# Tenellones A and B from a *Diaporthe* sp.:  Two Highly Substituted Benzophenone Inhibitors of Parasite cGMP-Dependent Protein Kinase Activity

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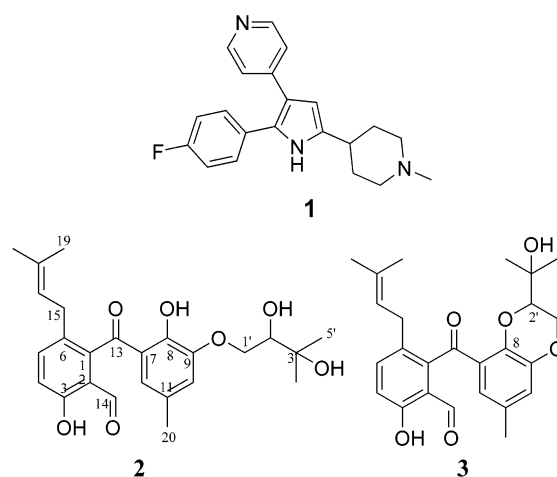
Parasite cGMP-dependent protein kinase (PKG) has been recently validated as a biochemical target for the treatment of coccidiosis. To discover new anticoccidial leads, we have screened our library of natural product extracts for inhibitors of parasite PKG. Bioassay-guided fractionation of the microbial extracts has led to the discovery of tenellones A (**2**) and B (**3**), two new highly substituted benzophenones. The isolation, structure, and activity of these compounds are described.

Apicomplexan parasites are the causative agents of various human and animal diseases. The most notable human diseases are malaria and toxoplasmosis caused by *Plasmodium falciparum* and *Toxoplasma gondii*, respectively. Coccidiosis, caused by *Eimeria* spp. of parasites, causes significant disease in chickens, leading to major economic losses through morbidity and mortality. Polyether ionophore anticoccidials, discovered 30 years ago, have been successfully used as prophylactic agents by the poultry industry. Not surprisingly, resistance to these and other existing anticoccidials has been observed, and new therapeutic agents with novel mechanisms of action are needed.

From empiric screening efforts we recently discovered a novel anticoccidial agent (compound **1**) with potent in vivo activity against *Eimeria* spp. and *T. gondii*.[1,2] It was determined that inhibition of parasite growth by synthetic compound **1** was due to the inhibition of parasite specific cGMP-dependent protein kinase (PKG), a serine and/or threonine kinase. On the basis of this discovery an in vitro assay using *E. tenella* PKG (EtPKG) was designed and used to screen our natural product extracts. A number of hits were then tested for efficacy in a whole cell *T. gondii* (TgWC) assay. We performed high-throughput bioassay-guided fractionation of those extracts with whole cell activity using the PKG enzyme assay. This effort led to the identification of two new benzophenones, tenellones A (**2**) and B (**3**), that inhibit PKG. These compounds were further evaluated in whole cell *T. gondii* assays.

The producing organism, an endophytic fungus isolated from a plant sample collected in Tenerife, Canary Islands (Spain), was identified as a *Diaphorte* sp. after sequencing of the ITS region of the ribosomal DNA and comparison with the GenBank database. The fungus was grown in CYS80 medium and extracted with methyl ethyl ketone. A two-step isolation procedure employing Sephadex LH20 followed by reversed-phase HPLC afforded compounds **2** (2.4 mg, 120 mg/L) and **3** (12.0 mg, 600 mg/L) as amorphous yellow powder.

Tenellone A (**2**) was assigned the molecular formula $C_{25}H_{30}O_7$ by ESI-FTMS ([M + Na]+ *m/z* 465.1886; Δ = −0.6 mmu), indicating the presence of 11 degrees of unsatura-



tion. The ^{13}C NMR spectra showed 25 carbon signals. The DEPT spectra suggested the presence of five methyl groups, two methylenes, one methine, five sp[2] methines, and one aldehyde. Of the remaining carbons, nine were sp[2] quaternary type including three oxygenated, one oxygenated quaternary, and a conjugated ketone. These assignments indicated the present of four exchangeable protons. The ^{1}H NMR spectrum showed the presence of two exchangeable phenolic protons (δ 11.51 and 12.09), one aldehyde proton (δ 9.70), four aromatic protons (two *ortho*-coupled:  δ 7.06 and 7.45, *J* = 8.6 Hz; and two *meta*-coupled protons:  δ 6.99 and 6.54, *J* = 1.8 Hz), one olefinic proton (δ 5.05) which was coupled to two methylene protons (δ 3.12, *J* = 7.4 Hz), and two allylic methyl groups (δ 1.46 and 1.56), indicating the presence of an isopentenyl group. The 2D NMR experiments (COSY, HMQC, and HMBC) confirmed the presence of the isopentenyl fragment, which was connected to an aromatic ring at C-6 (δ 129.8), as evidenced by HMBC correlations from the aromatic proton H-5 (δ 7.45) to the methylene carbon at δ 30.9. The HMBC correlations of aromatic proton H-4 (δ 7.06) to C-2 (δ 117.2) and C-6, and H-5 to C-1 (δ 140.5) and C-3 (δ 160.6), established the presence of a tetrasubstituted aromatic ring with the aldehyde substitution at C-2 and a phenolic group at C-3. The latter substitutions were confirmed by the HMBC correlations of the aldehyde proton to C-2 and C-3, and the phenolic hydroxyl protons at C-3 (δ 11.51) to C-2, C-3, and C-4.

**Table 1.** Biological Activities of Tenellones A (**2**) and B (**3**)

| compound | EtPKG (IC$_{50}$, $\mu$M) | TgWC (IC$_{50}$, $\mu$M) |
|---|---|---|
| **1** | <0.001 | 210 |
| **2** | 12.6 | 1.8 |
| **3** | 8.7 | ND |

The COSY spectrum showed that two methylene protons ($\delta$ 4.11 and 4.29) were coupled to a methine proton at $\delta$ 3.79. Two methyl groups showed HMBC correlations to the methine carbon at $\delta$ 75.2 and a quaternary carbon at $\delta$ 71.8, indicating the presence of a trioxygenated isopentane group, which was connected to an aromatic ring through an ether linkage that was confirmed by the HMBC correlation of one of the two methylene protons at $\delta$ 4.11 to C-9 ($\delta$ 147.6). The HMBC correlations of H-10 ($\delta$ 6.99) to C-8 ($\delta$ 151.3) and C-12 ($\delta$ 124.7), and H-12 ($\delta$ 6.54) to C-10 ($\delta$ 122.8) and C-8, established the second tetrasubstituted aromatic ring with the methyl group substitution at C-11 ($\delta$ 128.9). The structural assignment was confirmed by HMBC correlations of aromatic methyl protons (H$_3$-20) to C-10, C-11, and C-12. The HMBC correlation of H-12 to the ketone group at C-13 ($\delta$ 203.1) established the connectivity of the ketone group to C-7 of the aromatic ring. While there was no direct evidence to connect the ketone to the second aromatic ring, it was connected to C-1 on the basis of the $^{13}$C NMR chemical shift arguments of benzophenone and comparison with of pestalone, whose structure was established by X-ray crystallographic analysis.[3] On the basis of these data the structure **2** was assigned to tenellone A.

The molecular formula C$_{25}$H$_{28}$O$_6$ was assigned to tenellone B (**3**) by ESI-FTMS ([M + H]$^+$ *m/z* 425.1959; $\Delta$ = −1.17 mmu), suggesting 12 degrees of unsaturation. The molecular formula of **3** indicated that it has lost a H$_2$O unit from **2**. The structure of **3** was elucidated by 1D and 2D NMR spectral analysis and by comparison with that of **2**. The secondary hydroxy group at C-2' had cyclized to form an ether linkage at C-8, forming a 1,4-dioxane ring, which was confirmed by the HMBC correlations of one of two methylene proton at $\delta$ 4.32 to C-9 ($\delta$ 143.7) and the methine proton H-2' ($\delta$ 3.80) to C-8 ($\delta$ 142.3). Thus, structure **3** was assigned to tenellone B.

The compounds were evaluated for their ability to inhibit (i) *E. tenella* cGMP-dependent protein kinase (EtPKG)[1] activity using a radiometric assay and (ii) growth and viability of the related apicomplexan parasite *T. gondii* (TgWC) using a $\beta$-galactosidase whole cell reporter assay.[4,5] As a reference, the synthetic compound **1** had IC$_{50}$ values of <1 and 210 nM, respectively, in the EtPKG and TgWC assays (Table 1). Tenellone A (**2**) inhibited EtPKG activity with an IC$_{50}$ of 12.6 $\mu$M and had good activity in the cell-based TgWC assay (IC$_{50}$ = 1.8 $\mu$M). Tenellone B (**3**) was only evaluated in the EtPKG assay and displayed an IC$_{50}$ value of 8.7 $\mu$M. The higher level of cell-based activity of **2** may indicate an additional mode of action. Unfortunately, neither compound **2** or **3** had detectable anticoccidial activity when *Eimeria*-infected chickens were dosed ad lib at an inclusion rate of 100 ppm in the feed. While no antiparasitic activity was observed in chickens, there also were no signs of toxicity. Pestalone is reported[3] to exhibit in vitro toxicity against NCI's cell line (mean GI$_{50}$ 6 $\mu$M) and Gram-positive antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MIC = 37 ng/mL) and vancomycin-resistant *Enterococcus faecium* (MIC = 78 ng/mL). Prenylated benzophenones have been reported to show various biological activities such as antibacterial[6] and antifungal[7] and are inhibitors of HIV-1 reverse transcriptase,[8] acidic sphingomyelinase,[9] and 5$\alpha$-reductase.[10]

In summary, this paper describes the isolation and structure of two new benzophenones, tenellones A (**2**) and B (**3**), that are inhibitors of cGMP kinase activity of *E. tenella*. These compounds also exhibited cell-based activity against the related parasite *T. gondii*. Although these compounds lack in vivo activity, they represent new inhibitors of cGMP-dependent protein kinase and may be useful as biological tools.

## Experimental Section

**General Experimental Procedures.** Optical rotations were measured on a Perkin-Elmer 241 polarimeter. The UV spectra were recorded in MeOH on a Beckman DU-70 spectrophotometer. IR spectra were recorded on a Perkin-Elmer Spectrum One spectrometer. The NMR spectra were recorded on a Varian INOVA 500 FT-NMR spectrometer at 500 MHz for $^1$H and 125 MHz for $^{13}$C in CDCl$_3$. HRESIMS were obtained on a Thermo Quest FTMS spectrometer using electrospray ionization. HP1100 was used for analytical HPLC.

**Producing Organism and Fermentation.** The producing organism is an endophytic fungus identified as a *Diaporthe* sp. The strain was isolated from stems of *Aeonium cuneatum* Webb & Berthel (Crassulaceae) collected in El Pijaral (Tenerife, Canary Islands, Spain), following a surface sterilization technique conventionally used for the isolation of fungal endophytes.[11] Although the fungus remained sterile in pure culture stage, sequencing of the internal transcribed spacers (ITS) of the ribosomal DNA[12] revealed a high homology (>92%) with sequences from several *Diaporthe* species deposited in GenBank (accession codes AF001026, AF000567, AF000563, among others), allowing the identification of the strain as an undetermined species of *Diaporthe*. This identification was consistent with the macroscopic morphology of the fungus in culture. For production of the compounds, seed flasks were prepared from fresh slant PDA (potato dextrose agar, Difco) cultures as described by Peláez et al.[13] Two milliliter aliquots of the resulting cultures were used to inoculate 250 mL unbaffled Erlenmeyer flasks containing 50 mL of CYS80 production medium (sucrose 80 g/L, yellow corn meal 50 g/L, yeast extract Difco 1 g/L).[13] The production flasks were incubated at 25 °C in a rotary shaker at 220 rpm for 14 days.

**Extraction and Isolation.** The fungal culture (250 mL) was extracted with 250 mL of methyl ethyl ketone (MEK) by shaking on a reciprocating shaker for 1 h. A 20 mL aliquot of MEK extract was concentrated under reduced pressure to dryness, and the residue was redissolved in 5 mL of MeOH, chromatographed on a 70 mL Sephadex LH20 column, and eluted with MeOH at a flow rate of 12 mL/min, affording a single 48.1 mg active fraction eluting after one column volume. The active fraction was further chromatographed by preparative reversed-phase HPLC using a Zorbax RX C$_8$ (21.2 × 250 mm) column eluting with a 35 min gradient of 50−100% aqueous CH$_3$CN containing 0.1% TFA at a flow rate of 10 mL/min. Lyophilization of fractions eluting at 14 and 20 min afforded 2.4 mg (120 mg/L) of tenellone A (**2**) and 12.0 mg (600 mg/L) of tenellone B (**3**) as yellow amorphous powders.

**Tenellone A (2):** [$\alpha$]$^{22}_D$ +20.0° (*c* 0.1, CH$_3$OH); UV (CH$_3$OH) $\lambda_{max}$ 218 ($\epsilon$ 34 052), 266 (11 368), 344 (5534) nm; IR (ZnSe) $\nu_{max}$ 3426, 2974, 2928, 1653, 1637, 1463, 1372, 1334, 1266, 1168, 1038, 977, 752 cm$^{-1}$; $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 1.30 (3H, s, H$_3$-4'), 1.34 (3H, s, H$_3$-5'), 1.46 (3H, brs, H$_3$-18), 1.56 (3H, brs, H$_3$-19), 2.18 (3H, s, H$_3$-20), 3.12 (2H, d, *J* = 7.4 Hz, H$_2$-15), 3.79 (1H, dd, *J* = 7.0, 3.0 Hz, H-2'), 4.11 (1H, dd, *J* = 9.7, 7.0 Hz, H-1'), 4.29 (1H, dd, *J* = 9.7, 3.0 Hz, H-1'), 5.05 (1H, tm, *J* = 7.4, 1.4 Hz, H-16), 6.54 (1H, dd, *J* = 1.8, 0.7 Hz, H-12), 6.99 (1H, d, *J* = 1.8 Hz, H-10), 7.06 (1H, d, *J* = 8.6 Hz, H-4), 7.45 (1H, d, *J* = 8.6 Hz, H-5), 9.70 (1H, s, H-14), 11.51 (1H, s, OH-3), 12.09 (1H, brs, OH-8); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 17.6 (C-18), 20.8 (C-20), 25.5 (C-5'), 25.6 (C-19), 26.7 (C-4'), 30.9 (C-15), 71.8 (C-3'), 72.0 (C-1'), 75.2 (C-2'), 117.2 (C-2), 119.5 (C-4), 121.2 (C-7), 121.5 (C-16), 122.8 (C-10), 124.7 (C-12), 128.9 (C-11), 129.8 (C-6), 134.1 (C-17), 138.5 (C-5), 140.5 (C-1), 147.6 (C-9), 151.3 (C-8), 160.6 (C-3), 194.2 (C-14),

203.1 (C-13); HMBC (H→C, $^nJ_{CH}$ = 7 Hz): H-4 → C-2, 6; H-5 → C-1, 3, 15; H-10 → C-8, 9, 12, 20; H-12 → C-8, 10, 13, 20; H-14 → C-2, 3; H-18 → C-16, 17, 19; H-19 → C-16, 17, 18; H-20 → C-10, 11, 12; H-1′ → C-2′, 9; H-4′ → C-2′, 3′, 5′; H-5′ → C-2′, 3′, 4′; OH-3 → C-2, 3, 4; HRESI-FTMS $m/z$ 465.1886 (calcd for $C_{25}H_{30}O_7$+Na, 465.1889).

**Tenellone B (3):** $[\alpha]^{22}_D$ +40.0° (*c* 0.1, CH$_3$OH); UV (CH$_3$-OH) $\lambda_{max}$ 210 ($\epsilon$ 28 985), 266 (10 447), 342 (4426) nm; IR (ZnSe) $\nu_{max}$ 3431, 2976, 2929, 1651, 1606, 1585, 1476, 1377, 1334, 1283, 1257, 1217, 1165, 1070, 956, 747 cm$^{-1}$; $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 0.96 (3H, s, H$_3$-5′), 1.02, (3H, s, H$_3$-4′), 1.44 (3H, brs, H$_3$-18), 1.55 (3H, brs, H$_3$-19), 2.28 (3H, s, H$_3$-20), 3.11 (1H, dd, $J$ = 7.0, 16.0 Hz, H-15), 3.15 (1H, dd, $J$ = 7.0, 16.0 Hz, H-15), 3.80 (1H, t, $J$ = 8.4 Hz, H-2′), 3.81 (1H, dd, $J$ = 8.4, 16.0 Hz, H-1′), 4.32 (1H, dd, $J$ = 8.4, 16.0 Hz, H-1′), 5.08 (1H, mt, $J$ = 7.0 Hz, H-16), 6.95 (1H, brd, $J$ = 2.1 Hz, H-12), 6.98 (1H, d, $J$ = 8.7 Hz, H-4), 7.29 (1H, brs, H-10), 7.40 (1H, d, $J$ = 8.7 Hz, H-5), 9.82 (1H, s, H-14), 11.50 (1H, s, OH-3); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 17.6 (C-18), 20.5 (C-20), 24.3 (C-5′), 25.3 (C-4′), 25.5 (C-19), 30.7 (C-15), 64.4 (C-1′), 69.8 (C-3′), 80.4 (C-2′), 117.3 (C-2), 118.4 (C-4), 121.7 (C-16), 123.5 (C-10), 123.8 (C-12), 126.7 (C-7), 128.9 (C-6), 131.0 (C-11), 133.5 (C-17), 138.4 (C-5), 142.3 (C-8), 143.7 (C-9), 146.3 (C-1), 160.6 (C-3), 194.6 (C-13), 195.0 (C-14); HMBC (H→C, $^nJ_{CH}$ = 7 Hz) H-4 → C-2, 3, 6; H-5 → C-1, 2, 3, 15; H-10 → C-8, 12, 20; H-12 → C-8, 10, 20; H-14 → C-2, 3; H-15 → C-1, 5, 6, 16, 17; H-16 → C-15, 18, 19; H-18 → C-16, 17, 19; H-19 → C-16, 17, 19; H-20 → C-10, 11; H-1′ → C-2′, C-9; H-2′ → C-1′, 3′, 5′, 8; H-4′ → C-5′,C-3′,C-2′; H-5′ → C-4′,C-3′,C-2′; OH-3 → C-3,C-4; HRESI-FTMS $m/z$ 425.1959 (calcd for $C_{25}H_{28}O_6$+H, 425.1964).

**cGMP-Dependent Kinase Assay of *Eimeria tenella* (EtPKG).** The in vitro EtPKG radiometric enzyme assay was performed using a semipurified preparation of native *E. tenella* cGMP-dependent kinase, peptide substrate, and [$^{33}$P]-$\gamma$ATP. The enzyme transfers a phosphate group from ATP to the substrate peptide. Inhibition of the transfer of this phosphate group, using active extracts and compounds, was measured as detailed by Gurnett.[1]

***Toxoplasma gondii* Whole Cell Activity Assay (TgWC).** Whole cell antiparasitic activity was determined using a strain of *T. gondii* stably expressing bacterial $\beta$-galactosidase.[4] Host human foreskin fibroblasts grown to confluence in 96-well microtiter plates were infected with the tagged strain of parasite. Parasite infection and development in the presence of test extracts was allowed to proceed for 5 days, at which time chlorophenol red-$\beta$-D-galactopyranoside (CPRG), a colorimetric $\beta$-galactosidase substrate, was added. Following an additional 2−4 h incubation period, $\beta$-galactosidase enzyme activity and therefore parasite viability were measured by reading absorbance at 570 nm.[5]

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