Highly Substituted Terphenyls as Inhibitors of Parasite cGMP-Dependent Protein Kinase Activity

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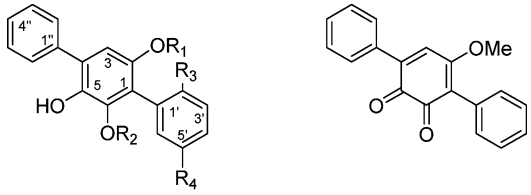
Parasite cGMP-dependent protein kinase (PKG) is one of the validated biochemical targets for the treatment of coccidiosis. We screened our library of natural product extracts for inhibitors of parasite PKG for the discovery of anticoccidial leads. Terferol (**1**) and three new terphenyls (**2**, **3**, and **4**) were isolated using bioassay-guided fractionation of the microbial extract of a *Phoma* sp. by a high-throughput two-step isolation method employing LH-20 and reversed-phase HPLC. These compounds inhibited parasite PKG with IC_{50} values in the range 0.9−5.8 μM.

Plasmodium falciparum and *Toxoplasma gondii* are two members of Apicomplexan parasites that cause malaria and toxoplasmosis, respectively. The third member of the Apicomplexan family, *Eimeria* species, inflicts coccidiosis in chickens, leading to major economic losses through morbidity and mortality. Coccidiosis has been treated prophylactically using polyether ionophores discovered 30 years ago. It is not a surprise that resistance to these and other existing anticoccidials has been observed, and new therapeutic agents with novel mechanisms of action are needed.

We have validated parasite cGMP-dependent kinase (PKG), a serine and/or threonine kinase, as a biochemical target for potential treatment of coccidiosis and developed an in vitro assay using *Eimeria tenella* PKG (EtPKG) for screening of both natural product extracts and synthetic chemical libraries for the discovery of leads.[1,2] Using this approach, we recently identified tenellones as inhibitors of EtPKG.[3] Continued screening and high-throughput bioassay-guided fractionation of an extract of a *Phoma* species led to the identification of terferol (**1**)[4] and three new terphenyls (**2**−**4**) that inhibit EtPKG. Many of these purified compounds have been further evaluated in whole cell *T. gondii* assays.

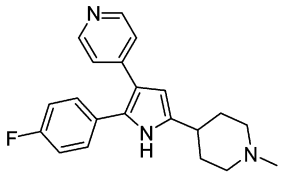
The producing organism, a *Phoma* species, was recovered from lichens in Martinique (France). It could not be identified to species level due to the lack of sporulation. The fungus was grown in CYS80 medium and extracted with methyl ethyl ketone. A two-step isolation procedure employing Sephadex LH-20 followed by reversed-phase HPLC afforded terferol **1** (2.1 mg, 105 mg/L) and compounds **2** (0.8 mg, 40 mg/L), **3** (1.4 mg, 70 mg/L), and **4** (1.6 mg, 80 mg/L) as amorphous yellow powders. The structure of **1** was determined by literature comparison of UV, IR, ^{1}H NMR, and mass spectral data.[4]

Compound **2** was assigned the molecular formula $C_{19}H_{16}O_5$ by HRESI-FTMS ($[M + H]^+$ *m*/*z* 325.1084), indicating the presence of 12 degrees of unsaturation. The ^{13}C NMR spectrum displayed 19 carbon signals, two of which overlapped. The presence of nine sp^2 carbons and one methoxy (δ 60.6) when combined with the nine quaternary carbons indicated the presence of a terphenyl structure similar to that of terferol (**1**). The ^{1}H NMR spectrum displayed nine aromatic proton signals, appearing from δ 6.74 to 7.56, and a methoxy signal at δ 3.38, indicating the presence of four phenolic hydroxyl groups. The monosubstituted phenyl unit



1: R_1= Me, R_2= R_3 = R_4 = H (Terferol)
2: R_1= H, R_2 = Me, R_3 = R_4 = OH
3: R_1= H, R_2 = Me, R_3 = OH, R_4 = H

4



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was established on the basis of the coupling patterns of a group of aromatic protons appearing at δ 7.56 (2H, dd, J = 8.0, 1.3 Hz), 7.37 (2H, dd, J = 8.0, 7.4 Hz), and 7.27 (1H, tt, J = 7.4, 1.3 Hz). The presence of three aromatic protons [*ortho* coupled appearing at δ 6.84 and 6.74, J = 8.5 Hz, and the latter proton was *meta* coupled with a proton appearing at 6.82, J = 3.0 Hz], along with the HMBC correlations from the aromatic proton H-3′ (δ 6.84) to the oxygenated aromatic carbon at δ 150.2, and from the aromatic protons H-4′ (δ 6.74) and H-6′ (δ 6.82) to the second oxygenated aromatic carbon at δ 146.5, suggested the presence of a trisubstituted phenyl ring with *para*-hydroxyl groups. The presence of one remaining aromatic proton as a singlet at δ 6.71 indicated that the third phenyl ring was pentasubstituted just like terferol (**1**). The 2D NMR experiments (COSY, HMQC, and HMBC) confirmed the presence of the terphenyl structure. HMBC correlations from H-3 (δ 6.71) to the carbon signal at δ 137.4 and H-2″/6″ (δ 7.56) to the carbon at δ 129.0 indicated the connection of the monosubstituted phenyl ring to C-4 (δ 129.0) of the central pentasubstituted phenyl ring. HMBC correlations of H-3 to carbons at δ 117.6, 140.0, and 146.7, along with the observed NOE from the methoxy protons to H-6′ (δ 6.82) in NOE difference experiment, suggested that C-1′ of the trisubstituted phenyl was connected to C-1 (δ 117.6) of the pentasubstituted phenyl ring. The methoxy protons gave a HMBC correlation to C-6 (δ 145.0), which allowed unambiguous assignment of the methoxy group to C-6. Thus, on the basis of the above analysis, structure **2** was assigned.

The molecular formula $C_{19}H_{16}O_4$ was established for compound **3** by HRESI-FTMS ($[M + H]^+$ *m*/*z* 309.1118). Compound **3**

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Table 1. Biological Activities of Compounds **1**−**4**[a]

compound	EtPKG (IC_{50}, μM)	TgWC (IC_{50}, μM)
1	3.9	26.8
2	5.8	9.5
3	5.2	10.4
4	0.9	ND
5[b]	<0.001	0.21

[a] IC_{50} data are an average of three experiments. [b] A synthetic compound.

possessed one oxygen atom less than compound **2**, indicating the absence of one hydroxyl group. The presence of a monosubstituted phenyl group and a pentasubstituted phenyl ring was obvious in compound **3** by comparison of its NMR data with those of compound **2**, especially HMBC correlations of H-3 (δ 6.81) to four carbons at δ 117.2, 137.3, 140.0, and 146.7. Four aromatic protons, at δ 7.06 (1H, dd, J = 8.0, 1.0 Hz), 7.32 (1H, td, J = 8.0, 1.7 Hz), 7.04 (1H, td, J = 8.0, 1.0 Hz), and 7.40 (1H, dd, J = 8.0, 1.7 Hz), indicated the presence of an *ortho*-substituted phenyl ring. The COSY correlations of these four protons also indicated that they were contiguous, suggesting that compound **3** was the C-5′ deoxy congener of terphenyl **2**. The HMBC correlation of H-6′ to C-2′ (δ 153.6) confirmed the above assignment. In addition, H-6′ showed a HMBC correlation to C-1 (δ 117.2), indicating the connectivity of the disubstituted phenyl ring to the pentasubstituted phenyl ring at C-1. NOE difference experiments displayed a NOE enhancement from the methoxy protons to H-6′ (δ 7.40), corroborating the structural assignment of **3**.

The molecular formula $C_{19}H_{14}O_3$ was revealed by HRESI-FTMS ($[M + H]^+$ m/z 291.1004) for compound **4**, indicating 13 degrees of unsaturation. The presence of a quinone feature was suggested by IR (ν_{max} 1653 cm^{-1}) and UV [λ_{max} (CH_3OH/THF, 1:1) 261, 327 nm] absorptions. The ^1H NMR spectrum of **4** was similar to that of **1** and indicated the presence of two monosubstituted phenyl groups. Four pairs of overlapping carbons (δ 127.9, 128.5, 129.1, and 130.5), together with associated protons in the ^1H NMR spectrum, supported this assignment. The ^{13}C NMR spectrum of compound **4** showed low-field signals at δ 183.1 and 187.1, which were assigned to two quinone carbonyls. The NOE difference spectrum showed a NOE from methoxy protons to H-3 (δ 6.92), indicating the presence of an *ortho*-quinone moiety. NOE enhancements from methoxy protons (δ 3.85) to H-2′/6′ (δ 7.35), and H-3 to H-2″/6″ (δ 7.54), indicated that two monosubstituted phenyl groups were located at C-1 (δ 128.7) and C-4 (δ 130.0) of the pentasubstituted phenyl ring. The HMBC correlation of H-3 (δ 6.92) to C-5 (δ 183.1), only one of the two quinone carbonyls, and C-1″ (δ 132.8) confirmed the above assignments and the structure of compound **4**.

Compounds **1**−**4** 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 β-galactosidase whole cell reporter assay.[5,6] The synthetic compound **5** was used as a reference and had IC_{50} values of <0.001 and 0.21 μM respectively in the EtPKG and TgWC assays (Table 1). Terferol (**1**) inhibited EtPKG activity with an IC_{50} of 3.9 μM. It was about 8-fold less potent in the cell-based TgWC assay (IC_{50} = 26.8 μM). Compound **2** displayed IC_{50} values of 5.8 and 9.5 μM in EtPKG and TgWC assays, respectively. Compound **3** exhibited similar activities and exhibited IC_{50} values of 5.2 and 10.4 μM in the EtPKG and TgWC assays, respectively. The *ortho*-quinoid terphenyl compound **4** was evaluated only in the EtPKG assay and displayed an IC_{50} value of 0.9 μM.

Recently, terphenyls have drawn a lot of attention,[7] and various biological activities including antioxidant,[8] chelation-mediated glutamate neurotoxicity inhibition,[9] antibody IgE−antibody suppressant,[10] insecticidal and antibacterial,[11] and HIV-1 integrase inhibition[12] have been reported.

In summary, this paper describes the isolation and structure elucidation of three new terphenyls (**2**−**4**) that are inhibitors of cGMP kinase activity of *E. tenella*. Compounds **1**−**3** also exhibited cell-based activity in *T. gondii*. Although these compounds could not be considered for further development due to poor in vitro potencies, they are expected to be of value in our understanding of the growing field of kinase inhibitors.

Experimental Section

General Experimental Procedures. HP1100 was used for analytical HPLC. 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. HRESIMS were obtained on a Thermo Quest FTMS spectrometer using electrospray ionization. The NMR spectra were recorded on a Varian INOVA 500 FT-NMR spectrometer at 500 MHz for ^1H and 125 MHz for ^{13}C in $CDCl_3$.

Producing Organism and Fermentation. The producing organism (MF7021) was a fungus isolated from unidentified lichen species growing on dead wood, which was collected in Baie de Sans-Sousi, Martinique Island, France. The fungus was isolated using a particle washing method as previously described.[13] Morphological analysis of PDA (Difco) cultures led to the characterization of the fungus as a *Phoma* sp. (anamorphic Pleosporales, Ascomycetes) based on the production of hyaline and ellipsoid ameroconidia by phialides lining the cavity of pycnidia, which appeared single or aggregated throughout the colony surface.[14] Sequence analysis of the internal transcribed spacers of the ribosomal DNA[15] indicated a 80−90% similarity with a number of GenBank sequences from isolates belonging to the Pleosporales but did not allow us to identify the strain at the species level. The culture has been deposited as accession number MF7021 in the permanent Merck Culture Collection. Liquid inocula of isolates were prepared from fresh slant cultures on PDA (Difco) as described by Pelaez et al.[16] Two-milliliter aliquots of the inoculum culture were used to inoculate 250 mL Erlenmeyer flasks containing 50 mL of CYS80 liquid production medium (sucrose 80 g/L, yellow corn meal 50 g/L, yeast extract Difco 1 g/L). The flasks were incubated for 14 days at 22 °C on a rotary shaker at 220 rpm.

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 to dryness under reduced pressure, and the residue was redissolved in 5 mL of MeOH and chromatographed on a 70 mL Sephadex LH-20 column eluted with MeOH at a flow rate of 12 mL/min, affording two adjacent active fractions (18.1 mg). 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 linear gradient of 40−100% aqueous CH_3CN containing 0.1% TFA at a flow rate of 10 mL/min. Lyophilization of fractions eluting at 11, 15, 19, and 23 min afforded 0.8 mg (40 mg/L) of compound **2**, 1.4 mg (70 mg/L) of compound **3**, 2.1 mg (105 mg/L) of terferol **1**, and 1.6 mg (80 mg/L) of compound **4** as yellow amorphous powders.

Terferol 1. Compound **1** was identified by comparison of UV, IR, ^1H, and mass spectral data with the published data.[4]

Compound 2: UV (CH_3OH/THF, 1:1) λ_{max} 261 (7538), 310 (6447) nm; IR (ZnSe) ν_{max} 3318, 2959, 2893, 1474, 1457, 1412, 1365, 1343, 1322, 1191, 1120, 1060, 1036, 962, 925 cm^{-1}; ^1H NMR ($CDCl_3$) δ 6.71 (1H, s, H-3), 3.38 (1H, s, OCH_3-7), 6.84 (1H, d, J = 8.5 Hz, H-3′), 6.74 (1H, dd, J = 8.5, 3.0 Hz, H-4′), 6.82 (1H, d, J = 3.0 Hz, H-6′), 7.56 (2H, dd, J = 8.0, 1.3 Hz, H-2″, 6″), 7.37 (2H, dd, J = 8.0, 7.4 Hz, H-3″, 5″), 7.27 (1H, tt, J = 7.4, 1.3 Hz, H-4″); ^{13}C NMR ($CDCl_3$) δ 117.6 (C-1), 146.7 (C-2), 112.9 (C-3), 129.0 (C-4), 140.0 (C-5), 145.0 (C-6), 60.6 (C-7), 121.0 (C-1′), 146.5 (C-2′), 117.8 (C-3′), 116.5 (C-4′), 150.2 (C-5′), 117.7 (C-6′), 137.4 (C-1″), 128.9 (C-2″, 6″), 128.1 (C-3″, 5″), 127.1 (C-4″); HMBC (H → C) H-3 → C-1″, 1, 2, 5; OCH_3-7 → C-6; H-3′ → C-1′, 5′; H-4′ → C-6′, 2′; H-2″,6″ → C-4″, 4; H-3″, 5″ → C-3″/5″,1″; H-4″ → C-2″/6″; HRESI-FTMS m/z 325.1084 (calcd for $C_{19}H_{16}O_5$+H, 325.1076).

Compound 3: UV (CH_3OH/THF, 1:1) λ_{max} 263 (7,402), 283 (5,646), 313 (4,517) nm; IR (ZnSe) ν_{max} 3302, 2957, 2886, 1474, 1457, 1440, 1365, 1343, 1322, 1289, 1189, 1120, 1056, 1024, 959, 922, 842 cm^{-1}; ^1H NMR ($CDCl_3$) δ 6.81 (1H, s, H-3), 3.41 (3H, s, OCH_3-7), 7.06 (1H, dd, J = 8.0, 1.0 Hz, H-3′), 7.32 (1H, td, J = 8.0, 1.7 Hz, H-4′),

7.04 (1H, td, $J = 8.0$, 1.0 Hz, H-5′), 7.40 (1H, dd, $J = 8.0$, 1.7 Hz, H-6′), 7.64 (2H, dd, $J = 8.0$, 1.3 Hz, H-2″, 6″), 7.44 (2H, dd, $J = 7.9$, 7.4 Hz, H-3″, 5″), 7.34 (1H, tt, $J = 7.4$, 1.3 Hz, H-4″); ^{13}C NMR ($CDCl_3$) δ 117.2 (C-1), 146.7 (C-2), 112.8 (C-3), 129.1 (C-4), 140.0 (C-5), 145.0 (C-6), 60.7 (C-7), 120.0 (C-1′), 153.6 (C-2′), 116.8 (C-3′), 129.8 (C-4′), 120.8 (C-5′), 132.0 (C-6′), 137.3 (C-1″), 129.0 (C-2″/6″), 128.2 (C-3″/5″), 127.2 (C-4″); HMBC (H → C) H-3 → C-1″, 1, 3, 2; OCH_3-7 → C-6; H-3′ → C-2′, 5′; H-4′ → C-6′, 2′; H-5′ → C-3′, 1′; H-6′ → C-1, 4′, 2′; H-2″,6″ → C-4″, 4, 2″/6″; H-3″,5″ → C-3″/5″,1″; H-4″ → C-2″/6″; HRESI-FTMS *m/z* 309.1118 (calcd for $C_{19}H_{16}O_4$+H, 309.1127).

Compound 4: UV (CH_3OH/THF, 1:1) λ_{max} 261 (9744) 327 (5317) nm; IR (ZnSe) ν_{max} 3303, 2959, 2889, 1653, 1474, 1457, 1440, 1366, 1344, 1322, 1190, 1120, 1058, 1035, 961, 924, 846 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 6.92 (1H, s, H-3), 3.85 (3H, s, OCH_3-7), 7.35 (2H, m, H-2′, 6′), 7.44 (1H, m, H-3′, 5′), 7.42 (1H, m, H-4′), 7.54 (2H, m, H-2″, 6″), 7.47 (2H, m, H-3″, 5″), 7.48 (1H, m, H-4″); ^{13}C NMR ($CDCl_3$) δ 128.7 (C-1), 155.2 (C-2), 132.8 (C-3), 130.0 (C-4), 183.1 (C-5), 187.1 (C-6), 61.4 (C-7), 132.4 (C-1′), 130.5 (C-2′), 127.9 (C-3′), 128.7 (C-4′), 127.9 (C-5′), 130.5 (C-6′), 132.8 (C-1″), 129.1 (C-2″/6″), 128.5 (C-3″/5″), 130.0 (C-4″); HMBC (H → C) H-3 → C-1″, 1, 5; OCH_3-7 → C-2; H-2′/6′ → C-1, 4′; H-2″/6″ → C-4, 4″; HRESI-FTMS *m/z* 291.1004 (calcd for $C_{19}H_{14}O_3$+H, 291.1021).

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]-γATP. The enzyme transfers a phosphate group from ATP to the substrate peptide. Inhibition of the transfer of this phosphate group, by active extracts and compounds, was measured as detailed by Gurnett.[1]

Whole Cell *Toxoplasma gondii* (TgWC). Whole cell antiparasitic activity was determined using a strain of *T. gondii* stably expressing bacterial β-galactosidase.[5] 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-β-D-galactopyranoside (CPRG), a colorimetric β-galactosidase substrate, was added. Following an additional 2−4 h incubation period, β-galactosidase enzyme activity and therefore parasite viability were measured by reading absorbance at 570 nm.[6]

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