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Azaphilones from an Acid Mine Extremophile Strain of a *Pleurostomophora* sp

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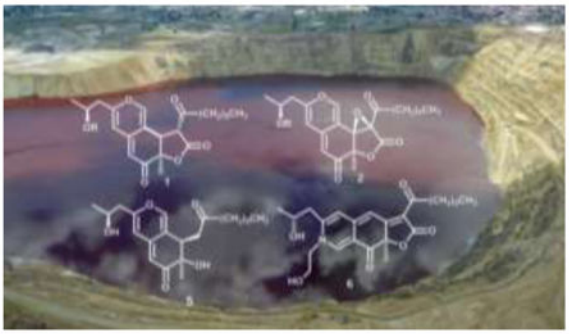
Abstract

An extremophilic fungus identified as a *Pleurostomophora* sp. was isolated from the Berkeley Pit, an acid mine waste lake. When grown in liquid culture the fungus produced berkchaetoazaphilones A-C (**1**, **2**, and **5**), the red pigment berkchaetorubramine (**6**), and the known compound 4-(hydroxymethyl)-quinoline. These compounds were evaluated as inhibitors of matrix metalloproteinase-3, caspase-1 and of proinflammatory cytokine production in induced THP-1 cells. Berkchaetoazaphilone B (**2**) inhibited IL-1β, TNFα, and IL-6 production in the induced inflammasome assay and was cytotoxic toward human retinoblastoma cell line *Y79* (IC_{50} = 1.1 μM), and leukemia cell lines *CCRF-CEM* and *SR*, and the melanoma cell line *LOX IMVI* (IC_{50} = 10 μM).

Graphical Abstract



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Supporting Information. Experimental details including ^{1}H NMR, ^{13}C NMR, COSY, NOESY, HSQC and HMBC spectra for compounds **1**, **2**, **5** and **6** and ECD spectra for **1** and **2**, discussion of 5-membered ring conformation, and details of testing data. The Supporting Information is available free of charge on the ACS Publications website at DOI:

Berkeley Pit Lake, an abandoned open-pit copper mine, is part of the largest EPA Superfund site in North America.[1] For the past 15 years this acidic, metal-rich lake has been a rich source of bioactive secondary metabolites produced by extremophilic fungi isolated from Pit water and sediments.[2] A filamentous fungus isolated from a water sample collected at a depth of 15 m was identified as a *Pleurostomophora* sp.[3] by 28 S rRNA sequence comparison (closest match, 319 base pairs).[4] The organic extracts of this fungus grown in potato dextrose broth inhibited the signal transducing enzymes matrix metalloproteinase-3 (MMP-3) and caspase-1. Inhibition of these enzymes guided compound isolation for this study.

According to the National Cancer Institute, most cancer deaths are associated with metastatic cancer[5] and drugs that block metastasis are currently not available.[6] MMP-3 promotes epithelial mesenchymal transition (EMT), a critical component of metastatic cancer. EMT is a hallmark of cells undergoing proliferation and differentiation and is characterized by loss of cell adhesion, repression of cell adhesive E-cadherin (E-cad) expression, destruction of the basal lamina, and increased cell mobility.[5–8] MMP-3 promotes tumor dissemination and hyperplasia by inducing EMT through activation of MMP-9 (which destroys the basal lamina) and through direct cleavage and repressed synthesis of E-cad.[9] Small molecule inhibitors of MMP-3 could mitigate EMT and its role in carcinogenesis.

Inflammation also contributes to EMT and the promotion of the tumor microenvironment, and anti-inflammatory drugs have been shown to reduce the risk of cancer.[10] Chronic inflammation involves macrophage accumulation either through recruitment or proliferation and is often associated with cancer initiation and promotion.[11] Within the macrophage, the NLRP3 inflammasome assemblage plays an important role in innate immunity.[11,12] It activates caspase-1 upon binding, which subsequently activates the pro-inflammatory cytokines IL-1β and IL-18 which can lead to chronic inflammation and the production of reactive oxygen species (ROS). ROS can induce oxidative damage to DNA, which can lead to the initiation and progression of carcinogenesis.[11] In this study, the induced THP-1 cell assay was used to assess the ability of caspase-1/MMP-3 inhibitors to block production of pro-inflammatory cytokines in a cellular system that is analogous to a tumor-associated macrophage (TAM) (Supporting Information, S30).[2a,b] MMP-3 and caspase-1 inhibition assays were used to select for microbial metabolites with activity against specific cancer cell lines associated with the up-regulation of at least one of these enzymes.

The $CHCl_3$ extract of a 10 day shake culture of *Pleurostomophora* sp. yielded the new compounds berkchaetoazaphilone A (**1**), berkchaetorubramine (**6**), and the known compound 4-(hydroxymethyl)-quinoline.[2e] Following acidification (pH 2.5), the $CHCl_3$ extract yielded berkchaetoazaphilone B (**2**) and the MeOH soluble mycelial extract yielded berkchaetoazaphilone C (**5**). The structures of these compounds were established by mass spectrometry and extensive NMR spectroscopy, molecular modeling, ECD, and comparison of spectroscopic data to those of known, related azaphilones.

Compound **1** had a molecular formula of $C_{25}H_{34}O_6$ and nine sites of unsaturation, which were established by the HRESIMS $[M+H]^+$ ion of *m/z* 431.2436. The three carbonyl absorbances in the IR spectrum were attributed to a saturated γ-lactone (1779 cm^{-1}), a

saturated ketone (1718 cm^{-1}) and an α,β-unsaturated ketone (1670 cm^{-1}). The out-of-plane bending region exhibited a single sharp absorption at 800 cm^{-1}, more typical of a tri-substituted olefin than of an aromatic ring.[13] Although only 24 carbons were observed in the ^{13}C NMR spectrum ($CDCl_3$ or C_6D_6), correlations in the HSQC spectrum confirmed the presence of two overlapping methylene carbons. The ^{13}C NMR spectrum (Table 1) also showed evidence of both the saturated and the *α,β*-unsaturated ketone carbonyls (δ_C 202.3 and 191.5, respectively), an ester carbonyl (δ_C 168.5) and six olefinic carbons (δ_C 159.6, 147.5, 144.1, 114.6, 108.5, 106.0). As the three carbonyls, three olefins and the γ-lactone ring accounted for seven of the nine degrees of unsaturation, compound **1** was determined to be tricyclic. The two remaining rings were highly conjugated with an electron donating group at one end and an electron withdrawing group (the α,β-unsaturated ketone carbonyl) at the other end. These data suggested a fused γ-lactone azaphilone.[2b] Fungally derived tricyclic azaphilones usually adopt either an angular or a linear fused scaffold (Figure 1).[14] Angular azaphilones include the chaetoviridins, sassafrin A (**7**), and monochaetin.[14–16] Linear azaphilones include rubropunctatin and monascorubrin.[14] Both scaffolds typically have a R_1 unit that consists of a proximal ketone carbonyl and variable hydrocarbon chain, and a three carbon R_2 unit.[14] Comparison of the ^1H NMR and COSY data with that of known azaphilones indicated that compound **1** had the same angular scaffold as sassafrin A (**7**).[14,15]

These data also provided evidence of a 1-oxodecyl moiety in the R_1 position and a 2-hydroxy-propyl moiety in the R_2 position (Figure 1). HMBC data provided adequate support for both of these assignments. Methine H-8 (δ_H 3.83) and H-14 (δ_h 3.70) showed correlations to the γ-lactone carbonyl C-13 and to the saturated ketone carbonyl C-15. Methylene H_2-16 (δ_H 3.13 and 2.48) showed strong correlations to C-15, establishing the position of the long chain hydrocarbon substituent on the lactone ring. H-4 showed correlations to methylene C-10, which confirmed the position of the propyl side chain. These key HMBC correlations shown in Figure 2 helped to establish the structure of compound **1** as shown. Comparison of the spectroscopic data of **1** with those of saffranin A,[15] monochaetin,[16] biscogniazaphilone B,[17] and monascuskaolin[18] supported this structure. The relative and absolute configurations of **1** and related azaphilones will be addressed in a later section.

Compound **2** gave an $[M+H]^+$ ion of *m/z* 445.2217 (HRESIMS). It had a molecular formula of $C_{25}H_{32}O_7$ with one more oxygen and degree of unsaturation than **1**. Although the NMR spectra (Table 1) and key HMBC correlations were similar to those of **1**, the NMR data of **2** indicated the presence of two oxygen-bearing non-protonated carbons at δ_C 72.1 and 66.8 and the absence of the two spin-coupled methine protons at H-8 and H-14. These data suggested the presence of a C-8-C-14 epoxide, which is unprecedented in this family of compounds.

In an HMBC experiment optimized for 8 Hz, methyl singlet H_3-9 (δ_H 1.57) and singlet olefins H-1 and H-4 (δ_H 7.44, 6.12, respectively) showed correlations to epoxide C-8 (δ_C 72.1). When the HMBC experiment was optimized for 3 Hz, methylene H-16 (δ_H 2.80) showed a correlation to epoxide C-14 (δ_C 66.8), which established the structure as berkchaetoazaphilone B (**2**). When treated with pyridine and acetic anhydride, compound **2**

formed a diacetate (**3**). Inspection of the spectroscopic data suggested the formation of a C-11 acetate and an enol acetate at C-15 (the configuration of the enol acetate was not determined). Epoxides exert a strong inductive electron withdrawing effect which would render H_2-16 more acidic and facilitate enol-acetate formation.[19]

Fractionation of the MeOH extract of the mycelia yielded compound **5** which had a molecular formula of $C_{24}H_{36}O_5$ (HRESIMS) with seven sites of unsaturation. It had one less carbon than either **1** or **2** and two less sites of unsaturation. The IR spectrum showed evidence of the saturated and α-β-unsaturated ketone carbonyls (1710 and 1673 cm^{-1} respectively), but not of the γ-lactone. The ^{1}H NMR spectrum of **5** included resonances that supported the presence of the bicyclic azaphilone skeleton and the two aliphatic side chains found in compounds **1** and **2**. The ^{13}C NMR spectrum of **5** showed no evidence of lactone carbonyl C-13 and indicated that the C-14 methine (compound **1**) was replaced by a methylene. The ^{1} H NMR spectrum of **5** indicated the presence of a CH-CH_2 moiety, C-8-C-14. These data, as well as all HMBC and COSY correlations, were accommodated by **5**. When the ^{1}H NMR spectrum of **5** was recorded in $CDCl_3$, H_3-9, H_3-12 and methylene protons overlapped. When recorded in C_6D_6, H_3-9 was well resolved at δ_H 1.35. The NOESY spectrum recorded in C_6D_6 showed a cross-peak between H_3-9 and H-8 (δ_H 3.52), indicating a *cis*- relationship consistent with compounds **1** and **2**. Compound **5** could be functionally derived by opening the lactone ring in **1**, with subsequent decarboxylation of the resulting β-ketoacid to give **5**.

Defining the stereoconfiguration of compound **1** was an interesting exercise. Numerous attempts were made to crystallize compounds **1** and **2**, but X-ray quality crystals have remained elusive. We could find no reports in the literature of X-ray data for tricyclic azaphilones related to **1** with H-8-H-14 present, so NOE correlations, chiral derivatives, and ECD spectra provided evidence for 3D assignments. The 2D-NOESY spectrum showed a correlation between H-8 and H_3-9 which could only be accommodated by if they were on the same side of the ring. However, establishing the relationship between H-8 and H-14 was not as straightforward. In five-membered rings, coupling constants can be similar in magnitude for both *cis*- and *trans*- vicinal protons, and NOE correlations can be present in both cases.[20] For example, H-8 and H-14 were assigned as *cis*- in sassafrin A (**7**) (J = 12.4)[15] and as *trans*- in biscogniazaphilone B[17] and monascuskaolin (J = 12.0).[18] Energy minimized conformers of both the *cis*- and *trans*- diastereomers of **1** were generated to facilitate correlation of spectroscopic data with structure.[21] H-14 showed an NOE correlation to H-8 and H_2-16, but not to H_3-9. However, energy minimized structures of both H-14 epimers indicated that H_3-9 and H-14 were too far apart for NOE correlations whether they had a *cis*- (4.68 Å) or *trans*- (4.12 Å) relationship (Supporting Information, Figure S27). However, the magnitude of the $^3J_{8–14}$ coupling constant (J = 12.2) could be correlated to the dihedral angle between H-8 and H-14 using a modified Karplus equation appropriate for five-membered rings (Figure S28).[22] When a five-membered ring assumes an envelope conformation, $J_{cis} > J_{trans}$, with J_{max} of 10–11 Hz when the two protons are eclipsing (dihedral angle = 0º). However, when the ring assumes a skewed conformation as in **1**, $J_{trans} > J_{cis}$, with J_{max} approaching 12–13 Hz when the dihedral angle approaches 180º. Energy minimized models of **1** showed a dihedral angle of 164º for *trans*-H-8-H-14 with $^3J_{8–14}$ of 12

Hz, and a dihedral angle of 22° for *cis*-H-8-H-14 with $^3J_{8-14}$ of 8.5–9 Hz. This analysis supported the *trans*- relationship and the configuration as shown.[22]

The absolute configuration of C-7 in **1**, **2**, and **5** was assigned as *R* based on their ECD spectra (Figure S26), which showed positive Cotton effects at 373 nm (Δε +4.42), 348 nm (Δε +7.66), and 362 nm (Δε +2.58) respectively, which is consistent with other related azaphilones[15,18,23,25] The absolute configuration of C-11 was determined using a modified Mosher's method.[24] Compound **1** did not yield a reasonable product so compound **2** was treated with *R*- or *S*-methoxy-(trifluoromethyl) phenylacetyl (MTPA) chloride in pyridine to give the corresponding *S*- or *R*- esters respectively. Molecular modeling of the resulting esters and consideration of the δ values (Figure 3) indicated that the absolute configuration at C-11 was *S*, which should be consistent for **1**, **2**, **5**, and **6**. These data generated the structure of **1** as shown.

The relationship between the epoxide (C-8-C-14) and methyl C-9 in compound **2** was established by an NOE correlation between H_3-9 and olefinic proton H-5 which could only be accommodated if the epoxide and C-9 were *anti*- to each other. Extensive molecular modeling studies indicated that the distance between H_3-9 and H-5 was 3.70 Å for the *anti*-relationship and 4.15 Å for the *syn*- relationship, which exceeds the effective range of NOE (Figure S29).[21] Further evidence for the *anti*- configuration was provided by the NOE correlation between olefinic H-16 and H-1 in diacetate **3**. Energy minimized structures were generated for the E- and Z-Δ^{15} isomers of the *syn*- and *anti*- configurations of **3**. The distance between H-1 and H-16 was 2.82–3.34 Å for the *anti*- configuration and 3.96–4.02 Å for the *syn*- configuration, providing further support for the *anti*- configuration as shown.

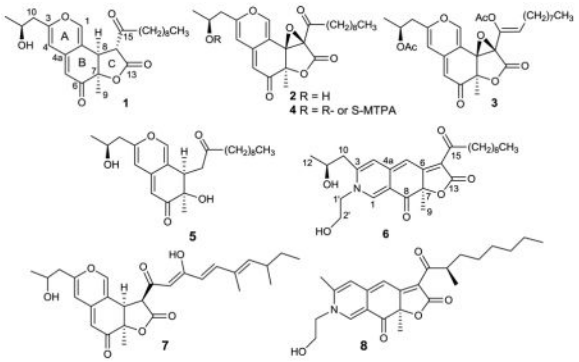
Comparison of the spectroscopic data of several related azaphilones suggested that specific ^{1}H NMR chemical shifts (H-8, H-14 and H_3-9) and ECD data could be used to predict stereoconfiguration (Table 2). H_3-9 is shielded in compounds with *trans*-ring junctions like cohaerins G and H[25] and chermesinone B[26], and deshielded in compounds with *cis*-ring junctions like sassafrin A (**7**), monascuskaolin, and compounds **1** and **2**.[15,18] A similar chemical shift pattern is also seen for H_3-9 in the related bicyclic compounds like **5**, monapurone A,[27] and cohaerins I and K.[25] If this pattern is indeed predictive, then longirostrerone C should have a *trans*-ring junction rather than *cis*-, as was reported in the literature.[28] It is interesting to note that the closely related longirostrerone B was assigned a *trans*- ring junction. The ring junction of helotialin A should also be reassigned as *trans*-.[29] The authors noted that their assignment of the *cis*- ring junction was based on the similarity of their compound to cohaerin F,[29] which was later revised to the configuration shown.[25]

Comparison of ECD data in Table 2 also suggested a trend. The absolute configuration of C-7 in these closely related azaphilones can be assigned by positive Cotton effect around 370 nm (350–390 nm).[15,18,23,25] This trend is observed regardless of substituent groups. For example, sassafrin A (**7**) which has a strong chromophore in the lipid tail attached to C-14 has a value of +367 nm, while compounds that lack a strong chromophore - including compounds **1** and **2**, the cohaerins, monapurone and the cohaerins - also show a positive Cotton effect from 350–390 nm.

As mentioned above the magnitude of $^{3}J_{8–14}$ could be used to assign a *cis*- or *trans*-relationship between H-8-H-14 in this class of azaphilones.[22] We hope to validate these hypotheses as they could be useful tools in the structure elucidation of an interesting, bioactive class of molecules.

The $CHCl_3$ extract also yielded a red pigment (**6**) that had a molecular formula of $C_{27}H_{37}NO_6$ with 10 degrees of unsaturation, as established by the HRESIMS $[M+H]^+$ ion of *m/z* 472.2634. When run in MeOH-d_4, the ^{13}C NMR spectrum showed 27 carbons with two ketone carbonyl carbons (δ_C 196.6 and 199.0), one ester carbonyl carbon (δ_C 173.9) and eight olefinic carbons. The remaining degrees of unsaturation required three rings. The NMR data also indicated the presence of both the hydroxypropyl (C-10 – C-12) and the 1-oxodecyl substituents (C-15 – C-24) found in **1**, **2**, and **5**. However, compound **6** also had a single nitrogen connected to a -CH_2-CH_2-OH group (δ_H 4.29 m, 4.11 m; and 3.87 m, 2H) and one more additional degree of unsaturation than **1**. In the ^{1}H NMR spectrum the chemical shifts of H-1, H-4 and H-5 (δ_H 8.30, 7.01, and 6.69, respectively) were more typical of a linear azaphilone skeleton.[30] The nitrogen containing analogs of several linear and angular azaphilones have been synthesized and the two skeletons can be distinguished by the chemical shifts of these protons.[30]

The HMBC spectrum showed 3-bond correlations from H-1 (δ_H 8.30) to ketone carbonyl C-8 (δ_C 196.6), C-3 (δ_C 154.7) and to C-1′ (δ_C 58.1), which connected the hydroxyethyl side chain to the ring system. H-4 (δ_H 7.01) showed 3-bond correlations to C-5 (δ_C 98.6) and C-10 (δ_C 41.4), which provided connectivity between the ring system and the hydroxypropyl moiety. H-5 (δ_H 6.69) showed 3-bond correlations to C-7 (δ_C 87.1) and C-4 (δ_C 120.0). Methyl H_3-9 showed 3-bond correlations to C-7 and C-8, and a 4-bond correlation to ester carbonyl C-13. Finally, side chain methylene H_2-16 (δ_H 2.96) showed a 3-bond correlation to C-14 (δ_C 122.6) which connected the oxodecyl moiety to the ring system and established the structure of the red pigment as **6**. These spectroscopic data compared favorably to those of sequoiamonascin D (**8**), a red pigment isolated from the redwood endophyte *Aspergillus parasiticus*.[31] The configuration of C-11 was assumed to be consistent with compounds **1**, **2**, and **5**.



The known compound 4-(hydroxymethyl) quinoline was identified by careful examination of NMR data and by HRESIMS which yielded an $[M+H]^+$ ion of *m/z* 160.0763, and a

molecular formula of $C_{10}H_9NO$. This compound has been previously isolated from the myxobacterium, *Archangium gephyra*[32] and a wood rotting *Polyporus* fungus.[33]

Compounds **1**, **2** and **6** inhibited both caspase 1 (IC_{50} values: 150, 25, and 50 μM, respectively) and MMP-3 (IC_{50} values: 130, 15, and 45 μM, respectively). The induced THP-1 assay was used to assess their abilities to block production of proinflammatory cytokines in a cellular system. In this assay, bacterial LPS and titanium dioxide (TiO_2) were used to stimulate the production of specific proinflammatory cytokines and the assemblage of the NLRP3 inflammasome in THP-1 cells (Supporting Information, S30).[2a,b] Compounds **1**, **2**, and **6** were administered to induced THP-1 cells and the production of cytokines post-administration was determined. Compound **2** was the most potent: at 10 μM it completely inhibited the production of interleukin 6 (IL-6) and IL-33, and mitigated production of tumor necrosis factor-alpha (TNF-α) and IL-1β by 95%. Compound **1** which lacks the epoxide, shows similar activity at 100 μM, but no effect at 10 μM.

Compounds **1** and **2** were sent to the NCI-Developmental Therapeutics Program and to Memorial Sloan-Kettering Cancer Center (MSKCC) for evaluation as cytotoxic or antiproliferative agents against selected and established cancer cell lines. In the NCI-DTP 60, **1** and **5** were inactive, but **2** had $\log_{10}$ GI_{50} of 10 μM against leukemia cell lines MOLT-4, RPMI-8226 and SR, and melanoma cell line LOX IMVI and $\log_{10}$ GI_{90} of 10 μM against leukemia cell line CCRF-CEM. MSKCC human cell lines include retinoblastoma Y79. Compounds were tested using two different protocols: the Alamar Blue Viability Assay[34, 35] and the Nuclei Count Proliferation Assay. Compound **2** had an IC_{50} of 1.1 μM against Y79 (Supporting Information, S31–32).

Experimental Section

General Experimental Procedures

Instrumentation has been previously described.[2]

Collection, Fermentation, Extraction, and Isolation Procedures

The collection of water samples from the Berkeley Pit, the isolation of the various organisms, and the pilot growths and biological testing of the extracts have been previously described.[2] The fungus was identified (best fit) as a *Pleurostomophora* sp. by Microbial Identification, Inc. Microbial ID, Inc. originally identified the organism as *Chaetomium funicola* (6.58% difference), but alignment of 319 base pairs of the 28S rRNA gene showed a 99% identity with *Pleurostomophora richardsiae*. It has therefore been designated as a *Pleurostomophora* sp. GenBank Accession # KT728826; NRRL repository # 66319.

It was grown in 8 × 500 mL of DIFCO potato dextrose broth in 1 L Erlenmeyer flasks (shaken 180 rpm for 10 days). At harvest time, 20 mL of MeOH was added to each flask and the broth cultures were filtered through cheesecloth to separate the mycelial mat. The filtrate was extracted three times with 500 mL of $CHCl_3$, and the extract was reduced in vacuo to yield *$CHCl_3$ extract A* (0.0590 g, oil). The filtrate was then acidified to pH 2.5 with 50% H_2SO_4 and re-extracted with $CHCl_3$ to yield *$CHCl_3$ extract B* (0.1030 g, oil). The mycelia mat was extracted with 100% MeOH.

$CHCl_3$ extract A was fractionated by flash silica gel column chromatography using a stepwise gradient system of increasing polarity starting with 100% hexanes to 100% IPA (six fractions) followed by 100% MeOH. A red oily pigment that eluted from the flash column with 100% IPA was berkchaetorubramine (**6**) (4.6 mg). The fraction that eluted with 5% IPA/hexanes was further resolved using semi-preparative silica gel HPLC [Varian Dynamax Microsorb 100-5] in gradient mode from 5% IPA/hexanes to 25% IPA/hexanes over 60 min to yield the known 4-(hydroxymethyl) quinoline (1.2 mg). The fraction that eluted with 10% IPA/hexanes was further resolved using the HPLC conditions as described to yield berkchaetoazaphilone A (**1**) (12.8 mg). *$CHCl_3$ extract B* was fractionated by flash silica gel chromatography as described above. Berkchaetoazaphilone B (**2**, 66.4 mg) eluted with 50% IPA/hexanes. The mycelia MeOH extract (3.2 g) was fractionated by size exclusion column chromatography [LH-20, 1:1 $CHCl_3$-MeOH]. Fraction E was further resolved by gradient flash silica gel chromatography (100% $CHCl_3$ to 100% MeOH). The fraction eluting with 20% MeOH was chromatographed with silica gel HPLC in gradient mode [Varian Dynamax, 10% IPA/hexanes to 50% IPA/hexanes] to yield berkchaetoazaphilone C (**5**) (2.5 mg).

Berkchaetoazaphilone A **(1)**: colorless oil, $[\alpha]^{20}_D$ +4.8 (*c* 0.65, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 249 (3.79), 360 (4.05) nm; ECD (*c* 2.32 × 10^{-5} M, MeOH) λ_{max} (Δε) 373 (+4.42), 330 (−7.22), 253 (+0.82) nm; IR ($CHCl_3$) ν_{max} 3550, 3010, 2928, 1779, 1718, 1670, 1623, 1553, 1376, 1248 $cm^{-1;}$ ^1H NMR and ^{13}C NMR see Table 1; HRESIMS *m/z* 431.2436 $[M + H]^+$ (calcd for $C_{25}H_{35}O_6$, 431.2434).

Berkchaetoazaphilone B **(2)**: colorless oil, $[\alpha]^{20}_D$ +41 (*c* 0.10, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 245 (3.30), 354 (3.45) nm; ECD (*c* 4.50 × 10^{-5} M, MeOH) λ_{max} (Δε) 348 (+7.66), 312 (−5.42), 283 (+0.57), 245 (+7.77), 213 (−25.26) nm; IR ($CHCl_3$) ν_{max} 3417, 3025, 2929, 2856, 1789, 1718, 1635, 1550, 1263, 1122 $cm^{-1;}$ ^1H NMR and ^{13}C NMR see Table 1; HRESIMS *m/z* 445.2217 $[M + H]^+$ (calcd for $C_{25}H_{33}O_7$, 445.2226).

Acetylation of 2—Compound **2** (1.0 mg) was dissolved in pyridine (40 μL) and Ac_2O (40 μL) and stirred for 24 hours. After that time the solvents were removed to give diacetate **3** as an oil. ^1H NMR ($CDCl_3$) δ_H 7.02 (s, H-1), 6.01 (s, H-4), 5.41 (t, *J*=7.6 Hz, H-16), 5.38 (s, H-5), 5.14 (m, H-11), 2.65 (dd, *J*=14.7, 7.3 Hz, H-10), 2.54 (dd, *J*=14.7, 5.6 Hz, H-10), 2.05 (3H, s, OAc), 1.99 (3H, s, OAc), 1.56 (3H, s, H-9), 1.29 (3H, d, *J*= 6.2 Hz, H-12), 1.24 (bs), 0.86 (3H, t, *J*= 7.1 Hz, H-24); ^{13}C NMR ($CDCl_3$) δ_C 190.0 (C, C-6), 170.1 (C, OAc), 168.5 (C, OAc), 166.4 (C, C-13), 158.6 (C, C-3), 144.7 (C, C-4a), 144.4 (CH, C-1), 134.2 (C, C-15), 123.6 (CH, C-16), 109.7 (C, C-8a), 108.5 (CH, C-4), 106.2 (CH, C-5), 83.5 (C, C-7), 71.0 (C, C-8), 67.6 (CH, C-11), 66.7 (C, C-14), 38.8 (CH_2, C-10), 31.9 (CH_2, C-22), 29.3 (CH_2, C-21), 29.2 (CH_2, 2C, C-20, C-19), 28.7 (CH_2, C-18), 25.2 (CH_2, C-17), 22.7 (CH_2, C-23), 21.0 (CH_3, OAc), 20.4 (CH_3, OAc), 19.8 (CH_3, C-12), 18.8 (CH_3, C-9), 14.1 (CH_3, C-24); ESIMS *m/z* 529 $[M + H]^+$.

Mosher's Analysis of 2—Compound **2** (1.0 mg) was dissolved in dry pyridine (40 μL) and either the R- or S- stereoisomer of α-methoxy-α-trifluoromethylphenylacetyl chloride (4 μL) added. The mixtures were stirred for 24 hours. After that time, MeOH (400 μL), was added and the solvents removed. The reaction mixtures were then each passed through a

small silica gel column and eluted with hexane and increasing amounts of IPA to give the products (**4**). *The product of esterification with R-MTPA-chloride is the S-MTPA-ester and the product of S-MTPA-chloride is the R-MTPA-ester.*

(*S*)-MTPA ester—^{1}H NMR (selected shifts) ($CDCl_3$) δ_H 1.38 (d, *J* = 6.4, H-12), 1.59 (s, H-9), 2.70 (m, H-10), 3.45 (OCH_3), 5.43 (m, H-11), 7.32 – 7.41 (m, aromatics); ESIMS *m/z* 661 $[M + H]^+$.

(*R*)-MTPA ester—^{1}H NMR (selected shifts) ($CDCl_3$) δ_H 1.45 (d, *J* =6.4, H-12), 1.58 (s, H-9), 2.65 (m, H-10), 3.50 (OCH_3), 5.42 (m, H-11), 7.32 – 7.41 (m, 5 H, aromatics); ESIMS *m/z* 661 $[M + H]^+$.

Berkchaetoazaphilone C **(5)**: colorless oil, $[\alpha]^{20}_D$ +183 (*c* 0.23, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 246 (3.50), 353 (3.67) nm; ECD (*c* 4.95 × 10^{-4} M, MeOH) λ_{max} (Δε) 362 (+2.58), 322 (−2.57), 253 (+0.79), 228 (−0.442) nm; IR ($CHCl_3$) ν_{max} 3448, 3018, 2856, 1710, 1673, 1616, 1548, 1456, 1371, 1170, 927 $cm^{-1;}$ ^{1}H NMR and ^{13}C NMR ($CDCl_3$) see Table 1; ^{1}H NMR (C_6D_6) δ_H 7.17 (s, H-1), 5.48 (s, H-5), 5.44 (s, H-4), 4.53 (bs, OH), 3.53 (m, H-11), 3.52 (dd, *J* = 10.3, 2.6, H-8), 3.10 (dd, *J* = 18.0, 2.6, H-14), 2.23 (dd, *J* = 18.0, 10.3, H-14), 1.85 (m, 3H, H-10, H-16), 1.73 (dt, 16.7, 7.2, H-16), 1.35 (s, 3H, H-9), 1.34-1.1 (m, H-17 – H-23), 0.92 (t, 3H, 6.9, H-24), 0.80 (d, 3H, 6.2, H-12); HRESIMS *m/z* 405.2634 $[M + H]^+$ (calcd for $C_{24}H_{37}O_5$, 405.2641).

Berkchaetorubramine (**6**): red oil, $[\alpha]^{20}_D$ +5.1 (*c* 0.75, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 228 (3.94), 269 (3.87), 423 (3.80), 486 (3.92) nm; IR ($CHCl_3$) ν_{max} 3800, 3009, 2928, 2855, 1716, 1637, 1544, 1464$cm^{-1;}$ ^{1}H NMR and ^{13}C NMR see Table 1; HRESIMS *m/z* 472.2723 $[M + H]^+$ (calcd for $C_{27}H_{38}NO_6$, 472.2699).

Induced Inflammasome Assay; Toxicity Assay; and Cytokine Assays

These assays have been described previously.[2a,b] (Supporting Information).

Antiproliferative/cytotoxicity Dose Response Studies of the Azaphilone Compounds; Image acquisition and analysis; and Data Analysis & IC_{50} determinations

These assays have been previously described.[2d] (Supporting Information).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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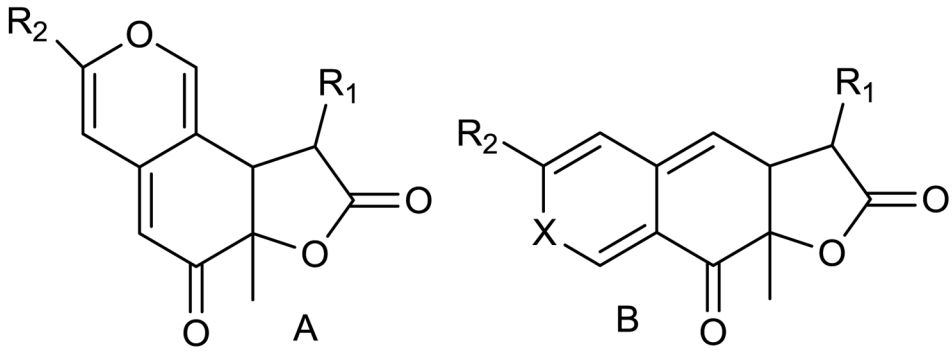


Figure 1.
Angular (A) and linear (B) fused azaphilone tricyclic systems isolated from fungi, X = O or N.

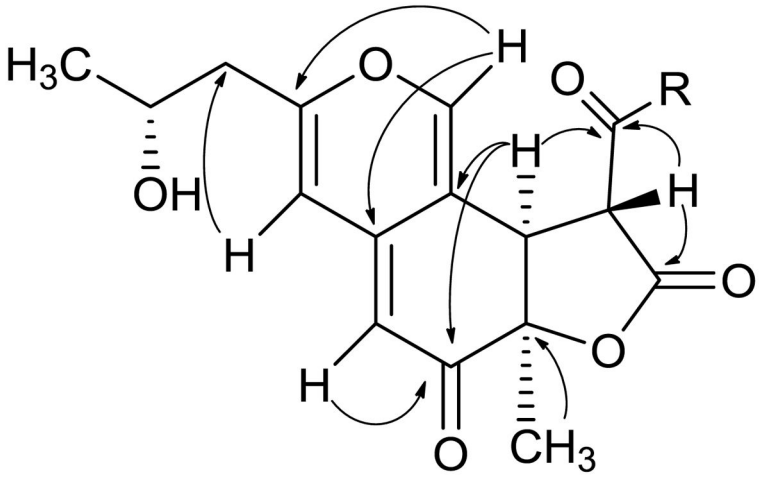


Figure 2.
Key HMBC correlations for berkchaetoazaphilone A (**1**).

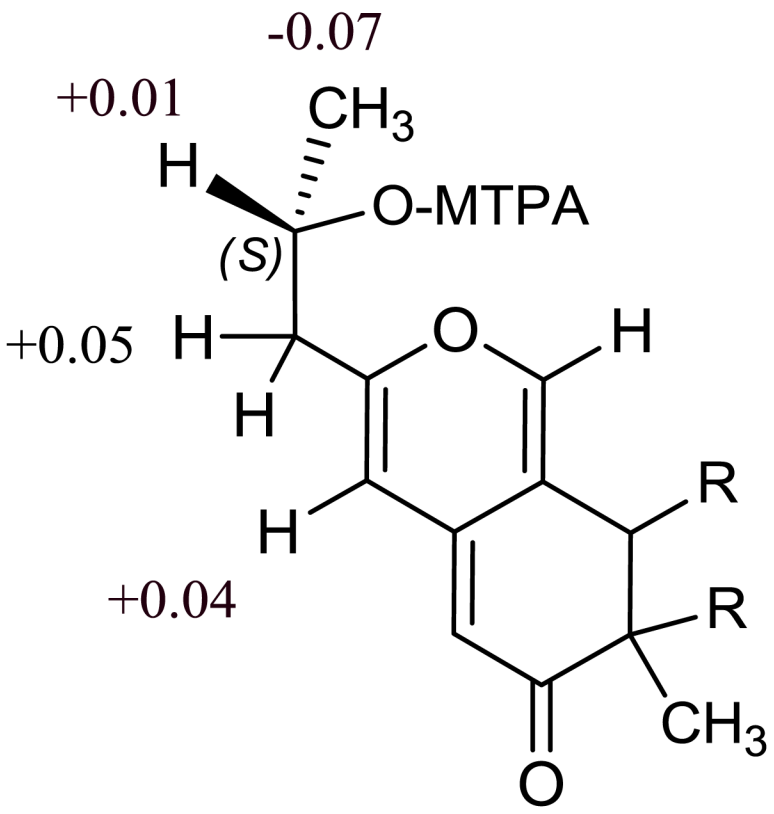


Figure 3.

Selected δΔ values around C-11 of the (R) and (S)-MTPA esters of compound **4**. [Δδ=chemical shift of (R)-MTPA ester minus chemical shift of (S)-MTPA ester in ppm].

Table 1

^{1}H and ^{13}C NMR Data for 1, 2, 5 ($CDCl_3$)[a] and 6 (MeOH-d_4)[a]

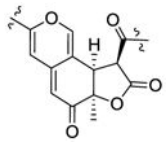
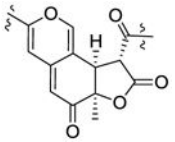
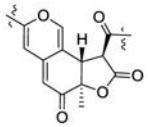
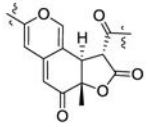
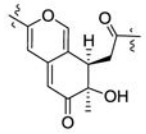
	berkchaetoazaphilone A (1)		berkchaetoazaphilone B (2)		berkchaetoazaphilone C (5)		berkchaetorubramine (6)	
no.	**δ_C, type**	**δ_H (*J* in Hz)**	**δ_C, type**	**δ_H (*J* in Hz)**	**δ_C, type**	**δ_H (*J* in Hz)**	**δ_C, type**	**δ_H (*J* in Hz)**
1	147.5, CH	7.35, bs	147.7, CH	7.44, bs	146.5, CH	7.36, s	144.9, CH	8.30, s
3	159.6, C		161.0, C		158.9, C		154.7, C	
4	108.5, CH	6.06, s	107.6, CH	6.12, s	108.4, CH	6.03, s	120.0, CH	7.01, s
4a	144.1, C		145.0, C		145.3, C		153.7, C	
5	106.0, CH	5.35, bs	105.2, CH	5.35, bs	103.4, CH	5.35, s	98.6, CH	6.69, s
6	191.5, C		189.1, C		198.5, C		174.2, C	
7	82.8, C		84.1, C		73.1, C		87.1, C	
8	42.7, CH	3.83, d (12.2)	72.1, C		40.5, CH	3.35, dd (10.1, 3.0)	196.6, C	
8a	114.6, C		108.3, C		120.5, C		102.3, C	
9	22.6, CH_3	1.55, s	18.7, CH_3	1.57, s	26.7, CH_3	1.28, s	30.8, CH_3	1.65, s
10	42.8, CH_2	2.49, m	42.7, CH_2	2.52, d (6.2)	42.9, CH_2	2.47, m	41.4, CH_2	2.81, m
11	65.6, CH	4.14, m	65.4, CH	4.14, m	65.6, CH	4.14, m	68.4, CH	4.44, bm
12	23.2, CH_3	1.29, d (6.5)	23.7, CH_3	1.27, d (6.1)	23.7, CH_3	1.26, d (6.4)	23.9, CH_3	1.30, d (6.1)
13	168.5, C		165.5, C				173.9, C	
14	56.4, CH	3.70, d (12.2)	66.8, C		40.4, CH_2	3.00, dd (18.6, 3.0) 2.28, m	122.6, C	
15	202.3, C		195.6, C		209.8, C		199.0, C	
16	42.8, CH_2	3.13, dd (18.4, 4.0) 2.48, dd (18.4, 7.9)	42.0, CH_2	2.80, dt (17.5, 7.0) 2.58, dt (17.5, 7.0)	43.5, CH_2	2.31, m 2.23, m	40.8, CH_2	2.96, m 2.81, m
17	23.7, CH_2	1.24, bs	23.2, CH_2	1.23, bs	23.5, CH_2	1.47, bs	23.5, CH_2	1.29, bs
18	28.9, CH_2	1.24, bs	28.9, CH_2	1.23, bs	29.2, CH_2	1.24, bs	30.6, CH_2	1.29, bs
19	29.2, CH_2	1.24, bs	29.2, CH_2	1.23, bs	29.4, CH_2	1.24, bs	30.7, CH_2	1.29, bs
20	29.4, CH_2	1.24, bs	29.3, CH_2	1.23, bs	29.4, CH_2	1.24, bs	30.8, CH_2	1.29, bs
21	29.4, CH_2	1.24, bs	29.3, CH_2	1.23, bs	29.2, CH_2	1.24, bs	30.8, CH_2	1.29, bs
22	31.8, CH_2	1.24, bs	31.8, CH_2	1.23, bs	31.8, CH_2	1.24, bs	33.2, CH_2	1.29, bs
23	23.0, CH_2	1.24, bs	22.6, CH_2	1.23, bs	22.6, CH_2	1.24, bs	26.6, CH_2	1.29, bs
24	14.1, CH_3	0.85, t (6.9)	14.1, CH_3	0.85, t (7.0)	14.1, CH_3	0.85, t (6.8)	14.6, CH_3	0.90, t (7.0)

	berkchaetoazaphilone A (1)		berkchaetoazaphilone B (2)		berkchaetoazaphilone C (5)		berkchaetorubramine (6)	
no.	δ_C, type	δ_H (*J* in Hz)	δ_C, type	δ_H (*J* in Hz)	δ_C, type	δ_H (*J* in Hz)	δ_C, type	δ_H (*J* in Hz)
1′							58.1, CH_2	4.29, bm 4.11, bm
2′							61.8, CH_2	3.87, bm

[a]All assignments are based on COSY, NOE, HSQC and HMBC experiments, 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR.

Table 2

Comparison of ^{1}H NMR and ECD data and stereoconfiguration in angular tricyclic azaphilones.

Compound	H-8	H_3-9	C-7	H-14	ECD (nm)	
Sassafrin A[15]	3.92 (12.4)	1.60	R	3.67 (12.4)	+367, −321, +274	
Sassafrin B[15]	3.98 (12.4)	1.59	R	3.68 (12.4)	+373, −322, +263	
Longirostrerone C[28]	3.89 (12.9)	1.42	R	4.14 (12.9)	+366, +332, −276	
Monascuskaolin[18]	3.86 (12.0)	1.58		3.71 (12.0)		
Biscogniazaphilone B[17]	3.86 (12.0)	1.58		3.73 (12.0)		
Berkchaetoazaphilone A (1)	**3.83 (12.2)**	**1.55**	**R**	**3.73 (12.2)**	**+373, −330, +258**	
Cohaerin G[25]	3.80 (12.5)	1.39	R	4.08 (12.5)	+350, −272	
Cohaerin H[25]	3.90 (13.2)	1.43	R	4.11 (12.9)	+350, −273	
Monochaetin[16]	3.76 (12.8)	1.36	R	4.07 (12.8)		
Chermesinone B[26]	3.81 (12.9)	1.36	S[a]	4.09 (12.9)		
Monapurone A[27]	3.31	1.28	R		+369, −326, +258	
Berkchaetoazaphilone C (5)	**3.35**	**1.28**	**R**		**+363, −322, +253**	
Helotialin A[29]	3.39	1.12	R		+370, −324, −261	

Compound	H-8	H_3-9	C-7	H-14	ECD (nm)	
Cohaerin I[25]	3.26	1.11	R		+366, +319, −245	
Cohaerin K[25]	3.36	1.15	R		+369, +319, −271	
Longirostrerone B[28]	3.34	1.14	R		+370, +318, −247	