Isolation, Structure Elucidation, and Antibacterial Activity of Methiosetin, a Tetramic Acid from a Tropical Sooty Mold (Capnodium sp.)

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Isolation, Structure Elucidation, and Antibacterial Activity of Methiosetin, a Tetramic Acid from a Tropical Sooty Mold (*Capnodium* sp.)

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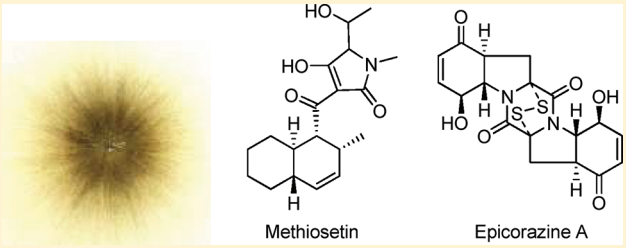
Supporting Information

ABSTRACT: Drug-resistant bacteria continue to make many existing antibiotic classes ineffective. In order to avoid a future epidemic from drug-resistant bacterial infections, new antibiotics with new modes of action are needed. In an antibiotic screening program for new drug leads with new modes of action using antisense *Staphylococcus aureus* Fitness Test screening, we discovered a new tetramic acid, methiosetin, from a tropical sooty mold, *Capnodium* sp. The fungus also produced epicorazine A, a known antibiotic. The structure and relative configuration of methiosetin was elucidated by 2D NMR and ESIMS techniques. Methiosetin and epicorazine A showed weak to modest antibacterial activity against *S. aureus* and *Haemophilus influenzae*. The isolation, structure elucidation, and antibacterial activity of both compounds are described.



Antibiotic resistance continues to pose a serious challenge to human lives.[1] Methicillin-resistant *Staphylococcus aureus* (MRSA) infections are responsible for over 18 000 deaths per year in the United States.[1] While infections by MRSA are well known, infections by a number of drug-resistant Gram-negative bacteria, such as *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, are cause for more concern. The problem of drug-resistant bacteria can only be addressed by the discovery of new classes of antibacterial agents. The majority of the existing antibacterial leads have been derived from natural sources, which continue to be important for new lead discovery. However, natural product discovery has become much more challenging due to the identification of known compounds, which is commonplace. Therefore, it is imperative to find a way to rapidly eliminate such rediscoveries at an early stage to improve the probability of success. One of the effective approaches to accomplish this goal is to employ new screening technologies for antibiotic discovery coupled with new sources of natural products. Recently, we have addressed both of these challenges. First, we have introduced a *S. aureus* target-based antisense whole-cell screening approach that provides hypersensitivity for target-based inhibitors and differentiates antibacterial agents at the crude extract stage.[2] Second, we have expanded our efforts to isolate microorganisms from different geographical regions and habitats and coupled culture growth with improved high-throughput fermentation methods.[3] The combination of the two approaches allowed for the discovery of a number of natural products[4−6] including platensimycin[7,8] and platencin[9,10] using a *fabH*/*fabF*-sensitized strain and a number of new natural products using an *rpsD*-sensitized antisense strain.[11−15]

The single target antisense screening approach was further developed into a *Staphylococcus aureus* Fitness Test (SaFT) in which 245 essential targets could be screened simultaneously. This technique can be used to screen crude extracts and often provides mechanistic information on active compounds. More importantly it provides a biological fingerprint that is a powerful dereplication tool, particularly when combined with HRESIFTMS.[16] The SaFT approach was used in the discovery of coelomycin[17] and the bacterial topoisomerase II inhibitor kibdelomycin.[18]

Whole cell empiric screening using wild-type *S. aureus* Smith strain followed by SaFT profiling led to identification of an extract from a tropical sooty mold (*Capnodium* sp., Capnodiaceae)[19] that produced a distinct SaFT profile, suggesting that the extract could contain a novel antibacterial agent. HRESIFTMS analysis of the extract confirmed that the extract contained an active compound not present in the Merck database. Bioassay-guided fractionation of the extract using *S. aureus* EP167 and *Haemophilus influenzae* growth inhibition assays led to the isolation of a new tetramic acid, methiosetin (**1**), and a known compound, epicorazine A (**2**). The isolation, structure elucidation,

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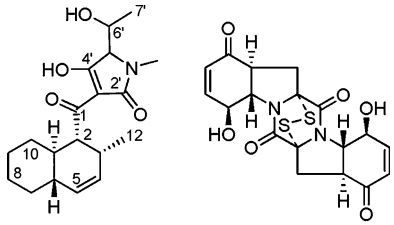
and antibacterial activity of methiosetin and epicorazine A are described.



1 (methiosetin) 2 (epicorazine A)

RESULTS AND DISCUSSION

The fungus (F-190,679) was isolated from palm leaf litter collected in Guatemala. The fungus was grown in various types of culture media but never produced any identifiable reproductive state. However, its mycelium was exceptionally dark and thick-walled, and its aerial hyphae exhibited wide monilioid filaments (Figure S1, A–D), similar to hyphae of sooty mold fungi in the Capnodiales.[19,20] Homology searching with the ITS region of the rDNA indicated that fungi in the Capnodiaceae were very close relatives to F-190,679, and the most similar fungus sequence in a public database was Capnodiales sp. M-Camp1 HQ634623 (ITS 95.6% identical), a fungus isolated from ant carton nests in Malaysia.[21] The recently described sooty mold fungus *Leptoxyphium madagascariense*[22] was the most similar named fungus (ITS 91.8% identical). Alignments and bootstrapped neighbor-joining analysis of the D1–D2 regions of the rDNA placed this fungus among a variety of sooty mold fungi from the Capnodiaceae (Supplemental Figure S2). Most of the named fungi represent conidial states of *Capnodium*[19,23] or, in the case of the unnamed vegetative isolates from carton nests of tropical ants, possibly vegetative states of *Capnodium* species. Therefore, we tentatively classify F-190,679 as a *Capnodium* sp.

A 1 L submerged fermentation broth was extracted with acetone. Most of the acetone was removed by concentration at reduced pressure, and the aqueous solution was slowly charged on an Amberchrome reversed-phase resin column. The column was eluted with a linear gradient of aqueous MeOH. Antibacterial activity was eluted in a broad elution zone. All fractions were evaluated for antibacterial activity using *S. aureus* and *H. influenzae* broth dilution assays. The pooled fractions with antibacterial activity were chromatographed on a Sephadex LH20 column. Elution of the column with MeOH led to the separation of antibacterial activity into two fractions, A and B. Fraction A showed predominant activity against *S. aureus* and was rechromatographed by reversed-phase HPLC to give the tetramic acid methiosetin (**1**, 49 mg/L) as an amorphous powder. Fraction B showed predominant activity against *H. influenzae* and was also chromatographed by reversed-phase HPLC to afford epicorazine A (**2**, 22 mg/L) as an amorphous powder. The structure and identity of epicorazine A (**2**) were confirmed by NMR spectral analysis and by comparison of an authentic sample obtained from the Merck sample repository; data for epicorazine A were not present in Merck mass spectral and SaFT databases. Epicorazine A, a metabolite characteristic of *Epicoccum nigrum*, is known to exhibit antibacterial activities against Gram-positive bacteria including *S. aureus* (MIC 20–50 μg/mL).[24] No activity against *H. influenzae* was previously reported.

Mass spectral analysis of methiosetin (**1**) produced a molecular formula of $C_{19}H_{27}NO_4$, suggesting seven degrees of unsaturation. The UV spectrum of **1** showed absorption maxima at λ_{max} 230 and 285 nm characteristic of tetramic acids. The IR spectrum of **1** showed absorption bands for hydroxy (3422 cm^{-1}) and conjugated ketone (1700 cm^{-1}) groups. The ^{13}C NMR spectrum of **1** in CD_3OD and $CDCl_3$ showed the presence of 19 resonances. All downfield ^{13}C resonances except those from the two olefinic carbon resonances were significantly broadened. The analysis of the DEPT spectrum suggested the presence of three methyl groups, two olefinic methines, six methines, and four methylenes, and the remaining four downfield ^{13}C resonances were assigned as quaternary. The broadened downfield carbons resonated at δ_C 190.2, 95.2, 175.4, and 104.2 (in CD_3OD). These resonances are characteristically produced by the tetramic acid moiety.[25] Two of the three methyl groups resonated as a doublet in the upfield region of the ^1H NMR spectrum. The third methyl group resonated as a broad singlet at δ_H 3.05 and correlated to δ_C 29.5 in the HMQC spectrum, indicating it to be an *N*-methyl group. The COSY correlation of **1** in both solvents provided a fragment consisting of a decalin and a threonine moiety. The downfield methyl doublet at δ_H 1.11 (in $CDCl_3$) showed HMBC correlations to the two downfield methine carbons C-5′ (δ_C 68.2) and C-6′ (δ_C 66.6); H-5′ (in $CDCl_3$) showed HMBC correlations to C-2′ (δ_C 174.1), C-4′ (δ_C194.0), and C-7′ (δ_C 17.4). These, together with the HMBC correlation of the *N*-methyl (δ_H 2.98) to C-2′ and C-5′, confirmed the presence of threonine as a part of the *N*-methyl tetramic acid moiety. The H-2 (δ_H 3.76) showed HMBC correlations to C-3′ and C-1, thus connecting the decalin unit to the tetramic acid moiety. The structure of the decalin moiety was confirmed by respective HMBC correlations (Table 1).

The relative configuration of the decalin moiety of **1** was established by the measurement of the magnitude of the scalar couplings. H-11 resonated as a doublet (J = 2.5 Hz) of a quartet (J = 12 Hz) in CD_3OD. A similar result was also obtained in $CDCl_3$. The presence of a quartet with a large coupling suggested that H-11 along with H-2 and H-6 were axially oriented, confirming a *trans*-ring fusion of the decalin. The third large coupling resulted from the vicinal axial proton at C-10, and the small coupling originated from the equatorial H-10. H-2 showed an intermediate J value (5.5–6 Hz) due to a coupling with H-3. 3D computer modeling of **1** with both epimeric structures at C-3 and measurement of the dihedral angles between H-2 and H-3 provided a dihedral angle of −32° for the equatorial H-3 and 160° for the axial H-3. The observed J value is more in agreement with the assignment of equatorial H-3, suggesting that the relative configuration at C-3 is similar to equisetin and other natural tetramic acids. Most of the decalin-containing tetramic acids possess an axial methyl group at C-2,[24,25] which originates from an *S*-adenosine methionine.[26] The C-2-desmethylated tetramic acids are rare and exemplified by Sch210971 and Sch213766.[27] Methiosetin (**1**) is most likely derived from the condensation of the heptapolyketide and threonine moiety.

Antibacterial Activity. Methiosetin (**1**) showed weak activity against *S. aureus* EP167 (MIC 256 μg/mL) and slightly better activity against *H. influenzae* (MIC 32 μg/mL). Epicorazine A showed 8-fold better activity against *S. aureus* (MIC 32 μg/mL) and displayed significantly better activity against *H. influenzae* (MIC 0.5 μg/mL). Methiosetin **1** did not show any growth inhibition of other bacterial strains (*S. aureus* Smith, *Enterococcus faecalis*, *Streptococcus pneumoniae*, *Escherichia coli*) at 64 μg/mL. Epicorazine A was not tested against other strains.

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Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Assignment of Methiosetin (1) in CD_3OD and $CDCl_3$[a]

	CD_3OD				$CDCl_3$	
no.	δ_C	mult	δ_H, mult (J in Hz)	HMBC (H→C)	δ_C	δ_H, mult (J in Hz)
1	190.2	C			191.3	
2	47.4	CH	3.76, dd (11, 5.5)	C-1, 3, 6, 11, 12	46.8	3.68, dd (11.5, 6.0)
3	34.4	CH	2.53, brm		33.1	2.53, brm
4	132.3	CH	5.56, ddd (10, 4.5, 2.5)	C-2, 3, 5, 6	130.9	5.54, ddd (10, 4.5, 2.5)
5	131.7	CH	5.41, brd (10)	C-3, 4, 6, 11	130.8	5.40, brd (10)
6	43.7	CH	1.75, m		42.2	1.76, m
7	34.4	CH_2	1.09, m		33.0	1.08, m
			1.78, m	C-6, 8, 11		1.75, m
8	27.6	CH_2	1.75, m		26.6	1.74, m
9	27.7	CH_2	1.34, m		26.5	1.35, m
			1.75, m			1.75, m
10	31.3	CH_2	0.88, dq (2.5, 12)		30.0	0.88, m
			1.84, brd (12)			1.84, m
11	37.3	CH	1.56, dq (2.5, 11.5)	C-2, 3, 6, 9	35.6	1.58, dq (3.0, 12)
12	16.2	CH_3	0.93, d (7.0)	C-2, 3, 4	17.8	0.94, d (7.5)
2′	175.4	C			174.1	
3′	104.3	C			102.6	
4′	195.2	C			194.0	
5′	72.7	CH	3.71, br		68.2	3.79, d (4.5)
6′	68.3	CH	4.18, dq (3.5, 7.0)	C-7′	66.6	4.18, dd (7.0, 4.5)
7′	20.5	CH_3	1.31, d (7.0)	C-5′, 6′	17.4	1.11, d (6.5)
N-Me	29.5	CH_3	3.05, s	C-2′, 5′	27.2	2.98, s

[a]HMBC correlations in both solvents were essentially identical except for the additional correlations of H-2 to C-1 and C-3′; H-5′ to C-2′, 4′, 6′, 7′; and H-6′ to C-4′, 5′.

A large number of decalin-containing tetramic acids have been reported as fungal metabolites. The structural variations are mostly at the C-3 side chain and the amino acid unit of the tetramic acid. These compounds are known to show a variety of activities including HIV-1 integrase inhibition (equisetin, phomasetin)[25] and as antibacterial agents (trichosetin,[28] equisetin and altersetin,[29] CJ-21,058,[30] CJ-17,572,[31] oxasetin,[32] and cissetin[33]). CJ-21,058 is also known to inhibit SecA activity (IC_{50} 15 μg/mL), responsible for ATP-dependent translocation of precursor proteins across the bacterial cell membrane.[30] Altersetin has been reported to exhibit weak in vivo antibacterial activity at 25 mg/kg by ip administration in a murine sepsis model.[29] None of these compounds show strong antibacterial activity. The activity of methiosetin (**1**) appears to be weaker than other tetramic acids but could be easily discovered by application of antisense hypersensitivity screens.

In summary, we have described the isolation of a new tetramic acid with weak antibacterial activity (MIC 32 μg/mL against *H. influenzae*) using a new discovery approach that allowed us to eliminate extracts with known compounds and prioritize antibacterial extracts with novel chemotypes. In this particular extract two structurally unrelated compounds were isolated that show varying degrees of antibacterial activities.

EXPERIMENTAL SECTION

General Experimental Procedures. An Agilent HP 1100 instrument was used for analytical HPLC. Optical rotation was recorded with a Perkin-Elmer 241 polarimeter. UV spectra were recorded on a Perkin-Elmer Lambda 35 spectrometer. CD spectra was recorded on a JASCO-J810 in MeOH. IR spectra was recorded with a Perkin-Elmer Spectrum One FT-IR spectrophotometer. All NMR spectra were recorded with a Varian Unity 500 (^{1}H, 500 MHz; ^{13}C, 125 MHz) spectrometer in CD_3OD or $CDCl_3$ using a 3 mm NMR probe. Chemical shifts are reported in δ (ppm) using residual solvent signals (for CD_3OD: δ_H 3.30 and δ_C 49.00; for $CDCl_3$: δ_H 7.26 and δ_C 77.00) as internal standards. ^{1}H, ^{13}C, COSY, DEPT, HMQC, and HMBC spectra were measured using standard Varian pulse sequences. LRMS data were recorded on an Agilent 1100 MSD with ES ionization, and HRESIFTMS were obtained on a Thermo Finnigan LTQ-FTMS spectrometer.

Producing Organism. *Capnodium* sp. (F-190,679) was isolated from palm leaf litter collected in Quetzalito, Guatemala, using a particle-filtration method that reveals slow growing fungi colonizing organic materials.[34] Frozen stock cultures in 10% glycerol (−80 °C) are maintained in the collection of Fundación MEDINA.

Total genomic DNA was extracted, and the 28S D1 and D2 variable and ITS regions of the rDNA were amplified and sequenced as previously described.[35] Consensus sequences of 28S D1 and D2 variable regions were aligned and visually adjusted with Clustal W.[36] Consensus neighbor-joining analyses were performed with PAUP 4.[37]

Fermentation. *Capnodium* sp. (F-190,679) was fermented by inoculating several mycelial agar plugs into a seed flask (50 mL of medium in a 250 mL unbaffled flask). The formulation for the seed media was as follows (g/L, unless specified): corn steep powder, 5.0; tomato paste, 40.0; oat flour, 10.0; glucose, 10.0; trace element mix, 10.0 mL/L; agar, 4.0;. adjusted pH to 6.8 with NaOH prior to sterilization. The formulation for the trace element mix consisted of (g/L) $FeSO_4{\cdot}7H_2O$, 1.0; $MnSO_4{\cdot}H_2O$, 1.0; $CuCl_2{\cdot}2H_2O$, 0.025; $CaCl_2$, 0.1; H_3BO_3, 0.056; $(NH_4)_6Mo_6O_{24}{\cdot}4H_2O$, 0.019; and $ZnSO_4{\cdot}7H_2O$, 0.2, prepared in 0.6 N HCl. The flasks were incubated on a rotary shaker at 220 rpm at 22 °C with 80% relative humidity.

After growing the seed stage flask for 4 to 7 days, a 1 mL aliquot was used to inoculate each flask of the production medium (50 mL of medium in a 250 mL unbaffled flask). The formulation consisted of (g/L) mannitol, 75.0; yeast extract, 1.0; oat flour, 1.0; MES, 16.2; NH_4Cl, 3.0; and $MgSO_4{\cdot}7H_2O$, 0.58. The pH was adjusted to 6.0 with NaOH prior to sterilization. The flasks were incubated at 22 °C with 80% relative humidity on a rotary shaker at 220 rpm for 14 days.

Isolation of Methiosetin (1) and Epicorazine A (2). A 1 L fermentation broth (pH 2) was extracted with 1 L of acetone and filtered through a bed of Celite. The filtrate was concentrated under reduced pressure to remove most of the acetone. The remaining aqueous solution (1.05 L) was charged on a 50 mL medium grade Amberchrome reversed-phase column and eluted with a 100 min linear gradient of 5−100% aqueous MeOH. Antibacterial activity was eluted in 70−90% MeOH fractions, which were concentrated under reduced pressure and then lyophilized to yield 2 g of solid. The entire active fraction from the Amberchrome chromatography was dissolved in 100 mL of methanol, loaded onto a 2 L Sephadex LH20 column, and eluted with methanol at a flow rate of 5 mL/min. Fractions 26−28 (0.67 column volume, fraction A) and fractions 38−40 (1 column volume, fraction B) showed activity. Fraction A was concentrated to yield 150 mg of material. A 15 mg aliquot of this material was chromatographed by reversed-phase HPLC on a Zorbax RX C_8 (9.1 × 250 mm) column eluting at a flow rate of 4 mL/min with a 25 min linear gradient of 30% to 95% aqueous acetonitrile each containing 0.1% TFA. Fractions eluting at 12 to 14 min were concentrated and lyophilized to give 4.9 mg of white solid material (**1**, 49 mg/L) as an amorphous powder. Fraction B was concentrated to yield 120 mg of a solid. A 60 mg aliquot of this solid was chromatographed by reversed-phase HPLC using a Zorbax RX C_8 (21 × 250 mm) column eluting at a flow rate of 12 mL/min with a 37 min gradient of 20% to 90% aqueous acetonitrile each containing 0.1% TFA. Fractions eluting

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at 18 min were concentrated and lyophilized to give 11 mg (22 mg/L) of a white solid, epicorazine A (**2**).

*Methiosetin (**1**):* $[\alpha]^{23}_{D}$ +12.4 (*c* 0.97, MeOH); UV (MeOH) λ_{max} 230 (ε 4397), 285 (ε 4420) nm; CD (MeOH) $\Delta\varepsilon_{250}$ 2000, $\Delta\varepsilon_{275}$ 0, $\Delta\varepsilon_{285}$ −1000; IR (ZnSe) ν_{max} 3422, 2929, 1700, 1651, 1610, 1453, 1376, 1341, 1261, 1212, 1125, 1088, 992, 948 cm^{-1}; ESIMS *m/z* 444 $[M + H]^+$; HRESIFTMS *m/z* 334.2004 (calcd for $C_{19}H_{27}NO_4$ + H, 334.2018); for ^1H and ^{13}C NMR data, see Table 1.

Minimum Inhibitory Concentration (MIC). The MIC against each of the strains was determined by National Committee for Clinical Laboratory Standards (NCCLS)—now called the Clinical Laboratory Standards Institute (CLSI)—method using 2-fold serial broth dilutions as previously described.[38] The cultures were incubated at 37 °C for 20 h, when activity was determined. MIC is defined as the lowest concentration of antibiotic that inhibited visible growth.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^1H and ^{13}C NMR data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ DEDICATION

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

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