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The Berkeleylactones, Antibiotic Macrolides from Fungal Coculture

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Abstract

A carefully timed coculture fermentation of *Penicillium fuscum* and *P. camembertii/clavigerum* yielded eight new 16-membered-ring macrolides, berkeleylactones A–H (**1**, **4**, **6–9**, **12**, **13**), as well as the known antibiotic macrolide A26771B (**5**), patulin, and citrinin. There was no evidence of the production of the berkeleylactones or A26771B (**5**) by either fungus when grown as axenic cultures. The structures were deduced from analyses of spectral data, and the absolute configurations of compounds **1** and **9** were determined by single-crystal X-ray crystallography. Berkeleylactone A (**1**) exhibited the most potent antimicrobial activity of the macrolide series, with low micromolar activity (MIC = 1–2 $\mu\text{g/mL}$) against four MRSA strains, as well as *Bacillus anthracis*, *Streptococcus pyogenes*, *Candida albicans*, and *Candida glabrata*. Mode of action studies have shown that, unlike other macrolide antibiotics, berkeleylactone A (**1**) does not inhibit protein synthesis nor target the ribosome, which suggests a novel mode of action for its antibiotic activity.

Graphical Abstract

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnat-prod.7b00133. Experimental details including ¹H NMR, ¹³C NMR, COSY, HSQC, and HMBC spectra for compounds **1**, **4**, **6–9**, **12**, and **13**, as well as cancer cell line data from NCI-DTP for **1**, **5**, **6**, and **9** (PDF)

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Notes

The authors declare no competing financial interest.

Microorganisms isolated from extreme environments have proven to be a good source of novel, bioactive compounds.^{1–3} In our early pilot studies of the extremophilic fungi isolated from the acidic, metal-rich waters of Berkeley Pit Lake, however, we found little evidence of antibiotic production. We therefore used enzyme inhibition assays targeting matrix metalloproteinase-3 (MMP-3), caspase-1, and caspase-3 to guide the isolation of compounds that block epithelial mesenchymal transition,^{4–6} inflammation,^{7–9} and apoptosis,^{10,11} respectively.

All of our previous studies have involved the isolation of secondary metabolites from fungi grown in pure culture. It has been shown, however, that “crosstalk” between microorganisms can activate silent gene clusters and lead to the formation of novel secondary metabolites.¹² Many studies have considered the effects of an actinomycete (or other bacteria) on fungal metabolism. For example, emericellamides A and B were produced by the marine-derived fungus *Emericella* sp. when cocultured with the marine actinomycete *Salinispora arenicola*.¹³ Coculture of *Aspergillus fumigatus* with *Streptomyces peucetius* yielded a series of novel *N*-formyl alkaloids.¹⁴

Fungal coculture, however, has received much less attention, and there are few reports in the literature, although it has also been shown to elicit the production of new secondary metabolites. *Bionectria ochroleuca* produced 2,2″-dimethylthielavin, a substituted trimer of 3,5-dimethylorsellinic acid, when grown in axenic culture. However, when *B. ochroleuca* was cocultured on solid agar with the fungus *Trichophyton rubrum*, 4″-hydroxysulfoxy-2,2″-dimethylthielavin was isolated from the zone of growth inhibition between the two fungi.¹⁵ Coculture of *Acremonium* sp. Tbp-5 and *Mycogone rosea* DSM 12973 led to the formation of new lipoaminopeptides, acremostatins A, B, and C.¹⁶ The antibacterial alkaloid aspercigin was derived from coculture of two *Aspergillus* species.¹⁷ In two separate coculture experiments, the mangrove fungi *Phomopsis* sp. K38 and *Alternaria* sp. E33 produced cyclo(L-leucyl-*trans*-4-hydroxy-L-prolyl-D-leucyl-*trans*-4-hydroxy-L-proline)¹⁸ and the antifungal tetrapeptides cyclo(gly-L-phe-L-pro-L-tyr) and cyclo(D-pro-L-tyr-L-pro-L-tyr).¹⁹

RESULTS AND DISCUSSION

In this study the effects of fungal coculture on the production of secondary metabolites and the elicitation of cryptic biosynthesis were explored. We selected *Penicillium fuscum* (Sopp) Raper & Thom and *P. camembertii/clavigerum* Thom,²⁰ two extrem-ophilic fungi that were

isolated from a single sample of surface water from Berkeley Pit Lake and established as pure cultures. Each fungus was initially grown as an axenic culture (potato dextrose broth), which was thoroughly extracted with CHCl_3 at time of harvest. The two fungi were then cocultured and extracted as described above, and the ^1H NMR spectral data of all three CHCl_3 extracts were compared (Figure S4, Supporting Information). It was clear from this comparison that there were compounds in the coculture that were not evident in either pure culture. The secondary metabolites of the axenic cultures were examined first. The most abundant compounds in the CHCl_3 extract of *P. camembertii/clavigerum* were citrinin and patulin, and that of *P. fuscum* was asperfuran. (A full report of the secondary metabolites of *P. fuscum* is in preparation.²¹) The ^1H NMR data of the mixed culture, however, showed that these compounds were now part of a more complex mixture of metabolites.

Although the CHCl_3 extract of the fungal coculture exhibited moderate inhibition of all three of our target enzymes, MMP-3 inhibitory activity was selected to guide isolation of macrolides **1**, **5**, **6**, and **9**. Analysis of ^1H NMR spectral data was then used for chemotype-guided isolation of structurally related compounds that were weaker inhibitors of MMP-3, which included macrolides **4**, **7**, **8**, **12**, and **13**. NMR spectral data for compound **1** was originally collected in both CDCl_3 and $\text{MeOH}-d_4$. CDCl_3 data provided better resolution and peak dispersal, so it was used for the structure elucidation of **1** (Table 1). Due to solubility issues with some of the more polar macrolides, however, the spectral data for all of the macrolides is reported in $\text{MeOH}-d_4$, to facilitate direct comparison. (Tables 2–5).

Berkeleylactone A (**1**) has a molecular formula of $\text{C}_{19}\text{H}_{32}\text{O}_7\text{S}$ with four sites of unsaturation, deduced from HRESIMS. The infrared spectrum showed a strong carbonyl absorbance at 1716 cm^{-1} as well as a broad O–H stretch at 3443 cm^{-1} , typical of a carboxylic acid, and strong C–O stretching vibrations typical of an ester (1277 , 1234 , and 1170 cm^{-1}).^{22a} The ^{13}C NMR data (CDCl_3) showed three carbonyl resonances (δ_{C} 172.3, 174.9, and 208.8), also indicating the presence of a ketone (δ_{C} 208.8). Compound **1** was readily methylated by diazomethane, yielding methyl ester **2**. In the HMBC data of **2**, the ester methyl showed correlations to the carbonyl carbon resonating at δ_{C} 174.9, establishing it as the carboxylic acid C-1'. The ^{13}C NMR data also provided evidence of three oxygen-bearing methines (δ_{C} 76.2, 73.3, and 70.4). Acetylation of methyl ester **2** yielded diacetate **3**, indicating the presence of two hydroxy groups, which accommodated the remaining two oxygens. As the three carbonyls required three of the four sites of unsaturation, we proposed that compound **1** was monocyclic.

Analysis of the ^1H NMR data of **1** (CDCl_3 , Table 1) in conjunction with $^1\text{H}-^1\text{H}$ COSY spectral data (Figure S7, Supporting Information) showed evidence of isolated spin system A: $\text{CH}-\text{CH}_2$ [$\text{H}-2$ (δ_{H} 4.01), H_2-3 (δ_{H} 3.20, 2.80)]; and B: $\text{CH}-\text{CH}_2$ [$\text{H}-2'$ (δ_{H} 4.53), H_2-3'

(δ_{H} 3.27, 2.98)]. HMBC data (Figure S9, Supporting Information) showed long-range correlations between the three protons of spin system A to both ketone C-4 (δ_{C} 208.8) and ester C-1 (δ_{C} 172.3). The three protons of spin system B showed similar correlations to carboxylic acid C-1' (δ_{C} 174.9). Three-bond coupling of methylene H-3' (δ_{H} 3.27) to methine C-2 (δ_{C} 41.3) and of methine H-2 (δ_{H} 4.01) to C-3' (δ_{C} 35.7) provided connectivity between spin systems A and B (Figure 1).

The position of the sulfide and the two hydroxy groups could also be determined using NMR spectral data and chemical shift arguments. In ^{13}C NMR, a hydroxy group has a stronger deshielding effect on the chemical shift of adjacent carbons than either a sulfide or methyl group (+41, +11, +9 ppm, respectively).^{22b} An oxygen-bearing methine generally resonates downfield of 70 ppm, while a sulfur-bearing methine resonates between 30 and 40 ppm. In the ^1H NMR, there is a similar trend, and the effects are additive. A proton attached to a sulfur-bearing carbon would resonate between 2.6 and 3.4 ppm.^{22c} If the carbon were also attached to a carbonyl moiety, the proton would resonate around 4.00 ppm.^{22c} These chemical shift arguments support the assignment of C-2 (spin system A) between ester C-1 (δ_{C} 172.3) and the sulfide moiety. They also support the assignment of C-3' (spin system B). The remaining hydroxy group is positioned at C-2' (δ_{C} 70.4). HMBC correlations between H-2 and C-3' and H-3' and C-2 as described above provide further support for these assignments (Figure 1).

Spin system C begins at hydroxy-bearing methine C-5 (δ_{C} 76.2) and ends with methyl C-16: $\text{CHOH}(\text{CH}_2)_9\text{-CHOCH}_3$. The ^1H - ^1H COSY data showed 3J -coupling between H-5 (δ_{H} 4.37) and H₂-6 (δ_{H} 1.83, 2H). Methylene H₂-6 was further spin-coupled to H₂-7 (δ_{H} 1.38, 0.97), which was coupled to H₂-8 (δ_{H} 1.26). In the HMBC spectrum, H₂-6 exhibited long-range coupling to ketone C-4, which provided connectivity to spin system A (Figure 1).

The terminus of spin system C could also be established using NMR spectral data. In the HMBC spectrum, oxygen-bearing methine H-15 (δ_{H} 4.94) showed long-range correlation to ester carbonyl C-1. These data not only confirmed that C-1 was the ester carbonyl but also provided connectivity between the terminus of spin system C and spin system A. COSY spectral data showed coupling between methine H-15 and methyl H₃-16 (δ_{H} 1.26) and to methylene H₂-14 (δ_{H} 1.55, 1.43). C-8 through C-13 consisted of a methylene chain that could be connected to both ends of spin system C through ^1H - ^1H COSY and HMBC correlations. These data could be accommodated by berkeleylactone A (**1**) as shown.

A single-crystal X-ray diffraction study confirmed the structure and allowed determination of the relative and absolute configurations of berkeleylactone A (**1**). The compound was crystallized from vapor diffusion using CHCl_3 and pentane. The absolute configuration of **1** (Figure 2) was determined and shown to be 2*R*, 5*S*, 15*R*, and 2'*S*.

The molecular formula of **4** was determined to be $\text{C}_{23}\text{H}_{36}\text{O}_{10}\text{S}$ based on HRESIMS. Compound **4** has four more hydrogens, four more carbons, three more oxygens, and two more sites of unsaturation than compound **1**. In the infrared spectrum, the carbonyl region of **4** showed overlapping absorbances between 1738 and 1716 cm^{-1} . The ^1H NMR data of **1** and **4** ($\text{MeOH-}d_4$, Table 2) were very similar except for the downfield shift of H-5 from δ_{H}

4.30 to δ_{H} 5.17 in compound **4** and the addition of two 2H multiplets at δ_{H} 2.66 and 2.60 ppm in **4**. The ^{13}C NMR data of **4** (Table 4) showed four additional carbon resonances: two methylenes (δ_{C} 29.8, 29.9) and two carbonyl carbons (δ_{C} 173.7, 176.1). Both methylenes showed HMBC correlations (Figure S16, Supporting Information) to the two carbonyl carbons, typical of a succinic acid moiety. These data indicated that **4** was a succinic acid derivative of **1**. The position of the succinate was established at C-5 by the downfield shift of H-5 and by HMBC correlations of H-5 to both ketone C-4 and ester C-1'' (δ_{C} 205.8 and 173.7, respectively), to give berkeleylactone B (**4**) as shown. It was assumed that **1** and **4** had the same relative and absolute configurations based on similarities in chemical shifts and coupling constants.

Compound **5** has a molecular formula of $\text{C}_{20}\text{H}_{30}\text{O}_7$ deduced from HRESIMS, with six sites of unsaturation. It was clear from the molecular formula that **5** lacked the 3-mercaptoplactate moiety found in compounds **1** and **4**. In the infrared spectrum, the carbonyl region of **5** was more complex, with overlapping carbonyl absorbances at 1745, 1734, 1716, and 1702 cm^{-1} . Comparison of the ^1H NMR and ^{13}C NMR spectral data of compound **4** with that of **5** (Tables 2 and 4) showed the presence of the succinate moiety as well as an isolated, trans-disubstituted double bond, C2–C3 [δ_{C} 133.3, δ_{H} 6.71, d ($J = 15.9$ Hz); δ_{C} 137.3, δ_{H} 7.32, d ($J = 15.9$ Hz)]. In the HMBC data, both olefinic H-2 and H-3 showed correlations to ketone C-4 (δ_{C} 197.7) and ester C-1 (δ_{C} 166.5). The upfield shift of these carbonyl carbons compared to those of **1** and **4** was consistent with α,β -unsaturation. These data suggested the structure of **5** as shown. Compound **5** was previously reported in 1977 as the antibiotic A26771B, a metabolite of *Penicillium turbatum*.²³ The NMR data of compound **5** and A26771B (CDCl_3) were virtually identical.^{23,24} There have been several total syntheses published for A26771B that have shown that the configurations at C-5 and C-15 are consistent with berkeleylactones A and B.²⁵ We have reported the ^1H and ^{13}C NMR spectral data of **5** (Tables 2 and 4) to facilitate direct comparison to those of the berkeleylactones.

Berkeleylactone C (**6**) has a molecular formula of $\text{C}_{20}\text{H}_{30}\text{O}_8$ deduced from HRESIMS, with six sites of unsaturation. From this formula it was clear that **6** has one more oxygen than **5**, although the NMR spectral data were very similar. The main difference was the replacement of methylene C-14 in **5** with an oxygen-bearing methine (δ_{C} 74.8, δ_{H} 3.50, m) in **6**. Methine H-14 was spin-coupled to both ester methine H-15 (δ_{H} 4.87, m) and methylene H₂-13 (δ_{H} 1.56, m, 1.44, m), which supported positioning of the hydroxy group at C-14. Since the absolute configuration was assumed to be the same as that of the other macrolides, extensive molecular modeling studies were performed in Spartan'06ES to confirm the configuration at C-14 from coupling constant data. Both the C-14*R* and the C-14*S* epimers were subjected to MMFF equilibrium conformation analysis to model the most stable conformer of each. The molecular modeling studies were inconclusive, probably due to the inherent flexibility of the lactone system. However, single-crystal X-ray data on the related compound **9** allowed us to ultimately assign the stereochemistry at C-14 as *R*.

Berkeleylactone D (**7**) has a molecular formula of $\text{C}_{20}\text{H}_{30}\text{O}_8$ deduced by HRESIMS. Compounds **6** and **7** are isomers and their NMR spectral data are very similar (Tables 2 and 4). The main difference is a shift in the position of the hydroxy group from C-14 to C-13, which was supported by ^1H – ^1H COSY correlations (Figure S24, Supporting Information).

Oxygen-bearing methine H-15 (δ_{H} 5.23, m) was readily identified by its chemical shift and by its ^1H – ^1H -COSY correlation to methyl doublet H₃-16 (δ_{H} 1.35). H-15 was also spin-coupled to methylene H₂-14 (δ_{H} 1.89, m, 1.83, m), which were further coupled to hydroxy-bearing methine H-13 (δ_{H} 3.81, m). Again, molecular modeling studies were run to try to assign the absolute configuration of C-13 but were inconclusive.

Berkeleylactone E (**8**) has a molecular formula of $\text{C}_{20}\text{H}_{32}\text{O}_7$ deduced from HRESIMS, with five sites of unsaturation. The NMR spectral data of **8** indicated the presence of the succinate moiety as well as a conjugated double bond, C2–C3 [δ_{C} 123.3, δ_{H} 6.10 dd (J = 15.7, 1.8 Hz); δ_{C} 148.3, δ_{H} 6.93 dd (J = 15.7, 4.9 Hz)], as in compounds **5**–**7** (Tables 3 and 5). However, there was no evidence of a ketone carbon in the ^{13}C NMR spectrum of **8**. Both olefinic protons H-2 and H-4 showed HMBC correlations to ester carbonyl C-1 (δ_{C} 167.8) and to an oxygen-bearing methine that resonated at δ_{C} 73.0 (Figure S31, Supporting Information). The attached proton (δ_{H} 4.55 m) showed HMBC correlations to C-2, C-3, and C-5 (δ_{C} 77.8) as well as COSY coupling to methine H-5 (δ_{H} 4.83 m) (Figure S29, Supporting Information). These data suggested that C-4 was reduced to an alcohol in macrolide **8**. The succinate moiety was again assigned to the C-5 position due to the chemical shift of H-5 and to a HMBC correlation between H-5 and succinate ester C-1''.

A molecular formula of $\text{C}_{16}\text{H}_{28}\text{O}_5$ was assigned to berkeleylactone F (**9**) by HRESIMS. The NMR spectral data of **9** were similar to those of **8** (Tables 3 and 5). These data showed the typical resonances associated with the unsaturated cyclic macrolide structure, with a C-4 alcohol instead of a ketone, but lacked evidence of the succinate moiety. The ^1H NMR data of **9** showed an upfield shift of H-5 to δ_{H} 3.61, which suggested a C-5 alcohol rather than a succinate ester. Compound **9** readily formed triacetate **10** when treated with Ac_2O –pyridine, indicating that **9** is a triol. The third hydroxy group could be assigned to methine C-14 [δ_{C} 75.0, δ_{H} 3.42 td (J = 8.5, 2.7 Hz)]. The COSY spectrum showed 3J -coupling of H-14 to ester methine H-15 [δ_{H} 4.75 dq (J = 8.5, 6.6)], which in turn was coupled to methyl H₃-16 [δ_{H} 1.33 d (J = 6.6)] (Figure S34, Supporting Information). Molecular modeling studies of **9** indicated the same relative configuration at C-14 as found in macrolide **6**. The absolute configuration of **9** was determined using a modified Mosher's method.²⁶ In order to determine the configurations at C-4 and C-14 and to confirm that the configurations of C-5 and C-15 are consistent with **1**, compound **9** was treated with *R*- or *S*-methoxy-(trifluoromethyl)phenylacetyl (MTPA) chloride in pyridine to give the corresponding *S*- or *R*-esters (*S*- and *R*-**11**), respectively. The results of this study are shown in Figure 3 and established the absolute configuration of **9** as 4*R*, 5*S*, 14*S*, 15*R*. A single-crystal X-ray diffraction study of triacetate **10** provided further confirmation of the structure and the relative and absolute configurations of berkeleylactone F (**9**) (Figure 4).

Berkeleylactone G (**12**) has a molecular formula of $\text{C}_{20}\text{H}_{32}\text{O}_8$, which was established by HRESIMS. The molecular formula of **12** has four more carbons and hydrogens, and three more oxygens than **9**, suggesting the presence of a succinate moiety. The NMR spectral data were very similar to those of **9**, with the addition of the ^1H and ^{13}C resonances associated with the succinate moiety as in **4**–**8** and the downfield shift of H-5 (δ_{H} 4.83), indicating the point of attachment (Tables 3 and 5). The COSY data (Figure S39, Supporting Information)

showed that H-5 was coupled to H-4 (δ_{H} 4.55), which was also coupled to olefin H-3 (δ_{H} 6.95). Further confirmation was provided by an HMBC correlation between H-5 and succinate C-1'' (δ_{C} 174.2).

The molecular formula of berkeleylactone H (**13**) was established as $\text{C}_{20}\text{H}_{32}\text{O}_8$, by HRESIMS. Compounds **13** and **12** are isomers, and the NMR spectral data are very similar, with one major exception (Tables 3 and 5). There was no spectral evidence of the C-16 methyl group found in all of the other berkeleylactones. Instead, it was replaced by hydroxymethylene C-16 (δ_{H} 3.61, δ_{C} 65.1). There is a strong COSY correlation between H₂-16 and ester methine H-15 (δ_{H} 5.05) that supports this assignment (Figure S44, Supporting Information). The absolute configurations at C-4, C-5, and C-15 again were assumed to be consistent with macrolide **9**.

Although MMP-3 inhibitory activity was used to guide compound isolation, we redirected our focus because of the similarities between the berkeleylactones and the antibiotic A26771B. The compounds were tested for activity against a panel of Gram-positive and Gram-negative bacteria and three *Candida* isolates at concentrations of 1 μM to 1 mM/well (Table 6). Berkeleylactone A (**1**) exhibited the strongest activity against Gram-positive bacteria and was even more active against four MRSA strains than it was against a methicillin-susceptible strain of *Staphylococcus aureus* (Table 7). Neither the berkeleylactones nor A26771B (**5**) was active against Gram-negative bacteria. The activities of compounds **1** and **5** were compared to those of several known antibiotics against three methicillin-resistant strains of *S. aureus* [Table 8, comparative data provided by Hartford Hospital, Center for Anti-Infective Research and Development (CAIRD)].

Berkeleylactone A (**1**) does not conform to either of the structure–activity paradigms associated with the macrolide antibiotics. Macrolide antibiotics with 14-, 15-, or 16-membered rings have been isolated from a number of bacteria, particularly actinomycetes. Unlike **1**, all of these antibiotics possess specific sugar moieties that have been considered essential to antibiotic activity. First-generation macrolide antibiotics include the natural product erythromycin, a 14-membered lactone first developed in 1952.²⁷ Semisynthetic derivatives of erythromycin, including clarithromycin, are typical of the second-generation macrolides. They retained the 3-*O*-cladinose and 5-desosamine sugar moieties, both of which were considered critical components of activity. The β -keto-macrolides (ketolides) were the third generation of macrolides and include telithromycin²⁸ and cethromycin.²⁹ These compounds exhibited improved activity against a number of resistant isolates including the MLS_B (macrolide-lincosamides-group B streptogramin resistant) bacteria and demonstrated that the 3-*O*-cladinose was not necessary for activity.^{28,29}

To date, over 40 16-membered macrolide antibiotics have been isolated from different species of *Streptomyces*. These have been classified as either the carbomycin-leucomycin group or tylosin-chalcomycin group.^{30,31} The former group is distinguished by the presence of the disaccharide mycarosyl-mycaminose at C-5, while the latter is distinguished by a mycinose at the C-14 methylene (Figure S46, Supporting Information).

Conventional macrolide antibiotics block bacterial protein biosynthesis by binding to the 23S rRNA of the 50S subunit and interfering with the elongation of nascent peptide chains during translation.³² We tested berkeleylactone A (**1**) along with erythromycin, josamycin, and tylosin in two assay systems to initiate mode of action studies. First, it was evaluated in the extension inhibition assay (toeprinting) to allow the direct monitoring of the ribosome stalling on mRNA.³³ The inducible genes of macrolide antibiotic resistance, including *ermB* (erythromycin ribosome methylase B), are regulated by cofactor-dependent programmed translation arrest. In the case of antibiotic resistance, ORF *ermBL* is constitutively translated and the macrolide resistance gene *ermB* is constitutively attenuated. Macrolide antibiotics stall the ribosome during translation of *ermBL*, which allows expression of *ermB* and subsequent antibiotic resistance.³⁴ Toeprinting can assess the ability of a specific antibiotic to stall the ribosome at a specific mRNA codon.³³ It can also give direct evidence of the specific mode of action of an antibiotic. As shown in Figure 5, unlike the known macrolide antibiotics, berkeleylactone A (**1**) did not induce stalling of the ribosome at the *ermBL* ORF.

We then examined the effect of berkeleylactone A (**1**) on cell-free translation of GFP protein. Although the known macrolide antibiotics erythromycin, josamycin, and tylosin effectively inhibited the synthesis of GFP, compound **1** had no effect at 50 or 250 μ M (Figure 6), which also indicated that it does not target protein synthesis.^{33,34}

The second antibiotic structure–activity paradigm was developed from data generated from fungally derived 12-, 14-, and 16-membered macrolides. These include the 12-membered-ring patulolides,^{35,36} pandangolides,^{37,38} cladospolides,³⁹ and sporiolides,⁴⁰ as well as the 14-membered-ring pestalotioprolides, seircuprilide, and nigrosporiolide.⁴¹ Antibiotic A26771B (**5**, *P. turbatum*) is the only fungally derived monocyclic hexadecenoic acid 16-membered-ring macrolide antibiotic^{23,24} previously reported in the literature: pyrenophorin^{42,43} and vermiculine^{44,45} are symmetrical dimers of octenoic acid. Of these compounds, A26771B (**5**) has an activity profile similar to that of **1**, as it targets Gram-positive bacteria and *Candida* sp.^{23,24} Patulolides A and B are weakly active against selected bacteria and fungi,³⁶ while pyrenophorin⁴³ and vermiculine^{44,45} are primarily antifungal agents. On the basis of these observations, it was proposed that antimicrobial activity of fungal macrolides was associated with a double bond flanked by two carbonyl carbons.³⁹ However, berkeleylactone A (**1**) demonstrates more potent antibiotic activity against Gram-positive bacteria and certain yeasts than A26771B (**5**), yet it lacks the double bond generally associated with antibiotic activity.^{23,24}

The compounds were also tested for MMP-3 inhibitory activity. Compounds **1**, **5**, **6**, and **9** had IC₅₀ values of 100, 50, 10, and 150 μ M, respectively. These compounds were then tested

in a single-dose assay (10 μ M) by the NCI-Developmental Therapeutics Program against 60 human cancer cell lines. Compound **1** targeted two leukemia cell lines, with 85% growth inhibition of K-562 and 2.4% lethality against RPMI-8226. A26771B (**5**) demonstrated 48% growth inhibition of RPMI-8226. Compound **6** showed 48% and 46% growth inhibition of leukemia cell lines CCRF-CEM and K-562, respectively. Compound **9** demonstrated 38% growth inhibition of cell line CCRF-CEM (Figures S47–50, Supporting Information).

EXPERIMENTAL SECTION

General Experimental Procedures

Optical rotations were recorded on a PerkinElmer 241 MC polarimeter using a 1.0 mL cell. IR spectra were recorded on a PerkinElmer Spectrum One FT-IR spectrometer. 1D and 2D NMR spectra were recorded with a Bruker Avance 400 MHz instrument at 400 MHz for ^1H NMR and 100 MHz for ^{13}C NMR. Chemical shift values (δ) are given in parts per million (ppm), and the coupling constants (J) are in hertz (Hz). All of the chemical shifts were recorded with respect to the deuterated solvent shift (CDCl_3 : δ_{H} 7.24 for the proton resonance and δ_{C} 77.0 for the carbon, $\text{MeOH}-d_4$: δ_{H} 3.31 for the proton resonance and δ_{C} 49.1 for the carbon). Both low- and high-resolution mass spectra were recorded on a Micromass LCT Premier XE mass spectrometer. X-ray structures were run on a Bruker D8 Venture instrument. All solvents used were spectral grade or distilled prior to use.

Collection, Extraction, and Isolation Procedures

The collection of water samples from Berkeley Pit Lake, the isolation of the various organisms, and the pilot growth and biological testing of the extracts have been previously described.³ The two fungal species *P. fuscum* and *P. camembertii/clavigerum*²⁰ were isolated from a surface water sample taken from the Berkeley Pit Lake. Each fungus was grown in potato dextrose broth (shaken, room temperature, 200 rpm) for 7 days. At time of harvest, MeOH was added to each culture, the mycelia were removed by gravity filtration, and the filtrate was extracted with CHCl_3 .

For the coculture experiment, *P. fuscum* (Sopp) Raper & Thom was grown in pure culture in potato dextrose broth (10 $\times$ 400 mL). After 24 h, an agar cube (8 mm³) impregnated with *P. camembertii/clavigerum* mycelium was added to each flask, and the resulting coculture was shaken for 6 more days (200 rpm, room temperature). At time of harvest, MeOH (50 mL/flask) was added, the mycelia were removed by gravity filtration, and the broth was extracted with CHCl_3 (3 $\times$ 2 L). The CHCl_3 was removed *in vacuo* to yield 663 mg of crude extract. This extract was active in the MMP-3, caspase-1, and caspase-3 enzyme inhibition assays.

The CHCl_3 extract was fractionated by flash silica gel column chromatography using a stepwise gradient of an isopropyl alcohol (IPA)–hexanes system of increasing polarity starting with 5% IPA to 100% IPA (10%, 20%, 50% IPA), followed by 100% MeOH. Fraction 1 (5% IPA–Hex) yielded pure citrinin (26.5 mg) and **5** (21.4 mg). Fraction 3 (20% IPA) was further resolved using semipreparative silica gel HPLC [Varian Dynamax Microsorb 100-5] in gradient mode from 10% IPA–hexanes to 20% IPA–hexanes over 60

min to yield **6** (6.0 mg) and **8** (10.4 mg). Fraction 4 (50% IPA) was further resolved in a similar manner to yield **1** (23.3 mg) and **7** (5.8 mg). Fraction 5 (50% IPA) was also further resolved as described to yield **4** (2.4 mg), **9** (20.7 mg), **12** (10.8 mg), and **13** (1.7 mg).

A second coculture experiment was run on a smaller scale (500 mL) under the same conditions described but with the addition of methyl oleate to the broth (1.25 g/500 mL). Under these growth conditions the production of compound **4** was enhanced from 0.6 mg/L to 4.0 mg/L.

Berkeleylactone A (1)—colorless solid, $[\alpha]_D^{25} +0.5$ (*c* 0.170, CHCl₃); IR (CHCl₃) $\nu_{\max}$ 3443, 2932, 2860, 1716, 1277, 1234, 1170, 1094 cm⁻¹; ¹H NMR see Tables 1 and 2; ¹³C NMR see Table 4; HRESIMS m/z [M – H][–] 403.1799 (calcd for C₁₉H₃₁O₇S, 403.1791).

Methylation of Berkeleylactone A (1)

Compound **1** (0.5 g) was dissolved in Et₂O (100 μ L), and a solution of CH₂N₂–Et₂O added dropwise until the solution stayed yellow. After that time the solvent was removed to give **2** as an oil (0.5 g): ¹H NMR (CDCl₃) δ_C 4.95 (1H, m, H-15), 4.48 (1H, dd, *J* = 5.6, 3.7 Hz, H-2'), 4.34 (1H, t, *J* = 4.1 Hz, H-5), 4.01 (1H, dd, *J* = 8.2, 6.1 Hz, H-2), 3.79 (3H, s, OMe), 3.25 (1H, m, H-3), 3.21 (1H, m, H-3'), 2.95 (1H, dd, *J* = 14.3, 5.8 Hz, H-3'), 2.72 (1H, dd, *J* = 18.5, 6.1 Hz, H-3), 1.84 (2H, m, H-6), 1.26 (3H, d, *J* = 6.2 Hz, H-16); ESIMS 419 [M + 1].

Acetylation of Compound 2

Compound **2** (0.5 mg) was dissolved in pyridine (30 μ L) and Ac₂O (30 μ L) and stirred for 24 h. The solvents were removed *in vacuo* to give compound **3** as an oil (0.5 mg): ¹H NMR (CDCl₃) δ_H 5.31 (1H, dd, *J* = 7.3, 3.8 Hz, H-2'), 5.05 (1H, t, *J* = 4.8 Hz, H-5), 4.99 (1H, p, *J* = 6.2 Hz, H-15), 3.88 (1H, dd, *J* = 11.3, 3.3 Hz, H-2), 3.75 (3H, s, OMe), 3.25 (1H, dd, *J* = 7.6, 3.6 Hz, H-3), 3.20 (1H, m, H-3'), 3.02 (1H, dd, *J* = 14.4, 7.5 Hz, H-3), 2.80 (1H, dd, *J* = 18.1, 3.4 Hz, H-3'), 2.15 (3H, s, OAc), 2.08 (3H, s, OAc), 1.25 (3H, d, *J* = 6.3 Hz, H-16); HRESIMS m/z [M + Na]⁺ 525.2119 (calcd for C₂₄H₃₈O₉NaS, 525.2134).

Berkeleylactone B (4)—colorless oil, $[\alpha]_D^{25} -1.5$ (*c* 0.67, CHCl₃); IR (CHCl₃) $\nu_{\max}$ 3436, 3028, 2933, 2860, 1726, 1459, 1375, 1268, 1167, 1091, 909 cm⁻¹; ¹H NMR see Table 2; ¹³C NMR see Table 4; HRESIMS m/z [M + H]⁺ ion at 505.2078 (calcd for C₂₃H₃₇O₁₀S, 505.2107).

A26771B (5)—colorless oil, $[\alpha]_D^{25} -13$ (*c* 0.055, CHCl₃); IR (CHCl₃) $\nu_{\max}$ 3440, 3020, 2835, 1745, 1715, 1287, 1048 cm⁻¹; ¹H NMR see Table 2; ¹³C NMR see Table 4; HRESIMS m/z [M – H][–] 381.1912 (calcd for C₂₀H₂₉O₇, 381.1913).

Berkeleylactone C (6)—colorless oil, $[\alpha]_D^{25} -0.9$ (*c* 0.0300, CHCl₃); UV (MeOH) $\lambda_{\max}$ (log *e*) 224 (3.5) nm; IR (CHCl₃) $\nu_{\max}$ 3416, 2928, 1744, 1702, 1288, 1163, 1043 cm⁻¹; ¹H NMR see Table 2; ¹³C NMR see Table 4; HRESIMS m/z [M – H][–] 397.1846 (calcd for C₂₀H₂₉O₈, 397.1862).

Berkeleylactone D (7)—colorless oil, $[\alpha]_D^{25} -18.0$ (c 0.0051, CHCl_3); IR (CHCl_3) ν_{max} 3274, 2914, 1739, 1366, 1217 cm^{-1} ; ^1H NMR see Table 2; ^{13}C NMR see Table 4; HRESIMS m/z $[\text{M} - \text{H}]^-$ 397.1862 (calcd for $\text{C}_{20}\text{H}_{29}\text{O}_8$, 397.1862).

Berkeleylactone E (8)—colorless oil, $[\alpha]_D^{25} +9.0$ (c 0.031, CHCl_3); IR (CHCl_3) ν_{max} 3444, 3020, 1737, 1727, 1366, 1047 cm^{-1} ; ^1H NMR see Table 3; ^{13}C NMR see Table 5; HRESIMS m/z $[\text{M} - \text{H}]^-$ 383.2069 (calcd for $\text{C}_{20}\text{H}_{31}\text{O}_7$, 383.2070).

Berkeleylactone F (9)—colorless solid, $[\alpha]_D^{25} +1.3$ (c 0.101, CHCl_3); IR (solid) ν_{max} 3200, 2916, 2850, 1706, 1275, 1043, 732 cm^{-1} ; ^1H NMR see Table 3; ^{13}C NMR see Table 5; HRESIMS m/z $[\text{M} + \text{H}]^+$ 301.2025 (calcd for $\text{C}_{16}\text{H}_{29}\text{O}_5$, 301.2015).

Acetylation of Berkeleylactone F (9)

Compound **9** (0.5 mg) was dissolved in pyridine (30 μL) and Ac_2O (30 μL) and stirred for 24 h. After that time the solvents were removed to give **10** as an oil (0.5 mg): ^1H NMR (CDCl_3) δ 6.83 (1H, dd, $J = 15.9, 5.5$ Hz, H-3), 6.02 (1H, dd, $J = 15.8, 1.7$ Hz, H-2), 5.72 (1H, dt, $J = 5.4, 2.0$ Hz, H-4), 4.98–5.06 (1H, m, H-15), 4.91 (1H, ddd, $J = 7.9, 5.1, 2.3$ Hz, H-5), 4.86 (1H, td, $J = 7.6, 3.9$ Hz, H-14), 2.12 (3H, s, Ac), 2.05 (3H, s, Ac), 2.04 (3H, s, Ac), 1.23 (3H, d, $J = 6.3$ Hz, H-16); ESIMS m/z $[\text{M} + 1]^+$ 427, m/z $[\text{M} + \text{Na}]^+$ 449.

Chiral Derivatization of Berkeleylactone F (9)

Compound **9** (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 h. After that time, MeOH (400 μL) was added and the solvents were 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 (**11**): (*S*)-MTPA ester: ^1H NMR (selected shifts) (CDCl_3) δ 7.32–7.41 (m, aromatics), 6.85 (1H, dd, $J = 15.9, 4.5$ Hz, H-3), 6.10 (1H, dd, $J = 15.9, 1.8$ Hz, H-2), 5.09 (1H, m, H-5), 5.05 (1H, m, H-14), 5.02 (1H, m, H-15), 4.65 (1H, m, H-4), 1.23 (3H, d, $J = 6.2$, H-16); ESIMS 949 $[\text{M} + 1]$. (*R*)-MTPA ester: ^1H NMR (selected shifts) (CDCl_3) δ 7.32–7.41 (m, aromatics), 6.62 (1H, dd, $J = 15.9, 5.0$ Hz, H-3), 5.82 (1H, m, H-4), 5.61 (1H, dd, $J = 15.9, 1.6$ Hz, H-2), 5.16 (1H, m, H-5), 4.98 (1H, m, H-14), 4.93 (1H, m, H-15), 1.08 (3H, d, $J = 6.0$, H-16); ESIMS 949 $[\text{M} + 1]$.

Berkeleylactone G (12)—colorless oil, $[\alpha]_D^{25} -3.5$ (c 0.051, CHCl_3); IR (CHCl_3) ν_{max} 3421, 3020, 1717, 1423, 1170, 1044, 929 cm^{-1} ; ^1H NMR see Table 3; ^{13}C NMR see Table 5; HRESIMS m/z $[\text{M} - \text{H}]^-$ 399.2006 (calcd for $\text{C}_{20}\text{H}_{31}\text{O}_8$, 399.2019).

Berkeleylactone H (13)—colorless oil, $[\alpha]_D^{25} -23.5$ (c 0.017, CHCl_3); IR (CHCl_3) ν_{max} 3403, 3020, 1716, 1508, 1423, 1047, 929 cm^{-1} ; ^1H NMR see Table 3; ^{13}C NMR see Table 5; HRESIMS m/z $[\text{M} - \text{H}]^-$ 399.2024 (calcd for $\text{C}_{20}\text{H}_{31}\text{O}_8$, 399.2019).

X-ray Crystallographic Data for Macrolide 1

Colorless rods of **1** were obtained by diffusing pentane into a chloroform solution of **1**. X-ray diffraction data for **1** were collected at 100 K using Mo $K\alpha$ radiation ($\lambda = 0.71073$ Å).

Data have been corrected for absorption using the SADABS⁴⁶ area detector absorption correction program. Using Olex2,⁴⁷ the structure was solved with the ShelXT⁴⁸ structure solution program using direct methods and refined with the ShelXL⁴⁸ refinement package using least-squares minimization. All non-hydrogen atoms were refined with anisotropic thermal parameters. Hydrogen atoms attached to heteroatoms were found from the residual density maps and refined with isotropic thermal parameters. All other hydrogens atoms were refined in calculated positions using a ridged group model. The absolute structure was determined by refinement of the Flack parameter,⁴⁹ based on anomalous scattering, with a final Flack parameter of 0.00(2). All calculations and refinements were carried out using APEX2,⁵⁰ SHELXTL,^{48,51} and Olex2⁴⁷ software. Crystallographic data for **1** have been deposited with the Cambridge Crystallographic Data Centre. Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: + 44 (0)1223-336033, or e-mail: deposit@ccdc.cam.ac.uk).

Crystallographic data for 1—C₁₉H₃₂O₇S, *M* = 404.50, monoclinic, space group *P*2₁, *a* = 10.6258(10) Å, *b* = 5.2403(5) Å, *c* = 18.8604(17) Å, β = 102.984(2)°, *V* = 1023.34(17) Å³, *Z* = 2, *T* = 100 K, μ(Mo Kα) = 0.195 mm⁻¹, ρ_{calcd} = 1.313 g mL⁻¹, 2θ_{max} = 68.870, 44 910 reflections collected, 8604 unique (*R*_{int} = 0.0656, *R*_{sigma} = 0.0528), *R*1 = 0.0470 (*I* > 2σ(*I*)), *wR*2 = 0.1022 (all data), Flack parameter = 0.00(2), CCDC number 1040078.

X-ray Crystallographic Data for Berkeleylactone F Acetate **10**

X-ray diffraction data for **10** were collected at 100 K using Cu Kα (λ = 1.541 78) radiation. Data have been corrected for absorption using the SADABS⁴⁶ area detector absorption correction program. Using Olex2,⁴⁷ the structure was solved with the ShelXT⁴⁸ structure solution program using direct methods and refined with the ShelXL⁴⁸ refinement package using least-squares minimization. All non-hydrogen atoms were refined with anisotropic thermal parameters. All hydrogens were placed in calculated positions using a ridged group model with isotropic thermal parameters *U*(H) = 1.2 *U*_{eq}(C) for C(H) groups and *U*(H) = 1.5 *U*_{eq}(C) for all C(H,H,H) groups. The absolute structure was determined by refinement of the Flack parameter,⁴⁹ based on anomalous scattering, with a final Flack parameter of 0.05(8). Further analysis of the absolute structure was carried out using likelihood methods⁵² was performed using PLATON.⁵³ The results were a final Hooft parameter of 0.04(6). All calculations and refinements were carried out using APEX2,⁵⁰ SHELXTL,^{48,51} Olex2,⁴⁷ and PLATON.⁵³

Crystallographic data for 10—C₂₂H₃₄O₈, *M* = 426.49, orthorhombic, space group *P*2₁2₁2₁, *a* = 7.4360(3) Å, *b* = 9.5511(3) Å, *c* = 32.8373(11) Å, *V* = 2332.17(14) Å³, *Z* = 4, *T* = 100 K, μ(Cu Kα) = 0.760 mm⁻¹, ρ_{calcd} = 1.215 g mL⁻¹, 2θ_{max} = 133.306, 18 279 reflections measured, 4118 unique (*R*_{int} = 0.0345, *R*_{sigma} = 0.0279), *R*1 = 0.0535 (*I* > 2σ(*I*)), *wR*2 = 0.1368 (all data), Flack parameter = 0.05(8), Hooft parameter = 0.04(6).

Signal Transduction Assays

The signal transduction Drug Discovery Kits for the matrix metalloproteinase-3, caspase-1, and caspase-3 enzymes were purchased from Enzo Life Sciences.

Antibiotic Testing

Minimum inhibitory concentrations (MICs) were assessed for each bacterium in the assay series using a broth microdilution approach based on CLSI standards and the use of the colorimetric reporter Alamar Blue. Stock solutions of test compounds were made at 50 mM in DMSO. Serial 2-fold dilutions of the stocks were prepared in test wells with a maximum concentration of 500 μ M (test concentrations therefore being 500, 250, 125, 64, 32, 16, 8, 4, 2, 1 μ M, etc.). MIC data are reported in μ M and also converted into μ g/ mL for comparison to other literature data.⁵⁴

Cell-Free Translation

The plasmid pY71-sfGFP⁵⁵ (5 ng) was translated in the PUREexpress *in vitro* protein synthesis system (New England Biolabs). Translation reactions were assembled in a total volume of 5 μ L, which contained 2 μ L of the kit solution A, 1 μ L of the solution B, and 5 ng of the pY71-sfGFP plasmid. When needed, the appropriate volume of the antibiotic solution was dried at the bottom of the tube prior to combining the reaction components. The final concentrations of erythromycin, josamycin, and tylosin in the translation reactions were 50 μ M. Compound **1** was tested at 50 and 250 μ M. The reactions were transferred into the wells of a 384-well clear bottom/black wall plate. The plate was covered with the lid and placed in a microplate reader (Tecan). The reactions were incubated at 37 °C, and the fluorescence readings were taken every 20 min for 2 h (excitation, 488 nm; emission, 520 nm).

Extension Inhibition Assay

The assay was carried out using the *ermBL* gene⁵⁶ following the procedure as described in the literature.^{57,58} Control antibiotics (erythromycin, josamycin, and tylosin) were present in the reaction at 50 μ M; berkeleylactone A (**1**, code named DNA76) was present at 50 or 250 μ M. The primer extension products were resolved in a 6% sequencing gel alongside the sequencing reactions prepared using the same template.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Giddings, L-A., Newman, DJ. Bioactive Compounds from Terrestrial Extremophiles. In: Tiquia-Arashiro, SM., Mormile, M., editors. Springer Briefs in Microbiology; Extremophilic Bacteria. Springer; Heidelberg: 2015. p. 6-61.p. 65-73.
- Giddings, L-A., Newman, DJ. Bioactive Compounds from Marine Extremophiles. In: Tiquia-Arashiro, SM., Mormile, M., editors. Springer Briefs in Microbiology; Extremophilic Bacteria. Springer; Heidelberg: 2015. p. 4-39.p. 54-121.
- Some of the previous reports of the isolation of secondary metabolites from Berkeley Pit fungi include:(a) Stierle DB, Stierle AA, Patacini B, McIntyre K, Girtsman T, Bolstad E. J Nat Prod. 2011; 74:2273–2277. [PubMed: 21916432] (b) Stierle AA, Stierle DB, Girtsman T. J Nat Prod. 2012; 75:344–350. [PubMed: 22295871] (c) Stierle, AA., Stierle, DB. Studies in Natural Products Chemistry. Rahman, Atta-Ur, editor. Vol. 39. Elsevier Science; Amsterdam: 2013. p. 1-44.(d) Stierle AA, Stierle DB, Mitman GG, Snyder S, Antczak C, Djaballah H. Nat Prod Commun. 2014; 9:87–90. [PubMed: 24660471] (e) Stierle AA, Stierle DB. Nat Prod Commun. 2014; 9:1037–1044. [PubMed: 25230522]
- Thiery JP, Acioque H, Huang RYJ, Nieto MA. Cell. 2009; 139:871–890. [PubMed: 19945376]
- Radisky DC, Levy DD, Littlepage LE, Liu H, Nelson CM, Fata JE, Leake D, Godden EL, Albertson DG, Nieto MA, Werb Z, Bissell MJ. Nature. 2005; 436:123–127. [PubMed: 16001073]
- Comoglio PM, Trusolino L. Nat Med. 2005; 11:1156–1159. [PubMed: 16270068]
- Franchi L, Eigenbrod T, Muñoz-Planillo R, Nuñez G. Nat Immunol. 2009; 10:241–256. [PubMed: 19221555]
- Schlosser S, Gansauge F, Ramadani M, Beger HG, Gansauge S. FEBS Lett. 2001; 491:104–108. [PubMed: 11226429]
- Coffelt SB, de Visser KE. Nature. 2014; 507:48–49. [PubMed: 24572360]
- McIlwain DR, Berger T, Mak TW. Cold Spring Harbor Perspect Biol. 2013; 5:a008656.
- Clark RSB, Kochanek PM, Watkin SC, Chen M, Dixon CE, Seidberg NA, Melick J, et al. J Neurochem. 2000; 74:740–753. [PubMed: 10646526]
- Netzker T, Fischer J, Weber J, Mattern DJ, König CC, Valiante V, Schroeckh V, Brakhage AA. Front Microbiol. 2015; 6:1–13. [PubMed: 25653648]
- Oh DC, Kauffman CA, Jensen PR, Fenical W. J Nat Prod. 2007; 70:515–520. [PubMed: 17323993]
- Zuck KM, Shipley S, Newman DJ. J Nat Prod. 2011; 74:1653–1657. [PubMed: 21667925]
- Bertrand S, Schumpp O, Bohni N, Monod M, Gindro K, Wolfender JL. J Nat Prod. 2013; 76:1157–1165. [PubMed: 23734767]
- Degenkolb T, Heinze S, Schlegel B, Strobel G, Gräfe U. Biosci, Biotechnol, Biochem. 2002; 66:883–886. [PubMed: 12036069]
- Zhu F, Chen G, Chen X, Huang M, Wan X. Chem Nat Compd. 2011; 47:767–769.
- Li C, Wang J, Luo C, Ding W, Cox DG. Nat Prod Res. 2014; 28:616–621. [PubMed: 24571709]
- Huang S, Ding W, Li C, Cox DG. Pharmacogn Mag. 2014; 40:410–414.
- Microbial ID, Inc., Delaware, originally identified the organisms based on the alignment of 321 base pairs of the 28S rRNA gene. PW-2A was identified as *Eupenicillium pinetorum* (GenBank match 100%, *Eupenicillium pinetorum* DQ473557, which has since been renamed *Penicillium fuscum* (Sopp) Raper & Thom). It has therefore been designated as *Penicillium fuscum* (*Eupenicillium pinetorum*, synonym), GenBank accession no. KT828537. It has been deposited as NRRL 66320. PW2B was identified as *Penicillium camembertii* Thom (*P. clavigerum-camembertii*), GenBank accession no. KT828538. It has been deposited as NRRL 66321.
- Manuscript describing compounds from PW2A in preparation.
- Silverstein, R., Bassler, G., Morrill, T. Spectrometric Identification of Organic Compounds. 5th. Sawicki, D., editor. John Wiley and Sons; New York: 1991. p. 120(b) Ibid., p 236(c) Ibid., pp 208–209
- Michel KH, Demarco PV, Nagarian RJ. J Antibiot. 1977; 7:571–575.
- Hase TA, Nylund EL. Tetrahedron Lett. 1979; 28:2633–2636.
- Kobayashi Y, Okui H. J Org Chem. 2000; 65:612–615. [PubMed: 10813983]

26. Seco JM, Quinoa E, Riguera R. *Chem Rev.* 2004; 104:17–117.
27. Kibwage IO, Hoogmartens J, Roets E, Vanderhaeghe H, Verbist L, Dubost M, Pascal C, Petitjean P, Levot G. *Antimicrob Agents Chemother.* 1985; 28:630–633. [PubMed: 4091529]
28. Scheinfeld N. *J Drug Dermat.* 2004; 3:409–13.
29. Rafie S, MacDougall C, James CL. *Pharmacotherapy.* 2010; 30:290–303. [PubMed: 20180612]
30. Suzuki M, Takamaki T, Miyagawa KI, Ono H, Higashide E, Uchida M. *Agric Biol Chem.* 1979; 43:1331–1336.
31. Okabe, M., Okamoto, R. *Antibiotics II: Antibiotics by Fermentation.* Ikoma, T., editor. Vol. 3. Gordon and Breach Science; Philadelphia: 1979. p. 95–124.
32. Xu HW, Qin SS, Liu HM. *Curr Top Med Chem.* 2014; 14:21–39. [PubMed: 24236728]
33. Gaynor M, Mankin AS. *Curr Top Med Chem.* 2003; 3:949–961. [PubMed: 12678831]
34. Ramu VL, Mankin AS. *Mol Microbiol.* 2009; 71:811–824. [PubMed: 19170872]
35. Sekiguchi J, Kuroda H, Yamada Y, Okada H. *Tetrahedron Lett.* 1985; 26:2341–2342.
36. Rodphaya D, Sekiguchi J, Yamada Y. *J Antibiot.* 1986; 39:629–635. [PubMed: 3733512]
37. Smith CJ, Abbanat D, Bernan VS, Maiese WM, Greenstein M, Jompa J, Tahir A, Ireland CM. *J Nat Prod.* 2000; 63:142–145. [PubMed: 10650098]
38. Jadulco R, Proksch P, Wray V, Sudarsono, Berg A, Gräfe U. *J Nat Prod.* 2001; 64:527–530. [PubMed: 11325242]
39. Hirota A, Sakai H, Isogai A. *Agric Biol Chem.* 1985; 49:731–735.
40. Shigemori H, Kasai Y, Komatsu K, Tsuda M, Mikami Y, Kobayashi J. *Mar Drugs.* 2004; 2:164–169.
41. Liu S, Dai H, Makhoulfi G, Heering C, Janiak C, Hartmann R, Mándi A, Kurtán T, Müller WEG, Kassack MU, Lin W, Liu Z, Proksch P. *J Nat Prod.* 2016; 79:2332–2340. [PubMed: 27556865]
42. Ishibashi K. *Nippon Nogei Kagaku Kaishi.* 1961; 35:257–262.
43. Nozoe S, Hirai K, Tsuda K, Ishibashi K, Shirasaka Y. *Tetrahedron Lett.* 1965; 51:4675–4677.
44. Fuska J, Nemec P, Kuhr I. *J Antibiot.* 1972; 25:208–211. [PubMed: 4559271]
45. Boekman RK, Fayos J, Clardy JA. *J Am Chem Soc.* 1974; 96:5954–5956. [PubMed: 4414095]
46. Sheldrick, GM. *SADABS; Area Detector Absorption Correction.* University of Göttingen; Göttingen, Germany: 2001.
47. Dolomanov OV, Bourhis LJ, Gildea RJ, Howard JAK, Puschmann H. *J Appl Crystallogr.* 2009; 42:339–341.
48. Sheldrick GM. *Acta Crystallogr, Sect A: Found Adv.* 2015; A71:3–8.
49. Flack HD, Bernardinelli G. *J Appl Crystallogr.* 2000; 33:1143–1148.
50. Bruker. *APEX2.* Bruker AXS Inc.; Madison, WI, USA: 2007.
51. Sheldrick GM. *Acta Crystallogr, Sect A: Found Crystallogr.* 2008; A64:112–122.
52. Hooft RWW, Straver LH, Spek AL. *J Appl Crystallogr.* 2008; 41:96–103. [PubMed: 19461838]
53. Spek AL. *Acta Crystallogr, Sect D: Biol Crystallogr.* 2009; D65:148–155.
54. Viswanathan K, Frey KM, Scocchera EW, Martin BD, Swain PW, Alverson JB, Priestley ND, Anderson AC, Wright DL. *PLoS One.* 2012; 7:e29434. [PubMed: 22347365]
55. Albayrak C, Swartz JR. *Nucleic Acids Res.* 2013; 41:5949–5963. [PubMed: 23589624]
56. Gupta P, Liu B, Klepacki D, Gupta V, Schulten K, Mankin AS, Vázquez-Laslop N. *Nat Chem Biol.* 2016; 12:153–158. [PubMed: 26727240]
57. Orelle C, Szal T, Klepacki D, Shaw KJ, Vazquez-Laslop N, Mankin AS. *Nucleic Acids Res.* 2013; 41:e144. [PubMed: 23761439]
58. Vazquez-Laslop N, Thum C, Mankin AS. *Mol Cell.* 2008; 30:190–202. [PubMed: 18439898]

Figure 2.
X-ray crystal structure of berkeleylactone A (**1**).

Figure 3.

Selected δ values of the (*S*)- and (*R*)-MTPA esters of berkeleylactone F (**11**) [δ = chemical shift of the (*S*)-MTPA ester minus the chemical shift of the (*R*)-MTPA ester in ppm].

Figure 4.
X-ray crystal structure of berkeleylactone F triacetate (**10**).

Figure 5.

The extension inhibition assay allows the direct monitoring of the formation of stalled ribosome complexes (SRCs) on mRNA. It can assess the ability of a specific antibiotic to stall the ribosome at a specific mRNA codon. In this case, the effect of berkeleylactone A (**1**, DNA76) was compared to the known macrolide antibiotics erythromycin, josamycin, and tylosin. Unlike the known antibiotics, compound **1** did not induce SRC formation.

Figure 6. Cell-free translation of GFP protein compared the effects of berkeleylactone A (**1**) and three known macrolide antibiotics—erythromycin, josamycin, and tylosin—on protein synthesis. The known macrolides were tested at 50 μ M, and compound **1** was tested at 50 and 250 μ M. Compound **1** did not inhibit protein synthesis, and its effect was comparable to that of the control (no antibiotic).

Table 1¹H NMR, ¹³C NMR, and HMBC Data for Berkeleylactone A (1) in CDCl₃

position	δ_{C} , type	δ_{H} , mult (<i>J</i> in Hz)	HMBC correlations
1	172.3, C		4.94, 4.01, 3.20, 2.80,
2	41.3, CH	4.01, t (7.0)	3.27, 2.98, 3.20, 2.80
3	40.9, CH ₂	3.20, dd (18.5, 7.0) 2.80, dd (18.5, 6.6)	4.01
4	208.8, C		4.37, 4.01, 3.20, 2.80, 1.83
5	76.2, CH	4.37, t (4.0)	1.83
6	32.3, CH ₂	1.83, m, 2H	4.37
7	20.7, CH ₂	1.38, m 0.97, m	4.37, 1.83
8	26.0, CH ₂	1.26	1.83
9	25.3, CH ₂	1.26	
10	26.6, CH ₂	1.26	
11	26.6, CH ₂	1.26	
12	26.6, CH ₂	1.26	
13	22.9, CH ₂	1.32	
14	34.5, CH ₂	1.55, m; 1.43, m	4.94, 1.26
15	73.3, CH	4.94, br dq (10.8, 6.2)	1.55, 1.46, 1.26
16	19.8, CH ₃	1.26, d (6.2)	
1'	174.9, C		4.53, 3.27, 2.98
2'	70.4, CH	4.53, dd (5.8, 3.7)	3.27, 2.98
3'	35.7, CH ₂	3.27, dd (14.6, 3.7) 2.98, dd (14.6, 5.8)	4.53, 4.01

Table 2

¹H NMR Data for Macrolides 1 and 4–7 (400 MHz, MeOH-*d*₄)^a

no.	1	4	5	6	7
δ_{H}	δ_{H}	δ_{H}	δ_{H}	δ_{H}	δ_{H}
mult (J in Hz)	mult (J in Hz)	mult (J in Hz)	mult (J in Hz)	mult (J in Hz)	mult (J in Hz)
2	3.90, dd (10.8, 4.0)	3.86, dd (11.5, 3.4)	6.71, d (15.9)	6.72, d (15.9)	6.71, d (15.9)
3	3.21, dd (18.2, 10.8) 2.88, dd (18.2, 4.6)	3.24, dd (18.1, 11.5) 2.89, dd (18.1, 3.4)	7.32, d (15.9)	7.33, d (15.9)	7.34, d (15.9)
5	4.30, t (5.1)	5.17, dd (6.0, 3.5)	5.37, dd (5.9, 4.9)	5.34, t (5.7)	5.40, dd (6.1, 4.7)
6	1.78, m	1.86, m	1.95, m 1.87, m	1.89, m, 2H	1.97, m 1.84, m
7	1.31, m 1.13, m	1.30, m	1.34, m	1.47, m	1.46, m 1.20, m
8	1.34, m	1.34, m	1.34, m	1.34, m	1.34, m
9	1.34, m	1.34, m	1.34, m	1.34, m	1.34, m
10	1.34, m	1.34, m	1.34, m	1.34, m	1.34, m
11	1.34, m	1.34, m	1.34, m	1.34, m	1.34, m
12	1.34, m	1.34, m	1.34, m	1.34, m	1.44, m
13	1.34, m	1.34, m	1.34, m	1.56, m 1.44, m	3.81, m
14	1.64, m 1.42, m	1.64, m 1.42, m	1.72, m 1.58, m	3.50, m	1.89, m 1.83, m
15	4.95, m	4.97, m	5.13, m	4.87, m	5.23, m
16	1.27, d (6.2)	1.27, d (6.2)	1.30, d (6.4)	1.35, d (6.4)	1.35, d (6.4)
2'	4.38, dd (6.7, 4.0)	4.35, dd (6.6, 4.3)			
3'	3.21, dd (13.5, 4.0) 2.93, dd (13.5, 6.7)	3.21, dd (13.7, 4.3) 2.93, dd (13.7, 6.6)			
2''		2.66, m	2.70, m	2.70, m	2.70, m
3''		2.60, m	2.62, m	2.62, m	2.62, m

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

Table 3¹H NMR Data for Berkeleylactones 8, 9, 12, and 13 (400 MHz, MeOH-*d*₄)^a

	8	9	12	13
no.	δ_{H} , mult (<i>J</i> in Hz)	δ_{H} , mult (<i>J</i> in Hz)	δ_{H} , mult (<i>J</i> in Hz)	δ_{H} , mult (<i>J</i> in Hz)
2	6.10, dd (15.7, 1.8)	6.05, dd (15.8, 1.7)	6.09, dd (15.8, 1.6)	6.17, dd (15.7, 1.8)
3	6.93, dd (15.7, 4.9)	6.95, dd (15.8, 5.0)	6.95, dd (15.8, 4.9)	6.97, dd (15.7, 4.5)
4	4.55, m	4.43, m	4.55, m	4.58, m
5	4.83, m	3.61, m	4.83, m	4.84, m
6	1.64, m 1.55, m	1.59, m 1.26, m	1.70, m 1.52, m	1.71, m 1.50, m
7	1.33, m	1.34, m	1.34, m	1.33, m
8	1.69, m 1.51, m	1.34, m	1.34, m	1.33, m
9	1.33, m	1.34, m	1.34, m	1.33, m
10	1.33, m	1.34, m	1.34, m	1.33, m
11	1.33, m	1.34, m	1.34, m	1.33, m
12	1.33, m	1.34, m	1.34, m	1.33, m
13	1.33, m	1.60, m 1.37, m	1.37, m 1.28, m	1.33, m
14	1.33, m	3.42, td (8.5, 2.7)	3.41, td (7.9, 2.7)	1.58, m 1.52, m
15	5.05, m	4.75, dq (8.5, 6.6)	4.77, m	5.05, ddt (9.9, 5.5, 2.6)
16	1.26, d (6.3)	1.33, d (6.6)	1.33, d (6.2)	3.61, d (5.5)
2''	2.72, m		2.63, brs	2.63, brs
3''	2.65, m		2.63, brs	2.63, brs

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

Table 4

¹³C NMR Data for Compounds 1 and 4–7 (100 MHz, MeOH-*d*₄)

no.	1	4	5	6	7
δ_C , type	δ_C , type	δ_C , type	δ_C , type	δ_C , type	δ_C , type
1	174.2, C	174.2, C	166.5, C	166.2, C	166.2, C
2	42.4, CH	41.8, CH	133.3, CH	133.0, CH	133.2, CH
3	43.1, CH ₂	43.6, CH ₂	137.3, CH	137.3, CH	137.9, CH
4	210.2, C	205.8, C	197.7, C	197.5, C	197.9, C
5	76.8, CH	78.7, CH	79.5, CH	79.3, CH	79.3, CH
6	33.6, CH ₂	30.6, CH ₂	30.1, CH ₂	30.0, CH ₂	30.0, CH ₂
7	22.9, CH ₂	23.4, CH ₂	23.5, CH ₂	24.9, CH ₂	23.4, CH ₂
8	27.9, CH ₂	27.9, CH ₂	28.3, CH ₂	28.1, CH ₂	28.5, CH ₂
9	27.2, CH ₂	27.2, CH ₂	28.4, CH ₂	28.1, CH ₂	28.5, CH ₂
10	28.1, CH ₂	27.8, CH ₂	29.3, CH ₂	28.5, CH ₂	29.2, CH ₂
11	28.1, CH ₂	28.3, CH ₂	29.0, CH ₂	29.3, CH ₂	26.2, CH ₂
12	26.4, CH ₂	26.6, CH ₂	28.7, CH ₂	23.7, CH ₂	36.8, CH ₂
13	24.5, CH ₂	24.4, CH ₂	24.8, CH ₂	33.4, CH ₂	68.1, CH
14	36.1, CH ₂	36.0, CH ₂	35.6, CH ₂	74.8, CH	42.6, CH ₂
15	73.4, CH	73.1, CH	74.0, CH	76.0, CH	71.0, CH
16	20.4, CH ₃	20.3, CH ₃	20.2, CH ₃	17.9, CH ₃	20.6, CH ₃
1'	175.9, C	176.0, C			
2'	71.5, CH	71.5, CH			
3'	36.7, CH ₂	36.7, CH ₂			
1''		173.7, C	173.6, C	173.6, C	173.6, C
2''		29.8, CH ₂	29.8, CH ₂	29.8, CH ₂	29.8, CH ₂
3''		29.9, CH ₂	29.9, CH ₂	29.9, CH ₂	29.9, CH ₂
4''		176.1, C	175.9, C	175.8, C	175.9, C

Table 5¹³C NMR Data for Compounds 8, 9, 12, and 13 (100 MHz, MeOH-*d*₄)

	8	9	12	13
no.	δ _C , type	δ _C , type	δ _C , type	δ _C , type
1	167.8, C	167.5, C	167.3, C	168.1, C
2	123.3, CH	122.5, CH	123.1, CH	123.1, CH
3	148.3, CH	149.9, CH	148.7, CH	148.7, CH
4	73.0, CH	75.7, CH	74.6, CH	73.0, CH
5	77.8, CH	75.2, CH	77.9, CH	77.8, CH
6	30.4, CH ₂	30.3, CH ₂	30.4, CH ₂	29.3, CH ₂
7	24.8, CH ₂	25.0, CH ₂	24.1, CH ₂	25.2, CH ₂
8	27.3, CH ₂	28.9, CH ₂	27.4, CH ₂	27.3, CH ₂
9	28.6, CH ₂	27.5, CH ₂	29.1, CH ₂	27.6, CH ₂
10	27.5, CH ₂	29.1, CH ₂	27.6, CH ₂	27.6, CH ₂
11	28.4, CH ₂	27.3, CH ₂	27.5, CH ₂	28.6, CH ₂
12	27.6, CH ₂	24.1, CH ₂	24.9, CH ₂	28.6, CH ₂
13	25.3, CH ₂	33.3, CH ₂	33.4, CH ₂	25.0, CH ₂
14	36.8, CH ₂	75.0, CH	72.9, CH	31.5, CH ₂
15	72.5, CH	74.6, CH	75.1, CH	76.5, CH
16	20.9, CH ₃	18.2, CH ₃	18.2, CH ₃	65.1, CH ₂
1''	174.2, C		174.2, C	174.2, C
2''	29.3, CH ₂		28.7, CH ₂	30.0, CH ₂
3''	29.9, CH ₂		29.9, CH ₂	30.5, CH ₂
4''	176.2, C		176.2, C	176.3, C

Table 6

Antibiotic Testing Data of Macrolides 1, 4–9, 12, and 13

	<i>Staphylococcus aureus</i> (13709)		<i>Streptococcus pyogenes</i>		<i>Candida glabrata</i>		<i>Bacillus subtilis</i>		<i>C. albicans</i>		<i>B. anthracis</i>	
	μM	$\mu\text{g/mL}$	μM	$\mu\text{g/mL}$	μM	$\mu\text{g/mL}$	μM	$\mu\text{g/mL}$	μM	$\mu\text{g/mL}$	μM	$\mu\text{g/mL}$
(1) berkeleylactone A	2	1	8	3	16	6	32	13	64	26	8	3
(4) berkeleylactone B	8	4	250	119	64	31	64	31	>250	>119	16	8
(5) A26771B	8	3	125	48	125	48	32	12	250	96	16	6
(6) berkeleylactone C	16	6	64	26	64	26	64	26	125	50	16	6
(7) berkeleylactone D	32	13	125	50	>1000	>400	250	100	>1000	>400	64	26
(8) berkeleylactone E	125	45	>250	>90	>250	>90	>250	>90	>250	>90	>250	>90
(9) berkeleylactone F	64	19	500	150	>1000	>300	>1000	>300	>1000	>300	250	75
(12) berkeleylactone G	64	24	>125	>50	>125	>50	>125	>50	>125	>50	64	24
(13) berkeleylactone H	>250	>100	>250	>100	>250	>100	>250	>100	>250	>100	>250	>100

Antibiotic Testing Data of Selected Berkeleylactones and A26771B (5) against Methicillin-Resistant Strains of *S. aureus*

Table 7

compound	<i>S. aureus</i> CAIRD116		<i>S. aureus</i> CAIRD142		<i>S. aureus</i> CAIRD148		<i>S. aureus</i> (NE277)	
	μM	$\mu\text{g/mL}$	μM	$\mu\text{g/mL}$	μM	$\mu\text{g/mL}$	μM	$\mu\text{g/mL}$
(1) berkeleylactone A	2	1	4	2	2	1	2	1
(4) berkeleylactone B	32	15	32	15	16	8	16	8
(5) A26771B	16	6	16	6	16	6	16	6
(8) berkeleylactone E	>250	>90	>250	>90	>250	>90	>250	>90
(12) berkeleylactone G	125	47	125	47	125	47	125	47
(13) berkeleylactone H	>250	>100	>250	>100	>250	>100	>250	>100

Comparison of Antibiotic Activity of Compounds 1 and 5 and Several Known Antibiotics against Methicillin-Resistant Strains of *S. aureus*^a

Table 8

CAIRD isolate#	description	antibiotics								
		<u>1</u>	<u>5</u>	<u>linezolid</u>	<u>vancomycin</u>	<u>erythromycin</u>	<u>clindamycin</u>	<u>levofloxacin</u>	<u>doxycycline</u>	<u>cefazolin</u>
		MIC (µg/mL)								
116		1	6	2	1	>32	>16	>16	8	256
142		2	6	4	2	>32	>16	0.25	16	256
148	USA100 HA-MRSA	1	6	2	0.5	>32	>16	>16	0.25	256

^a Antibiotic data for linezolid, vancomycin, erythromycin, clindamycin, levofloxacin, doxycycline, and cefazolin were provided by Hartford Hospital, Center for Anti-Infective Research and Development (CAIRD).