

The Berkeleyamides, Amides from the Acid Lake Fungus *Penicillium rubrum*

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We previously reported several novel bioactive hybrid polyketide-terpenoid metabolites from a deep water *Penicillium rubrum* isolated from Berkeley Pit Lake, Butte, Montana. In this paper we report the structures of four new amides, berkeleyamides A–D (**1**, **4**, **5**, **7**), isolated from extracts of this fungus. The structures of these compounds were deduced by analysis of NMR data, chemical derivatization, and comparison of their spectroscopic data to those of known compounds.

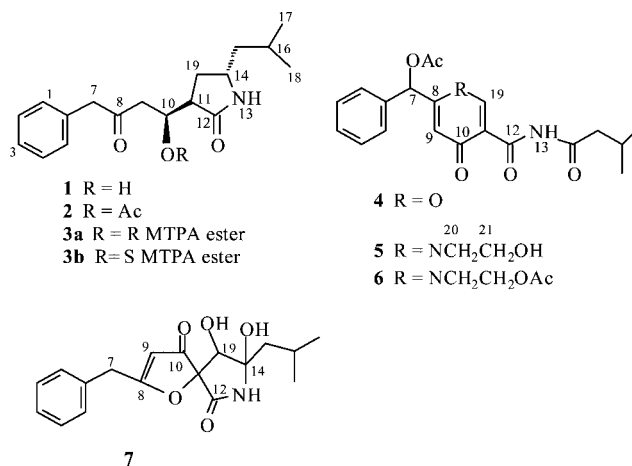
The Berkeley Pit Lake System is part of the largest EPA Superfund site in North America.¹ We have isolated over 70 unique microbes from this evolving extreme ecosystem and are in the process of studying the bioactive secondary metabolites as possible anticancer or antimicrobial agents. Several interesting compounds have been isolated from these microbes on the basis of their activities in different bioassay-guided fractionation schemes.^{2–6}

One of the first microbes to be studied from this environment was isolated from a water sample taken from a depth of 885 feet and identified as *Penicillium rubrum* Stoll.^{3,7} The organic extracts of this fungus inhibited the signal transducing enzymes matrix metalloproteinase-3 and caspase-1. We have previously reported several novel bioactive compounds isolated from the extracts of this fungus: the berkeleyacetals,⁷ berkeleydione, and berkeleytrione, all of which inhibited MMP-3 and caspase-1.³

The berkeleyamides were also isolated on the basis of inhibition of MMP-3 and caspase-1. MMP-3 is up-regulated in many tumors.^{8,9} Specific MMP inhibitors block the activity of proteolytic enzymes (MMPs) used by tumor cells to promote metastatic spread.^{10,11} Caspase-1 inhibitors have shown promise in halting tumor progression, delaying the onset of Huntington's disease¹² and amyotrophic lateral sclerosis,¹³ mitigating the effects of stroke¹⁴ and multiple sclerosis,^{15,16} and inhibiting the proliferation of acute myelogenous leukemia (AML) progenitor cells.¹⁷ Specific caspase-1 inhibitors might provide a new class of antileukemia or anti-inflammatory drugs with multipotent action.¹⁸ Inhibition of these two enzymes guided isolation of the berkeleyamides.

The fungus was grown in liquid cultures using acidified potato dextrose broth (pH 2.7) for 21 days. At harvest time the fungus was killed with the addition of MeOH. The culture was filtered through cheesecloth to remove the mycelial mat. The filtrate was extracted with CHCl₃, and the extract was reduced *in vacuo* to an oil. The CHCl₃ extract inhibited both MMP-3 and caspase-1 in the assay systems in the micromolar range. It was fractionated by flash silica gel column chromatography followed by HPLC to yield berkeleyamides **1**, **4**, **5**, and **7**.

Examination of mass spectra, IR, ¹H NMR, ¹H–¹H COSY, HSQC, and HMBC spectra provided the necessary information to determine the structures and the relative configurations of the berkeleyamides. HRESIMS established a molecular formula of C₁₈H₂₅NO₃ for berkeleyamide A (**1**), with seven double-bond equivalents (DBE). The IR spectrum indicated either an OH or NH stretch and carbonyl stretching frequencies indicative of both a ketone (1715 cm⁻¹) and an amide (1685 cm⁻¹). The ¹³C NMR and DEPT spectra (see Table 1) also provided support for the presence of the ketone (δ 208.8) and amide (δ 177.2), as well as a monosubstituted aromatic ring (δ 133.5, 129.5 (2C, CH), 128.8 (2C,



CH) and 127.2(CH)), four methylene carbons (δ 50.6, 46.2, 46.0, and 28.7), four methines (δ 66.8, 50.5, 45.3, and 25.2), and two methyl carbons (δ 22.9 and 22.2). The benzene ring and two carbonyl functionalities accommodated six sites of unsaturation and indicated the presence of an additional ring. The ¹H NMR spectrum clearly showed five aromatic protons (δ 7.28–7.16m), an isolated methylene group (δ 3.72), and two overlapping methyl doublets.

Analysis of the COSY spectrum indicated the presence of three discrete spin systems: a monosubstituted benzene ring, an isolated methylene (H₂-7, δ 3.72), and an extended system of protons attached to sp³-hybridized carbons: [–CH₂–CH–CH–CH₂–CH–CH₂–CH(CH₃)₂]. Careful analysis of the data provided a clear stepwise path along the backbone connecting methylene H₂-9 (δ 2.83, 2.68) to H-10 (δ 4.36), which was spin-coupled to methine H-11 (δ 2.44). H-11 was coupled to methylene H₂-19 (δ 2.29, 1.69), which coupled to methine H-14 (δ 3.62), which coupled to methylene H₂-15 (δ 1.28). H₂-15 was finally coupled to a terminal isopropyl moiety, H-16–H-18.

The HMBC spectrum provided several key correlations that supported this partial structure and generated the complete carbon backbone of berkeleyamide A. Long-range correlations from isolated methylene protons H₂-7 to aromatic carbons C-1, C-5 (δ 129.5, 2C), and C-6 (δ 133.5) and to ketone carbon C-8 (δ 208.8) indicated its benzylic position. The terminal methylene of the extended spin system H₂-9 also showed long-range correlations to the ketone carbon, which bridged these two spin systems. Both carbon and proton chemical shifts indicated that C-10 was oxygen bearing (δ_C 66.8, δ_H 4.36) and that C-14 was nitrogen bearing (δ_C 50.5, δ_H 3.62). Acetylation of **1** gave a monoacetate (**2**), which showed the expected downfield shift of H-10 from δ 4.36 to δ 5.48. In addition, methine H-11 showed HMBC correlations to both amide C-12 and oxygen-bearing methine C-10, and the NH proton

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Table 1. ^{13}C and ^1H NMR Data for Berkeleyamides A (1), B (4), C (5), and D (7) in CDCl_3^a

no.	1		4		5		7	
	δ_{C}	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)
1, 5	129.5	7.28 m	129.2	7.38 m	129.4	7.38 m	129.2	7.33 m
2, 4	128.8	7.16 m	127.4	7.38 m	127.9	7.28 m	129.0	7.33 m
3	127.2	7.16 m	129.8	7.38 m	129.9	7.28 m	127.0	7.33 m
6	133.5		134.2		134.5		133.2	
7	50.6	3.72 s, 2H	72.4	6.54 s	71.4	6.83 s	37.4	3.98 d (17.4), 3.96 d (17.4)
8	208.8		166.4		149.8		197.8	
9	46.2	2.83 dd (17.4, 3.1) 2.68 dd (17.4, 9.0)	114.8	6.59 s	120.6	6.85 s	104.4	5.35 bs
10	66.8	4.36 ddd (9.0, 5.3, 3.1)	177.6		177.4		199.4	
11	45.3	2.44 ddd (9.3, 7.2, 5.3)	119.3		117.1		95.3	
12	177.2		160.4		163.2		164.1	
13		6.02 bs		11.53 bs		13.22 bs		6.78 bs
14	50.5	3.62 qd (7.4, 4.1)	173.3		173.2		84.9	
15	46.0	1.37 m, 2H	47.3	2.58 d, 2H (6.9)	47.8	2.32 d, 2H (7.0)	45.5	1.88 m, 2H
16	25.2	1.57 m	24.9	2.17 m	25.6	2.17 m	24.0	1.92 m
17	22.9	0.88 d, 6H (6.6)	22.4	0.96 d, 6H (6.7)	22.4	0.97 d, 6H (6.6)	23.9	1.00 d, 3H (5.2)
18	22.2	0.88 d, 6H (6.6)	22.4	0.96 d, 6H (6.7)	22.4	0.97 d, 6H (6.6)	23.8	0.98 d, 3H (5.2)
19	28.7	β 2.29 dt (12.8, 7.6) α 1.69 ddd (12.9, 9.1, 4.1)	163.0	8.69 bs	150.4	8.97 bs	75.1	4.41 d (10.0)
Ac			169.0		169.4			
Ac-Me			20.7	2.18 s, 3H	20.9	2.17 s, 3H		
20					56.1	4.11 m, 2H		
21					61.1	3.85 m, 2H		
OH,C-19								3.04 d (10.0)
OH,C-14								5.48 bs

^a All assignments are based on COSY, NOE, HSQC, and HMBC experiments.

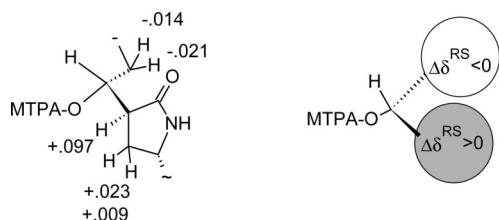


Figure 1. Selected $\Delta\delta$ values around C-10 of the (*R*) and (*S*)-MTPA esters of compound 3 [$\Delta\delta$ = chemical shift of (*R*)-MTPA ester minus chemical shift of (*S*)-MTPA ester in ppm].

showed correlations to amide C-12 and to nitrogen-bearing C-14. It remained to connect N-13 to C-12 to generate a γ -lactam and the proposed structure for 1.

Establishing the overall configuration of this compound proved more challenging than was anticipated. The relative configuration of the amide ring was established by 1D NOE difference spectroscopy. Irradiation of H-14 enhanced both H_{β} -19 (δ 2.29) and H-13. Irradiation of H-11 enhanced H_{α} -19 (δ 1.69) and H-10 (4.6%), which clearly established the trans relationship of H-11 and H-14. We attempted to determine the absolute configuration of berkeleyamide A (1) using a modified Mosher method.¹⁹ Treatment of 1 with (*R*)- and (*S*)-methoxy(trifluoromethyl)phenylacetyl (MTPA) chloride in pyridine gave the corresponding *R*- or *S*-esters respectively (3a, 3b). Molecular modeling of the esters and consideration of the $\delta\Delta$ values (see Figure 1) indicated that the absolute configuration at C-10 was *S*. However, molecular modeling studies of the two possible diastereomers did not provide sufficient evidence to determine unambiguously if the overall structure was (10*S*), (11*R*), (14*S*) or (10*S*), (11*S*), (14*R*).

HRESIMS gave a molecular formula of $\text{C}_{20}\text{H}_{21}\text{NO}_6$ for berkeleyamide B (4), with 11 sites of unsaturation and two more carbons than 1. The proton NMR spectrum of berkeleyamide B exhibited resonances for both the monosubstituted aromatic ring and the terminal isobutyl moiety found in berkeleyamide A (1). A preliminary look at the data showed that 14 of the 20 carbons were sp^2 hybridized and that the two additional carbons in 4 could be assigned to an acetate moiety (δ_{C} 169.0, 20.7 and δ_{H} 2.18 s, 3H). The IR spectrum supported the presence of an acetate moiety with a carbonyl absorption at 1754 cm^{-1} , an amide functionality with

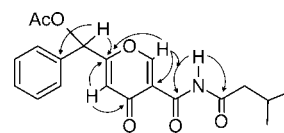


Figure 2. Important HMBC correlations in 4.

an absorption at 1697 cm^{-1} , and a series of absorption frequencies (1654 , 1616 , and 1574 cm^{-1}) that suggested the presence of a 4-pyrone.²⁰

The structure of 4 was established by consideration of these data and extensive analysis of the HMBC spectrum. The most important long-range correlations are shown in Figure 2. The unusually deshielded methine proton H-7 (δ_{H} 6.54) attached to oxygen-bearing C-7 (δ 72.4) showed HMBC correlations to the acetate carbonyl, to aromatic carbons C-6, C-1/C-5, and C-2/C-4, and to olefinic carbons C-8 (δ 166.4) and C-9 (δ 114.8). These connections helped explain the downfield shift of H-7. Olefinic proton H-9 (δ 6.59) also showed correlations to methine C-7, to carbonyl C-10 (δ 177.6), and to olefinic carbons C-11 and C-19 (δ 119.3, 163.0, respectively). These last two carbons showed HMBC correlations to olefinic proton H-19 (δ 8.69). These HMBC correlations were useful in generating a disubstituted 4-pyrone ring. The NMR chemical shifts of the disubstituted pyrone ring compared favorably with those of the 2,5-disubstituted-4-pyrone, microsphaerone A (S10, Supporting Information), isolated from the fungus *Microsphaeropsis* sp.²¹

Olefinic proton H-19 also showed HMBC correlation to amide carbonyl C-12 (δ 160.4), whose chemical shift was indicative of α,β -unsaturation. The amide proton (δ 11.53) showed long-range correlations to both amide carbons C-12 and C-14 (δ 160.4, 173.3), which generated an imide functionality. The NMR absorbances of the 4-pyrone-imide moiety also compared favorably to those reported for himeic acid A, a compound isolated from a marine-derived *Aspergillus* sp.²² The C-14 resonance showed correlations to methylene H₂-15 (δ 2.58) and methine H-16 (δ 2.17), which coupled to both methyl H-17 and H-18, generating the isobutyl end group typical of these compounds. These data generated the structure proposed for berkeleyamide B.

HRESIMS gave a molecular formula of $\text{C}_{22}\text{H}_{26}\text{N}_2\text{O}_6$ for berkeleyamide C (5) with 11 sites of unsaturation. This formula contained

two more carbons, one more nitrogen, and five more hydrogens than **4**. The ^1H and ^{13}C NMR (Table 1) spectra for compounds **4** and **5** were very similar, although the NMR spectra of **5** showed two additional coupled methylenes (δ_{C} 61.1, 56.1 and δ_{H} 3.85 and 4.11, respectively). In the ^{13}C NMR spectrum, the olefinic carbons exhibited less extreme shift variations, suggesting that the ring was a 4-pyridone rather than a 4-pyrone. These differences from **4** could be accommodated by an *N*-ethyl-2-hydroxy 4-pyridone ring in **5**. The IR data provided additional support for this assignment: the absorbance at 1634 cm^{-1} is typical of the carbonyl stretch of 4-pyridone rings.²³

The long-range correlations in the HMBC of compound **5** were in full agreement with the proposed structure. In addition to all of the correlations seen in **4**, HMBC correlations from H_2 -20 (δ 4.11) to the β -carbons of the pyridone ring were observed (δ 149.8, 150.4). Acetylation of **5** gave the monoacetate **6** with the expected downfield shift of the methylene protons H_2 -21 (δ 3.85 to 4.24). A similarly substituted 4-pyridone ring has been reported in the recently revised structure of aspernigrin A isolated from *Aspergillus niger* and *Cladosporium herbarum*.²⁴ The NMR signals of this ring compared favorably with berkeleyamide C (S11, Supporting Information).

Although the terminal benzyl and isobutyl moieties suggested that berkeleyamide D (**7**) belonged to the same family of compounds as berkeleyamides A–C, the NMR data indicated many structural differences. Berkeleyamide D (**7**) had a molecular formula of $\text{C}_{18}\text{H}_{21}\text{NO}_5$ established by HRESIMS. This formula required nine units of unsaturation. With only 10 sp^2 -hybridized carbons it was clear that **7** contained an additional ring. The benzylic and isobutyl moieties accounted for $\text{C}_{11}\text{H}_{16}$ and four of the nine DBE. The central portion of the molecule accommodated $\text{C}_7\text{H}_5\text{NO}_5$ and five DBE.

The presence of an amide was indicated by the IR absorbance at 1684 cm^{-1} and by the carbonyl absorbance in the ^{13}C NMR spectrum at δ 164.1 (Table 1). An IR absorbance at 1736 cm^{-1} and a carbonyl absorbance of δ 199.4 indicated the presence of a ketone. ^{13}C NMR data also indicated the presence of two unusually deshielded oxygen-bearing quaternary carbons (δ 95.3 and 84.9), an oxygen-bearing methine (δ 75.1), and a highly asymmetric olefin (δ 104.4, 197.8) influenced by an electron-withdrawing functionality at one end and an electron-donating group at the other. As the amide, ketone, and olefin accounted for only three DBE, two fused rings were required to accommodate the last two DBE. Of the two methines present in this moiety, one was olefinic C-9 (δ_{C} 104.4, δ_{H} 5.35) and the other was oxygen-bearing C-19 (δ_{C} 75.1, δ_{H} 4.41, d, $J = 10.0$). H-19 showed J -coupling to an –OH proton (δ 3.04, d, $J = 10.0$), indicating that it was a secondary alcohol. This spectrum also showed proton resonances for NH and/or OH protons at δ 6.78, bs and 5.48, bs. The construction of the molecule from these pieces required extensive analysis of the NMR data.

The benzylic methylene C-7 (δ 37.4) provided a convenient starting point to generate the structure of **7**. H_2 -7 (δ 3.98, d, $J = 17.4$ and 3.96, d, $J = 17.4$) showed HMBC correlations to several of the aromatic carbons, as well as to olefinic carbons C-8 and C-9 (δ 197.9 and 104.4, respectively). Long-range COSY correlations could be seen from H_2 -7 and aromatic H-1 and H-5 to olefinic H-9. H-9 showed HMBC correlations to the carbons resonating at δ 197.8, 199.4, and 95.3. The C-19-OH, H-19, and H-13 (δ 6.78) also showed HMBC correlations to quaternary C-11 (δ 95.3). Hence, only one carbon remained to be assigned. H-19 and all three of the OH/NH groups showed long-range coupling to quaternary C-14 (δ 84.9). These connectivity data could be accommodated by the [C-7–C-14] unit shown in **7**. The isobutyl methylene H_2 -15 (δ 1.88) also showed connectivity to C-14, which completed the carbon skeleton of compound **7**. Important HMBC correlations are shown in Figure 3.

Berkeleyamide D (**7**) showed some interesting derivatization patterns when treated with a variety of silylating agents. When **7**

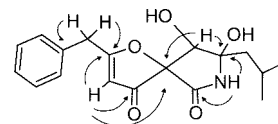


Figure 3. Important HMBC correlations in **7**.

was treated with *N*-trimethylsilylimidazole (TMSIM), a very active trimethylsilyl donor, the ESIMS spectrum gave a parent ion for the addition of three trimethylsilyl (TMS) groups, indicating three OH/NH groups, or enolizable systems. When **7** was treated with the less active silylating agent *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA), ESIMS indicated the addition of only two TMS. This was strong evidence that **7** had three silylatable groups, but only two were the more easily silylated hydroxy groups.²⁵ Further evidence for the proposed novel azaspirocyclic ring system in **7** was obtained by comparison of the NMR data to those of other known fungal metabolites. The pseurotins A–F, isolated from fungal strains of *Pseudeurotium ovalis*,²⁶ and the more recently reported azaspirene, isolated from the fungus *Neosartorya* sp.,²⁷ contain a similar azaspirocyclic system. The carbon and proton chemical shifts around the lactam ring in these compounds are very close to those of the lactam ring in **7**. However, as the furanone ring in these latter compounds is alkylated, the chemical shifts of this ring are a bit different. Therefore, the furanone ring shifts were compared to the furanone ring system of phelligrudin E isolated from the fungus *Phellinus igniarius*.²⁸ The NMR data of these two systems compared nicely (S12, Supporting Information).

The presumed biosynthetic building blocks for the pseurotins and azaspirene are a polyacetate precursor and phenylalanine.²⁹ If this biosynthetic scheme is used as a model, berkeleyamide D (**7**) could be produced from a phenyl triketide precursor and leucine as shown in Scheme 1. When this fungus was grown with ^{15}N -labeled leucine, the ESIMS of **7** showed a 2% incorporation of ^{15}N , as evidenced by the increase of the $M + 1$ peak of the parent ion $[\text{M} + \text{Na}]^+$.

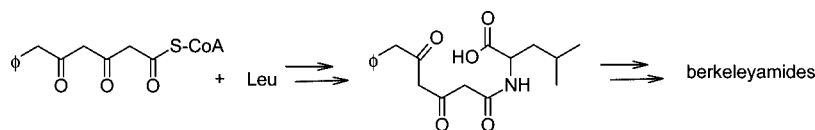
Consideration of the structures of berkeleyamides **1**, **4**, and **5** indicated that they could come from similar biosynthetic precursors. Conjugation of the phenyl triketide and leucine and reduction of the carbonyl at C-10 followed by cyclization could give **1** directly. The biosynthesis of **4** and **5** from these two precursors is a little more convoluted. The origin of C-19 in all of the berkeleyamides is of special interest. It could come from the carbonyl carbon of the leucine group through a hypothetical cyclic intermediate. C-19 could also come from methylation by *S*-adenosylmethionine, which is common in polyketide metabolism and is observed in pseurotin biosynthesis.²⁹

All of these compounds were isolated on the basis of their activity in enzyme inhibition assays. All four compounds were active against both caspase-1 and MMP-3 in the low micromolar range. Berkeleyamide A (**1**) and berkeleyamide D (**7**) exhibited the greatest potency, with IC_{50} values of 0.33 and 0.61 μM , respectively. These compounds were submitted to the NCI/NIH Developmental Therapeutics Program for testing against their suite of 60 human cancer cell lines. Although the compounds were accepted for the single-dose assay, they did not meet the criterion for inclusion in the five-dose screen.

Experimental Section

General Experimental Procedures. Optical rotations were recorded on a Perkin-Elmer 241 MC polarimeter using a 1.0 mL cell. UV spectra were recorded on a Hewlett-Packard 8453 spectrophotometer. ^1H and ^{13}C NMR spectra were run on a Bruker DPX-300 spectrometer. ^1H NMR spectra were recorded at 300 MHz, and the ^{13}C NMR spectra were recorded at 75 MHz unless otherwise noted. All of the 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. Mass

Scheme 1. Possible Biosynthetic Pathway for the Production of the Berkeleyamides



spectra were provided by the University of Montana mass spectrometer facility in Missoula, Montana. HPLC separations were made on a Varian Dynamax (250 × 21.4 mm) column. All solvents used were spectral grade.

Collection, Extraction, and Isolation Procedures. The collection and isolation of Berkeley Pit fungi has previously been described.^{2,3,7} The Berkeley Pit *Penicillium rubrum* isolate was grown in potato dextrose broth (26 flasks with 300 mL of broth per flask, acidified to pH 2.7 with concentrated H₂SO₄) for 6 days on a shaker table and then 15 days in still culture. The fungus was then killed with MeOH (50 mL per flask), mycelia were removed by filtration, and the broth was extracted with CHCl₃ (3 × 1 L). Removal of the CHCl₃ *in vacuo* gave 1.13 g of crude extract. This extract showed very good activity in our enzyme inhibition assays. The crude extract was chromatographed on a flash Si gel column with a gradient system that began with pure hexanes to which increasing amounts of isopropyl alcohol were added, ending with pure isopropyl alcohol. The column was washed with MeOH. The fraction that eluted with 10% isopropyl alcohol/hexanes was further purified with silica gel HPLC using an isopropyl alcohol/hexanes gradient to give berkeleyamide A (**1**, 15.6 mg), berkeleyamide B (**4**, 22.9 mg), berkeleyamide C (**5**, 5.4 mg), and berkeleyamide D (**7**, 20.3 mg).

Berkeleyamide A (1): oil, [α]_D²⁵ -1.0 (c 0.017, MeOH); IR (CHCl₃) ν_{max} 3428, 3012, 2960, 1715, 1685, 1368, 1100, 911 cm⁻¹; ¹H NMR and ¹³C NMR, see Table 1; HRESIMS *m/z* 304.1927 [M + H]⁺ (calcd for C₁₈H₂₆NO₃, 304.1913).

Acetylation of Berkeleyamide A (1). Compound **1** (2.0 mg) was dissolved in pyridine (50 μL) and Ac₂O (50 μL) and stirred for 24 h, after which the solvents were removed *in vacuo* to give **2** as an oil (2.1 mg): ¹H NMR (CDCl₃) δ 7.13–7.35 (m, 5 H, aromatics), 5.93 (br s, NH), 5.48 (q, *J* = 6.5 Hz, H-10), 3.71 (s, 2H, H-7), 3.64 (m, H-14), 3.01 (dd, H-9), 2.85 (m, H-9), 2.69 (m, H-11), 2.11 (m, H-19), 1.96 (s, 3H, Ac), 1.68 (m, H-19), 1.54 (m, H-16), 1.21–1.43 (m, 2H, H-15), 0.89 (d, *J* = 6.6 Hz, 6 H, H-17, H-18).

Chiral Derivatization of Berkeleyamide A (1). Berkeleyamide A (**1**, 1.0 mg) was dissolved in dry pyridine (100 μL), and either the *R* or *S* stereoisomer of α-methoxy-α-trifluoromethylphenylacetyl chloride (6 μL) was added. The mixtures were stirred for 24 h under N₂. MeOH (400 μL) was added to terminate the reaction, and the solvents were removed *in vacuo*. The reaction mixtures were then each passed through a small Si gel column and eluted with CH₂Cl₂ to give the products. **(R)-MTPA ester (3a):** ¹H NMR (CDCl₃) δ 7.32–7.42 (m, 5 H, aromatics), 5.78 (q, *J* = 6.6 Hz, H-10), 5.57 (br s, NH), 3.68 (bs, 2H, H-7), 3.64 (m, H-14), 2.95 (dd, *J* = 16.9, 6.1 Hz, H-9), 2.89 (dd, *J* = 16.9, 6.7 Hz, H-9), 2.77 (m, H-11), 2.05 (m, H-19), 1.57 (m, H-19), 1.34–1.49 (m, 3H, H-15, H-16), 0.84 (d, *J* = 6.7 Hz, 6H, H-17, H-18). **(S)-MTPA ester (3b):** ¹H NMR (CDCl₃) δ 7.32–7.41 (m, 5H, aromatics), 5.79 (q, *J* = 6.1 Hz, H-10), 5.53 (br s, NH), 3.72 (br s, 2H, H-7), 3.64 (m, H-14), 3.09 (dd, *J* = 16.9, 6.9 Hz, H-9), 2.91 (dd, *J* = 16.9, 6.0 Hz, H-9), 2.67 (m, H-11), 2.04 (m, H-19), 1.54 (m, H-19), 1.34–1.49 (m, 3H, H-15, H-16), 0.85 (d, *J* = 6.6 Hz, 6H, H-17, H-18).

Berkeleyamide B (4): oil, [α]_D²⁵ +39.6 (c 0.023, CHCl₃); IR (CHCl₃) ν_{max} 3025, 2962, 1754, 1697, 1654, 1616, 1574, 1506, 1411, 1189, 1037, 910 cm⁻¹; ¹H NMR and ¹³C NMR, see Table 1; HRESIMS *m/z* 372.1403 [M + H]⁺ (calcd for C₂₀H₂₂NO₆, 372.1369).

Berkeleyamide C (5): oil, [α]_D²⁵ +24.9 (c 0.007, CHCl₃); IR (CHCl₃) ν_{max} 3448, 2955, 2866, 1735, 1681, 1634, 1502, 1215, 1033 cm⁻¹; ¹H NMR and ¹³C NMR, see Table 1; HRESIMS *m/z* 415.1870 [M + H]⁺ (calcd for C₂₂H₂₇N₂O₆, 415.1869).

Acetylation of Berkeleyamide C (5). Compound **5** (2.0 mg) was dissolved in pyridine (50 μL) and Ac₂O (50 μL) and stirred for 24 h, after which the solvents were removed *in vacuo* to give **6** as an oil (2.1 mg): ¹H NMR (CDCl₃) δ 13.23 (bs, NH), 8.44 (bs, 1H, H-19), 7.26–7.42 (m, 5H, aromatics), 6.84 (s, 1H, H-9), 6.83 (s, 1H, H-7), 4.24 (br m, 4 H, H-20, H-21), 2.45 (d, *J* = 7.0 Hz, 2H, H-15), 2.30 (s, 3H, OAc), 2.17 (s, 3H, OAc), 2.11 (m, 1H, H-16), 0.97 (d, *J* = 6.6 Hz, 6H, H-17, H-18); HRESIMS *m/z* 457.1979 [M + H]⁺ (calcd for C₂₄H₂₉N₂O₇, 457.1975).

Berkeleyamide D (7): oil, [α]_D²⁵ -56.9 (c 0.007, MeOH); UV (MeOH) λ_{max} (log ε) 272 (3.8), 206 (4.1); IR (CHCl₃) ν_{max} 3289, 3024, 2961, 2871, 1736, 1684, 1582, 1385, 1155, 1121, 961 cm⁻¹; ¹H NMR and ¹³C NMR, see Table 1; EIMS *m/z* (rel) 313 (2), 295 (4), 217 (65), 57 (100); HREIMS *m/z* 313.1302 [M - 18]⁺ (calcd for C₁₈H₁₉NO₄, 313.1314); HRESIMS *m/z* 354.1318 [M + Na]⁺ (calcd for C₁₈H₂₁NO₅Na, 354.1317), 314.1383 [M - 17]⁺ (calcd for C₁₈H₂₀NO₄, 314.1392).

Silylation of Berkeleyamide D (7) with TMSIM. Compound **7** (0.5 mg) was treated with trimethylsilylimidazole (500 μL) and heated for 2 min at 50 °C, and then the mixture was subjected to ESIMS. ESIMS *m/z* 570 [C₁₈H₂₁NO₅ - 3H + 3(TMS) + Na]⁺.

Silylation of Berkeleyamide D (7) with BSTFA. Compound **7** (0.5 mg) was treated with *N,O*-bis(trimethylsilyl)trifluoroacetamide (500 μL) and heated for 2 min at 50 °C, and then the mixture was subjected to ESIMS. ESIMS *m/z* 498 [C₁₈H₂₁NO₅ - 2H + 2(TMS) + Na]⁺.

Growth of *Penicillium rubrum* with ¹⁵N-Labeled Leucine. *Penicillium rubrum* was grown in acidified potato dextrose broth (20 flasks with 300 mL of broth per flask and 30 flasks with 700 mL) as described above. The flasks were grown for 14 days, and ¹⁵N leucine (10 mg of powder to each small flask and 20 mg to each large flask; Cambridge Isotope Laboratories) was added. The cultures were allowed to grow for an additional 7 days. The fungus was then killed and extracted with CHCl₃ as described above. Removal of the CHCl₃ gave 1.80 g of crude extract. This extract was fractionated by flash Si gel chromatography followed by Si gel HPLC using isopropyl alcohol/hexanes mixtures to give berkeleyamide B (**4**, 9.6 mg) and berkeleyamide D (**7**, 5.5 mg). The ESI mass spectra of these compounds were compared to the spectra from the unlabeled samples. Berkeleyamide B (**4**): ESIMS *m/z* (intensity) 394.1 [M + Na]⁺ (100%), 395.1 [M + Na + 1]⁺ (25.1%), ¹⁵N-labeled sample 394.1 (100%), 395.1 (27.0%). Berkeleyamide D (**7**): ESIMS *m/z* (intensity) 354.1 [M + Na]⁺ (100%), 355.1 [M + Na + 1]⁺ (23.8%), ¹⁵N-labeled sample 354.1 (100%), 355.1 (26.0%).

Signal Transduction Assays. MMP-3 colorimetric (AK-400) and caspase-1 (AK-701) drug discovery kits were used for this assay (BIOMOL International). Crude extracts were tested at 1000 μg/mL, and column fractions were tested at 500 μg/mL.

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Supporting Information Available: ¹H and ¹³C NMR spectra for the berkeleyamides A–D (**1**, **4**, **5**, **7**) and the comparison of selected NMR data to known compounds are available free of charge via the Internet at <http://pubs.acs.org>.

References and Notes

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