JOC Notes

Berkelic Acid, A Novel Spiroketal with Selective Anticancer Activity from an Acid Mine Waste Fungal Extremophile

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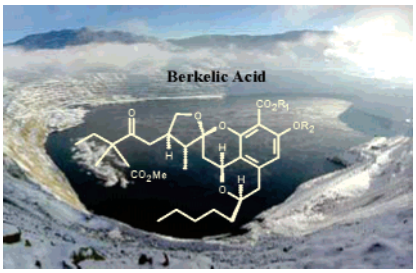

Berkeley Pit Lake is an abandoned open-pit copper mine filled with 30 billion gallons of acidic, metal-contaminated water. This harsh environment is proving to be a source of unusual microorganisms that produce novel bioactive metabolites. Bioassay-guided fractionation using signal transduction enzyme assays led to the isolation of the novel spiroketal, berkelic acid **1**, and of the known γ-pyrone, spiciferone A **4**. Berkelic acid has shown selective, nanomolar activity against OVCAR-3, an ovarian cancer cell line in the National Cancer Institute cell line screen. The isolation and characterization of these compounds are reported here.

The Berkeley Pit Lake mine waste system in Butte, Montana is part of the largest EPA Superfund site in North America. It includes Berkeley Pit Lake, a mile wide, 1500 foot deep abandoned open-pit copper mine. As infiltrating groundwater seeps into the Pit, rich veins of pyrite and other minerals dissolve, constantly generating acid in the process. There are over 30 billion gallons of water in the Pit, with an inflow rate of 4 million gallons/day. The water is acidic (pH 2.5) and contaminated with high concentrations of metal sulfates (including 1200 ppm iron, 240 ppm copper, 290 ppm aluminum, and 650 ppm zinc).[1] Unfortunately, the Pit Lake system sits at the headwaters of the Columbia River. If the water rises another 200 feet, it will reach the critical overflow level. At the current rate of rise, the critical level will be reached in approximately 10 years.

Despite the low pH and high metal content of Berkeley Pit Lake, it has proven a surprisingly rich source of diverse fungi, protists, algae, protozoans, and bacteria.[2] Although conditions within the Pit Lake System are toxic for "normal" aquatic biota, these same conditions represent an ideal environment for certain types of extremophiles. We have previously reported the isolation of several unique metabolites from this environment.[3−6]

One of the *Penicillium* species isolated from the surface waters of the Pit was active in two signal transduction enzyme inhibition assays: matrix metalloproteinase-3 (MMP-3) inhibition and caspase-1 inhibition. These assays are effective tools for assessing the bioactivities of crude extracts and guiding isolation of the pure enzyme inhibitor. MMPs are a family of zinc-containing, calcium-dependent endopeptidases that hydrolyze the extracellular matrix of connective tissues and basement membranes.[7,8] Specific MMP inhibitors represent a new therapeutic approach to the treatment of cancers that act by blocking the activity of proteolytic enzymes (MMPs) used by tumor cells to promote metastatic spread. [9,10] Recent studies show that these inhibitors might also halt tumor progression and could be used as low toxicity complements to cytotoxic therapies. MMPs are also implicated in the occurrence of rheumatoid arthritis and multiple sclerosis.[8]

Caspase-1 was the first of a novel type of cysteine proteases responsible for converting interleukin-1β to its mature form in monocytes.[11] Mature IL-1β is a key mediator of inflammation. Caspase-1 is believed to be analogous to CED-3, a cell death protein in *Caenorhabditis elegans*.[12] Caspase-1 inhibitors have shown promise in delaying the onset of Huntington's disease[13] and amyotropic lateral sclerosis[14] and in mitigating the effects of stroke[15] and multiple sclerosis.[16,17] All of these diseases

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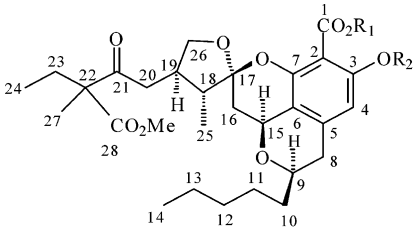
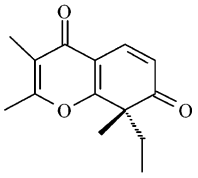
exhibit certain autoimmune phenomena. It has also been implicated in the physiological production of interferon-gamma-inducing factor (IGIF). It, therefore, appears to play a critical role in the regulation of multiple proinflammatory cytokines. Specific caspase-1 inhibitors might provide a new class of anti-inflammatory drugs with multipotent action.[18] These assays were used to guide isolation of the compounds described in this paper.

Fungus pitna 4, identified as a *Penicillium* species, was grown in 32 × 200 mL liquid cultures using potato dextrose broth in 500 mL Erlenmeyer flasks (still) for 21 days. At harvest time, the fungus was killed with the addition of 20 mL of MeOH. The culture was filtered through cheesecloth to remove the mycelial mat. The filtrate was extracted three times with 1 L of $CHCl_3$, and the extract was reduced in vacuo to an oil (1.3761 g). The $CHCl_3$ extract inhibited both MMP-3 and caspase-1 in the assay systems, so it was fractionated on a flash silica gel column using hexane and hexane/IPA mixtures to IPA/MeOH mixtures. The large flash fractions were again tested for enzyme inhibitory activity, and the active component was further fractionated by preparative HPLC on a 21 mm prep Sigel column (4 mL/min) with a hexane/IPA gradient to give pure spiciferone A, **4** (15.0 mg) and berkelic acid, **1** (32.1 mg) which eluted at 47 min. The isolation and characterization of three bisabolane sesquiterpenes and a coumarin derivative from this fungal culture have been described previously.[5]

Although berkelic acid (**1**) did not give a parent ion in EIMS, esterification with diazomethane yielded a dimethyl ester **2** that gave a strong parent ion in HREIMS and established the molecular formula of the parent compound as $C_{29}H_{40}O_9$. Only 28 carbons were observed when the ^{13}C NMR spectrum was run in $CDCl_3$, but all 29 carbons were displayed when the spectrum was recorded in CD_3OD (Table 1) and C_6D_6. DEPT analysis indicated that **1** contained 10 quaternary carbons, 5 methines, 9 methylenes, and 5 methyl carbons. The two remaining protons are attached to heteroatoms. HSQC experiments (CD_3OD and $CDCl_3$) provided all one-bond $^1H-^{13}C$ connectivities. The data from gradient HMBC experiments (CD_3OD and $CDCl_3$) provided sufficient information to establish long-range connectivities.



1 R_1= H, R_2= H
2 R_1= Me, R_2= H
3 R_1= Me, R_2= Ac



spiciferone A **4**

An initial examination of the ^1H and ^{13}C NMR spectra of berkelic acid **1** suggested the presence of the following structural units: a saturated ketone (C-21), a methyl ester (C-28), a carboxylic acid (C-1), a penta-substituted phenol ring (C-2−C-7), a ketal carbon (C-17), an isolated methyl group (C-27), and an isolated ethyl group (C-23−C-24). The IR spectrum of **1** confirmed the presence of three carbonyl carbons at ν 1740, 1712, and 1690 cm^{-1}, which were eventually assigned to the nonenolic β-keto methyl ester, the ketone, and the aryl carboxylic acid absorbances, respectively. The molecular formula indicated that **1** had 10 degrees of unsaturation. Because the phenolic ring and three carbonyl carbons accounted for seven degrees of unsaturation and all of the nine sp2 carbons, **1** was determined to be a tetracyclic molecule.

TABLE 1. NMR Data for Berkelic Acid, 1[a]

position	^{13}C (δ)	^1H δ, mult (J in Hz)	HMBC (H → C)
1	173.6		
2	101.0		
3	163.4		
4	109.4	6.27, br s	C: 2,[b] 3,[b] 6,[b] 5, 8
5	142.3		
6	113.7		
7	153.0		
8α	35.4	2.77, dd (17.4, 4.3)	C: 5,[b] 6[b]
8β		2.54, dd (17.4)	C: 5,[b] 6,[b] 9,[b] 10[b]
9	76.5	3.79, m	
10	37.4	1.63, m, 2H	
11	26.2	1.55, m, 2H	
12	33.0	1.4, m, 2H	
13	23.7	1.4, m	
14	14.4	0.92, br t, 3H (6.4)	C: 12, 13
15	69.4	4.72, dd (12.4, 5.4)	C: 6, 9, 16, 17
16α	35.0	2.02, t (12.4)	C: 6, 15,17[b]
16β		2.16, dd (12.4, 5.4)	C: 6, 15, 17[b]
17	110.7		
18	49.2	1.82, m	C: 17, 19, 25
19	40.4	2.66, m	C: 17[b], 18, 21, 26
20	42.6	2.87, dd (17.5, 3.0)	C: 18, 19, 20, 21, 25, 26
		2.53, m	C: 19, 20, 21
21	208.7		
22	61.0		
23	28.9	1.93, m	C: 21, 22, 24, 27, 28
		1.84, m	C: 22, 24, 27, 28
24	9.0	0.83, t, 3H (7.7)	C: 21, 22, 23, 27, 28
25	11.9	1.07, d (6.7)	C: 17, 18, 19, 20
26α	74.1	4.30, t (8.3)	C: 20
26β		3.50, t (8.3)	C: 19, 20
27	19.0	1.32, s, 3H	C: 21, 22, 23, 28
28	174.8		
OMe	52.9	3.73, s, 3H	C: 28
OH[b]		11.82, s	C: 2,[b] 3,[b] 4[b]

[a] 500 MHz, CD_3OD. [b] Correlations from HMBC ($CDCl_3$, 300 MHz).

The phenolic proton (δ 11.82 ppm) provided a convenient starting point to establish long-range connectivities through the analysis of the gradient HMBC spectrum ($CDCl_3$). This proton exhibited coupling to quaternary aromatic C-2 and C-3 and methine C-4. The chemical shifts of C-2 and C-4 were typical of aromatic carbons *ortho* to a hydroxyl moiety, while that of C-3 suggested it was hydroxylated. The single aromatic proton H-4 also showed coupling to C-3 as well as three-bond coupling to aromatic carbons C-2 and C-6. The chemical shift of H-4 (δ 6.27 ppm) suggested the presence of an alkoxyl substituent, either *ortho* or *para* to C-4. H-4 also showed three-bond coupling to methylene carbon C-8, while both H-8α and H-8β showed connectivity to aromatic C-5 and C-6. As these data indicated that methylene C-8 was *ortho* to C-4, the alkoxyl substituent could be placed at C-7. The higher field of the two methylene protons (H-8β) was further coupled to both oxygen-bearing methine C-9 and methylene C-10. Analysis of $^1H-^1H$ COSY also showed clear scalar coupling between the H-8 and

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H-9 protons, H-9 and H-10 methylene protons, and on down the line to terminal methyl H-14 protons, which helped to generate the C-10−C-14 *n*-pentyl side chain.

The HMBC spectrum also showed a critical connection between H-15 and both aromatic C-6 and methine C-9, which could establish the second ring of the tetracycle. Unfortunately, the chemical shifts of aromatic C-6 and C-17 were too close for definitive assignment (δ 112.1 and δ 112.2), so the HMBC experiment was rerun in CD_3OD. The chemical shifts of C-6 (δ 113.7) and C-17 (δ 110.7) could now be clearly distinguished, and connectivities could now be assigned definitively. In CD_3OD, H-15 showed a connectivity to both C-6 and C-17, as well as methylene C-16, which not only confirmed the second ring of the tetracycle, but also established part of the third ring. IR data indicated the presence of an aryl carboxylic acid. Because $^1H-^{13}C$ coupling data had already established the hydroxyl moiety at C-3 and connected C-5 to methylene C-8, C-2 was a reasonable location for the carboxylic acid moiety.

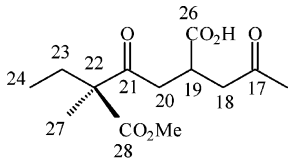
C-17 showed a three-bond coupling to methyl protons H-25 and methylene protons H-26α and H-26β. In the $CDCl_3$ HMBC spectrum, two-bond coupling from C-17 to both H-16α and H-16β and three-bond coupling to methine proton H-19 could be seen. These coupling data generated a five-membered ring oxocycle. The chemical shift of C-17 (δ 110.7 ppm) was suggestive of a ketal, which could be created by connecting C-17 to C-7 through an ether linkage, completing the tetracyclic backbone of **1**. The chemical shifts of H-16α and H-16β are noteworthy. In a cyclohexane ring, equatorial protons usually resonate upfield of axial protons because of anisotropy. In spiroketal systems, however, methylene protons adjacent to the spiro center show the reverse effect.[19,20] The magnitude of the coupling constants is also helpful in determining which proton is axial. In the case of berkelic acid, H-16β (t, J = 12.4 Hz) showed typical axial−axial coupling to H-15, while coupling between H-15 and H-16β is typically axial−equatorial (J = 5.4 Hz). Clearly designating the orientation of the H-16 methylene protons was important in establishing the relative stereochemistry of berkelic acid.

$^1H-^1H$ COSY showed scalar coupling between H-19 and methylene protons H-20. In the HMBC spectrum, these protons (H-20) showed two-bond coupling to ketone C-21, which could be further coupled to methyl protons H-27. The H-27 methyl protons showed two-bond connectivity to quaternary C-22 and to ester carbonyl C-28. Methylene protons H-23 also showed two-bond connectivity to C-22 as well as to terminal methyl C-24 and three-bond connectivity to ketone C-21. These data established the structure of berkelic acid as **1**. Examination of the ^1H NMR, ^{13}C NMR, HMBC, and COSY spectral data of the two derivatives of berkelic acid **1**, methyl berkelate **2** and methyl berkelate monoacetate **3**, indicated that they were also consistent with the structure proposed.

The relative stereochemistry of **1** was established by NOE difference spectroscopy. A 12% enhancement was observed between H-9 and H-15, indicating a 1,3-diaxial relationship between these protons. Irradiation of H-15 also resulted in enhancement of equatorial H-16α. Mutual enhancement of H-26α and H-19 and of methyl H-25 and H-19 indicated their cis relationships. Irradiation of methyl H-25 also resulted in enhancement of H-16α and H-20 in both berkelic acid **1** and in the derivative **3**, which supported the stereochemistry of the spiroketal. NOE data also confirmed the proximity of the aromatic proton H-4 and methylene protons H-8. All of these enhancements were consistent with distances calculated from an AM1 optimized structure.

As an additional support of the structure of berkelic acid **1**, the mass spectral, ^1H and ^{13}C NMR, HSQC, and HMBC data were submitted to ACD labs for analysis by their ACD/structure elucidator program.[21] The results of this analysis generated several hundred possible structures, with the best fit analysis identical to the structure proposed based on similarity of the calculated ^1H and ^{13}C NMR chemical shifts.

A second compound isolated from the chloroform extract of this fungus was identical to the known compound spiciferone A **4**. Spiciferone A, as well as several derivatives, has been previously isolated from *Cochliobolus spicifer*.[22,23] One of these derivatives, spicifernin closely resembles C-17 through C-28 of berkelic acid.[24] The biosynthesis of spicifernin has been studied and was found to be of acetate biogenesis with methylation from S-adenosyl methionine. The structure of berkelic acid **1** appears to involve a further elongation of the acetate chain.



spicifernin **5**

Berkelic acid effectively inhibited MMP-3 in the micromolar range (GI50 = 1.87 μM) and caspase-1 in the millimolar range (GI50 = 0.098 mM). Berkelic acid (**1**) was tested in the National Cancer Institute (NCI) antitumor screen against 60 human cell lines. It showed selective activity toward ovarian cancer OVCAR-3 with a GI_{50} of 9.13 E−8.00 (91 nM concentration). We have been using signal transduction enzyme inhibition assays to target compounds with potential anticancer activity. The NCI provides access to information concerning specific molecular targets (in our case, MMP-3 and caspase-1) and how they might relate to specific cancers. We searched the molecular target data for both caspase-1 and MMP-3 on the NCI Developmental Therapeutics Program website. These data show up- or down-regulation of the molecular target for each human cancer cell line in the NCI screen. There were no apparent patterns for caspase-1 and ovarian cancer cell lines in any of the experimental data cited. MMP-3, however, showed an intriguing correlation within the ovarian cancer cell lines. In several experiments (experiment id. 89914, 89913, 20375, 20374, 6381, and 9829), MMP-3 was up-regulated in OVCAR-3, but not in the other ovarian cancer cell lines. [25]

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Experimental Section

Berkelic acid (1): $[\alpha]^{20}_{D}$ −83.5° (*c* 0.0113, MeOH); UV (MeOH) λ_{max} (log ϵ) 319 (3.56), 257 (3.92), 216 (4.31); IR ($CHCl_3$) ν_{max} 3243, 3027, 2927, 1740, 1712, 1690, 1633, 1587, 1457, 1249, 1074, 1002, 888 cm^{-1}; ^1H NMR and ^{13}C NMR (CD_3OD), see Table 1; ^1H NMR ($CDCl_3$) δ 11.82 (s, OH), 6.41 (br s, H-4), 4.76 (dd, J = 12.2, 5.7 Hz, H-15), 4.43 (t, J = 8.8 Hz, H-26α), 3.80 (m, H-9), 3.73 (s, 3H, OMe), 3.58 (t, J = 8.8 Hz, H-26β), 2.84 (dd, J = 17.0, 2.5 Hz, H-20_1), 2.77 (dd, J = 17.6, 4.0 Hz, H-8α), 2.59 (dd, J = 17.6, 11.0 Hz, H-8β), 2.50 (m, H-19), 2.42 (dd, J = 17.0, 10.3 Hz, H-20_2), 2.20 (dd, J = 12.2, 5.7 Hz, H-16β), 2.05 (t, J = 12.2 Hz, H-16α), 1.87 (m, H-18), 1.61 (m, H-10_1), 1.50 (m, H-10_2), 1.50 (m, 2H, H-11), 1.30 (m, 4H, H-12, H-13), 0.88 (t, 3H, H-14); ^{13} C NMR ($CDCl_3$) δ 206.1 (C-21), 173.4 (C-28), 170.5 (C-1), 162.5 (C-3), 149.8 (C-7), 142.2 (C-5), 112.2 (C-17), 112.1 (C-6), 110.5 (C-4), 98.6 (C-2), 75.2 (C-9), 73.5 (C-26), 67.2 (C-15), 59.7 (C-22), 52.5 (OMe), 48.2 (C-18), 41.6 (C-20), 39.3 (C-19), 36.2 (C-10), 34.3 (C-8), 34.2 (C-16), 31.7 (C-12), 27.9 (C-23), 25.0 (C-11), 22.6 (C-13), 18.4 (C-27), 14.0 (C-14), 12.0 (C-25), 8.7 (C-24); EIMS *m*/*z* 417 (20), 402 (28), 288 (32), 234 (25), 97 (100).

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Supporting Information Available: Experimental details including ^1H NMR,^{13}C NMR, COSY, HMBC, and HSQC spectra for berkelic acid **1** and derivatives. This material is available free of charge via the Internet at http://pubs.acs.org.

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