on the basis of the NOESY correlations (Figure 3). The cross-peak for H-2/H_α-11 (δ_H 2.89) was much more intense than that for H-2/H_β-11 (δ_H 3.07). Intense NOESY correlations H-10′/H_α-11 and H-10′/H-5′ demonstrated the relative configuration of the three contiguous chiral carbon centers C-10′, C-10a′ and C-5′ (Figure 3).

The molecular formula of torrubiellin B (**2**) was determined by HRESIMS as $C_{30}H_{20}O_{10}$, which has two more oxygen atoms than **1**. The ^{1}H and ^{13}C NMR spectra suggested close structural resemblance to **1**, although the ^{1}H NMR spectrum of **2** in DMSO-d_6 (used due to the solubility reason) did not exhibit any peaks for phenolic OH. Interpretation of the 2D NMR (COSY, HMQC and HMBC) data revealed that the BCDEFG-ring structure was identical to **1**. The significant differences were the ^{1}H and ^{13}C NMR spectroscopic data for the A-ring, consisting of a methine (δ_H 7.27, s; δ_C 110.0) and five quaternary carbons. The methine proton was assigned to H-5 on the basis of its HMBC correlations to four quaternary carbons (C-10a, C-6, C-7 and C-8a) and C-10 (δ_C 182.6). In addition, a weak ^4J correlation from H-5 to C-9 (δ_C 190.9) further supported the anthraquinone structure. Although HMBC correlation to C-8 was absent, the chemical shifts of C-6 (δ_C 153.6), C-7 (δ_C 139.9) and C-8 (δ_C 151.8) were consistent with the 6,7,8-trihydroxy functionalities. The NOESY spectrum of **2** exhibited similar key correlations as **1**, H-2/H_α-11, H-10′/H_α-11 and H-10′/H-5′, which indicated the identical relative configuration. Torrubiellin B (**2**) was therefore identified as the 6,7-dihydroxy derivative of **1**.

The co-occurrence of anthraquinone monomers **3–5** strongly suggested that **1** and **2** were biogenetically derived from two anthraquinones (ABC-ring and EFG-ring). Although a variety of dimeric anthraquinones have been reported as fungal secondary metabolites, the linkage pattern as shown for torrubiellins, condensation at C-4–C-5′ and C-11–C-10a′, is quite rare. The only but closely related compounds are uredinorubellins I and II, which were isolated from the plant pathogenic fungus *Ramuralia uredinicola*.[10] Uredinorubellin II is the 8′,8′a-dihydro-8′a-hydroxy derivative of **1**, while uredinorubellin I possesses additionally 6-OH functionality. Rubellins A–D, isolated from *Ramuralia collo-cygni* and reported before uredinorubellins, are F-ring modified (degraded) analogs probably derived from uredinorubellins.[10–12]

As torrubiellins A and B possess a rare chemical skeleton, they were subjected to several bioassay protocols in our research center (Table 2). Torrubiellin B (**2**) exhibited higher biological activity than **1** in all the tests. Although torrubiellin B (**2**) exhibited significant antimalarial (*Plasmodium falciparum*), antifungal (*Candida albicans*) and antibacterial (*Bacillus cereus*) activities, it also showed comparable magnitude of cytotixicity to cancer cell lines. Related compounds, uredinorubellins I and II, are reported to exhibit photodynamic activity influencing cell viability in three mammalian cell lines, HIG82, HT29 and J774A.1, as well as antibacterial activity against *Staphylococcus aureus*.[10]

METHODS

General experimental procedures

Melting points were measured with an IA9100 digital melting point apparatus (Electrothermal, Essex, UK). Optical rotations were measured with a P-1030 digital polarimeter (JASCO, Tokyo, Japan). UV spectra were recorded on a Cintra 404 spectrophotometer (GBC Scientific Equipment, Braeside, VIC, Australia). FTIR spectra were taken on an ALPHA spectrometer (Bruker, Bremen, Germany). NMR spectra were recorded on DRX400 and AV500D spectrometers (Bruker). ESITOF mass spectra were measured with a micrOTOF mass spectrometer (Bruker).

Fungal material

The fungus used in this study was isolated from a leafhopper (Hemiptera) collected in Khlong Lan National Park, Kamphaeng Phet Province, Thailand, by one of the authors (KT). This fungus was deposited in the BIOTEC Culture Collection (BCC) on 27 November 2007 as BCC 28517. On the basis of the sequence data of the ITS rDNA, the strain was assigned to the genus *Torrubiella* within the family Cordycipitaceae.[1]

Fermentation and isolation

The fungus BCC 28517 was maintained on potato dextrose agar at 25 °C. The agar plugs (1 × 1 cm) were cut into small pieces and inoculated into 4 × 250 ml Erlenmeyer flasks containing 25 ml of potato dextrose broth (potato starch 4.0 g l^{-1}, dextrose 20.0 g l^{-1}) and incubated at 25 °C for 4 days on a rotary shaker (200 r.p.m.). Each primary seed culture was transferred into a 1 l Erlenmeyer flask containing 250 ml of potato dextrose broth, and incubated at 25 °C for 4 days on a rotary shaker (200 r.p.m.). Each 25 ml portion of the seed culture was added into 40 × 1 l Erlenmeyer flasks containing 250 ml of M102 medium (sucrose 30 g l^{-1}, malt extract 20 g l^{-1}, Bacto-peptone 2.0 g l^{-1}, yeast extract 1.0 g l^{-1}, KCl 0.5 g l^{-1}, $MgSO_4 \cdot 7H_2O$ 0.5 g l^{-1} and KH_2PO_4 0.5 g l^{-1}), and the final fermentation was carried out at 25 °C for 15 days on rotary shakers (200 r.p.m.). The culture was filtered to separate broth (filtrate) and mycelia (residue). The filtrate was extracted with EtOAc (2 × 6 l), and the combined organic layer was concentrated under reduced pressure to obtain a dark-brown gum (2.36 g, broth extract). The wet mycelia were macerated in

Table 1 NMR data for 1 ($CDCl_3$, 500 MHz) and 2 (DMSO-d_6, 400 MHz)

Position	Torrubiellin A (**1**)			Torrubiellin B (**2**)		
	δ_C, mult.	δ_H, mult. (J in Hz)	HMBC	δ_C, mult.	δ_H, mult. (J in Hz)	HMBC
1	163.3, qC			162.5, qC		
2	120.9, CH	7.13, s	1, 4, 9a, 11	121.0, CH	7.21, s	1, 4, 9a, 11
3	154.0, qC			153.8, qC		
4	137.3, qC			136.9, qC		
4a	129.5, qC			129.9, qC		
5	120.0, CH	7.82, d (8.1)	7, 8a, 10	110.0, CH	7.27, s	6, 7, 8a, 9, 10, 10a
6	137.2, CH	7.72, t (8.1)	8, 10a	153.6, qC		
7	124.6, CH	7.32, d (8.3)	5, 8, 8a	139.9, qC		
8	162.4,[a] qC			151.8, qC		
8a	115.8, qC			110.3, qC		
9	192.5,[b] qC			190.9, qC		
9a	115.2, qC			115.6, qC		
10	183.1, qC			182.6, qC		
10a	133.8, qC			125.8, qC		
11	42.2, CH_2	3.07, dd (17.4, 1.2)	2, 3, 4, 8a′, 10′, 10a′	42.2, CH_2	2.93, d (17.5)	3, 8a′, 10′, 10a′
		2.89, d (17.4)	2, 3, 4, 5′, 10′, 10a′		2.77, d (17.5)	2, 3, 4, 5′, 10′, 10a′
1′	162.4,[a] qC			161.6, qC		
2′	119.0, CH	6.78, s	1′, 4′, 9a′, 11′	118.1, CH	6.76, s	1′, 4′, 9a′, 11′
3′	148.1, qC			148.3, qC		
4′	121.9, CH	6.53, s	2′, 4a′, 9a′, 10′, 11′	123.0, CH	6.63, s	2′, 4a′, 9′, 9a′, 10′, 11′
5′	46.6, CH	5.48, dd (2.9, 2.6)	3, 4, 11, 6′, 7′, 10′, 10a′	46.7, CH	5.34, dd (2.8, 2.5)	3, 4, 11, 6′, 7′, 10′, 10a′
6′	142.4, CH	6.48, dd (10.0, 2.6)	4, 8′, 10a′	144.2, CH	6.45, dd (10.0, 2.5)	5′, 8′, 10a′
7′	123.0, CH	6.16, dd (10.0, 2.9)	5′, 8a′	122.9, CH	6.16, dd (10.0, 2.8)	5′, 8a′
8′	172.3, qC			171.7, qC		
8a′	102.6, qC			104.7, qC		
9′	190.1,[b] qC			191.0, qC		
9a′	111.5, qC			112.3, qC		
10′	71.9, CH	3.89, br s	4′, 5′, 8a′, 9a′, 10a′	71.0, CH	3.74, s	11, 4′, 4a′, 5′, 8′, 8a′, 9a′, 10a′
11′	22.0, CH_3	2.32, s	2′, 3′, 4′	22.2, CH_3	2.28, s	2′, 3′, 4′
1-OH		12.62, s	1, 2, 9a		—[c]	
8-OH		12.12, s	7, 8, 8a		—[c]	
1′-OH		11.95, s	1′, 2′, 9a′		—[c]	
8′-OH		15.05, s	7′, 8′, 8a′		—[c]	

[a]The carbon resonances were superimposed.
[b]Assignment of carbons can be interchanged.
[c]The resonances of these phenolic protons were not observed.

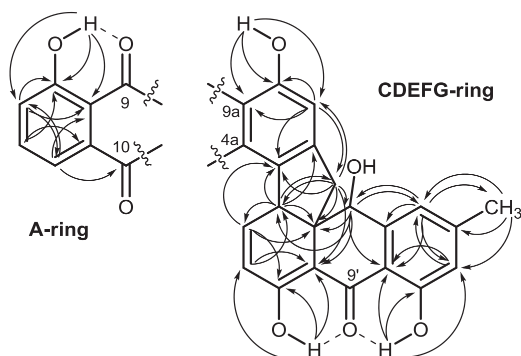

Figure 2 HMBC correlations for **1**.

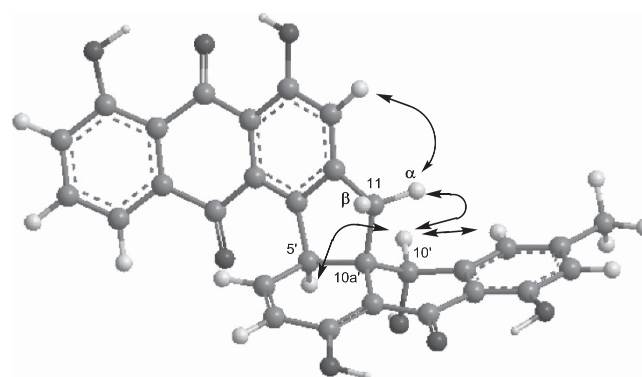

Figure 3 Key NOESY correlations for **1**.

MeOH (1 l, RT, 2 days) and filtered. Hexanes (800 ml) and H_2O (40 ml) were added to the filtrate, and the layers were separated. The aqueous MeOH layer was concentrated by evaporation, and H_2O (150 ml) was added to the residue, which was then extracted with EtOAc (2 × 1.2 l). The EtOAc solution was concentrated under reduced pressure to obtain a brown gum (3.66 g, mycelial extract). The mycelial extract was triturated in MeOH (2 ml)/CH_2Cl_2 (5 ml) and filtered. The filtrate was concentrated to leave a brown gum (3.50 g), which was subjected to fractionation by column chromatography on Sephadex LH-20

Table 2 Biological activities of 1 and 2

	1	*2*
Cytotoxicity (IC$_{50}$, μg ml^{-1})[a]		
KB	2.78	0.48
NCI-H187	7.05	0.20
MCF-7	16.9	3.20
Antimalarial activity (IC$_{50}$, μg ml^{-1})[b]		
Plasmodium falcipatum K1	3.10	0.33
Antitubercular activity (MIC, μg ml^{-1})[c]		
Mycobacterium tuberculosis H37Ra	>50	>50
Antifungal activity (IC$_{50}$, μg ml^{-1})[d]		
Candida albicans	>50	1.66
Antibacterial activity (MIC, μg ml^{-1})[e]		
Bacillus cereus	>50	6.25

[a]The IC$_{50}$ values of a standard compound, doxorubicin hydrochloride, against KB (oral epidermoid carcinoma), NCI-H187 (small-cell lung cancer) and MCF-7 (breast cancer) cells were 0.30, 0.045 and 6.47 μg ml^{-1}, respectively.
[b]Standard antimalarial compounds dihidroartemisinin and mefloquine hydrochloride showed IC$_{50}$ values of 0.61 ng ml^{-1} and 0.013 μg ml^{-1}, respectively.
[c]The MIC values of isoniazid (standard anti-TB drug) were 0.023–0.047 μg ml^{-1}.
[d]Amphoteric B was used as a standard compound (IC$_{50}$ 0.072 μg ml^{-1}).
[e]Vancomycin hydrochloride was used as a standard compound (MIC 4.0 μg ml^{-1}).

(3.5 × 50 cm, MeOH) to obtain four pooled fractions, wherein fractions 3 (20.9 mg) and 4 (34.2 mg) were identified as **2**. Fraction 2 (240 mg) was subjected to preparative high performance liquid chromatography (Grom-Sil 120 ODS-4 HE, 20 × 150 mm, 5 μm; isocratic elution with MeCN/H_2O = 65:35, 0–13 min, then MeCN/H_2O = 90:10; flow rate 8 ml min^{-1}) to furnish **4** (3.4 mg, t_R 7 min), **5** (2.6 mg, t_R 10 min), **2** (27.2 mg, t_R 12 min), **3** (13.9 mg, t_R 15 min) and **1** (6.1 mg, t_R 20 min). The broth extract was also fractionated using similar procedures to obtain **2** (12.5 mg), **3** (2.3 mg) and **1** (2.0 mg).

*Torrubiellin A (**1**):* dark-brown solid; m.p. 215–217 °C; $[\alpha]_D^{25}$ +148 (*c* 0.075, $CHCl_3$); UV/vis (MeOH) λ_{max} (log ε) 227 (4.45), 253 (4.21), 277 sh (4.01), 289 sh (3.93), 396 (3.96), 416 (3.97), 438 sh (3.89), 463 sh (3.78) nm; IR (ATR) ν_{max} 1626, 1572, 1281 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) data, see Table 1; HRMS (ESI-TOF, positive) *m/z* 509.1228 $[M+H]^+$ (calculated for $C_{30}H_{21}O_8$, 509.1231).

*Torrubiellin B (**2**):* dark-brown solid; m.p. 251–253 °C (dec.); $[\alpha]_D^{27}$ +189 (*c* 0.025, $CHCl_3$); UV/vis (MeOH) λ_{max} (log ε) 218 (4.40), 287 (4.31), 395 (4.20), 417 (4.18), 460 sh (3.98) nm; IR (ATR) ν_{max} 3424, 1623, 1588, 1572, 1275 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6) and ^{13}C NMR (100 MHz, DMSO-d_6) data, see Table 1; HRMS (ESI-TOF, negative) *m/z* 539.0971 $[M-H]^-$ (calculated for $C_{30}H_{19}O_{10}$, 539.0984).

Biological assays

Cytotoxic activities against human cancer cell lines (KB, NCI-H187 and MCF-7), antifungal (*Candida albicans*) and antibacterial (*Bacillus cereus*) activities were evaluated using the resazurin microplate assay.[13] Antiplasmodial activity (*Plasmodium falciparum* K1, multi-drug resistant strain) was performed using the microculture radioisotope technique.[14] Antimycobacterial activity (*Mycobacterium tuberculosis* H37Ra) was performed using the green fluorescent protein microplate assay.[15]

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)