JOURNAL OF NATURAL PRODUCTS

Note

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Diarylcyclopentendione Metabolite Obtained from a *Preussia typharum* Isolate Procured Using an Unconventional Cultivation Approach

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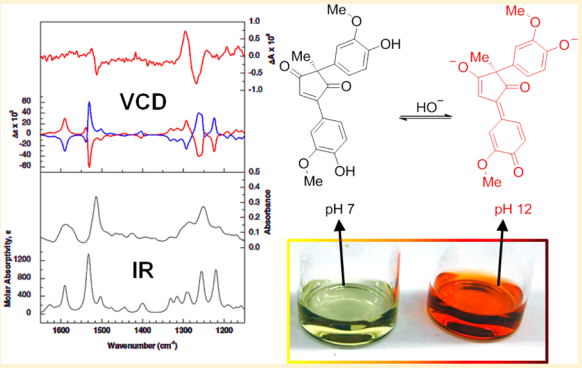
Supporting Information

ABSTRACT: An uncommon 2,5-diarylcyclopentenone compound, preussidone (**1**), and a new biphenyl compound, 1′,5-dimethoxy-3,5′-dimethyl-2,3′-oxybiphenyl-1,2′-diol (**4**), together with two known biphenyl compounds, 5-methoxy-3,5′-dimethyl-2,3′-oxybiphenyl-1,1′,2′-triol (**2**) and cyperin (**3**), were obtained from a *Preussia typharum* isolate that was procured using a panel of unconventional media formulations. The structures of the new compounds were established by NMR and mass spectrometry, while the absolute configuration of **1** was assigned by quantum chemical ECD and VCD calculations. The antimicrobial and DPPH radical scavenging activities of **1**−**4** were tested. Compounds **2** and **4** exhibited DPPH radical scavenging activities that were comparable to the positive control ascorbic acid.

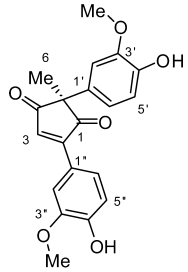


Cosmopolitan fungi such as *Acremonium*, *Aspergillus*, *Fusarium*, *Penicillium*, *Phoma*, and *Trichoderma* are the sources of many thousands of secondary metabolites,[1] which exhibit a range of unique chemical motifs.[2] However, genomics data provide compelling evidence that the secondary-metabolite biosynthetic capabilities of fungi extend well beyond these and other intensely studied phylogenetic lineages.[3] Therefore, efforts to enhance the prevalence of uncommon and/or locally endemic fungal isolates within culture libraries should provide access to new natural products. A variety of successful strategies have been reported that have focused on creating culture conditions that select for the growth of particular subsets of fungi, while interfering with the growth of other organisms.[4,5] Methods such as these have helped with the procurement of various fungal isolates that have been the sources of many intriguing natural products.[6,7] However, these techniques tend to work best as part of "informed" isolation strategies when the selection of a defined group of targeted organisms has been determined *a priori*.

In contrast, our group's efforts to obtain new fungal isolates is not directed toward the procurement of selected fungal taxa, but rather it is guided by the necessity to eliminate or severely restrict the culturability of cosmopolitan species. Consequently, our laboratory has addressed this need by developing several unusual media formulations (Supporting Information, Table S1), which we now routinely use to acquire new fungal isolates. Several of the media contain markedly enhanced concentrations of organic or inorganic substrates, which we presume confer intense stresses upon many frequently encountered fungal species. This approach has enabled the isolation of a variety of unique low-abundance fungal propagules, which are desirable accessions for incorporation into our microbial library. We recently used this panel of unusual media to investigate a soil sample collected from a bottomland forest (Oliver's Woods) located near the University of Oklahoma campus.[8] Using this approach, 169 morphologically distinct fungal isolates were obtained. Among the 22 media and culture conditions used in the panel, HSUC (high sucrose) medium (50 g sucrose, 1.5 g agar, 100 mL H_2O) proved to be a valuable tool for obtaining many morphologically distinctive isolates. One of the HSUC-derived isolates that exhibited an unusual phenotype was determined to be *Preussia typharum* (Sacc.) Cain. A number of metabolites have been reported from *Preussia* spp. including anthraquinones,[9] a diphenyl ether,[10] culpin,[11] preussin,[12] auranticins A and B,[13] preussomerins A−F,[14−16] the cycloartane triterpene S19159,[17] spirobisnaphthalenes,[16] and thiopyranchromenones.[4] In the process of testing different media

Received: July 5, 2012
Published: October 9, 2012

for small-scale bioactivity screening of the *P. typharum* isolate, we made the fortuitous discovery that the fungus was capable of vigorous growth on Cheerios breakfast cereal supplemented with a 0.3% sucrose solution. Considering that the LC-MS trace of the ethyl acetate extract of the fungus grown on Cheerios medium exhibited a constellation of metabolites that was not attributable to most of the previously reported compounds (Supporting Information, Figure S1), we selected this strain for a detailed investigation. In this report, we describe the isolation, structural determination, and absolute configuration assignments, as well as DPPH radical scavenging activities, of metabolites **1**–**4** from the *P. typharum* isolate.



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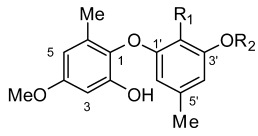

2: R_1=OH, R_2=H
3: R_1=H, R_2=H
4: R_1=OH, R_2=Me

Compound **1** was determined to have the molecular formula $C_{20}H_{18}O_6$ based on HRESIMS, which corresponded to 12 units of unsaturation. The ^{13}C NMR (Table 1) and HSQC spectra indicated the presence of three methyl groups, seven methines, and 10 quarternary carbons. A set of two 1,3,4-trisubstituted phenyl groups was identified based on the characteristic ABC coupling patterns exhibited by their respective proton resonances (δ_H 6.5–8.0, refer to Table 1 for ^1H NMR data). The relative assignments of the methoxyl and hydroxyl groups were confirmed based on ^1H–^{13}C HMBC correlation data revealing $^3J_{H-C}$ couplings from 3′-OCH_3 (δ_H 3.81) and 3″-OCH_3 (δ_H 3.92) to C-3′ (δ_C 148.4) and C-3″ (δ_C 148.7), respectively (Figure 1). The remaining portion of the molecule consisted of $C_6H_4O_2$ and accounted for the remaining four units of unsaturation. A set of ^1H–^{13}C HMBC $^{2-3}J_{H-C}$ couplings from the remaining methyl (δ_H 1.54) and olefinic (δ_H 7.62) singlet hydrogens to two ketones (δ_C 203.7 and 205.7), one quaternary olefinic carbon (δ_C 156.1), and one sp^3 carbon resonance (δ_C 56.3) (Figure 1) established that these atoms formed a disubstituted methylcyclopentenedione system. Finally, ^1H–^{13}C HMBC correlations from H-2′/H-6′ and H-2″/H-6″ to C-5 and C-2, respectively, were used to establish the attachments of both diaryl groups to the cyclopentenedione core. Thus the planar structure of **1** was established as 2,4-bis(4-hydroxy-3-methoxyphenyl)-2-methylcyclopent-4-ene-1,3-dione. We have given metabolite **1** the trivial name preussidone in recognition of its biogenic source.

Table 1. ^1H and ^{13}C NMR Data for 1 and 4 (400 and 100 MHz, δ ppm)

	1[a]		**4**[a]	
no.	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1	205.7		135.6	
2	156.1		151.7	
3	139.1	7.62, s	101.1	6.42, d (2.4)
4	203.7		158.3	
5	56.3		107.8	6.32, d (2.4)
6	19.8	1.54, s	133.3	
4-OCH_3			55.7	3.75, s
6-CH_3			16.5	2.05, s
1′	130.6		147.0	
2′	111.0	6.93, d (2.1)	134.5	
3′	148.4		149.3	
4′	147.0		107.8	6.47, d (1.2)
5′	115.9	6.77, d (8.3)	128.7	
6′	120.1	6.72, dd (2.1, 8.3)	108.3	5.95, d (1.2)
3′-OCH_3	56.3	3.81, s	56.7	3.82, s
5′-CH_3			21.5	2.10, s
1″	122.0			
2″	113.3	7.77, d (2.1)		
3″	148.7			
4″	151.4			
5″	116.3	6.96, d (8.2)		
6″	125.1	7.78, dd (2.1, 8.2)		
3″-OCH_3	56.4	3.92, s		

[a]Recorded in acetone-d_6 at 25 °C.

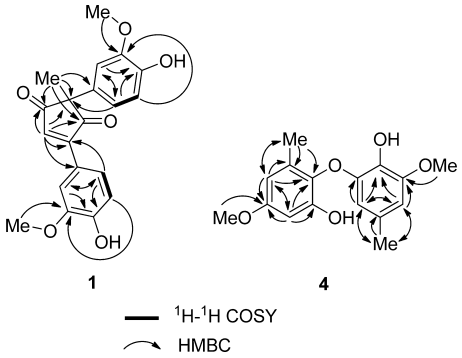

Figure 1. Key ^1H–^1H COSY and ^1H–^{13}C HMBC correlations for **1** and **4**.

The unusual oxidation state and substitution pattern of the cyclopentenedione in **1** prohibited using existing empirical rule sets to reliably deduce the absolute configuration of C-5. Therefore, we employed *ab initio* ECD and VCD spectral calculation methods to compare theoretical and experimentally derived data sets for **1**. Density functional theory (DFT) calculations performed at the B3LYP/6-31+G** level were used to generate ECD and VCD spectra for a set of the eight lowest-energy conformers of **1**. The resulting ECD spectra were combined by Boltzmann weighting to give a composite spectrum for each enantiomer. The calculated ECD spectrum of the *R* enantiomer (blue-shifted 15 nm) agreed well with the experimental data (Figure 2A). Similarly, the calculated VCD spectra (prepared using ComputeVOA software, BioTools, Inc.) were comparable to the experimental VCD spectrum of **1**, which also supported an *R* configuration for the stereogenic C-5 position (Figure 2B).

2,5-Diarylcyclopenteneones and their biosynthetically related pulvic acid analogues[19−24] are principally found in lichens and basidiomycetes; however, a set of similar metabolic intermediates, including the aspergillides[25] and asterredione,[26] have also been reported from two *Aspergillus terreus* isolates. Compound **1** varies from these natural products since it combines several chemical features that had not been previously observed incorporated into a single compound within this metabolite family. Accordingly, the proposed biosynthetic formation of **1** is decidedly speculative (Supporting Information, Scheme S1), but it does provide a blueprint

dx.doi.org/10.1021/np300473h | *J. Nat. Prod.* 2012, 75, 1819–1823

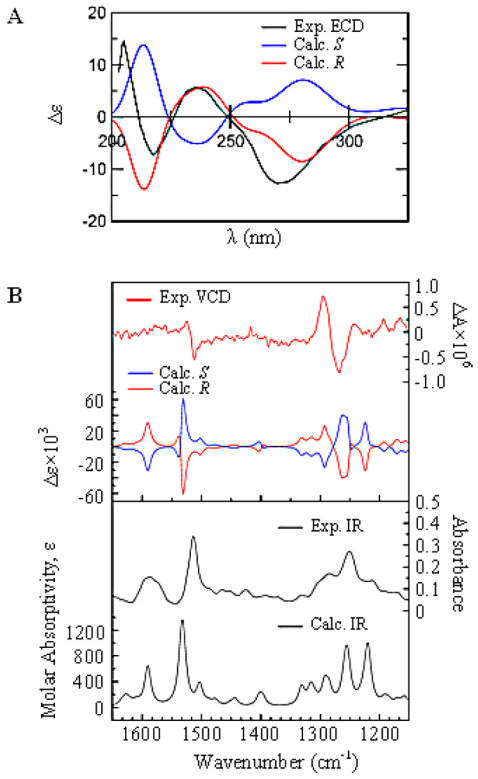

Figure 2. Stereochemical assignment of **1** by quantum chemical ECD (A) and VCD (B) calculations.

for envisaging how the condensation of two units of 3-(3,4-dihydroxyphenyl)-2-hydroxyacrylic acid could produce **1**.

This group of metabolites[19−24] has been implicated in the formation of brown and blue discolorations associated with certain fruiting-body injuries in basidiomycetes. We observed that compound **1** held at pH 2 and 7 afforded almost no discoloration to a 30 μg/mL methanolic solution, whereas at pH 12, the solution turned a rust-red color (red-shift of the UV−vis spectrum from λ 380 to 486 nm; Supporting Information, Figure S2). We hypothesize this can occur as illustrated in Figure S2, wherein an extended conjugated system is formed following the deprotonatation of **1** under basic conditions.

Two known biphenyl metabolites, 5-methoxy-3,5′-dimethyl-2,3′-oxybiphenyl-1,1′,2′-triol (**2**) and cyperin (**3**), were also purified from the fraction containing **1**. A fourth metabolite (**4**) was likewise obtained, which exhibited ^{1}H and ^{13}C NMR signatures that were comparable to **2** and **3**. However, efforts to dereplicate the compound proved unsuccessful, leading us to conclude that **4** was a new biphenyl metabolite. HRESIMS provided evidence that the molecular formula of **4** was $C_{16}H_{18}O_5$, which was indicative of 8 degrees of unsaturation. The IR absorption spectrum of **4** revealed a broad band (3402 cm^{-1}) indicating the presence of hydroxyl groups in the compound. The ^{1}H and ^{13}C NMR spectra of **4** were nearly superimposable to those reported for **2**,[12] with the major exceptions being the new proton (δ_H 3.82) and carbon (δ_C 56.7) resonances corresponding to a methoxyl group (Table 1). A $^{3}J_{H-C}$ HMBC correlation from the new proton singlet to C-3 confirmed the location of the methoxyl group, as shown in Figure 1. Therefore, the structure of **4** was established as 1′,5-dimethoxy-3,5′-dimethyl-2,3′-oxybiphenyl-1,2′-diol.

The antimicrobial[27] and DPPH radical scavenging[28] activities of **1**−**4** were tested. Compounds **2** and **4** exhibited modest DPPH radical scavenging activities, with IC_{50} values of 11.6 and 7.9 μM, respectively. In contrast, the positive control, ascorbic acid, exhibited an IC_{50} value of 5.6 μM. No activity was observed for **1** and **3** at concentrations up to 30 μM. None of these compounds exhibited antimicrobial activities against bacteria (methicillin-resistant *Staphylococcus aureus* and *Klebsiella pneumoniae*) or a fungus (*Aspergillus fumigatus*) at concentrations up to 30 μM.

EXPERIMENTAL SECTION

General Methods. Optical rotations were measured on a Rudolph Research Autopol III automatic polarimeter. UV data were measured with a Hewlett-Packard 8452A diode array spectrophotometer. ECD spectra were recorded on a model 202-01 AVIV circular dichroism spectrometer. VCD spectra were recorded on a BioTools, Inc. ChiralIR-2X VCD spectrometer, equipped with a dual PEM accessory. IR spectra were measured on a Bruker Vector 22 FT-IR spectrometer. NMR data were obtained on a Varian VNMR spectrometer (400 MHz for ^{1}H and 2D NMR, 100 MHz for ^{13}C) with a broad band resonance probe at 25 ± 0.5 °C. Electrospray-ionization mass spectrometry data were collected on an Agilent 6538 high-mass-resolution QTOF mass spectrometer. HPLC separations were performed on a Shimadzu system using a SCL-10A VP system controller and Gemini 5 μm C_{18} column (110 Å, 250 × 21.2 mm and 250 × 10.0 mm) with flow rates of 10 or 4 mL/min. All solvents were of ACS grade or better.

Fungal Strain. A sample of finely degraded organic matter was collected from the base of a very large (1.2 m diameter) cottonwood tree recently blown over by a tornadic storm (Tree ID #99) in the Oliver Wildlife Preserve on the outskirts of Norman, OK, in July 2010. The sample was diluted 1:28 (vol/vol) with sterile water and then spread over the surfaces of 10 cm diameter Petri plates containing 22 different agar formulations. The fungi were allowed to grow for several weeks at room temperature, and morphologically distinct colonies were selected from the plates and transferred to fresh Petri plates containing Czapek agar. A second round of strain dereplication was performed based on the patterns of color, growth rate, and morphology of the pure isolates on Petri plates containing Czapek agar. In total, 169 morphologically distinct fungi were obtained (Supporting Information, Table S1). The fungus investigated in this study was obtained from the Petri plates containing HSUC agar, a medium that affords extremely low water activity and high osmotic pressures. The isolate was identified as *Preussia typharum* (Sacc.) Cain according to its morphological characteristics (Supporting Information).[29] This was further supported by sequence data generated for ribosomal internal transcribed spacers and the 5.8S rRNA gene (ITS1-5.8S-ITS2),[30] which were definitive for a *Preussia* sp. (GenBank accession JX143871).

Fermentation, Extraction, and Isolation. Spores were directly inoculated into 300 Erlenmeyer flasks (1 L) that each contained 8 g of Cheerios and 48 mL of a 3 g/L sucrose solution. The flasks were incubated statically for 30 days at room temperature. The cultures were combined and extracted three times with ethyl acetate (20 L each time), which was then evaporated *in vacuo* to generate the crude extract (100 g). The extract was separated into nine fractions by silica gel column chromatography (eluted with a gradient of hexanes/dichloromethane to dichloromethane/MeOH). Fraction 7 was further separated into three subfractions (7-1 to 7-3) by C_{18} vacuum column chromatography. Subfraction 7-1 was subjected to Sephadex LH20 column chromatography (eluted with 1:1 dichloromethane/MeOH). The resulting subfraction 7-1-4 was subjected to semipreparative reversed-phase HPLC (Gemini 5 μm, C_{18}, 110 Å, 250 × 10.0 mm, 60% MeOH in H_2O, 4.0 mL/min) to yield purified **1** (6.7 mg, 0.0067% yield), **2** (22 mg, 0.022% yield), **3** (27 mg, 0.027% yield), and **4** (6.7 mg, 0.0067% yield).

Preussidone (1): yellow solid; $[\alpha]^{24}_{D}$ −68 (*c* 0.3, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 210 (4.45), 270 (4.09), 380 (4.00); UV

dx.doi.org/10.1021/np300473h | J. Nat. Prod. 2012, 75, 1819−1823

(MeOH, HCl, pH = 2) λ_{max} (log ε) 210 (4.43), 270 (4.06), 380 (3.98); UV (MeOH, KOH, pH = 12) λ_{max} (log ε) 212 (4.49), 292 (4.17), 486 (2.81); IR (film) ν_{max} 3430, 2969, 2934, 1735, 1685, 1559, 1509, 1458, 1421, 1374, 1260, 1207, 1129, 1100, 1029 cm^{-1}; CD (EtOH) λ_{max} ($\Delta\varepsilon$) 218 (−2.7), 236 (2.1), 271 (−4.6), 364 (1.6), 422 (−1.0); ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 355.1179, $[M + H]^+$ (calcd for $C_{20}H_{19}O_6$, 355.1176).

*1′,5-Dimethoxy-3,5′-dimethyl-2,3′-oxybiphenyl-1,2′-diol (**4**):* brown oil; UV (EtOH) λ_{max} (log ε) 214 (4.29), 282 (3.49); IR (film) ν_{max} 3402, 2937, 2843, 1617, 1516, 1496, 1464, 1351, 1318, 1270, 1203, 1150, 1087, 983, 933, 819 cm^{-1}; HRESIMS m/z 291.1226, $[M + H]^+$ (calcd for $C_{16}H_{19}O_5$, 291.1227).

Quantum Chemical ECD Calculations. DFT calculations were performed using the Gaussian09 program[31] with the B3LYP functional and 6-31+G** basis set. The geometry was optimized starting from various initial conformations, with vibrational frequency calculations confirming the presence of minima. Time-dependent DFT calculations were performed on eight lowest-energy conformations for each configuration using 20 excited states. ECD spectra were calculated by fitting Gaussian functions with a half-bandwidth of 0.2 eV to the rotatory strengths. The results reported here are for rotatory strengths calculated in the dipole length formalism. The dipole velocity forms yielded negligible differences. The spectra of the conformers were combined using Boltzmann weighting, with the three lowest-energy conformations accounting for about 90% of the weights. The calculated spectrum was blue-shifted by 15 nm to facilitate comparison to the experimental data.

Quantum Chemical VCD Calculations. A conformational search was carried out with ComputeVOA for the *R* configuration at the molecular mechanics level. Geometry, frequency, and IR and VCD intensity calculations of the 22 lowest-energy conformers resulting from the conformational search were carried out at the DFT level (B3LYP functional/6-31G(d) basis set) with Gaussian 09.[31] The calculated frequencies were scaled by 0.98, and the IR and VCD intensities were converted to Lorentzian bands with a 6 cm^{-1} half-width for comparison to experimental data. The VCD studies were carried out under contractual agreement at BioTools, Inc., Jupiter, FL.

Biological Assays. The antimicrobial assays were performed as previously described.[27] For the DPPH radical scavenging assay,[28] samples (10 mM in DMSO) and ascorbic acid (10 mM in DMSO, positive control) were dissolved in 2-propanol, and the solution (50 μL) or the vehicle (50 μL of 2-propanol, negative control) was dispensed into wells of a 96-well microtiter tray. An aliquot of 150 μL of the DPPH solution in 2-propanol (40 uM) was added to each well. The mixture was shaken and left to stand for 30 min. After the reaction, the absorbance of each well was measured at 520 nm using a microplate reader (Infinite M200, Tecan Group Ltd.), and the percent inhibition calculated by comparisons to control wells. All the samples were tested in triplicate. The reported IC_{50} values denote the concentration of sample required to scavenge 50% of the DPPH free radicals.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

NMR (^1H and ^{13}C NMR, HSQC, HMBC, and COSY) data for compounds **1** and **4**. Fungal strain identification, summary of fungal isolates from different media sources, and associated data. This information is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Research reported in this publication was supported by the National Institute of General Medical Sciences of the National Institutes of Health under award number RO1GM092219. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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