Pyridone and Tetramic Acid Alkaloids from the Spider Pathogenic Fungus *Torrubiella* sp. BCC 2165

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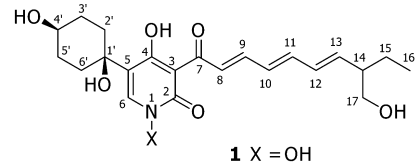
Torrubiellones A−D (**1**−**4**), new pyridone and tetramic acid alkaloids, were isolated from the spider pathogenic fungus *Torrubiella* sp. BCC 2165. Torrubiellone A (**1**) exhibited antimalarial activity with an IC_{50} value of 8.1 μM, while it showed very weak cytotoxic activity.

Torrubiella is a genus of arthropod-pathogenic fungi that primarily attacks spiders and scale insects.[1] While some other genera of arthropod-pathogenic fungi have been investigated as sources of novel bioactive compounds,[2−4] relatively few reports describe chemical investigations of *Torrubiella*; compounds reported include paecilodepsipeptide A and a naphthopyrone glucoside from *T. luteorostrata* BCC 9617,[5] torrubiellutins A−C from *T. luteorostrata* BCC 12904,[6] and isocoumarin glucosides from *T. tenuis* BCC 12732.[7] Very recently, a reclassification of the genus *Torrubiella* (family Clavicipitaceae) based on a multigene phylogeny was reported.[1] Two scale insect pathogens, *T. luteorostrata* and *T. tenuis*, were assigned to a newly proposed genus *Conoideocrella* (family Clavicipitaceae), while most of the spider-pathogenic species were retained as *Torrubiella*, but assigned to the family Cordycipitaceae. To our knowledge, there has been no report on compounds from the latter fungal sources. As part of our research program on arthropod-pathogenic fungi,[3] we examined the secondary metabolite profiles of 16 strains of spider-pathogenic *Torrubiella* species, which were collected at various locations in Thailand. Among these, an extract from *Torrubiella* sp. BCC 2165 showed a unique ^{1}H NMR spectroscopic profile. This latter strain was selected for large-scale fermentation and analysis. We report here the isolation and structure elucidation of four new alkaloids, torrubiellones A−D (**1**−**4**).



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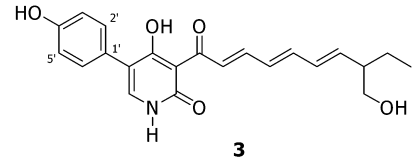
2 X = H



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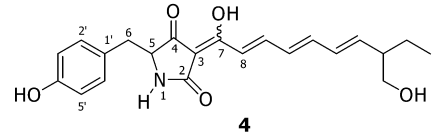

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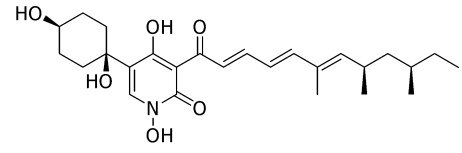

militarinone A

Torrubiellone A (**1**) was obtained as a yellow, amorphous substance, and the molecular formula was established as $C_{22}H_{29}NO_7$ by HRESIMS. The IR spectrum exhibited absorption maxima at 3418, 1716, and 1645 cm^{-1}. The ^{13}C NMR, DEPT135, and HMQC spectroscopic data indicated the presence of 22 carbons categorized as a conjugated ketone resonating at δ_C 193.8, three quaternary sp^2 carbons at δ_C 173.9, 157.1, and 117.9, seven sp^2 methines, an oxygenated quaternary carbon, an oxygenated methine, an oxygenated methylene, a methine at δ_C 47.9, two pairs of equivalent methylenes at δ_C 33.9 × 2 and 30.9 × 2, a methylene at δ_C 23.7, and a methyl group (Tables 1 and 2). The COSY data revealed the presence of a conjugated triene (CH-8−CH-13). The all-*E*-geometry was evident from the vicinal coupling constants of 15.0, 14.8, and 15.2 Hz, respectively for $J_{8,9}$, $J_{10,11}$, and $J_{12,13}$. The HMBC correlations from H-8 and H-9 to the ketone (δ_C 193.8) indicated that the triene was attached to the ketone (C-7). The COSY data implied that the other side of the triene (C-13) was connected to a methine (δ_H 2.22, m; δ_C 47.9; CH-14), which was further linked to a hydroxymethyl group and an ethyl group; from this, the side-chain structure (C-7−C-17) was established. The COSY and HMBC data also revealed that the other aliphatic moiety, two pairs of equivalent methylenes (C-2′/C-6′ and C-3′/C-5′), a secondary alcohol (CH-4′), and a tertiary alcohol (C-1′) constitute a 1,4-dihydroxycyclohexyl group. The rest of the four sp^2 carbons were assigned to a heteroaromatic ring. The downfield-shifted OH (δ_H 17.45, br s) showed HMBC correlations to quaternary carbons at δ_C 173.9 (C-4), 117.9 (C-5), and 105.5 (C-3). The chemical shifts of this chelated OH and the sp^2 carbons C-3, C-4, and C-7 strongly suggested that these three carbons formed a keto−enol system. HMBC correlations from the sp^2 methine at δ_H 8.16 (s, H-6) to C-4, tertiary alcohol carbon of the cyclohexyl moiety (C-1), and a quaternary carbon at δ_C 157.1 (C-2) indicated the location of the sp^2 methine (CH-6). The molecular formula of **1** required the involvement of a nitrogen atom and a hydroxyl group (δ_H 10.50, br s), and together with the carbon chemical shifts of the heteroaromatic sp^2 carbons (C-2-C-6), an *N*-hydroxy-2-pyridone structure was proposed. The relative configuration of the cyclohexyl moiety was addressed on the basis of the NOESY data. Correlations between H_{ax}-2′/H_{ax}-6′ (δ_H 2.40) and H-4′ and between H_{eq}-2′/H_{eq}-6′ (δ_H 1.62) and 1′-OH indicated the *cis* relationship of the two hydroxyl groups (1′-OH and 4′-OH). These data are consistent with the equatorial orientation of the pyridone group and 4′-OH. The

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Table 1. ^{13}C NMR (125 MHz) Data for Torrubiellones A−D (**1**−**4**) in Acetone-d_6

position	1	2	3	4
2	157.1, qC	162.1, qC	161.8, qC	175.5, qC
3	105.5, qC	106.1, qC	106.3, qC	100.2, qC
4	173.9, qC	179.1, qC	178.1, qC	194.4, qC
5	117.9, qC	119.4, qC	113.8, qC	63.1, CH
6	135.6, CH	138.8, CH	139.9, CH	36.5, CH_2
7	193.8, qC	193.6, qC	193.7, qC	173.1, qC
8	127.4, CH	127.7, CH	127.9, CH	120.4, CH
9	145.8, CH	145.1, CH	144.9, CH	143.8,[a] CH
10	129.5, CH	129.6, CH	129.6, CH	129.2, CH
11	144.0, CH	143.4, CH	143.2, CH	144.1, CH
12	131.3, CH	131.3, CH	131.3, CH	131.3, CH
13	143.6, CH	143.2, CH	143.0, CH	143.7,[a] CH
14	47.9, CH	47.9, CH	47.9, CH	47.9, CH
15	23.7, CH_2	23.7, CH_2	23.7, CH_2	23.7, CH_2
16	11.1, CH_3	11.1, CH_3	11.1, CH_3	11.1, CH_3
17	64.8, CH_2	64.8, CH_2	64.8, CH_2	64.8, CH_2
1′	70.4, qC	70.3, qC	124.4, qC	126.9, qC
2′, 6′	33.9, CH_2	34.0, CH_2	130.2, CH	130.7, CH
3′, 5′	30.9, CH_2	30.9, CH_2	115.0, CH	115.1, CH
4′	69.1, CH	69.2, CH	157.0, qC	156.2, qC

[a] Carbon assignment may be interchanged.

absolute configuration of the methine C-14 remains unassigned. The structure of torrubiellone A (**1**) is most closely related to militarinone A, which was previously isolated from *Paecilomyces militaris* RCEF 0095.[8] The only differences are the side-chain structures. The ^1H and ^{13}C NMR spectroscopic data of **1** were very similar to those reported for militarinone A.[8]

The molecular formula of torrubiellone B (**2**) was determined by HRESIMS as $C_{22}H_{29}NO_6$, containing one less oxygen atom than **1**. The ^1H and ^{13}C NMR spectroscopic data of **2** were very similar to those of **1**, except for the small chemical shift differences of the pyridone carbons and H-6 (Table 1). The methine H-6 (δ_H 7.79) resonated as a broad singlet, and it showed a COSY correlation to a broad singlet at δ_H 10.40. Similar to **1**, key HMBC correlations were observed from H-6 to C-2, C-4, and C-1′ and from 4-OH to C-3, C-4, and C-5. In addition, a weak $^4J_{CH}$ correlation from 4-OH to the ketone C-7 was observed, which further confirmed the side-chain linkage at C-3. Therefore, torrubiellone B (**2**) was assigned as the *N*-deoxy analogue of **1**. The ^1H and ^{13}C NMR spectroscopic data of **2** were similar to those of *N*-deoxymilitarinone A.[9]

Torrubiellone C (**3**) possessed the molecular formula $C_{22}H_{23}NO_5$ (HRESIMS). The ^1H and ^{13}C NMR spectroscopic data of **3** were similar to those of **2** with only some chemical shift differences of C-5 (δ_C 113.8; δ_C 119.4 for **2**) and resonances for the cyclohexyl moiety (C-1′−C-6′) replaced by those assignable to a 4-hydroxyphenyl group. The phenolic OH exhibited HMBC correlations to C-3′/C-5′ and C-4′. HMBC correlations from H-6 to C-1′ and from H-2′/H-6′ to C-5 confirmed the attachment of the 4-hydroxyphenyl group to C-5. Torrubiellone C (**3**) is structurally related to militarinone D[10] and farinosone A,[11] with a different side chain.

The NMR spectroscopic data of torrubiellone D (**4**) were in part similar to those of **3**. The molecular formula was established as $C_{22}H_{25}NO_5$ by HRESIMS, which had two more hydrogen atoms than **3**. Analyses of the 2D NMR spectroscopic data confirmed that the side chain (C-7−C-17) of **4** was identical to other analogues except for the upfield shift of C-7 (δ_C 173.1). Compound **4** also possessed a 4-hydroxyphenyl group, although the chemical shifts of proton and carbons of this unit were significantly different from those of **3**. The spacer linking the side chain and 4-hydroxyphenyl group was composed of two carbonyl carbons resonating at δ_C 194.4 (C-4) and 175.5 (C-2), a quaternary sp^2 carbon at δ_C 100.2 (C-3), a methine at δ_C 63.1 (CH-5; δ_H 4.10, dd, J = 6.7, 4.3 Hz), a methylene at δ_C 36.5 (CH_2-6; δ_H 3.03 and 2.84), and an amide NH resonating at δ_H 7.79 (br s). The COSY correlations demonstrated that the methine (CH-5) was attached to the methylene (CH_2-6) and the amide nitrogen (NH). HMBC correlations from the methylene protons (H_2-6) to C-1′ and C-2′/C-6′ and from H-5 to C-1′ indicated the linkage to the 4-hydroxyphenyl group. HMBC correlations from H-5, H_2-6, and NH to the conjugated ketone at δ_C 194.4 (C-4) revealed that this carbonyl carbon was attached to the methine (CH-5). The amide NH also showed HMBC correlation to the upfield-shifted sp^2 quaternary carbon (C-3). Although no HMBC correlation to the carbonyl carbon at δ_C 175.5 was observed, the molecular formula of **4** (HRESIMS) required the amide linkage to form a tetramic acid. The structure of torrubiellone D (**4**) is most closely related to militarinone C,[10] which possesses the same tetramic acid and 4-hydroxyphenyl moiety but differ only in the side chain. Similar to militarinone C and other known 3-acylated tetramic acids, the keto−enol tautomerization (C-4, C-3, and C-7) was inverted to the C-4 keto form from the 3-acyl-4-hydroxypyridone series. On the other hand, there was an isomerism of the *exo*-enol tautomers due to the rotation of the enol double bond.[10] In the ^1H NMR spectrum of **4**, two pairs of signals with a 5:1 ratio were observed for NH (δ_H 7.79/7.84) and H-8 (δ_H 7.11/7.14).

Biosynthetic pathways of similar phenylalanine-derived alkaloids, militarinones and tenellin, were previously proposed.[10,12] Thus, torrubiellone D (**4**) may be a biosynthetic intermediate for **1**−**3**. It should be noted that closely related compounds were also isolated from entomopathogenic fungi of the family *Cordycipitaceae*,[13] tenellin and pyridovericin from *Beauveria bassiana*,[14,15] militarinones from *Paecilomyces militaris*,[8,10] and farinosones from *P. farinosus*.[9,11]

Compounds **1**, **2**, and **4** were evaluated against *Plasmodium falciparum* K1, *Mycobacterium tuberculosis* H37Ra, three cancer cell lines (KB, MCF-7, and NCI-H187), and nonmalignant Vero cells. Torrubiellone A (**1**) exhibited weak antimalarial activity, with an IC_{50} of 8.1 μM (dihydroartemisinin IC_{50} was 0.004 μM), while it was inactive against the TB mycobacterium. Compound **1** was not cytotoxic to KB and MCF-7 cells at a concentration of 119 μM (50 μg/mL), but it was very weakly active against NCI-187 cells (IC_{50} 69 μM) and Vero cells (IC_{50} 87 μM). Torrubiellones B (**2**) and D (**4**) were inactive in these assays except that **4** was weakly cytotoxic to KB cells (IC_{50} 44 μM).

Experimental Section

General Experimental Procedures. Optical rotations were measured with a JASCO P-1030 digital polarimeter. UV spectra were recorded on a GBS Cintra 404 spectrophotometer. FTIR spectra were taken on a Bruker VECTOR 22 spectrometer. NMR spectra were recorded on Bruker DRX400 and AV500D spectrometers. ESITOF mass spectra were measured with Micromass LCT and Bruker micrOTOF mass spectrometers.

Fungal Material. *Torrubiella* sp. was isolated from a spider (Araneae) in Nam Nao National Park, Phetcha Bun Province, Thailand. The living culture was deposited in the BIOTEC Culture Collection (BCC) on October 9, 1998, as BCC 2165. It was identified on the basis of morphology and ITS rDNA sequence data.

Fermentation and Isolation (Batch 1). The fungus BCC 2165 was maintained on potato dextrose agar at 25 °C. The agar was cut into small plugs and inoculated into 2 × 250 mL Erlenmeyer flasks containing 25 mL of potato dextrose broth (PDB; potato starch 4.0 g/L, dextrose 20.0 g/L). After incubation at 25 °C for 18 days on a rotary shaker (200 rpm), each primary culture was transferred into a 1 L Erlenmeyer flask containing 250 mL of the same liquid medium (PDB) and incubated at 25 °C for 18 days on a rotary shaker (200 rpm). These secondary cultures were pooled, each 25 mL portion was transferred into 20 × 1 L Erlenmeyer flasks containing 250 mL of PDB, and final fermentation was carried out at 25 °C for 52 days under static conditions. The cultures were filtered to separate broth (filtrate) and mycelia (residue). Culture broth (5 L) was extracted with EtOAc, and the combined organic phase was concentrated to obtain a brown gum (700 mg). This broth extract was passed through a column on Sephadex LH-20 (3.5 × 50 cm, MeOH) to obtain nine pooled fractions: fraction 1−9. Fraction 4 (283 mg) was subjected to column chromatography on silica gel (2.5 × 20 cm, step gradient elution with acetone/CH_2Cl_2) to furnish **2** (10 mg). Fraction 8 (22 mg) was purified by

Table 2. ^{1}H NMR (500 MHz) Data for Torrubiellones A−D (**1**−**4**) in Acetone-d_6

position	1	2	3	4
NO*H*	10.50 br s			
N*H*		10.40 br s	10.54 br s	7.79 br s
4-O*H*	17.45 s	18.29 s	17.83 s	
5				4.10 dd (6.7, 4.3)
6	8.16 s	7.79 br s	7.62 br s	3.03 dd (14.1, 4.3)
				2.84 dd (14.1, 6.7)
8	8.07 d (15.0)	8.11 d (15.0)	8.14 d (15.1)	7.11 d (15.3)
9	7.65 dd (15.0, 11.6)	7.62 dd (15.0, 11.5)	7.62 dd (15.1, 11.5)	7.47 dd (15.3, 11.4)
10	6.54 dd (14.8, 11.6)	6.52 dd (14.6, 11.5)	6.53 dd (14.7, 11.5)	6.54 dd (14.6, 11.4)
11	6.88 dd (14.8, 10.8)	6.84 dd (14.6, 10.9)	6.83 dd (14.7, 10.8)	6.83 dd (14.6, 10.9)
12	6.37 dd (15.2, 10.8)	6.36 dd (15.1, 10.9)	6.36 dd (15.2, 10.8)	6.37 dd (15.2, 10.9)
13	5.97 dd (15.2, 8.8)	5.95 dd (15.1, 8.8)	5.95 dd (15.2, 8.8)	5.97 dd (15.2, 8.7)
14	2.22 m	2.22 m	2.22 m	2.22 m
15	1.61 m; 1.33 m	1.64 m; 1.33 m	1.61 m; 1.31 m	1.61 m; 1.31 m
16	0.88 t (7.5)	0.88 t (7.4)	0.88 t (7.4)	0.88 t (7.4)
17	3.53 d (6.0)	3.52 d (5.7)	3.53 br m	3.52 d (6.0)
17-O*H*	3.63[a] m	3.68[b] br s	3.67 br m	−[c]
1′-O*H*	4.16 br s	4.03 s		
2′, 6′	2.40 dt (4.6, 13.5); 1.62 m	2.35 m; 1.62 m	7.34 d (8.6)	7.02 d (8.4)
3′, 5′	1.80 m; 1.75 m	1.79 m; 1.75 m	6.87 d (8.6)	6.71 d (8.4)
4′	3.62[a] m	3.62[b] m		
4′-O*H*	3.63[a] m	3.65[b] m	8.52 br s	8.29 br s

[a,b] The ^{1}H NMR resonances are overlapped. [c] The OH resonance was probably overlapped with the H_2O peak.

column chromatography on silica gel (1.5 × 20 cm, step gradient elution with EtOAc/CH_2Cl_2) to furnish **3** (4 mg).

Fermentation and Isolation (Batch 2). The fungus BCC 2165 was cultured using the same conditions as batch 1, but in 4 times larger scale (80 × 250 mL). The cultures were filtered to separate broth (filtrate) and mycelia (residue). Culture broth (20 L) was extracted three times with EtOAc, and the combined organic phase was concentrated to obtain a brown gum (2.1 g, extract A). The mycelium was macerated in MeOH (4.6 L, 2 days) and filtered. The residue was extracted again with MeOH (2 L). The filtrate was defatted by partitioning with an equal volume of hexane. The MeOH phase was evaporated, the residue was diluted with H_2O (200 mL) and extracted with EtOAc (3 × 600 mL), and the organic layer was concentrated under reduced pressure to leave a dark brown gum (800 mg, extract B). Extract A was passed through a column on Sephadex LH-20 (3.5 × 50 cm, MeOH) to obtain eight pooled fractions: A-1−A-8. Fraction A-4 (711 mg) was subjected to a column on Sephadex LH-20 (3.5 × 50 cm, MeOH) to obtain four subfractions: fractions A-4-1−A-4-4. Fraction A-4-2 (607 mg) was repeatedly fractionated by Sephadex LH-20 column chromatography, and the fraction (130 mg) containing **1** was further purified by preparative HPLC using a reversed-phase column (Phenomenex Luna 10u C18(2) 100A, 21.2 × 250 mm, 10 μm; mobile phase MeOH/H_2O, 45:55, flow rate 15 mL/min) to furnish **1** (87 mg, t_R 15 min). Fraction A-4-3 (138 mg) was repeatedly fractionated by Sephadex LH-20 column chromatography to give **4** (18 mg). Extract B was passed through a column on Sephadex LH-20 (3.5 × 50 cm, MeOH) to obtain nine fractions: fractions B-1−B-9). Fraction B-5 (54 mg) was further separated by column chromatography on Sephadex LH-20 and preparative HPLC (MeOH/H_2O, 50:50) to furnish **2** (4 mg, t_R 21 min).

Torrubiellone A (1): yellow, amorphous; $[\alpha]^{26}_D$ −18 (*c* 0.105, MeOH); UV (MeOH) λ_{max} (log ε) 207 (4.36), 279 (4.28) nm; IR (KBr) ν_{max} 3418, 1716, 1645, 1613, 1441 cm^{-1}; ^{1}H NMR (500 MHz, acetone-d_6) and ^{13}C NMR (125 MHz, acetone-d_6) data, see Tables 1 and 2; HRMS (ESI-TOF) *m*/*z* 420.2017 $[M + H]^+$ (calcd for $C_{22}H_{30}NO_7$, 420.2020).

Torrubiellone B (2): yellow, amorphous; $[\alpha]^{26}_D$ −15 (*c* 0.085, MeOH); UV (MeOH) λ_{max} (log ε) 206 (4.25), 233 (4.11), 329 sh (3.96), 380 (4.03) nm; IR (KBr) ν_{max} 3423, 1649, 1445 cm^{-1}; ^{1}H NMR (500 MHz, acetone-d_6) and ^{13}C NMR (125 MHz, acetone-d_6) data, see Tables 1 and 2; HRMS (ESI-TOF) *m*/*z* 404.2067 $[M + H]^+$ (calcd for $C_{22}H_{30}NO_6$, 404.2068), 426.1880 $[M + Na]^+$ (calcd for $C_{22}H_{29}NO_6Na$, 426.1887).

Torrubiellone C (3): yellow, amorphous; $[\alpha]^{26}_D$ −22 (*c* 0.175, MeOH); UV (MeOH) λ_{max} (log ε) 251 (4.15), 371 (4.16) nm; IR (KBr) ν_{max} 3424, 1643, 1612, 1467 cm^{-1}; ^{1}H NMR (500 MHz, acetone-d_6) and ^{13}C NMR (125 MHz, acetone-d_6) data, see Tables 1 and 2; HRMS (ESI-TOF) *m*/*z* 382.1643 $[M + H]^+$ (calcd for $C_{22}H_{24}NO_5$, 382.1649).

Torrubiellone D (4): yellow, amorphous; $[\alpha]^{26}_D$ −182 (*c* 0.12, MeOH); UV (MeOH) λ_{max} (log ε) 226 (4.30), 286 (4.07), 333 sh (4.14), 348 (4.15) nm; IR (KBr) ν_{max} 3397, 1650, 1597, 1516, 1446, 1238 cm^{-1}; ^{1}H NMR (500 MHz, acetone-d_6) and ^{13}C NMR (125 MHz, acetone-d_6) data, see Tables 1 and 2; HRMS (ESI-TOF) *m*/*z* 406.1622 $[M + H]^+$ (calcd for $C_{22}H_{25}NO_5Na$, 406.1625).

Biological Assays. The assay for activity against *Plasmodium falciparum* (K1, multi-drug-resistant strain) was performed using the microculture radioisotope technique.[16] The IC_{50} value of the standard antimalarial compound dihydroartemisinin was 0.0040 μM. Growth inhibitory activity against *Mycobacterium tuberculosis* H37Ra and cytotoxicity to Vero cells (African green monkey kidney fibroblasts) were assessed using the green fluorescent protein microplate assay (GFPMA).[17] Ellipticine was used as the standard of cytotoxicity (IC_{50} 2.1 μM). Anticancer activities against KB cells (oral human epidermoid carcinoma), MCF-7 cells (human breast cancer), and NCI-H187 cells (human small-cell lung cancer) were evaluated using the resazurin microplate assay.[18] The IC_{50} values of the standard compound doxorubicin hydrochloride were 0.37 μM for KB cells, 2.3 μM for MCF-7 cells, and 0.098 μM for NCI-H187 cells.

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Supporting Information Available: NMR spectra of compounds **1**−**4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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