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Inhibition and Kinetics of *mycobacterium tuberculosis* and *Mycobacterium Smegmatis* Mycothiol-*S*-conjugate Amidase by Natural Product Inhibitors

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Abstract—The current rise in mycobacterial-related infections and disease, coupled with drug resistance, underlines the continuing need for new antimycobacterials. To this end, we have screened ~1500 extracts derived from marine plants and invertebrates and terrestrial fungi for their ability to inhibit a newly described mycobacterial detoxification enzyme mycothiol-*S*-conjugate amidase (MCA). As described in this paper, our screening and chemistry efforts thus far have led to the identification of 13 natural product inhibitors that represent six different structural classes. By conducting enzyme inhibition assays using varied inhibitor and substrate concentrations, we have determined the mode of inhibition of *Mycobacterium tuberculosis* MCA for four of these compounds. We show that two types of bromotyrosine-derived natural products are competitive inhibitors of MCA; while oceanapiside, an α,ω-bis-aminohydroxy glycosphingolipid, and the fungal metabolite gliotoxin, a dithiadiketopiperazine, are simple and mixed non-competitive inhibitors, respectively. Correlation of these results with the chemical structures suggests that MCA is a metalloenzyme and that the oximinoamide and spiro-isoxazoline amide groups present in the competitive inhibitors are substrate mimics.

Introduction

The current rise in mycobacterial-related infections and disease, coupled with drug resistance, has resulted in renewed interests in the identification of new classes of anti-mycobacterials.[1a–c] To this end, we have been engaged in the identification of inhibitors of a novel detoxification enzyme, mycothiol-*S*-conjugate amidase (MCA),[2] which is unique to actinomycetes. Mycothiol[3–6] (MSH, **1**) is the major low molecular weight thiol in actinomycetes, and appears to play an analogous role to glutathione in eukaryotes and Gram negative bacteria in maintaining a reducing intracellular environment.[7] Like glutathione, MSH reacts with electrophiles and alkylating agents to form mycothiol-*S*-conjugates, which in turn serve as the substrate for MCA.[2] MCA cleaves the amide bond between cysteine (to the carboxy side of the amide) and D-glucosaminα(1–1)-*myo*-D-inositol (D-GI, to the amino side of the amide) in MSH, thereby freeing cysteine-*S*-conjugates for excretion from the cell, while D-GI is retained intracellularly and is reused in MSH biosynthesis. At the level of amino acid sequence, MCA lacks substantial homology with publicly available sequences of eukaryotic proteins, including other known enzymes that cleave amide bonds (such as proteases and deacetylases). With respect to prokaryotes, on the other hand, BLAST[8] searches reveal a number of mycobacterial MCA homologues, as well as several other non-mycobacterial proteins that share only modest sequence homology with MCA. However, none of the conserved regions among these putative bacterial amidases can be aligned to regions of secondary structure in known amidases. Consequently, when we embarked on this project, we had little understanding of the detailed workings of this enzyme. In the absence of a three-dimensional structure of MCA or an MCA homologue, we chose to begin looking for MCA inhibitors by screening natural product extracts[9] using a fluorescence-detected assay[2] for inhibition of MCA activity on the substrate mycothiol bimane (MSmB, **2**).[6] This screening effort yielded about 20 active extracts. As part of this

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work, we reported earlier a series of novel and known bromotyrosine-derived natural products that inhibit MCA with IC_{50}s in the low micromolar range,[10] which in turn led to recent studies using a panel of psammaplin A-inspired synthetic compounds from a combinatorial library constructed by Nicolaou and coworkers.[11,12] In this paper, we report the structures of the remaining natural product inhibitors of MCA that we have identified so far, and describe the inhibition profiles for four compounds representing the various structural classes identified. In view of our findings, implications for inhibitor design are discussed (Fig. 1).

Results and Discussion

We screened approximately 1500 crude organic extracts, 1200 of which were derived from marine plants and invertebrates and 300 from terrestrial fungal cultures, in a fluorescence-detected assay that measures the extent of cleavage of the substrate mycothiol bimane, **2**, by MCA.[2] This initial screening effort yielded about 20 active extracts that inhibited MCA at concentrations less than 50 micrograms per milliliter. We chose to pursue the chemistry of half of these extracts on the basis of potency and availability of sufficient material. The active compounds of each mixture were isolated by bioassay-guided fractionation. Purification schemes typically included an initial solvent partitioning step, followed by purification with LH20 or normal or reverse phase flash column chromatography. A final chromatographic separation of the active fractions, using the above methods or HPLC, was generally needed to obtain pure compounds. (See experimental section.) Once pure, the structures of the active compounds were determined using standard spectroscopic techniques including NMR and mass spectrometry.

Inhibition curves were measured for each of the pure compounds using natural MCA isolated from *Mycobacterium smegmatis*, and recombinant *Mycobacterium tuberculosis* MCA, which we have previously shown cleaves MSmB with rates identical to those of natural *M. smegmatis* MCA.[6] The structures of the 13 active natural products isolated thus far are illustrated in Figure 2, and Table 1 summarizes our results. Although we screened an approximately equal number of extracts from three sources, namely marine plants, marine invertebrates and terrestrial fungi, 10 of the 13 active compounds come from marine sponges, and the remaining three are known fungal metabolites. We did not detect any active compounds from marine plants. As we published previously, three types of bromotyrosine-derived natural products inhibit MCA at micromolar concentrations.[10,11] These include compounds containing a spirocyclic isoxazoline ring system, including spiro[4.5]decatrienes as seen in compounds **3**[10] and **4**,[13] and spiro[4.6]undecatrienes present in psammaplysins A (**7**) and B (**8**);[14] as well as the reduced bromophenyl oximinoamides seen in compounds **5**[10] and **6**.[15] Common to all of the bromotyrosine-derived compounds are a central amide group, an oxyimine to the carboxy side of the amide, and polar substituents to the amino side of the amide.[11]

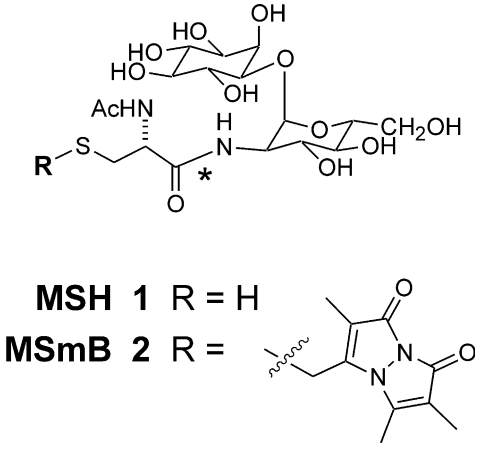

Figure 1. Mycothiol (**1**) and mycothiol bimane (**2**). Mycothiol-*S*-conjugate amidase (MCA) cleaves the amide bond connecting the cysteine and the α-D-glucosamine units.

The α,ω-bis-aminohydroxy glycosphingolipid oceanapiside[16] (**9**) inhibited MCA at low micromolar concentrations (Table 1). Like the substrate mycothiol bimane, oceanapiside contains a D-glucopyranoside unit. To determine whether the presence of the D-glucose unit in oceanapiside was essential for MCA inhibition, we removed the pyranose from the C-3 hydroxyl of oceanapiside to form the aglycon, **10**. Dose–response curves for compound **10** showed a 50- to 100-fold decrease in activity for the aglycon relative to oceanapiside, indicating that the presence of the D-glucoside in oceanapiside contributes favorably to MCA binding.

Representing a completely different class of compounds, the known sulfated terpenes suvanine[17] (**11**) and halisulfate 1[18] (**12**) were isolated from extracts of *Coscinoderma matthewsi* and were modest inhibitors of *M. smegmatis* MCA with IC_{50} values of 60 and 40 μM, respectively. A mixture of 1,3-pyridinium polymers,[19] **13**, from *Amphimedon* sp. represent the most potent inhibitors of MCA, showing submicromolar IC_{50} values [based on the molecular weight (506 g mol^{-1}) of the predominant dimeric species]. Thus, four different classes of compounds that inhibit MCA were obtained from marine sponges. The remaining three inhibitors were isolated from cultures of terrestrial fungi and include the known compounds gliotoxin[20] (**14**), a well-studied toxin that features a dithiadioxopiperazine ring, the analogue *S*,*S*-dimethyl gliotoxin[21] (**15**), and the anthraquinone physcion[22] (**16**).

As seen in Figure 2, the structures of the natural products that inhibit MCA are diverse. To further investigate the mode of MCA inhibition of these active compounds, we carried out competition assays on compounds **5**, **7**, **9**, and **14**, which include the spiroisoxaline- and linear oximinoamide-containing bromotyrosine-derived compounds, the glycosylated sphingolipid, and the tricyclic piperazine-containing toxins, respectively. Double reciprocal plots (1/v vs 1/s) for compounds **5** and **7** are shown in Figure 3. In both cases, lines intersect on the

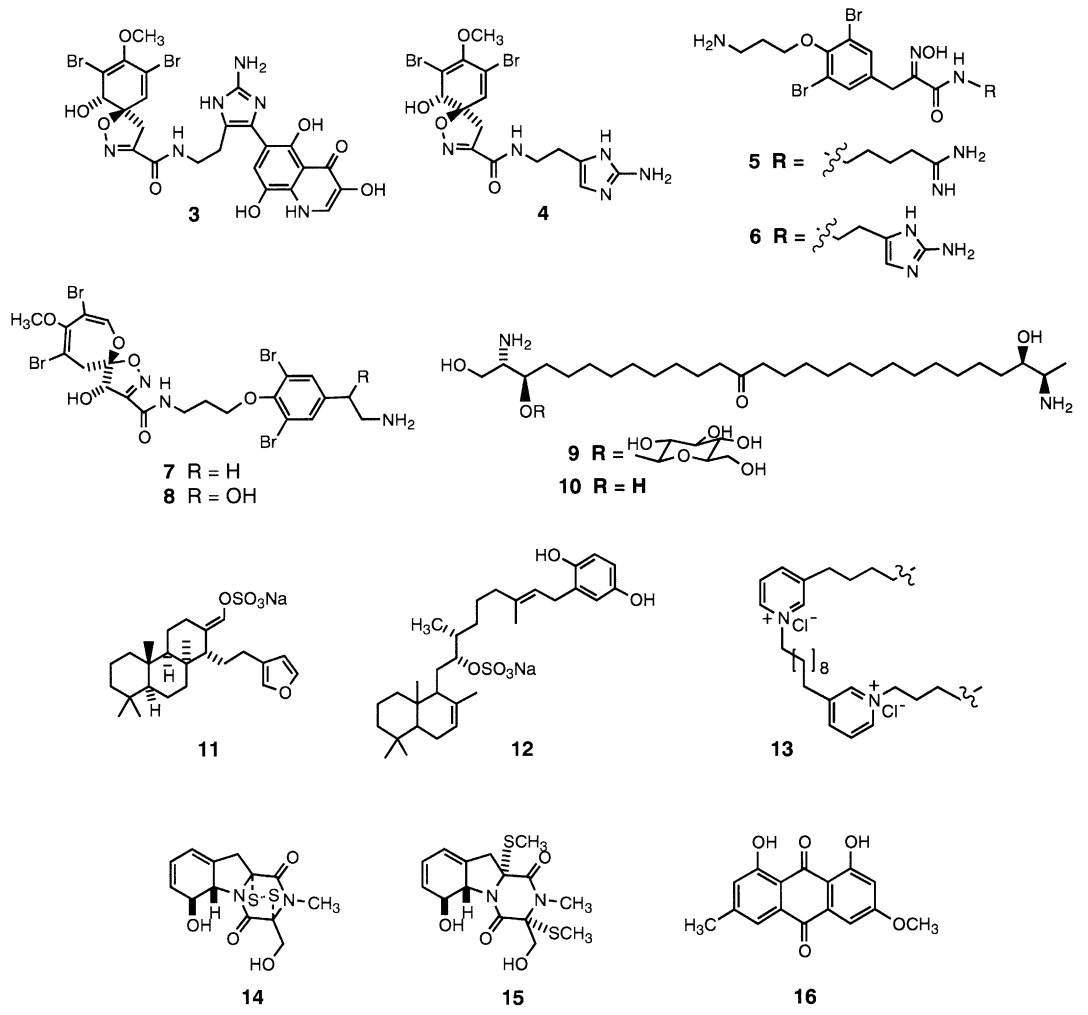

Figure 2. Natural products that inhibit mycothiol-*S*-conjugate amidase.

Table 1. Source, taxonomic identities and IC_{50} values for natural product inhibitors of *M. tuberculosis* and *M. smegmatis* mycothiol-*S*-conjugate amidase

Compound	Ref.	Common name or first author	Organism	Genus and species	NCI collection no.	IC_{50} (μM)	
						M. tb	*M. smeg*
3	10	Nicholas et al.	Marine sponge	*Oceanapia* sp.	C2385	3	2
4	13	Pseudoceratine	Marine sponge	*Oceanapia* sp.	C2385	100	100
5	10	Nicholas et al.	Marine sponge	*Oceanapia* sp.	C2385	3	2
6	15	Litaudon and Guyot	Marine sponge	*Oceanapia* sp.	C2385	30	37
7	14	Psammaplysin A	Marine sponge	*Pseudoceratina* sp.	C16115	30	30
8	14	Psammaplysin B	Marine sponge	*Pseudoceratina* sp.	C16115	30	20
9	16	Oceanapiside	Marine sponge	*Oceanapia* sp.	C2523	10	0.5
10	–	Oceanapiside aglycon	Marine sponge	—	C2523	100	50
11	17	Suvanine	Marine sponge	*Coscinoderma matthewsi*	C16199	nt	60
12	18	Halisulfate 1	Marine sponge	*Coscinoderma matthewsi*	C16199	nt	40
13	19	1,3-Pyridinium polymers	Marine sponge	*Amphimedon* sp.	C2355	0.1	0.1
14	20	Gliotoxin	Terrestrial fungus	*Mycena* sp.	F205435	50	50
15	21	*S*,*S*-Dimethyl gliotoxin	Terrestrial fungus	*Aspergillus* sp.	F205337	70	nt
16	22	Physcion	Terrestrial fungus	*Aspergillus* sp.	F205337	nt	50

1/v axis (in this case at the zero points of both axes) indicating that both natural products are competitive inhibitors of MCA. Oceanapiside (**9**), on the other hand, is a simple non-competitive inhibitor with lines of the double reciprocal plot intersecting on the 1/s axis (Fig. 4), indicating that **9** can interact equally well with free enzyme or an enzyme–substrate complex. The double reciprocal plot for gliotoxin (**14**) shows intersecting lines, but on neither axis, and therefore demonstrates that gliotoxin is a mixed non-competitive inhibitor and binds to free enzyme and enzyme–substrate complexes with dissimilar inhibition constants. (Parallel lines in a double reciprocal plot indicate uncompetitive inhibition where an inhibitor can only bind to the enzyme–substrate

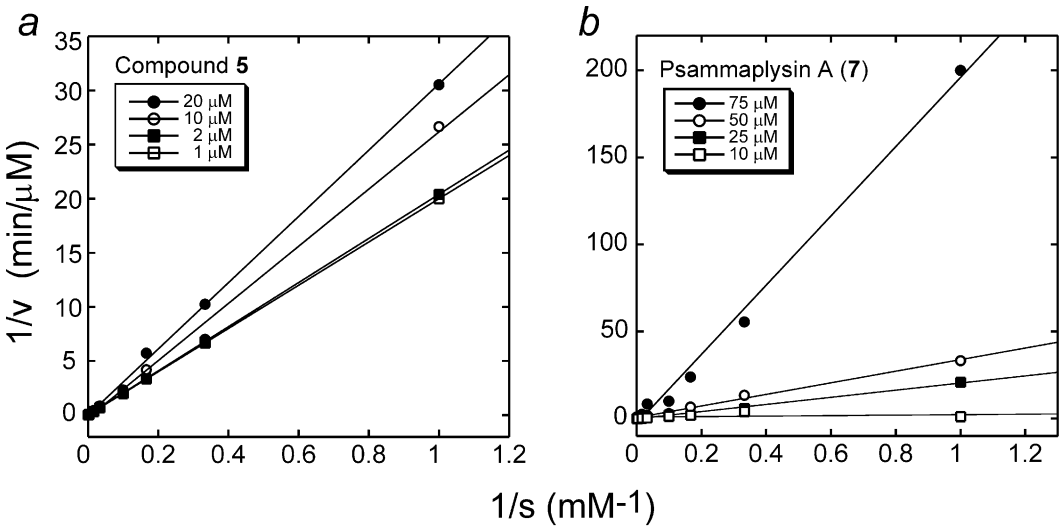

Figure 3. Competitive inhibition of *M. tuberculosis* MCA by bromotyrosine-containing natural products. Double reciprocal plots showing $1/v$ versus $1/s$ where v is the initial velocity and s the substrate concentration for (a) compound **5**, and (b) psammaplysin A (**7**). Initial rates of MCA activity were determined at seven substrate concentrations ranging from 1 μM to 1 mM synthetic MSmB. Varied inhibitor concentrations used for each compound are indicated in the legend within each plot.

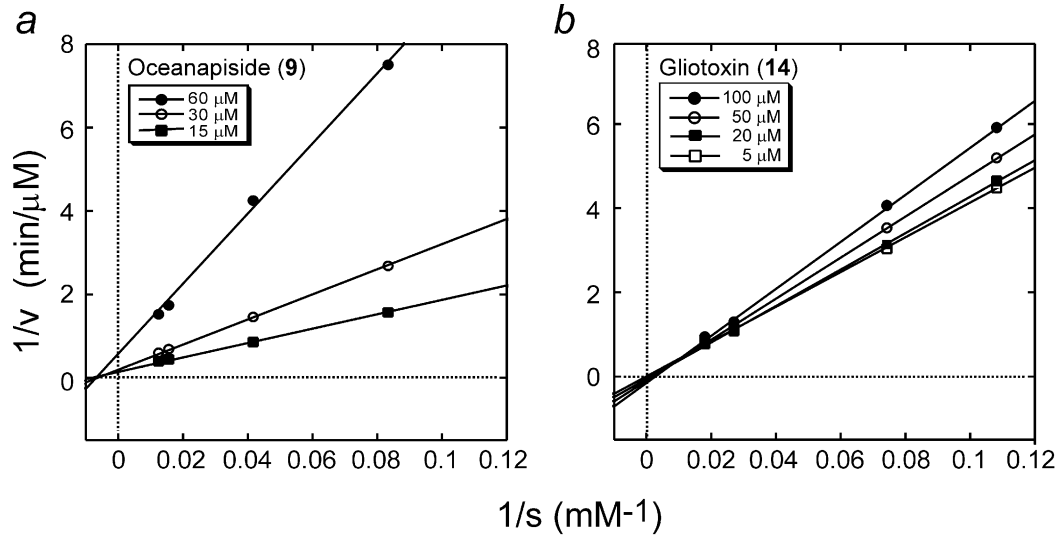

Figure 4. Non-competitive inhibition of *M. tuberculosis* MCA. Double reciprocal plots showing $1/v$ versus $1/s$ where v is the initial velocity and s the substrate concentration for (a) oceanapiside, **9**, and (b) gliotoxin, **14**. Initial rates of MCA activity were determined at four substrate concentrations ranging between 10 and 120 μM synthetic MSmB. Varied inhibitor concentrations used for each compound are indicated in the legend within each plot.

complex.) These results therefore demonstrate that compounds **5** and **7** combine only with free enzyme, while oceanapiside and gliotoxin can interact with various forms of free or substrate-bound MCA.[23] Since compounds **5** and **7** both contain a centrally located amide (Fig. 2) and compete directly with the substrate MSmB for MCA activity, we believe these compounds most likely mimic the amide bond connecting cysteine and glucosamine in mycothiol (Fig. 1), which is cleaved by MCA. In support of this notion, we note that bromotyrosine-containing natural products lacking an amide group, such as moloka'iamine[24] (3-[4-(2-amino-ethyl)-2,6-dibromo-phenoxy]-propylamine), are inactive in the MCA assay; unpublished data.)

Neither oceanapiside nor gliotoxin are competitive inhibitors, nor do they possess a linear amide bond. However, the findings that the inhibitory activity of the aglycon of oceanapiside is reduced but not abrogated, and that oceanapiside is not an *un*competitive inhibitor, suggests first that the presence of the D-glucose unit may assist with oceanapiside binding into the active site of the enzyme, and second, that the amino alcohol groups are sufficient for weak interactions with MCA. Interestingly, gliotoxin also contains an amide group in the form of a lactam, as well as a cluster of heteroatoms provided by the hydroxyl groups flanking the lactam. Gliotoxin is a well-studied fungal metabolite that shows a diverse range of biological activities, including antimicrobial, antifungal and antiviral properties, as well as DNA-damaging and immunomodulating activity.[25] Studies have demonstrated that gliotoxin activities hinge on the presence of the disulfide bond that can

form mixed disulfides with free thiols present in protein targets, or form reactive oxygen species in the presence of reducing agents (such as glutathione). For MCA, however, the fact that gliotoxin (**14**) and *S*,*S*-dimethyl gliotoxin (**15**) exhibit similar IC_{50} values (50 and 70 μM, respectively) indicates that the disulfide bridge is not required for inhibition of this enzyme, and that gliotoxin is not inhibiting MCA through mixed disulfide formation. (Absence of mixed disulfides was corroborated by mass spectrometry of a mixture of 50 nM MCA and 1 mM gliotoxin incubated at 31 °C overnight, which showed the presence of only recombinant MCA. No covalent modification by gliotoxin was detected.) Thus, the noncompetitive inhibitors **9** and **15**, and their respective homologues **10** and **14**, possess completely unrelated carbon skeletons comprising bis-amino alcohol lipids or tricyclic dithiadioxopiperazines. In both pairs of compounds, removal of key features from the active compounds, namely the D-glucose moiety in oceanapiside and the disulfide bond in gliotoxin, yield compounds that retain at least some inhibitory activity. Therefore, the presence of a cluster of heteroatoms within these compounds, as well as most of the inhibitors (Fig. 2), is the only real similarity among these otherwise structurally dissimilar inhibitors. (To test whether the the 1,3-hydroxy-2-amino moiety was sufficient for MCA inhibition, we prepared the peracetylated aglycon of oceanapiside. However, we were unable to test this derivative due to insolubility.)

Together with the conspicuous presence of an oximinoamide in natural products **3**–**8**, the structures and mode of inhibition of the active compounds shown in Figure 2 strongly suggest that MCA contains a metal ion in its active site that may be coordinated by the oximinoamides, the amino-alcohols, or the dithiadioxopiperazine moieties, thereby disrupting MCA activity. Although MCA lacks sequence homology with other known amidases, it is well known that many proteins that cleave amide bonds are metalloenzymes, many of which contain a catalytic zinc ion in the active site coordinated by the side chains of histidine and aspartate or glutamate residues.[26] In support of this hypothesis, MCA and its closest homologues also contain two short but highly conserved histidine- and aspartate-rich sequences. To our knowledge, one other example of a bacterial acetyl glucosamine deacetylase, LpxC, involved in Lipid A biosynthesis in *Escherichia coli* has been reported,[27] and the authors later demonstrated the amidase to be a zinc metalloenzyme.[28] (Note that MCA and LpxC are not homologous in sequence.) If MCA is also a zinc metalloenzyme, it is possible that the negatively charged sulfate groups on suvanine (**11**) and halisulfate 1 (**12**), and the cluster of oxygen atoms comprising the carbonyl oxygen and flanking hydroxyls[29] in the anthraquinone physcion (**16**), contribute to MCA inhibition by chelating the putative metal cation/s.[30]

In conclusion, by screening a relatively small number of marine and fungal extracts, we have identified 13 compounds encompassing several unrelated structural classes that inhibit MCA with micromolar IC_{50} values. Preliminary studies with psammaplysins A and B, oceanapiside, and the known toxin gliotoxin demonstrate that these compounds inhibit growth of *M. smegmatis* (MC^2155) in disk diffusion assays. (Additional assays against *M. tuberculosis* are underway.) Structural features of the active natural products and modified analogues suggest that MCA is a metalloenzyme, similar in function to other peptidases or endoproteases. Our findings should therefore prove valuable in the design and synthesis of both natural products-inspired and substrate-based inhibitors of MCA.

Experimental

General

Low- and high-resolution mass spectra were recorded on a Jeol SX102 mass spectrometer, and chemical ionization mass spectra on a Finnigan 4500. NMR spectra were recorded on a Varian Gemini300 or Bruker DMX500 spectrometer. Fluorescence-detected HPLC MCA inhibition assays were carried out with an Agilent Technologies 1100 HPLC system equipped with photodiode array and fluorescence detectors, and a refrigerated 96-well plate autosampler. Reaction mixtures were run on an analytical HPLC column (Waters Symmetry® C18, 5 μm particle size, 4.6×150 mm), equilibrated with 9% CH_3CN in 0.05% TFA before each injection, and eluting with a 5.5 min gradient of 9–18% CH_3CN in 0.05% TFA, followed by a 3-min wash with 60% CH_3CN in 0.05% TFA, at a flow rate of 1 mL/min; R_f AcCys-*S*-bimane 4.4 min; R_f MSmB 8.3 min. Semipreparative reverse phase (C18) HPLC (RP-HPLC) was carried out on the natural products using a GBC LC1150 pump, a GBC LC5100 photodiode array detector and a Waters μBondapak C18 column (7.8×300 mm) at a flow rate of 3 mL/min. Reverse phase C18 flash chromatography was carried out using C18 bulk silica (J. T. Baker, 40 μ diameter), and normal phase flash chromatography was carried out with Si gel (Sorbent Technologies, 60 Å pore size, 32–63 μ diameter). Size exclusion column chromatography was carried out using Sephadex LH20 (Pharmacia).

Screening for MCA inhibitors

Crude organic extracts (1:1 $MeOH/CH_2Cl_2$) were obtained from the National Cancer Institute Natural Products Repository in 96-well plates. Extracts were first tested for their ability to inhibit MCA cleavage (by at least 80%) of the fluorescent substrate mycothiol bimane (MSmB, **2**) at 100 μg/mL, followed by rescreening only the active extracts at 50 μg/mL. The active compounds of the crude mixtures were pursued for those extracts that inhibited MCA activity by greater than 50% at the lower concentration.

Source of crude extracts

Collections of each organism listed in Table 1 were frozen immediately upon collection and stored at −20 °C until being transported to NCI, Frederick, MD, USA.

Frozen specimens were lyophilized prior to extraction, and organic extracts were prepared by soaking the freeze dried animal material in 1:1 $MeOH/CH_2Cl_2$ three times, followed by solvent removal in vacuo of the combined extracts. Screening was performed on crude organic extracts in a 96-well plate format, and bulk extract was provided upon request.

Isolation of natural product inhibitors

***Oceanapia* sp. (C2385).** See ref 10 for details.

***Pseudoceratina* sp. (C16115).** An 1150-g sample of the marine sponge *Pseudoceratina* sp. (identified by Michele Kelly-Borges) was collected at a depth of 5 m from East Bakeldaob Island, Palau (Coral Reef Research Foundation), and processed as described above. Approximately 1 g of the crude $MeOH/CH_2Cl_2$ extract was partitioned in petroleum ether/10% aq MeOH, followed by partitioning in $CHCl_3$/10% aq MeOH. The active MeOH extract (595 mg) was loaded onto an LH20 column (2×85 cm) and eluted with MeOH. Following testing, active fractions were combined and the solvent was removed in vacuo to yield ~90 mg of an active mixture of at least two compounds. Further purification was accomplished by RP C18 chromatography using a water to methanol gradient to yield 16 mg of psammaplysin A (**7**) and 12 mg of psammaplysin B (**8**).

***Oceanapia* sp. (C2523).** A 1-kg sample of the marine sponge *Oceanapia* sp. was collected at depths ranging from 15 to 18 m in southwestern Australia. Five hundred milligrams of the crude organic extract was partitioned according to the same scheme described for psammaplysins A and B. The aqueous MeOH fraction contained the active components, and the MeOH-soluble fraction of this extract was purified over an LH20 column (2×80 cm) in MeOH. Modest activity (~20% inhibitory at 100 μg/mL) was observed throughout most of the fractions. Thus, rescreening at 10 μg/mL yielded only two active fractions that were purified to homogeneity on a C18 column eluting with a gradient of 0–100% MeOH in water to give 22 mg of oceanapiside (**9**). (This sequence was later repeated on a larger scale.)

***Coscinoderma matthewsi* (C16199).** A 920-g sample of the marine sponge *C. matthewsi* was collected at a depth of 20 m from Beqa Lagoon, Fiji, and identified by Michele Kelly-Borges. Five-hundred milligrams of the crude organic extract was partitioned in pet. ether/MeOH (10% H_2O) to give ~400 mg of an active methanolic fraction. Column chromatography using LH20 (2×80 cm) in MeOH gave two sets of active, but impure, fractions weighing approximately 45 and 30 mg, respectively. Each fraction was subsequently purified by RP-HPLC eluting with a gradient from 0 to 100% CH_3CN in 0.05% aq TFA at 1 mL/min. Two mg of suvanine and halisulfate 1 were obtained.

***Amphimedon* sp. (C2355).** A 750-g sample of the sponge *Amphimedon* sp. was collected from the Great Barrier Reef in the Indo-West Pacific. The crude organic extract was partitioned between pet. ether and MeOH to give an active MeOH-soluble fraction (370 mg). The MeOH-soluble portion of the active fraction (320 mg) was purified over a C18 column (3×25 cm) by elution with a six-step gradient of MeOH:H_2O mixtures going from 0 to 100% MeOH in 20% increments. The column was finally eluted with 1:4 $MeOH/CH_2Cl_2$ and neat CH_2Cl_2. Fractions eluting with 20–80% MeOH showed strong activity (100% inhibition at 50 μg/mL) and contained a heterogeneous mixture of pyridinium polymers as determined by NMR and mass spectrometry.

***Mycena* sp. (F205435).** 650 mg of the crude organic extract was partitioned between hexanes/MeOH (10% H_2O) to give ~500 mg of active MeOH-soluble material. Following dissolution in ~2 mL MeOH, the mixture was chromatographed over an LH20 column (2×80 cm) eluting with MeOH to give six (of 21) late-eluting, active fractions. Thin-layer chromatography (TLC) (Si gel) showed these fractions to contain only two components. Thus, one representative fraction (18 mg) was purified further by isocratic RP-HPLC eluting with 20% aq CH_3CN (0.05% TFA) at 1 mL/min to give 2.2 mg of pure gliotoxin (**15**). The second component was an inactive quinone.

***Aspergillus* sp. (F205337).** 950 mg of the crude organic extract of *Aspergillus* sp. was partitioned and purified over an LH20 column as described directly above for the *Mycena* extract from which gliotoxin was isolated. The combined active fractions (130 mg) were insoluble in MeOH; thus 1 g C18 was added to the insoluble mixture, followed by removal of the solvent in vacuo to yield a friable powder that was loaded directly onto a C18 column. Elution with a 10% step gradient of increasing MeOH yielded ~50 mg of *S*,*S*-dimethyl gliotoxin (**15**) (eluted with 40% MeOH in H_2O), confirmed by HRFABMS and ^1H NMR. A second set of active fractions eluting with 60% MeOH were combined and rechromatographed over a C18 column to yield 1.3 mg of physcion (**16**).

Oceanapiside aglycon (10). A solution of **9** (20 mg) was stirred in 2 M HCl in MeOH (2 mL) at 80 °C, and the hydrolysis was monitored by TLC (Si gel, 12:18 MeOH/$CHCl_3$); oceanapiside R_f 0.21, oceanapiside aglycon R_f 0.52. After 24 h, the hydrolysis had gone to near completion (~5% starting material remaining). After reducing the volume of the reaction mixture to ~1 mL on a rotary evaporator, the mixture was purified by flash column chromatography (Si gel) eluting with 12:18 $MeOH/CHCl_3$ to remove the glucose, followed by 12:18:1 $MeOH/CHCl_3/NH_4OH$ to elute the aglycon. Signals corresponding to D-glucose were absent in the ^1H NMR spectrum of **10** (see below).

Mass spectral data for pure compounds

Compounds 3–6. See ref 10 for experimental details.

Psammaplysin A (7). Clear oil; HRFABMS m/z $[M+H]^+$ 733.8340. $C_{21}H_{24}Br_2^{79}Br_2^{81}N_3O_6$ requires 733.8358. (Consistent with the presence of four bromine atoms, a pentet of molecular ions differing by 2 amu was observed in the mass spectrum.)

Psammaplysin B (8). Clear oil; FABMS m/z $[M+H]^+$ 749.5. $C_{21}H_{24}Br_2^{79}Br_2^{81}N_3O_7$ requires 749.83.

Oceanapiside (9). Clear oil; HRFABMS m/z $[M+H]^+$ 649.5003. $C_{34}H_{69}N_2O_9$ requires 649.5000.

Oceanapiside aglycon (10). White glass; FABMS m/z $[M+H]^+$ 487.45. $C_{28}H_{56}N_2O_4$ requires 487.45; ^1H NMR for **10** (CD_3OD) was identical to that previosly published.[16]

Suvanine (11). White powder; FABMS m/z $[M+H]^+$ 473.36. $C_{25}H_{38}NaO_5S$ requires 473.23.

Halisulfate 1 (12). White powder; FABMS m/z $[M+H]^+$ 571.30. $C_{31}H_{48}NaO_6S$ requires 571.30.

1,3-pyridinium polymers (13). Yellow oil; CIMS m/z $[M+H]^+ + HCl$ 226, $C_{13}H_{20}Cl$ requires 225.76 and corresponds to the molecular ion for the major fragment (under these conditions)1-chloro-3-oct-7-enyl-pyridine; FABMS m/z $[M+H]^+ + Cl_2$ 506.3195; requires 506.32.

Gliotoxin (14). White powder; EIMS m/z $[M]^+$ 326.0395. $C_{13}H_{14}N_2O_4S_2$ requires 326.39.

***S,S*-Dimethyl gliotoxin (15).** Yellow powder, HRFABMS m/z $[M+H]^+$ 357.0949; $C_{15}H_{21}N_2O_4S_2$ requires 357.0943.

Physcion (16). Bright orange solid, FABMS m/z $[M-H]^-$ 283.0612; $C_{16}H_{11}O_5$ requires 283.0606.

MCA inhibition assays

To determine their mode of inhibition, assays were conducted on inhibitors **5**, **7**, **9** and **14** in the presence of 20 nM natural *M. smegmatis* MCA[2] or recombinant *M. tuberculosis* MCA[6] in the presence of varying amounts of mycothiol bimane (**2**). Substrate concentrations ranged from 1 μM to 1 mM. Varied inhibitor concentrations were selected based on our measured IC_{50} values for the relevant inhibitor (see Table 1) and are shown in the inset of each plot (Figs 3 and 4). Assays were set up in 96-well plates by adding 36 μL of the various stock solutions of MSmB, 2 μL of the relevant stock solutions of inhibitors dissolved in either MeOH or 1:1H_2O/DMSO, and 2 μL of a 0.4-μM solution of MCA, in that order. (All reagents were kept on ice, but the plate was set up at room temperature. If the plates were refrigerated or set up on ice, the extent of cleavage of MSmB was highly variable.) Plates were incubated at 31 °C for 15 min, after which time the reactions were quenched immediately with an equal volume of cold 40 mM methanesulfonic acid (4 °C). Control reactions in which 2 μL of the relevant solvent were added to the reaction were run in parallel with each inhibitor and the data were normalized to these controls (see below). Following centrifugation of the 96-well plates (4000×g, 4 °C, 10 min), 40 μL of the quenched reaction mixtures were transferred to an unused 96-well plate which was loaded into a refrigerated 96-well plate autosampler for HPLC detection of the extent of cleavage of the fluorescent substrate MSmB. To determine IC_{50} values, initial velocities were plotted as a function of inhibitor concentration and the data were fit to the equation:

$$\upsilon_i/\upsilon_c = IC_{50}/([I] + IC_{50})$$

where υ_i and υ_c are the initial velocities in the presence of inhibitor or in the control reactions, respectively. Double reciprocal plots of $1/v$ versus $1/S$, where v is the initial velocity for each reaction condition and S is the substrate concentration, for each data set are shown in Figures 3 and 4.

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References and Notes

1. (a) For recent reviews, see: (a) Dye, C.; Williams, B. G.; Espinal, M. A.; Raviglione, M. C. *Science* **2002**, *295*, 2041. (b) Pozniak, A. *Ann. N.Y. Acad. Sci.* **2001**, *953*, 952. (c) Grange, J. M.; Zumla, A. *Lancet* **1999**, *353*, 996.
2. Newton, G. L.; Av-Gay, Y.; Fahey, R. C. *Biochemistry* **2000**, *39*, 10739.
3. Sakuda, S.; Zhou, Z.; Yamada, Y. *Biosci. Biotech. Biochem.* **1994**, *58*, 1347.
4. Spies, H. S.; Steenkamp, D. J. *Eur. J. Biochem.* **1994**, *224*, 203.
5. Newton, G. L.; Bewley, C. A.; Dwyer, T. J.; Horn, R.; Aharonowitz, Y.; Cohen, G.; Davies, J.; Faulkner, D. J.; Fahey, R. C. *Eur. J. Biochem.* **1995**, *230*, 821.
6. Nicholas, G. M.; Kováč, P.; Bewley, C. A. *J. Am. Chem. Soc.* **2002**, *124*, 3492.
7. Reviewed in Fahey, R. C. *Ann. Rev. Microbiol.* **2001**, *55*, 333.
8. Altschul, S. F.; Gish, W.; Miller, W.; Myers, E. W.; Lipman, D. J. *J. Mol. Biol.* **1990**, *215*, 403.
9. Obtained from the Natural Products Open Repository, National Cancer Institute.
10. Nicholas, G. M.; Newton, G. L.; Fahey, R. C.; Bewley, C. A. *Org. Lett.* **2001**, *3*, 1543.
11. Nicholas, G. M.; Eckman, L. L.; Ray, S.; Hughes, R. O.; Pfefferkorn, J.; Barluenga, S.; Nicolaou, K. C.; Bewley, C. A. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 2487.
12. Nicolaou, K. C.; Hughes, R.; Pfefferkorn, J. A.; Barluenga, S.; Roeker, A. J. *Chem. Eur. J.* **2001**, *7*, 4280.
13. Benharref, A.; Pais, M. *J. Nat. Prod.* **1996**, *59*, 177.
14. Roll, D. M.; Chang, C. W. J.; Scheuer, P. J.; Gray, G. A.; Shoolery, J. N.; Matsumoto, G. K.; Van Duyne, G. D.; Clardy, J. C. *J. Am. Chem. Soc.* **1985**, *107*, 2916.
15. Litaudon, M.; Guyot, M. *Tetrahedron Lett.* **1986**, *27*, 4455.
16. Nicholas, G. M.; Hong, T. W.; Molinski, T. F.; Lerch, M. L.; Cancilla, M. T.; Lebrilla, C. B. *J. Nat. Prod.* **1999**, *62*, 1678.

17. Manes, L. V.; Crews, P.; Kernan, M. R.; Faulkner, D. J.; Fronczek, F. R.; Gandour, R. D. *J. Org. Chem.* **1988**, *53*, 570.
18. Kernan, M. R.; Faulker, D. J. *J. Org. Chem.* **1988**, *53*, 4574.
19. Davies-Coleman, M. T.; Faulkner, D. J.; Dubowchik, G. M.; Roth, G. P.; Polson, C.; Fairchild, C. *J. Org. Chem.* **1993**, *58*, 5925.
20. Weindling, R. *Phytopathology* **1941**, *31*, 991.
21. Kirby, G. W.; Robins, D. J.; Sefton, M. A.; Talekar, R. R. *J. Chem. Soc., Perkin Trans. 1* **1980**, 119.
22. Reviewed inAnke, H.; Kolthoum, I.; Zahner, H.; Laatsch, H. *Arch. Microbiol.* **1980**, *126*, 223.
23. Shindler, J. S.; Bardsley, W. G. *Biochem. Pharm.* **1976**, *25*, 2689. Cornish-Bowden, A. In *Principles of Enzyme Kinetics*; Butterworths: London, 1976. Henderson, P. J. F. In *Enzyme Assays, A Practical Approach*; Eisenthal, R., Danson, M. J. Eds.; Oxford University Press: New York, 1998; p 277.
24. Hamann, M. T.; Scheuer, P. J.; Kelly-Borges, M. *J. Org. Chem.* **1993**, *58*, 6565.
25. Waring, P.; Beaver, J. *Gen. Pharmac.* **1996**, *27*, 1311 and references cited therein..
26. Reviewed in Vallee, B. L.; Auld, D. S. *Biochemistry* **1990**, *29*, 5647.
27. Young, K.; Silver, L. L.; Bramhill, D.; Cameron, P.; Eveland, S. S.; Raetz, D. R.; Hyland, S. A.; Anderson, M. S. *J. Biol. Chem.* **1995**, *270*, 30384.
28. Jackman, J. E.; Raetz, C. R.; Fierke, C. A. *Biochemistry* **1999**, *38*, 1902.
29. Note that similar distances (2.55–2.65 Å) separate the three neighboring oxygen atoms in physcion and the gliotoxins.
30. Due to the heterogenous nature of the 1,3-pyridinium polymers, along with their tendency to aggregate, we did not investigate their mode of inhibition of MCA. For recent insights into aggregation related to non-specific or 'promiscuous' inhibitors, see McGovern, S. L.; Caselli, E.; Grigorieff, N.; Shoichet, B. K. *J. Med. Chem.* **2002**, *45*, 1712.