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NOTE

Inhibition of metallo-β-lactamases by pyridine monothiocarboxylic acid analogs

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Metallo-β-lactamases (MBLs) are potent bacterial enzymes that can destroy almost all classes of β-lactams, including carbapenems. Strong hydrolytic activity against carbapenems is observed when these enzymes are produced chromosomally by clinically important pathogens such as *Stenotrophomonas maltophilia* and *Bacteroides fragilis*.[1–3] More importantly, many MBLs now appear as plasmid-encoded enzymes that can be transferred among the Enterobacteriaceae as well as the non-fermentors.[2,3] Over 80 unique MBLs have been described in the literature, with at least 23 members in each of the inosine monophosphate and VIM families of plasmid-encoded MBLs.[1] Although genes encoding these metalloenzymes may be found in the bacterial chromosome, they are not always expressed in *B. fragilis*.[4,5] The MBL genes encoded on mobile bacterial plasmids in *Pseudomonas aeruginosa*,[6] *Serratia marcescens*[7] and many other Gram-negative pathogens are also found with genes encoding other types of antibiotic resistance (for example, aminoglycosides),[3] resulting in multidrug-resistant pathogens of high clinical importance. Consequently, clinical failures of carbapenems as a result of MBLs continue to be a serious problem. For this reason, an inhibitor of MBLs could provide a distinct advantage when used in combination therapy with susceptible β-lactam antibiotics.

MBLs require an active site divalent zinc cation to hydrolyze the amide bond in the β-lactam ring of susceptible compounds. These enzymes are inactivated by chelating agents such as EDTA, 1,10-*o*-phenanthroline and dipicolinic acid (**1**, Figure 1),[8] but not by classical β-lactamase inhibitors such as clavulanic acid. Payne *et al.*[9] reported that MBLs were irreversibly inhibited by mercaptoacetic acid thiol ester derivatives by a mechanism based on the delivery of mercaptoacetic acid, forming a disulfide linkage with the active site serine. Conversely, mercaptophenylacetic acid thiol ester derivatives act as competitive inhibitors of the enzyme.[10]

Using an automated screen, 19 366 natural product extracts were tested for time-dependent inhibitory activity against the CcrA MBL from *B. fragilis*. Potent activity was identified from actinomycete culture LL-10G568.[11] We now report the bioassay-guided isolation, identification, inhibition parameters and structure–activity relationships for dipicolinic acid analogs **2**–**5** active against this purified MBL. Specificity of inhibition was also assessed by examining the inhibitory activity of the compounds against bacterial MBLs, bacterial serine-β-lactamases and mammalian-derived metalloenzymes.

The unidentified actinomycete was originally isolated from a soil sample collected in Seoul, South Korea. A first-stage seed (100 ml) was grown at 28 °C for 72 h in a medium containing glucose 1%, N-Z amine A 0.5%, $CaCO_3$ 0.1%, dextrin 2%, yeast extract 0.5% and Hodag FD-82 antifoam 0.3% v/v. This seed was used to inoculate a second-stage seed in the same medium (3-l, grown at 28 °C for 48 h, 500 r.p.m. agitation and 3 l min^{-1} aeration). The entire second stage seed was transferred to a 41-l jar fermentor containing 30 l of production medium, which consisted of glycerol 2%, KH_2PO_4 0.25%, NaCl 0.5%, soy peptone 0.5%, KCl 0.05%, $MgSO_4{\cdot}7\,H_2O$ 0.05% and $CaCO_3$ 0.1%. Antifoam agent was added as required. The production fermentation was conducted at 28 °C with 30 l min^{-1} aeration and 500 r.p.m. agitation. MBL inhibitory activity in the fermentation broth was measured daily and the fermentation was harvested after 67–71 h.

Isolation of the compounds was monitored by measuring the hydrolysis of nitrocefin (Becton and Dickinson & Co., Franklin Lakes, NJ, USA) in the presence of the CcrA enzyme. Owing to the lability of the compounds, purification processes were blanketed with nitrogen and kept chilled whenever possible. The 30-l fermentation broth was mixed with toluene (1.5% v/v) and 3 kg of celite 512, filtered, and the mycelia were washed with water and were discarded. The pH of the combined filtrate and wash (45 l) was adjusted to 7 with HCl, loaded onto a 5.5-l Diaion HP20 (Mitsubishi Chemical Corp., Tokyo, Japan) column and eluted. The combined 50 l column effluent and water wash were adjusted to pH 4 with $(NH_4)_2SO_4$ (80 g l^{-1}) and loaded onto a second HP20 column (4-l), which was eluted with 8 l of water.

Bioactive fractions (5 l) were adjusted to pH 8 with 0.2 M phosphate buffer and applied onto a 300-ml diethylaminoethyl cellulose-Sephadex

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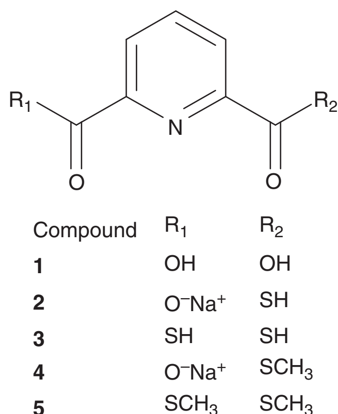

Figure 1 The structures of compounds **1**–**5**.

A-25 ion exchange column in the Cl^- form. The column was eluted with 600 ml of 0.02 M phosphate buffer at pH 6, followed by 1500 ml of a 0–100% linear gradient of the buffer containing 1 M NaCl and finally with 600 ml of buffer containing 1 M NaCl. Fractions were monitored by bioactivity and UV detection at 270 nm. Component **2** eluted near the end of the linear gradient, while component **3** eluted with 1 M NaCl in buffer. Active fractions were pooled and freeze-dried. Additional material could be recovered from the HP20 columns by elution with 40% aq acetone and ion exchange chromatography as described above.

Freeze-dried solids containing **2** were triturated with MeOH (2×250 ml) and filtered. The filtrate was concentrated to dryness and applied to a 1-liter Sephadex LH-20 column and eluted with 20% aq MeOH. Active fractions were pooled to give 65.6 mg of partially desalted **2**. This sample was rechromatographed on a 450-ml Bondesil C18 column using water as eluant to give 53.8 mg of **2** as a bright yellow powder. Fractions containing component **3** were treated in an identical manner and yielded 27.3 mg of a bright yellow solid that turned orange under vacuum. Both **2** and **3** were allowed to react with methyl iodide (one and two equivalents, respectively) to give the *S*-methyl derivatives **4** and **5**.

High-resolution fast atom bombardment–mass spectroscopy data confirmed the formula $C_7H_5NO_3S$ for **2**, which exists as a sodium salt, and $C_7H_5NO_2S_2$ for **3**. The IR spectrum of **2** contained strong bands at 1610 and 1385 cm^{-1} indicative of a carboxylate ion, which were missing in **3**. The uv spectrum of **2** in acid displayed maxima at 213, 270 and 329 nm, which shifted to 227 and 286 nm in base. Heteronuclear multiple bond coherence correlations were seen from the ring protons to the carbonyl in the NMR spectra of both **2** and **4** and a corresponding correlation was also seen from the *S*-methyl protons to the carbonyl. Interpretation of the spectroscopic data of the natural products and *S*-methyl derivatives and comparison with analogs of **1** readily led to assignment of **2** as pyridine-2-carboxylic acid-6-monothiocarboxylic acid and **3** as pyridine 2,6-di(monothiocarboxylic acid). Both **2** and **3** have been previously reported from *P. putida* and have been identified as their dimethyl esters.[12–14] Although **5** is a known compound, to our knowledge the mono-*S*-methyl ester **4** has not been previously reported in the literature.

Natural product inhibitors of the CcrA MBL were initially identified using an automated robotic assay (Biomek SL1000, Beckman Coulter, Inc., Brea, CA, USA). For the time-dependent automated screen, the enzyme was preincubated with an inhibitor at 25 °C for 7 min, then a 10-fold excess volume of prewarmed nitrocefin (100 μg ml^{-1}) was added

Table 1 Inhibitory activity of pyridine monothiocarboxylic acid analogs against metalloenzymes

	Enzyme	Original source	μM Zn^{2+}	IC50 (μM) (1)	(2)	(3)	(5)
Bacterial β-lactamases							
Metallo-β-lactamases	CcrA[a]	*Bacteroides fragilis*	0	4.2	0.29	0.14	250
			1.0	9.0	1.5	1.4	>250
	L1[b]	*Stenotrophomonas maltophilia*	0	7.8	1.2	0.60	340
			1.0	11	7.1	1.1	>340
Serine β-lactamases[c]	PC1	*Staphylococcus aureus*	0	ND	>250	>250	ND
	TEM-2	*Escherichia coli*	0	ND	>250	>250	ND
	SHV-1	*Escherichia coli*	0	ND	>250	>250	ND
	TEM-26	*Klebsiella pneumoniae*	0	ND	>250	>250	ND
	SME-1	*Serratia marcescens*	0	ND	>44[d]	>250	ND
	P99	*Enterobacter cloacae*	0	ND	>250	>250	ND
Mammalian Zn^{2+} enzymes	DHP	Porcine	0	ND	0.27	0.23	ND
			1.0	ND	0.55	1.4	ND
	CPase A[e]	Bovine	0	ND	56	16	ND
			1.0	ND	110	20	ND
	CPase B	Porcine	0	ND	5.5	2.0	ND
			1.0	ND	21	15	ND

Abbreviations: DHP, dehydropeptidase; ND, not determined.
[a]CcrA MBL, originally from *B. fragilis* strain TAL3636, was purified from inclusion bodies produced in *E. coli*.[19,20]
[b]The L1 MBL was purified from *S. maltophilia* strain 1712.[15]
[c]Purified β-lactamases were prepared as described previously.[15]
[d]Highest concentration tested.
[e]Purchased from Sigma-Aldrich (St Louis, MO, USA).

to initiate the β-lactamase reaction. Negative controls were followed in the presence of media or buffer; 1,10-*o*-phenanthroline at 50 μM was tested as the positive control. Spectrophotometric methods were used for all quantitative IC_{50} determinations. In assays without an enzyme-inhibitor preincubation period, the inhibitor and substrate were briefly incubated at 25 °C and reactions were initiated by addition of enzyme. For time-dependent assays, the enzyme and inhibitor were preincubated at 25 °C for 10 min, then a 10- to 50-fold excess volume of substrate was added to initiate the reaction. IC_{50} values were determined graphically. β-Lactamase activities were assayed as described previously.[15] Partially purified porcine renal dipeptidase (dehydropeptidase) was obtained from fresh hog kidneys by butanol extraction followed by ammonium sulfate precipitation.[16] Dehydropeptidase was assayed at 299 nm using imipenem (Merck & Co., Inc., Whitehouse Station, NJ, USA) as substrate at a final concentration of 50 μg ml^{-1}. Carboxypeptidases A (bovine pancreas) and B (porcine pancreas) were assayed according to literature methods using Hip-Arg[17] and Hip-Phe,[18] respectively, as the reporting substrate. MIC values were determined by standard methodology.[7]

Although **2**, **3** and **5** had previously been identified in the literature,[12–14] their metalloenzyme inhibitory activity properties had not been defined. The natural products **2** and **3** inhibited metalloenzymes from both bacterial and mammalian sources (Table 1), but were

ineffective against active-site-serine β-lactamases from Bush groups 1 and 2.[1] Dithioacid **3** had the strongest inhibitory activity against both bacterial MBLs CcrA from *B. fragilis* and L1 from *S. maltophilia*, whereas the monothioacid **2** was two-fold less active. Inhibitory activity for the MBLs was detected for **1**, but it was at least 10-fold less potent. In preliminary experiments, the mono-*S*-methyl ester **4** was assayed only against the CcrA MBL and was inactive. The di-*S*-methyl ester **5** had no inhibitory activity against either of the MBLs. These results suggested that the best dipicolinic acid-derived inhibitors of MBLs required a functional group capable of chelating an active site cation. When thioacid groups were blocked, less inhibitory activity was seen.

Inhibition of both MBLs by **2** and **3** was reversed 2–10-fold after the addition of 1.0 μM Zn^{2+} and by the addition of 50 μM 1,10-*o*-phenanthroline, indicating that these natural product-derived metalloenzyme inhibitors may act as chelating agents for divalent cations through the sulfur atom of thioacid group(s) on the pyridine ring. Preincubation of the CcrA MBL with **2** was not required for maximum inhibition. The IC_{50} of **2** with the CcrA enzyme was 0.29 μM with 10 min preincubation and 0.34 μM without preincubation. The same pattern occurred with **3**, a behavior consistent with that of a competitive inhibitor. These data, in addition to data demonstrating reversal of inhibition by 1,10-*o*-phenanthroline, implied that the natural product inhibitors were binding to the active site Zn^{2+} ions in the MBLs. No inhibition of the serine β-lactamases occurred at inhibitor concentrations as high as 250 μM, even after preincubation of the enzyme and the inhibitor. The lack of inhibitory activity of these derivatives against bacterial serine β-lactamases supported this hypothesis.

Good inhibition of the hog dehydropetidase (Table 1) was observed for both **2** and **3**. This mammalian Zn^{2+}-containing peptidase is a pseudo-β-lactamase that can hydrolyze carbapenems. Thus, similarity in inhibition profiles between the mammalian metallo-dehydropeptidase and the bacterial MBLs might be expected. Note, however, that carbapenem-hydrolyzing activity alone is not a sufficient requirement for inhibition potential by these natural products. The SME-1 serine β-lactamase that can hydrolyze carbapenems was not inhibited by the dipicolinic acid analogs, confirming that chelation is the operative mechanism. Both mammalian carboxypeptidases were modestly inhibited by **2** and **3** (Table 1). Inhibition of all the mammalian metalloenzymes was reversed to some degree when 1.0 μM Zn^{2+} was added, with 2–7-fold increased IC_{50} values. The synergistic activity of the inhibitors was limited in whole-cell assays. The addition of compound **2** to piperacillin (1:1 ratio) lowered the piperacillin MIC against *S. maltophilia* from >512 to 256 mg l^{-1}; however, the presence of a serine β-lactamase in the strain prevented further protection of piperacillin. In an *Escherichia coli* strain containing the CcrA MBL, the piperacillin MIC of 8 mg l^{-1} was decreased to 4 mg l^{-1} when combined (1:1) with either compound **2** or **3**. When the inhibitory activity of these analogs was compared for the bacterial and mammalian metalloenzymes, higher inhibitor potency was seen with the enzymes displaying β-lactamase activity. Such an observation demonstrates the moderate selectivity of **2** and **3** for metalloenzymes with substrate specificities that include carbapenems. Although these inhibitors do not demonstrate sufficient selectivity or potency to be used clinically, they may provide the basis for future medicinal chemistry efforts to identify a new MBL inhibitor.

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