JOURNAL OF NATURAL PRODUCTS

Article

pubs.acs.org/jnp

Caspase-1 Inhibitors from an Extremophilic Fungus That Target Specific Leukemia Cell Lines

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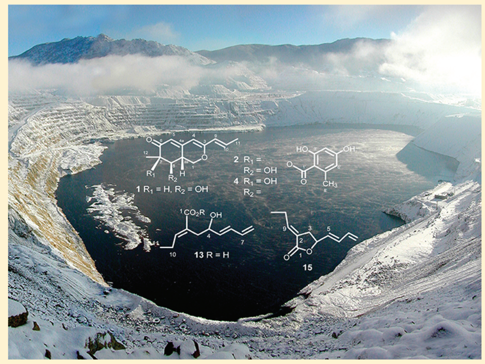
Ⓢ *Supporting Information*

ABSTRACT: Berkeley Pit Lake, Butte, Montana, is a 540 m deep abandoned open-pit copper mine filled with over 140 billion liters of acidic, metal-sulfate-contaminated water. This harsh environment has yielded several microorganisms that produce interesting biologically active compounds. Several polyketide metabolites including the new berkazaphilones A (**1**) and B (**2**) and octadienoic acid derivatives berkedienoic acid (**13**) and berkedienolactone (**15**), as well as previously reported azaphilone **4**, vermistatin (**6**), dihydrovermistatin (**7**), penisimplicissin (**8**), aldehyde **9**, and methylparaconic acid (**11**), were isolated from a culture broth of *Penicillium rubrum* taken from a depth of 270 m. The structures of these compounds were deduced by interpretation of spectroscopic data. The compounds were isolated either for their inhibition of the signal transduction enzyme caspase-1 or because of their structural similarity to these inhibitors. Selected compounds were further evaluated for their ability to inhibit interleukin-1β production by inflammasomes in induced THP-1 cells. Berkazaphilones B (**2**) and C (**4**) and vermistatin analogue penisimplicissin (**8**) exhibited selective activity against leukemia cancer cell lines in the National Cancer Institute 60 human cell line assay.



The Berkeley Pit Lake system is one of the largest contaminated sites in North America. The Pit itself is over 540 m deep with a surface area of 3.2 km^2 and is continually filling with metal-sulfate-rich, acidic water (pH 2.5), at a rate of 10 million L/day. This represents roughly 140 billion L of contaminated water and constitutes an important component of the largest EPA Superfund site in the United States.[1]

In 1995 we began to study the microbes inhabiting the waters of this Pit Lake as if they were inhabitants of a new and exotic ecosystem. Over the past 15 years we have studied the secondary metabolism of several microbes isolated from the water and sediments of this ecosystem under a variety of physicochemical conditions to determine whether or not they produce metabolites with desirable bioactivity. This approach has yielded interesting results.[2−8]

Bioactivity is currently assessed using 96-well plate assays that demonstrate the ability of crude extracts, column fractions, and pure compounds to inhibit specific signal transduction enzymes. We routinely target the enzymes matrix metalloproteinase-3 (MMP-3), caspase-1, and caspase-3. These assays are proving to be effective tools for assessing the bioactivities of crude extracts and guiding isolation of pure enzyme inhibitors. The lead compounds presented in this article were isolated because of their ability to inhibit caspase-1. After the structures of these compounds were elucidated, compounds with similar ^{1}H NMR spectral characteristics were also isolated to ascertain how subtle differences in structure might affect biological activity.

Caspase-1 was the first of a novel type of cysteine protease responsible for converting interleukin-1β to its mature form in monocytes. Caspase-1, also known as interleukin-1 converting enzyme (ICE), is responsible for the activation of IL-1β and IL-18 from precursor molecules.[9] Caspase-1 is activated upon binding to the inflammasome, a multiprotein complex that plays a key role in innate immunity by activating the proinflammatory pleiotropic cytokines interleukin 1-β and IL-18.[9] There is a strong correlation between dysregulated inflammasome activity and both inherited and acquired inflammatory diseases.[9]

Several researchers have demonstrated that caspase-1 inhibitors have shown promise in delaying the onset of Huntington's disease[10] and amyotropic lateral sclerosis[11] and in mitigating the effects of stroke[12] and multiple sclerosis.[13,14] All of these diseases exhibit autoimmune phenomena. Capsase-1 has also been implicated in the physiological production of interferon-gamma-inducing factor (IGIF). It therefore appears to play a critical role in the regulation of multiple proinflammatory cytokines.[15]

The up-regulation of caspase-1 and concomitant chronic inflammation have been associated with a number of different pathologies including the development of insulin resistance in obesity-related diabetes,[16] degeneration of retinal capillaries associated with diabetes and galactosemia,[17] the demyelination of neurons in multiple sclerosis,[11,18] and the formation of amyloid plaques in Alzheimer's disease.[19] High levels of

Special Issue: Special Issue in Honor of Gordon M. Cragg

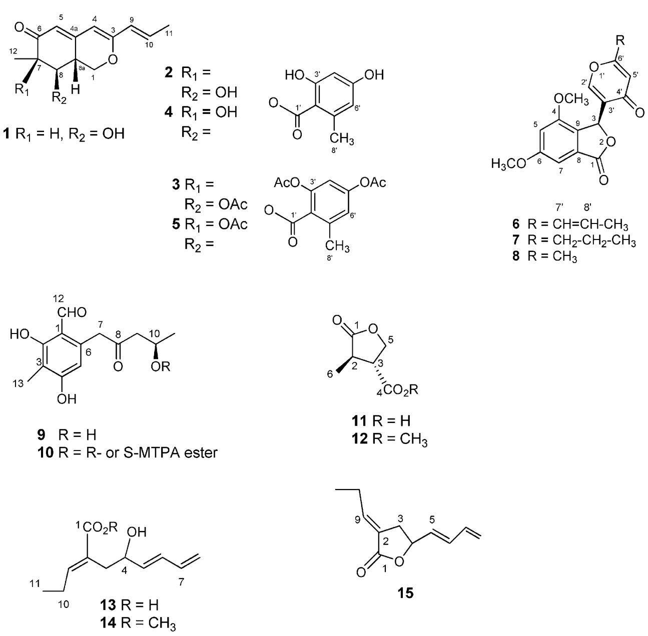
Received: May 16, 2011
Published: February 1, 2012

caspase-1 and interleukin-1β have been found in certain cancers[20] by many different researchers: acute myelogenous leukemia,[21] melanoma,[22,23] certain glioblastomas[24,25] and pancreatic cancers,[26−29] certain breast cancers,[30] and human cancer xenografts,[31] all of which may be exacerbated by chronic inflammation associated with activation of the inflammasome.

Caspase-1 inhibitors have been proposed as potential therapies for the above-mentioned cancers, as well as osteoarthritis and rheumatoid arthritis,[32,33] Alzheimer's disease,[19] amyotrophic lateral sclerosis,[9] and brain and nerve trauma.[34,35]

Caspase-1 is also down-regulated in many solid tumor cancers, and activation of caspase-1 in prostate cancer and ovarian cancer may be required for apoptotic breakdown of tumors. The development of new caspase-1 inhibitors will not only provide potential chemotherapeutics but also provide tools for the investigation of the intricacies of signal transduction.

One of the first microbes to be studied from the Pit Lake was isolated from a depth of 270 m and was subsequently identified as *Penicillium rubrum* Stoll on the basis of rRNA sequencing alignment data (300 base pairs). The fungus was grown in acidified potato dextrose broth (pH 2.7) for 21 days as a still culture. At time of harvest the mycelium was removed by filtration and the broth was thoroughly extracted with chloroform. This organic extract was active in all three enzyme inhibition assays, although in this study we focused on the caspase-1 inhibitors. Size exclusion chromatography (LH-20) followed by HPLC yielded the new berkazaphilones A and B (**1** and **2**) as well as the new octadienoic acid derivatives berkedienoic acid (**13**) and berkedienolactone (**15**) and the previously reported azaphilone (**4**), vermistatin (**6**), dihydrovermistatin (**7**), penisimplicissin (**8**), aldehyde **9**, and methylparaconic acid (**11**).



HREIMS of **1** gave a molecular formula of $C_{13}H_{16}O_3$, corresponding to a molecule with six sites of unsaturation. The ^{13}C NMR spectrum showed seven sp^2-hybridized carbons in the molecule, suggesting the presence of three double bonds and one carbonyl carbon. A bicyclic ring system accommodated the two remaining sites of unsaturation. The UV spectrum (λ_{max} 344 nm) indicated extended conjugation, and the IR spectrum showed the presence of a dienone moiety (ν_{max} 1644 cm^{-1})[36] that was supported by a carbon resonating at δ_C 198.2 ppm in the ^{13}C NMR spectrum. The DEPT spectrum showed that 15 protons were attached to carbons. The strong OH stretch in the IR spectrum (ν_{max} 3415 cm^{-1}) provided sufficient information to assign the remaining proton to the hydroxy group. The ^1H NMR (Table 1) and $^1H-^1H$ COSY spectra indicated the presence of two spin systems, $CH_3-CH-CHO-CH-CH_2O$ and a terminal $CH{=}CH-CH_3$, as well as two olefinic protons at δ_H 5.71 (d, J = 1.9 Hz) and 5.49 (s). The HMBC spectrum provided correlations to connect the first spin system to ketone C-6 (δ_C 198.2). Mutually ^3J-coupled methine H-7 (δ_H 2.41, J = 10.3, 6.6 Hz) and methyl doublet H_3-12 (δ_H 1.25, J = 6.6 Hz) showed strong HMBC correlations to carbonyl C-6 and to oxygen-bearing C-8 (δ_C 74.2 ppm). H_3-12 also showed correlations to methine C-7 (δ 50.0 ppm). $^1H-^1H$ COSY showed ^3J-coupling between H-7 and H-8 (δ_H 3.43, J = 10.3 Hz), between H-8 and methine H-8a (δ_H 2.84, J = 10.3 Hz), and between H-8a and oxygen-bearing methylene H-1 (1α = δ_H 3.72, J = 13.2 Hz; 1β = δ_H 4.79, J = 5.4 Hz). Methylene H-1β could be connected to the conjugated diene system through three-bond HMBC correlations to oxygen-bearing C-3 (δ_C 159.9) and C-4a (δ_C 150.5). HMBC correlations from olefinic H-4 (δ_H 5.49) to C-5 (δ_C 118.1) established the diene backbone, and from H-4 to C-9 (δ_C 125.4) connected the diene system to the terminal propylene moiety. H-4 also showed a HMBC correlation to C-8a (δ_C 40.9), which allowed the $\alpha,\beta,\gamma,\delta$-unsaturated ketone bicyclic ring system to be established. The configuration of the propylene olefin could be established as *E* on the basis of the magnitude of the 15.7 Hz coupling between H-9 and H-10.

The relative stereochemistry of **1** was established by interpretation of ^1H NMR and 1D NOE difference spectra. The broad triplet H-8 showed *ax/ax* coupling (J = 10.3 Hz) to both H-7 and H-8a, which also exhibited strong *ax/ax* coupling (J = 13.2 Hz) to H-1α. In the NOE difference spectrum irradiation of H-8a enhanced the resonance of H-1β, supporting the relative stereochemistry assignment as shown. These data were used to generate the proposed structure for berkazaphilone A (**1**).

Compound **1** belongs to the class of fungal metabolites known as the azaphilones. A comprehensive review of azaphilone analogues in 2010 listed over 170 compounds from 23 different fungal genera.[37] Most of the known azaphilones are oxygenated at both C-7 and C-8 and often form orsellinic or chlorinated orsellinic acid esters. Of the azaphilone analogues reviewed, only one other compound, pseudohalonectrin, was not oxygenated at C-7.[37]

HREIMS established the molecular formula of compound **2** as $C_{21}H_{22}O_7$, which showed compound **2** to have eight more carbons than **1** and five additional sites of unsaturation, indicative of an aromatic moiety. There were many similarities between the ^{13}C and ^1H NMR spectra of compounds **1** and **2** (Table 1). Two obvious differences were due to the replacement of the C-7 methine in compound **1** by a quaternary oxygen-bearing carbon (δ_C 89.3), leading to the replacement of the H_3-12 doublet of **1** with a singlet in compound **2** (δ_H 1.77). These data suggested that **2** had the same carbon skeleton as **1** with a substituent at C-7. HMBC and ^1H NMR spectral data provided the necessary information to support this deduction. A terminal *E*-configured propylene moiety, olefinic singlet H-4 (δ_H 5.51), and doublet H-5 (δ_H 5.78 ppm) were present as in **1**. ^3J-coupling data again showed

Table 1. ^{13}C and ^{1}H NMR Data for Compounds 1, 2, and 4, Berkazaphilones A−C, in $CDCl_3$ (δ ppm)[a]

	1		2		4	
atom	δ_C, type	δ_H, mult. (J in Hz)	δ_C, type	δ_H mult. (J in Hz)	δ_C, type	δ_H mult. (J in Hz)
1	68.6, CH_2	β 4.79, dd (10.9, 5.4) α 3.72, dd (13.2, 10.9)	68.4, CH_2	β 4.82, dd (11.1,5.3) α 3.78, dd (13.5, 11.1)	67.9, CH_2	β 4.38, dd (10.8, 5.4) α 3.85, dd (13.0, 10.8)
3	159.9, C		160.8, C		160.7, C	
4	102.8, CH	5.49, s	102.4, CH	5.51, s	102.8, CH	5.56, s
4a	150.5, C		152.5, C		151.7, C	
5	118.1, CH	5.71, d (1.9)	115.4, CH	5.78, d (1.6)	115.7, CH	5.79, d (1.7)
6	198.2, C		190.5, C		194.8, C	
7	50.0, CH	2.41, dq (10.3, 6.6)	89.3, C		74.1, C	
8	74.2, CH	3.43, bt (10.3)	74.6, CH	3.58, bt (10.1)	74.7, CH	5.29, d (9.9)
8a	40.9, CH	2.84, dddd (13.2, 10.3, 5.4, 1.9)	37.8, CH	2.82, dddd (13.5, 10.1, 5.3, 1.6)	34.9, CH	3.44, dddd (13.0, 9.9, 5.4, 1.7)
9	125.4, CH	5.87, dq (15.7, 1.7)	125.2, CH	5.85, dd (15.3,1.5)	125.2, CH	5.87, dd (15.4, 1.7)
10	133.2, CH	6.40, dq (15.7, 6.8)	134.4, CH	6.45, dq (15.3,6.9)	134.5, CH	6.41, dd (15.4, 7.0)
11	18.4, CH_3	1.84, dd (6.8, 1.7)	18.4, CH_3	1.84, dd (6.9,1.5)	18.5, CH_3	1.84, dd, (7.0, 1.7)
12	10.8, CH_3	1.25, d (3H, 6.6)	15.4, CH_3	1.77, s (3H)	20.4, CH_3	1.38, s (3H)
1′			172.0, C		170.6, C	
2′			104.7, C		104.4, C	
3′			166.0, C		165.9, C	
4′			101.5, CH	6.25, d (2.4)	101.6, CH	6.30, d (2.3)
5′			161.4, C		161.1, C	
6′			112.1, CH	6.18, d (2.4)	112.0, CH	6.27, d (2.3)
7′			145.0, C		144.6, C	
8′			24.7 CH_3	2.28, s (3H)	25.0, CH_3	2.58, s (3H)

[a]Assignments of the ^{13}C and ^{1}H signals were made on the basis of HSQC spectral data.

ax/ax interactions between H-8 and H-8a (J = 10.1 Hz) and between H-8a and H-1α (J = 13.5 Hz) and an *ax/eq* interaction between H-8a and H-1β (J = 5.3 Hz). Singlet H_3-12 showed three-bond correlations to ketone C-6 (δ_C 190.5) and methine C-8 (δ_C 74.6 ppm), as well as two-bond coupling to quaternary C-7. H-1β showed a two-bond correlation to C-8a (δ_C 37.8) and three-bond correlations to C-3 (δ_C 160.8) and C-4a (δ_C 152.5 ppm). Olefinic H-4 had three-bond correlations to C-8a and C-5 (δ_C 115.4), again establishing the bicyclic diene, and to C-9 (δ_C 125.2), connecting the ring system to the terminal propylene moiety. H-4 also showed two-bond correlations to C-3 and C-4a. H-5 (δ_H 5.78) showed three-bond correlations to C-4 (δ_C 102.4) and to C-7, supporting the location of the oxy substituent at C-7.

Eight carbons, seven hydrogens, three oxygens, and five sites of unsaturation remained to be assigned and attached to the oxygen at C-7. A carbon resonating at δ_C 172.0 (C-1′) and an IR absorbance of 1708 cm^{-1} showed the presence of an aromatic ester. The DEPT and ^{13}C spectra showed the presence of five quaternary carbons, including the carbonyl carbon, two methines, and a methyl carbon. Chemical shifts indicated that all but the methyl were sp^2-hybridized, consistent with an aromatic ester, and that two carbons were oxygen-bearing. HMBC data showed two-bond correlations from H-4′ (δ_H 6.25, J = 2.4 Hz) to both oxygen-bearing C-3′ (δ_C 166.0) and C-5′ (δ_C 161.4). H-6′ (δ_H 6.18, J = 2.4 Hz) showed a three-bond correlation to methyl C-8′ (δ_C 24.7), and H_3-8′ (δ_H 2.28) showed three-bond correlations to both C-6′ (δ_C 101.5) and C-2′ (δ_C 104.7). These data established the substituent as an orsellinic acid moiety and were consistent with literature data.[38]

Acetylation of compound **2** resulted in triacetate **3**. The orsellinic acid moiety was diacetylated as expected, and chemical shift data were consistent with the literature.[39] Acetylation of the hydroxyl group at C-8 induced a large downfield shift of H-8 from δ 3.58 to δ 5.00 (d, J = 9.9 Hz) and a downfield shift of H-8a from δ_H 2.82 to δ_H 3.34 ppm. H-8 was ^3J-coupled to H-8a and showed HMBC correlations to C-8a, C-1, C-7, and an acetate carbonyl. These data confirmed the structure proposed for compound **2**, berkazaphilone B, with the placement of the orsellinic moiety at C-7.

Compound **4** was isomeric with **2**, with a molecular formula of $C_{21}H_{22}O_7$ established by HREIMS. The ^{13}C NMR spectra of **2** and **4** were very similar (see Table 1), but the ^{1}H NMR spectrum had two distinct differences. Methylene proton H-1β showed a marked upfield shift, and methine H-8 showed a marked downfield shift in compound **4** when compared to **2**. These differences indicated that the orsellinic acid moiety was at C-8 rather than C-7. HMBC correlations were observed from H-8 (δ_H 5.29) to C-1′ (δ_C 170.6), confirming this deduction. Acetylation of **4** gave the expected diacetate **5**. The proposed compound has been previously reported, and the NMR and mass spectral data of compound **4**, berkazaphilone C, compared favorably to the data reported for azaphilone Sch 725680.[38] Unfortunately, the original authors did not provide the optical rotation for their compound, so we cannot be sure if the two are the same stereoisomer.[38]

Examination of mass spectral, NMR, and optical rotation data indicated that the major cytotoxic compound in the extract was the known fungal metabolite vermistatin (**6**), which was previously reported as a metabolite of *Penicillium vermiculatum*.[40] The NMR data of compounds **7** and **8** were similar to those of vermistatin, indicating strong structural similarities. The HREIMS of compound **7** gave a molecular formula of $C_{18}H_{18}O_6$, with two more hydrogens than vermistatin. Indeed, the only major difference between the NMR spectra of vermistatin **6** and compound **7** was the peaks associated with the terminal propylene moiety. It was apparent from the NMR data that it was reduced to an *n*-propyl moiety, designating **7** as 14,15-dihydrovermistatin. Dihydrovermistatin was previously reported from broth cultures of *Penicillium simplicissimum*.[41]

The third vermistatin analogue, compound **8**, had a molecular formula of $C_{16}H_{14}O_6$, with two less carbons than either vermistatin (**6**) or dihydrovermistatin (**7**). In this case, the ^{1}H NMR signals of both the terminal propylene and propyl moiety were absent and were replaced by a methyl singlet at δ 2.44. This compound was previously reported as penisimplicissin from broth cultures of *P. simplicissimum*.[41] Compounds **7** and **8** had the same sign and relative optical rotations as vermistatin (**6**), suggesting that they had the same configuration.[40]

The molecular formula of compound **9** was $C_{13}H_{16}O_5$, as determined from HREIMS. ^{13}C NMR and DEPT spectra indicated the presence of two carbonyl carbons: ketone C-8 at δ_C 208.8 (C) and aldehyde C-12 at δ_C 195.1 (CH). Six additional sp^2-hybridized carbons indicated the presence of an aromatic moiety with two oxygen-bearing carbons resonating at δ_C 165.2 and 164.6 ppm. These assignments accommodated all six sites of unsaturation associated with the molecular formula. Examination of the spectral data established the structure of compound **9** as shown, which was previously reported as a metabolite of *Aspergillus versicolor*.[42] We prepared the Mosher ester of **9** and found the same absolute configuration (*R*) as reported.[43] The *S* stereoisomer of compound **9** has also been reported from a *Pseudobotrytis* sp.[44]

EIMS established the molecular formula of compound **11** as $C_6H_8O_4$, associated with three sites of unsaturation. Its infrared spectrum indicated the presence of two carbonyl moieties: the broad −OH stretch (ν_{max} 3027 cm^{-1}) and carbonyl absorption at 1716 cm^{-1} indicated the presence of a saturated carboxylic acid, while the carbonyl absorption at 1774 cm^{-1} indicated the presence of a saturated γ-butyrolactone.[45] The presence of the acid was confirmed by methylation of compound **11** with diazomethane to yield the methyl ester, **12**. Analysis of the spectral data established the structure of **11**, which is the known compound α-methylparaconic acid.[46]

Compound **13** had a molecular formula of $C_{11}H_{16}O_3$, established by the HRESIMS spectrum, with four sites of unsaturation. The broad −OH stretch (ν_{max} = 3021 cm^{-1}) and the carbonyl absorption at 1683 cm^{-1} in the infrared spectrum were indicative of an α,β-unsaturated carboxylic acid. Methylation of **13** with diazomethane yielded the methyl ester **14**, which had a molecular formula of $C_{12}H_{19}O_3$, as determined by HREIMS.

The ^{1}H−^{1}H COSY spectrum of **13** provided connectivity data for two spin systems beginning with a terminal diene. The ^3J-coupling of the diene protons clearly correlated H-8a (δ_H 5.20) and H-8b (δ_H 5.08) to H-7 (δ_H 6.28), H-7 to H-6 (δ_H 6.20), H-6 to H-5 (δ_H 5.70), H-5 to oxygen-bearing methine H-4 (δ_H 4.31), and H-4 to methylene H$_2$-3 (δ_H 2.57). The second spin system consisted of a terminal propylidene moiety with ^3J-coupling from H-9 (δ_H 7.02) to methylene H$_2$-10 (δ_H 2.23) and from H-10 to the terminal methyl H$_3$-11 (δ_H 1.05). The HMBC spectrum provided the necessary information to connect these two spin systems to the carbonyl carbon with three-bond correlations from H-9 and H$_2$-3 to C-1. H-9 also exhibited correlations to C-3 and C-10. Methylene H$_2$-3 provided a point of connectivity with three-bond correlations to C-1, C-9, and C-5 and two-bond correlations to flanking carbons C-2 and C-4. H-4 also afforded nice bilateral connectivity with three-bond correlations to C-2 and C-6 and two-bond correlations to C-3 and C-5. The chemical shift of H-9 indicated an *E* configuration for $\Delta^{2,9}$.[47] The magnitude of the coupling constant between olefinic H-5 and H-6 (J = 14.3 Hz) also indicated an *E* configuration. These data generated the structure proposed for compound **13**, berkedienoic acid.

Compound **15** had a molecular formula of $C_{11}H_{14}O_2$ established by the HRESIMS [M + H]$^+$ peak at *m/z* 179.1082. The carbonyl absorption at 1751 cm^{-1} and accompanying C=C absorption at 1679 cm^{-1} in the infrared spectrum indicated the presence of an α,β-unsaturated γ-lactone.[45] These data and the similarities in the NMR spectra suggested that compound **15** was the γ-butyrolactone of compound **13**. The ^{1}H−^{1}H COSY and the ^{1}H NMR spectra provided connectivity data for a single extended spin system beginning with a terminal diene at one end. The ^3J-coupling of the diene protons correlated H-8a (δ_H 5.28) and H-8b (δ_H 5.18) to H-7 (δ_H 6.30), H-7 to H-6 (δ_H 6.30), H-6 to H-5 (δ_H 5.69), H-5 to oxygen-bearing methine H-4 (δ_H 4.98), and H-4 to methylene H$_2$-3 (δ_H 2.58, 3.05 ppm).

The terminal propylidene moiety produced ^3J-coupling from methyl H$_3$-11 (δ_H 1.07 ppm) to methylene H$_2$-10 (δ_H 2.17) and from H$_2$-10 to olefinic H-9 (δ_H 6.72). The coupling pattern for methylene H$_2$-10, however, was quite complex; the expected pentet pattern was actually a pentet of triplets, and the ^{1}H−^{1}H COSY spectrum showed connectivity to methylene H$_2$-3, which required five-bond coupling in our proposed structure. The coupling patterns for H$_2$-3a and -3b were also complex, and both were doublets of doublets of pentets. Bearing in mind that a pentet is actually a dddd with equivalent coupling constants, then these patterns become complex indeed. These data suggest that these unusually complex patterns are the result of allylic and homoallylic coupling. Studies on 2-butene and compounds containing a butenyl moiety showed similar correlations.[48]

Homoallylic coupling data from both the ^{1}H NMR and the ^{1}H−^{1}H COSY spectra provided the information to connect the two ends of the molecule. The coupling patterns for this molecule were complex but could be deconstructed to include homoallylic coupling between the methylenes H$_2$-10 and H$_2$-3.

The exocyclic double bond could be established as the *E* isomer by chemical shift arguments. A series of *E* and *Z* isomers of α-alkylidene-γ-butyrolactones were synthesized, and the chemical shifts of the olefinic protons analogous to H-9 were compared.[49] In all cases the chemical shift of H-9 in the *Z* isomer approached δ_H 6.0 ppm, while in the *E* isomer it was closer to δ_H 6.6 ppm.[49] These data were used to generate the structure proposed for compound **15**, berkedienolactone. A series of similar lactones including the tricyclic compound gallielalactone were isolated from an unidentified ascomycete.[50,51] Unfortunately, the reported monocyclic lactones most structurally similar to **15** were not isolated as pure compounds, so it was not possible to compare optical rotation data with that of berkedienolactone (**15**).

Compounds **1**, **2**, **4**, and **6**−**9** were evaluated for their ability to inhibit caspase-1 *in vitro*, and the most active compounds and closely related analogues were evaluated for their ability to inhibit the production of interleukin 1-β in THP-1 cells (promonocytic leukemia cell line). THP-1 cells produce high levels of IL-1β when induced with titanium nanowires and bacterial lipopolysaccharide (LPS). Caspase-1 inhibition was determined in a fluorometric assay normalized to 1.00, where 0 is *total enzyme inhibition* and 1.00 is *lack of enzyme inhibition*. Both berkazaphilones B (**2**) and C (**4**) had IC$_{100}$ values of 25 μM against caspase-1, while berkazaphilone A (**1**), penisimplicissin (**8**), and compound **9** were completely inhibitory at a

dx.doi.org/10.1021/np200414c | J. Nat. Prod. 2012, 75, 344−350

concentration of 250 μM. Vermistatin (**6**) and dihydrovermistatin (**7**) were not inhibitory at the concentrations tested.

Induced THP-1 cells were exposed to compounds **2**, **4**, and **6**–**8**, and the concentrations of IL-1β postexposure were determined. All of the compounds tested inhibited the production of IL-1β in THP-1 cells at a concentration of 250 μM. In dilution assays, however, only compounds **2** and **4** inhibited the production of IL-1β. Compounds **2** and **4** completely inhibited the production of IL-1β at concentrations of 5 and 50 μM, respectively.

Compounds **2**, **4**, **7**, and **8** were tested in the National Cancer Institute (NCI) antitumor screen against 60 human cell lines.[52] The compounds showed selective cytotoxicity toward leukemia cell lines only. Berkazaphilone B (**2**) exhibited a $\log_{10}$ GI_{50} of −5.67 against cell line RPMI-8226, and berkazaphilone C (**4**) exhibited a $\log_{10}$ GI_{50} of −6.42 against cell line SR. In the vermistatin family, penisimplicissin (**8**) exhibited a $\log_{10}$ GI_{50} of −6.70 against cell line CCRF-CEM and −5.83 against HL-60(TB), and dihydrovermistatin (**7**) was inactive at the concentrations tested. Vermistatin (**6**) had been previously tested by the NCI and was also inactive (Supporting Information).

The NCI Molecular Target database includes experiments that determine relative RNA levels for nearly 10 000 human clones, measured in microarray experiments for the NCI cell lines. It was interesting to note that in several microarray experiments caspase-1 was upregulated almost exclusively in different leukemia cell lines.[53−56]

■ EXPERIMENTAL SECTION

General Experimental Procedures. ^{1}H and ^{13}C NMR spectra were run on a Bruker DPX-300. Chemical shifts were recorded with respect to the deuterated solvent shift ($CDCl_3$, δ 7.24 for the proton resonance and δ 77.0 for the carbon). IR spectra were recorded on a Nicolet NEXUS 670 FT-IR spectrometer. Optical rotations were measured on a Perkin-Elmer 241 MC polarimeter using a 1 mL cell. Mass spectral data were provided by the Mass Spectrometry, Proteomics and Metabolomics Facility at Montana State University and the Mass Spectral Analysis Laboratory at the University of Montana. All solvents used were spectral grade or distilled prior to use.

Collection, Extraction, and Isolation Procedures. The collection and isolation of the Berkeley Pit fungi have previously been described.[7,8] The fungus was identified as *Penicillium rubrum* by Microbial Identification, Inc. The fungus was grown at room temperature in 26 × 300 mL of DIFCO potato dextrose broth (acidified to pH 2.7 with sulfuric acid) in 1 L Erlenmeyer flasks (shaken at 180 rpm for 6 days then still for 15 days). At time of harvest 50 mL of MeOH was added per flask. The combined cultures (7.8 L) were filtered through cheesecloth to remove the mycelia mat for a separate study. The filtrate from the combined cultures was extracted three times with 1 L of $CHCl_3$, and the extract was reduced *in vacuo* to an oil (1.14 g). This extract demonstrated inhibition of caspase-1 and MMP-3, antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli*, and brine shrimp lethality.

The $CHCl_3$ extract was fractionated using a flash Si gel column using hexanes, hexane/isopropyl alcohol (IPA) mixtures, to isopropyl alcohol/MeOH mixtures. The 50% IPA/hexane fraction was further fractionated by preparative HPLC on a Rainin 21 mm preparative Si gel column with a hexanes/isopropyl alcohol gradient. The 10% IPA fraction was further fractionated on Si gel to yield the three azaphilone derivatives **1** (5.1 mg), **2** (35.1 mg), and **4** (7.1 mg). The fraction that eluted with 50% IPA/hexane yielded the three cytotoxic compounds **6** (46.2 mg), **7** (4.0 mg), and **8** (18.0 mg). The 25% IPA/hexane fraction yielded aldehyde **9** (5.6 mg), lactone **11**, berkedienoic acid **13** (1.5 mg), and berkedienolactone **14** (2.0 mg)

*Berkazaphilone A (**1**):* $[\alpha]^{20}_{D}$ +20.5 (*c* 0.0019, MeOH); UV ($CHCl_3$) λ_{max} (log ε) 344 (4.36), 241 (3.75) nm; IR ($CHCl_3$) ν_{max} 3415, 3020, 2934, 1644, 1592, 1384, 1063, 876 cm^{-1}; ^{1}H NMR and ^{13}C NMR ($CDCl_3$) see Table 1; EIMS *m*/*z* 220 (50), 162 (48), 134 (100); HREIMS *m*/*z* 220.1099 [M^+] (calcd for $C_{13}H_{16}O_3$, 220.1099).

*Berkazaphilone B (**2**):* $[\alpha]^{20}_{D}$ −55.2 (*c* 0.0025, MeOH); UV (MeOH) λ_{max} (log ε) 349 (4.11), 270 (4.05) nm; IR (neat) λ_{max} 3400, 2933, 1708, 1669, 1633, 1585, 1326, 1267, 1172, 1118 cm^{-1}; ^{1}H NMR and ^{13}C NMR ($CDCl_3$) see Table 1; EIMS *m*/*z* 386 (15), 236 (20), 218 (38), 175 (83), 162 (94), 134 (100), 69 (85); HREIMS *m*/*z* 386.1363, $[M]^+$ (calcd for $C_{21}H_{22}O_7$, 386.1365).

Acetylation of Compound 2. Compound **2** (1.0 mg) was dissolved in pyridine (50 μL) and Ac_2O (50 μL) and stirred for 24 h. After that time the solvents were removed to give **3** as an oil (0.9 mg).

*Compound **3**:* ^{1}H NMR ($CDCl_3$) δ 6.82 (d, *J* = 2.0 Hz, H-6′), 6.77 (d, *J* = 2.0 Hz, H-4′), 6.38 (dq, *J* = 15.1, 6.9, H-10), 5.83 (dd, *J* = 15.1, 1.7 Hz, H-9), 5.82 (d, *J* = 1.6 Hz, H-5), 5.50 (s, H-4), 5.00 (d, *J* = 9.9 Hz, H-8), 4.26 (dd, *J* = 10.7, 5.1 Hz, H-1β), 3.71 (d, *J* = 13.2, 10.7 Hz, H-1α), 3.34 (dddd, *J* = 13.2, 9.9, 5.1, 1.6 Hz, H-8a), 2.33 (s, 3H, H-8′), 2.26 (s, 3H, OAc), 2.22 (s, 3H, OAc), 2.17 (s, 3H, OAc), 1.83 (dd, *J* = 6.9, 1.7 Hz, 3H, H-11), 1.61 (s, 3H, H-12); ^{13}C NMR ($CDCl_3$) δ 190.4 (C-6), 170.2 (OAc), 168.8 (OAc), 168.5 (OAc), 164.1 (C-1′), 159.9 (C-3), 151.8 (C-5′), 149.8 (C-4a), 148.6 (C-3′), 138.4 (C-7′), 133.4 (C-10), 125.1 (C-9), 124.1 (C-2′), 121.0 (C-6′), 116.4 (C-5), 114.1 (C-4′), 102.4 (C-4), 83.3 (C-7), 74.2 (C-8), 67.8 (C-1), 35.8 (C-8a), 21.1 (OAc), 20.8 (OAc), 20.6 (OAc), 19.8 (C-8′), 18.4 (C-11), 17.2 (C-12).

*Berkazaphilone C (**4**):* $[\alpha]^{25}_{D}$ +54.3 (*c* 0.0030 $CHCl_3$); ^{1}H NMR and ^{13}C NMR ($CDCl_3$) see Table 1; EIMS *m*/*z* 386; HREIMS *m*/*z* 386.1365 $[M]^+$ (calcd for $C_{21}H_{22}O_7$, 386.1365).

Acetylation of Compound 4. Compound **4** (1.0 mg) was dissolved in pyridine (50 μ L) and Ac_2O (50 μL) and stirred for 24 h. After that time the solvents were removed to give **5** as an oil (0.9 mg).

*Compound **5**:* ^{1}H NMR ($CDCl_3$) δ 6.93 (d, *J* = 1.6 Hz, H-6′), 6.86 (d, *J* = 1.6 Hz, H-4′), 6.42 (dq, *J* = 15.2, 6.9 Hz, H-10), 5.88 (bd, *J* = 15.2, 1.5 Hz, H-9), 5.78 (bd, *J* = 1.9 Hz, H-5), 5.56 (s, H-4), 5.17 (d, *J* = 10.0, H-8), 4.51 (dd, *J* = 10.8, 5.4 Hz, H-1β), 3.85 (dd, *J* = 13.5, 10.8 Hz, H-1α), 3.34 (m, H-8a), 2.41 (s, 3H, H-8′), 2.29 (s, 6H, OAc), 1.85 (dd, *J* = 6.9, 1.5 Hz, 3H, H-11), 1.41 (s, 3H, H-12); ^{13}C NMR ($CDCl_3$) δ 193.9 (C-6), 168.9 (OAc), 168.6 (OAc), 165.4 (C-1′), 160.4 (C-3), 149.6 (C-3′), 151.1, (C-5′), 152.1 (C-4a), 139.9 (C-7′), 134.3 (C-10), 122.5 (C-2′), 121.6 (C-6′), 125.2 (C-9), 116.0 (C-5), 114.7 (C-4′), 102.8 (C-4), 74.6 (C-7), 74.2 (C-8), 67.9 (C-1), 34.9 (C-8a), 21.1 (OAc, 2C), 21.1 (C-8′), 20.2 (C-12), 18.4 (C-11).

*Berkedienoic acid (**13**):* solid, IR ($CHCl_3$) ν_{max} 3021 (broad), 2971, 2877, 1683, 1637, 1419, 1004 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 7.02 (1H, t, *J* = 7.5 Hz, H-9), 6.28 (1H, ddd, *J* = 16.1, 10.4, 9.6 Hz, H-7), 6.20 (1H, dd, *J* = 14.3, 10.4 Hz, H-6), 5.70 (1H, dd, *J* = 14.3, 6.4 Hz, H-5), 5.20 (1 H, dd, *J* = 16.1, 1.4 Hz, H-8), 5.08 (1H, dd, *J* = 9.6, 1.4 Hz, H-8), 4.31 (1H, q, *J* = 6.4 Hz, H-4), 2.57 (2H, m, H-3), 2.23 (2H, quin, *J* = 7.5 Hz, H-10), 1.05 (3H, t, *J* = 7.5 Hz, H-11); ^{13}C NMR ($CDCl_3$) δ 172.7 (C, C-1), 149.9 (CH, C-9), 136.2 (CH, C-7), 135.5 (CH, C-5), 131.0 (CH, C-6), 126.8 (C, C-2), 117.8 (CH_2, C-8), 71.8 (CH, C-4), 34.3 (CH_2, C-3), 22.6 (CH_2, C-10), 13.0 (CH_3, C-11); HRESIMS $[M - H_2O + H]^+$ *m*/*z* 179.1076 (calcd for $C_{11}H_{15}O_2$, 179.1072).

*Methylation of **13***. Compound **13** (0.2 mg) was dissolved in Et_2O (100 μL), and a solution of CH_2N_2 in Et_2O was added dropwise until the yellow color persisted. The solution was stirred for 5 min, and the solvent removed under a stream of N_2 to yield the methyl ester **14** (0.2 mg); HREIMS *m*/*z* $[M + H]^+$ *m*/*z* 211.1325 (calcd for $C_{12}H_{19}O_3$, 211.1334).

*Berkedienolactone (**15**):* solid, $[\alpha]^{25}_{D}$ −37.0 (*c* 0.0020, MeOH); IR ($CHCl_3$) ν_{max} 3023, 2971, 2877, 1751, 1679, 1301, 1016 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 6.72 (1H, tt, *J* = 7.5, 2.8 Hz, H-9), 6.30 (2 H, m, H-7, H-6), 5.69 (1H, dd, *J* = 14.3, 6.7 Hz, H-5), 5.28 (1H, dd, *J* = 15.1, 1.38 Hz, H-8), 5.18 (1H, dd, *J* = 9.5, 1.38 Hz, H-8), 4.98 (1H, ddd, *J* = 8.1, 7.6, 6.2 Hz, H-4), 3.05 (1H, dddd, *J* = 16.3, 8.1, 2.8, 1.4 Hz, H-3), 2.58 (1H, dd pentet, *J* = 16.3, 6.2, 1.5 Hz, H-3), 2.17 (2H, m, H-10), 1.07 (3H, t, *J* = 7.5 Hz, H-11); ^{13}C NMR ($CDCl_3$) δ 170.7 (C, C-1), 142.5 (CH, C-9), 135.4 (CH, C-6), 133.3 (CH, C-7), 131.0 (CH, C-

5), 125.1 (C, C-2), 75.7 (CH, C-4), 119.6 (CH_2, C-8), 31.8 (CH_2, C-3), 23.6 (CH_2, C-10), 12.6 (CH_3, C-11); HRESIMS $[M + H]^+$ m/z 179.1082 (calcd for $C_{11}H_{15}O_2$, 179.1072).

***In Vitro* THP-1 Assay.** Human monocyte cell line THP-1 was purchased from ATCC (#TIB-202). The cells were suspended at (2−4) × 10^5 viable cells/mL in RPMI media supplemented with 10% fetal bovine serum, 0.05 mM 2-mercaptoethanol, sodium pyruvate, and an antimycotic/antibiotic cocktail containing penicillin, streptomycin, and amphotericin B (Mediatech,VWR). The cells were differentiated into macrophage-like cells by the phorbol ester PMA (1 μg/mL, Sigma) 24 h prior to experimentation. The transformed cells were removed from the flask by scraping and centrifuged at 450*g* for 5 min. The resulting cell pellet was suspended at 1.0 × 10^6 cells/mL and exposed to caspase-1 inhibitors at concentrations described below (0.5−0.005%), LPS [20 ng/mL], and TiO_2 nanowires (100 μg/mL). Experiments were conducted in 96-well plates for 24 h in 37 °C water-jacketed CO_2 incubators (ThermoForma).

Toxicity Assay. Cell viability was determined by MTS reagent using the CellTiter96 assay (Promega), according to the manufacturer's protocol. The plate was read at 490 nm.

Cytokine Assays. Human IL-1β DuoSet was obtained from R&D Systems, and ELISA assays were performed according to the manufacturer's protocol. The plate was read at 490 nm.

■ ASSOCIATED CONTENT

Supporting Information

^{1}H NMR, ^{13}C NMR, COSY, and HMBC spectra of berkazaphilones A (**1**) and B (**2**), berkedienoic acid (**13**), and berkedienolactone (**15**); ^{1}H NMR and ^{13}C NMR of berkazaphilone C (**4**); and NCI cell line data for compounds **2**, **4**, and **8** are 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

We thank Ms. B. Parker (University of Montana) for HRMS data and our colleagues from the Department of Chemistry, Montana State University: Dr. S. Busse for assistance with NMR spectroscopy and Dr. L. J. Sears for mass spectral data. We thank the National Science Foundation grant 9506620 for providing funding for NMR upgrades at the MSU facility and grant CHE-9977213 for acquisition of a NMR spectrometer. We gratefully acknowledge NIH grants R01CA139159, P20RR16455-04, and P20RR017670 (NCRR); 5P30NS055022; and RC2ES018742.

■ DEDICATION

Dedicated to Dr. Gordon M. Cragg, formerly Chief, Natural Products Branch, National Cancer Institute, Frederick, Maryland, for his pioneering work on the development of natural product anticancer agents.

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■ NOTE ADDED IN PROOF

Berkazaphilones B and C were also recently published as the pinophilins, *J. Nat. Prod.*, Article ASAP, DOI: 10.1021/np200523b, submitted 6/22/2011.