

# Molecular Discrimination of Type-I over Type-II Methionyl Aminopeptidases<sup>†</sup>

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Received October 20, 2004; Revised Manuscript Received June 23, 2005

**ABSTRACT:** Two residues that are conserved in type-I methionyl aminopeptidases (MetAPs) but are absent in all type-II MetAPs are the cysteine residues (*Escherichia coli* MetAP-I: C59 and C70) that reside at the back of the substrate recognition pocket. These Cys residues are 4.4 Å apart and do not form a disulfide bond. Since bacteria and fungi contain *only* type-I MetAPs while all human cells contain both type-I and type-II MetAPs, type-I MetAPs represent a novel antibiotic/antifungal target if type-I MetAPs can be specifically targeted over type-II. Based on reaction of the thiol-specific binding reagent 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) with the type-I MetAP from *E. coli* and the type-II MetAP from *Pyrococcus furiosus*, the type-I MetAP can be selectively inhibited. Verification that DTNB covalently binds to C59 in *EcMetAP-I* was obtained by mass spectrometry (MS) from reaction of DTNB with the C59A and C70A mutant *EcMetAP-I* enzymes. In addition, two inhibitors of *EcMetAP-I*, 5-iodopentaphosphonic acid (**1**) and 6-phosphonohexanoic acid (**2**), were designed and synthesized. The first was designed as a selective-C59 binding reagent while the second was designed as a simple competitive inhibitor of *EcMetAP-I*. Indeed, inhibitor **1** forms a covalent interaction with C59 based on activity assays and MS measurements, while **2** does not. These data indicate that type-I MetAPs can be selectively targeted over type-II MetAPs, suggesting that type-I MetAPs represent a new enzymatic target for antibacterial or antifungal agents.

Bacterial infections are a significant and growing medical problem in the United States and throughout the world, in part because an increasing number of disease-causing microbes have become resistant to antibiotics (1–4). Tuberculosis, staph, malaria, and childhood meningitis are just a few of the diseases that have become hard to treat with available antibiotics (3, 5). An important aspect of this problem is the development of antibiotic resistant bacteria and microorganisms that cause infections (3, 4). Even a single random gene mutation can have a large impact on an antibiotic's ability to kill a microorganism. Since most microbes replicate very rapidly, they can evolve rapidly; thus, a mutation that helps a microbe survive in the presence of an antibiotic will quickly become predominant throughout the microbial population. Microbes also commonly acquire genes, including those encoding for resistance, by direct transfer from members of their own species or from unrelated microbes. Since many of the broad-spectrum antibiotics contain  $\beta$ -lactam functional units that target enzymes involved in bacterial cell wall synthesis or pathways involved in cell replication, any new  $\beta$ -lactam antibiotic will likely be a structural variant of an existing compound, shortening its useful lifetime as an antibiotic (3, 5). To overcome this problem, new enzymatic targets must be located and small molecule inhibitors designed and synthesized to target these enzymes.

Methionyl aminopeptidases (MetAPs)<sup>1</sup> represent just such an enzymatic target since the biosynthesis of all prokaryotic

and eukaryotic proteins present in the cytosol starts with the initiator amino acid methionine. The cleavage of this N-terminal methionine residue by MetAPs plays a central role in protein synthesis and maturation (6, 7). The physiological importance of MetAP activity is underscored by the cellular lethality upon deletion of the MetAP gene in *Escherichia coli*, *Salmonella typhimurium*, and *Saccharomyces cerevisiae* (8–11). MetAPs are organized into two classes (types I and II) based on the absence or presence of an extra 62 amino acid sequence (of unknown function) inserted near the catalytic domain of type-II enzymes. The type-I MetAPs from *E. coli* (*EcMetAP-I*), *Staphylococcus aureus* (*SaMetAP-I*), and *Thermotoga maritima* (*TmMetAP-I*) and the type-II MetAPs from *Homo sapiens* (*HsMetAP-II*) and *Pyrococcus furiosus* (*PfMetAP-II*) have been crystallographically characterized (12–17). All five display a novel “pita-bread” fold with an internal pseudo 2-fold symmetry that structurally relates the first and second halves of the polypeptide chain to each other. Each half contains an antiparallel  $\beta$ -pleated sheet flanked by two helical segments and a C-terminal loop. Both domains contribute conserved residues to the metallo-active site. In all five structures, a bis( $\mu$ -carboxylato)( $\mu$ -aquo/hydroxo)dicobalt core is observed with an additional carboxylate residue at each metal site and a single histidine bound to Co1. Each of the

<sup>†</sup> This work was supported by the National Science Foundation (CHE-02408102, R.C.H.) and the National Institutes of Health (GM-56495, R.C.H.).

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<sup>1</sup> Abbreviations: *EcMetAP-I*, methionyl aminopeptidase from *Escherichia coli*; *PfMetAP-II*, methionyl aminopeptidase from *Pyrococcus furiosus*; *HsMetAP-II*, human methionyl aminopeptidase; DTNB, 5,5'-dithio-bis(2-nitrobenzoic acid); HEPEs, [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]; EDTA, ethylenediaminetetraacetic acid; ICP-AES, inductively coupled plasma atomic emission spectroscopy; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; WT, wild-type; MGMM, Met-Gly-Met-Met; MP-*p*-NA, Met-Pro-*p*-nitroanilide; MS, mass spectrometry.

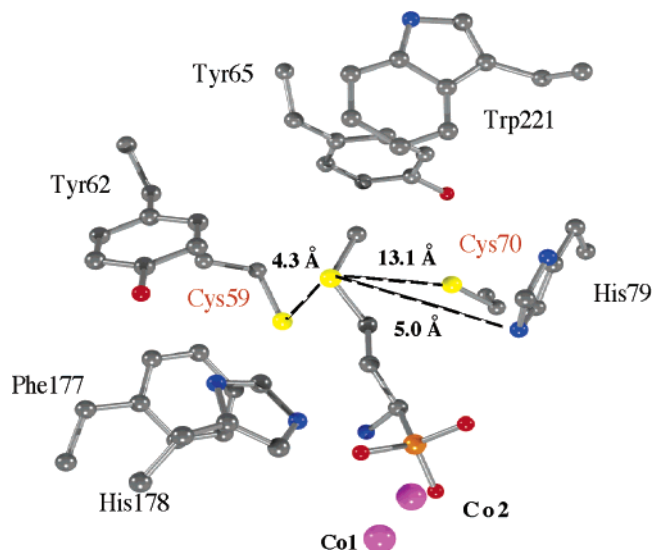


FIGURE 1: *EcMetAP-I* complex with methionine phosphonate bound. The sulfur atom of the methionine residue resides 4.3 Å from C59 and 13.1 Å from C70. Prepared from PDB file 1C23.

crystallographically characterized MetAPs has a closed pocket lined by hydrophobic residues adjacent to the metallo-active site. For *Ec*MetAP-I this pocket is composed of C59, C70, Y62, Y65, F177, and W221 (Figure 1); however, the residues present in this pocket for *Pf*MetAP-II are F50, N53, L160, I205, P234, and Y265. This pocket has been proposed to act as the binding site of the N-terminal methionine side chain required for substrate recognition and catalysis based on X-ray crystallography such as the structure of *Ec*MetAP-I bound by L-methionine phosphonate (Figure 1) (13, 14, 16).

Bacteria express only type-I MetAPs while archaea contain only the type-II enzyme (18). Eukaryotic cells, in contrast, have both type-I and type-II enzymes. Because type-I and type-II enzymes exhibit similar functions, there is redundancy in eukaryotic cells. For example, it has been shown that *S. cerevisiae* is viable if the gene encoding for the type-I MetAP is deleted but the type-II gene is present (18). Although type-I and type-II MetAPs are nearly structurally identical, one key difference has been identified that can be exploited to differentiate type-I from type-II enzymes (7), i.e. the two active site cysteine residues (e.g. C59 or C70 in *E. coli*) that exist only in type-I MetAPs (Figure 2) (13, 14, 16). Since bacteria contain only type-I MetAPs, compounds with selective inhibition for type-I MetAPs, by targeting the cysteine residues C59 and C70 (*E. coli* numbering), represent novel antibiotics. Herein, we provide “proof-of-concept” that Cys59 provides a molecular target to differentiate the type-I *Ec*MetAP enzyme from type-II MetAP enzymes and, therefore, small molecules that target Cys59 represent a new class of antibiotic.

## MATERIALS AND METHODS

**Purification of Recombinant EcMetAP-I and PfMetAP-II.** EcMetAP-I was purified as previously described (19, 20) and exhibited a single band on SDS-PAGE and a single symmetrical peak in matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS analysis indicating  $M_r = 29\,630 \pm 10$ . Protein concentrations were estimated from the absorbance at 280 nm using an extinction coefficient of  $16\,450\text{ M}^{-1}\text{ cm}^{-1}$ . PfMetAP-II was purified as previously

		59										70						
Type-I	<i>Ec</i>	C	L	G	Y	H	G	Y	P	K	S	V	C	I	S	I		
	<i>Hi</i>	C	L	N	Y	H	G	F	P	K	A	T	C	I	S	I		
	<i>St</i>	C	L	G	Y	H	G	Y	P	K	S	V	C	I	S	I		
	<i>Hp</i>	F	K	G	L	Y	G	F	P	N	S	V	C	M	S	L		
	<i>Sc1</i>	P	L	N	Y	Y	N	F	P	K	S	L	C	T	S	V		
	<i>Hs1</i>	P	L	N	Y	Y	N	F	P	K	S	C	C	T	S	V		
Type-II	<i>Pf</i>	F	-	-	-	-	-	-	-	-	-	-	P	V	N	L	S	I
	<i>Sc2</i>	F	-	-	-	-	-	-	-	-	-	-	P	T	G	L	S	L
	<i>Hs2</i>	F	-	-	-	-	-	-	-	-	-	-	P	T	G	C	S	L

(*Ec*) *E. coli*-I; (*Hi*) *H. influenzae*-I; (*St*) *S. typhimurium*-I. (All are Enterobacteria)

(*Hp*) *H. pylori*-I; (*Sc*) *S. cerevisiae*-I; (*Hs*) *Homo sapiens*-I.

(*Pf*) *P. furiosus*-II; (*Sc*) *S. cerevisiae*-II; (*Hs*) *Homo sapiens*-II.

FIGURE 2: Amino acid sequence alignment for selected MetAPs. C59 is conserved in Gram negative bacteria while C70 is conserved in both Gram positive and Gram negative bacteria. C59 is absent in type-I and type-II eukaryotic MetAPs. A 62 amino acid insert is present in type-II enzymes; thus the dashed lines do not indicate the number of amino acids missing between the conserved Cys residue.

reported (21). Protein concentrations were estimated from the absorbance at 280 nm using an extinction coefficient of  $21\,650\text{ M}^{-1}\text{ cm}^{-1}$ . The metal free enzyme (apo-*PfMetAP-II*) was prepared by concentrating the as-purified enzymes to a volume of  $\sim 5\text{ mL}$  after which EDTA was added to a final concentration of 10 mM. The resulting protein solution was dialyzed against 25 mM HEPES (2 L, pH 7.5) containing 10 mM EDTA and 150 mM KCl at  $4\text{ }^{\circ}\text{C}$  for 2 days with two buffer changes per day. This protein solution was then dialyzed against chelexed (Chelex-100 column) 25 mM HEPES buffer (2 L, pH 7.5) containing 150 mM KCl for 3 days against two buffer changes per day. The as-purified apo-*EcMetAP-I* enzyme was exchanged into 25 mM HEPES, pH 7.5, containing 150 mM KCl (Centricon-10, Millipore Corp). The apo-*PfMetAP-II* and apo-*EcMetAP-I* enzymes were inactive and found to contain no detectable metal ions via inductively coupled plasma atomic emission spectrometry (ICP-AES).

**Preparation of the C59A and C70A Altered *EcMetAP-I* Enzymes.** The C59A and C70A altered *EcMetAP-I* enzymes were generated using the Quick-Exchange mutagenesis kit (Stratagene, San Diego, CA). Briefly, the codons encoding the C59A and C70A mutations were placed in the wild-type (WT) expression system for *EcMetAP-I* containing the R175Q mutation (mutation eliminating a secondary thrombin cleavage site) using the following mutagenic primers: 5'-G GTT TCT GCT GCC CTC GGC TAT CAC G-3' (C59A) and 5'-CCGAAA TCC GTT GCC ATC TCT ATT AAT GAA GTG G-3' (C70A) with corresponding lower strand primers. Mutagenesis reactions and transformations were performed as prescribed in the manufacture's protocols with negligible modifications. Presence of the mutation was verified by DNA sequencing. Recombinant WT, C59A, and C70A *EcMetAP-I* were expressed in BL21 *E. coli* cells and purified as previously described (19, 20). Purified C70A and C59A *EcMetAP-I* exhibited a single band on SDS-PAGE and a single symmetrical peak in MALDI-TOF MS analysis indicating  $M_r = 29\,600 \pm 15$ . Protein concentrations were estimated from the absorbance at 280 nm using an extinction coefficient of  $16\,450\text{ M}^{-1}\text{ cm}^{-1}$ . Apo-*EcMetAP-I* C59A and C70A samples were exchanged into 25 mM HEPES, pH 7.5, containing 150 mM KCl (Centricon-10, Millipore Corp).

**Metal Content Measurements.** Metal analyses were performed on WT, C59A, and C70A *EcMetAP-I* samples that were typically 30  $\mu\text{M}$  using ICP-AES. Apo-*EcMetAP-I* samples were incubated under anaerobic conditions with  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  ( $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ :  $\geq 99.999\%$  Strem Chemicals, Newburyport, MA, or Aldrich) for 30 min prior to exhaustive dialysis under anaerobic conditions against Chelex-treated buffer as previously reported (19, 20).

**Synthetic Procedures.** All chemicals used in this study were purchased commercially and were of the highest quality available. The synthesis of 5-iodopentaphosphonic acid (**1**) as the disodium salt started with the diethyl ester of 5-bromopentaphosphonic acid. In this reaction, 5-bromopentaphosphonic acid diethyl ester (3.28 g, 12 mmol) was refluxed with hydroiodic acid (55–58%, 20 mL) for 24 h (22). The cooled postreaction mixture was diluted with water (100 mL) and extracted with dichloromethane ( $3 \times 50$  mL). The water was removed under vacuum, and the resulting residue was diluted with water (50 mL) and evaporated again. This procedure was repeated 3 times. The resulting dark red residue was dissolved in acetone (50 mL) and titrated with concentrated aqueous sodium hydroxide. A white precipitate formed and was filtered off, washed with acetone and diethyl ether, and dried in vacuo to give the disodium salt of 5-iodopentaphosphonate (3.70 g, 100%).  $^1\text{H}$  NMR ( $\text{D}_2\text{O}$ ): 3.15–3.20 (m, 2H), 1.80–1.94 (m, 2H), 1.50–1.70 (m, 4H).  $^{31}\text{P}$  NMR ( $\text{D}_2\text{O}$ ): 26.7. The synthesis of 6-phosphonohexanoic acid (**2**) was carried out according to the published procedure (23).

**Enzymatic Assay of *EcMetAP-I* and *PfMetAP-II*.** WT and the altered forms of *EcMetAP-I* were assayed for catalytic activity with the tetrapeptide MGMM as the substrate (8 mM) using an HPLC method as previously described (20). This method is based on the spectrophotometric quantification of the reaction product GMM following separation on a C8 HPLC column (Phenomenex, Luna; 5 m,  $4.6 \times 25$  cm). The kinetic parameter  $v$  (velocity) was determined at pH 7.5 by quantifying the tripeptide GMM at 215 nm in triplicate. Enzyme activities are expressed as units/mg, where one unit is defined as the amount of enzyme that releases 1  $\mu\text{mol}$  of product at 30  $^\circ\text{C}$  in 1 min. The inhibition constant ( $K_i$ ) for **2** was obtained by measuring the activity of a 1  $\mu\text{M}$  Co(II)-loaded *EcMetAP-I* solution with 1–15 mM MGMM in the presence of 0–15 mM **2**. These data were fit to a nonlinear competitive inhibition model using Sigma Plot Software.

A continuous assay was used to monitor the activity of Co(II)-loaded *EcMetAP-I* upon incubation with DTNB. In this assay, the hydrolysis of MP-*p*-NA was monitored spectrophotometrically at 405 nm based on the increase in absorbance of *p*-NA ( $\Delta\epsilon_{405}$  value of *p*-nitroaniline of  $10\,600\text{ M}^{-1}\text{ cm}^{-1}$ ) using a coupled assay (24). The reaction mixture consisted of a 10  $\mu\text{L}$  aliquot of a 5  $\mu\text{M}$  enzyme solution preincubated with 200  $\mu\text{M}$  DTNB, 2  $\mu\text{L}$  of a 2.0  $\mu\text{M}$  prolidase in 20 mM Tris containing 30% glycerol and 0.5 mM substrate in buffer (25 mM HEPES in Chelex-treated water at pH 7.5, 150 mM KCl) with a final volume of 150  $\mu\text{L}$ . Catalytic activities were determined with an error of  $\pm 10\%$ . In order to eliminate a possibility of inhibition of prolidase by DTNB, a control experiment was set up in which 5  $\mu\text{M}$  prolidase was incubated with 200  $\mu\text{M}$  DTNB. Samples were taken between 5 min and 5 h from the time of DTNB

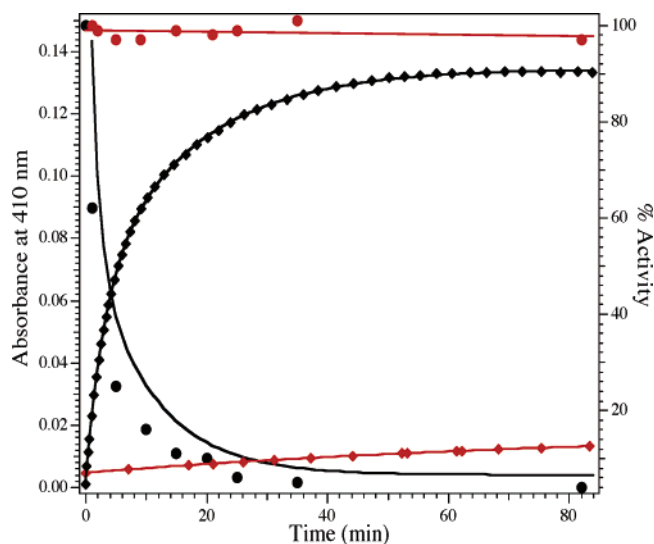


FIGURE 3: Activity as a function of time for *EcMetAP-I* (black circles) and *PfMetAP-II* (red circles) after reaction with 200  $\mu\text{M}$  DTNB. DTNB absorbance after reaction with *EcMetAP-I* (black diamonds) and *PfMetAP-II* (red diamonds). Reaction conditions: 1  $\mu\text{M}$  Co(II)-loaded MetAP in 50 mM HEPES buffer, pH 7.5, 150 mM KCl under anaerobic conditions.

addition and were checked for activity with 500  $\mu\text{M}$  MP-*p*-NA.

**MS Experiments.** *EcMetAP-I* samples before and after reaction with DTNB or **1** were analyzed by MALDI-TOF MS equipped with delayed ion extraction (25, 26). For the MALDI-TOF MS experiments, an ion source bias voltage of 24 kV and an extraction delay time of 1050 ns were employed. Ion extraction pulse voltages were selected to optimize mass resolution (via time lag focusing) for the largest  $m/z$  ion of interest in each sample. The ion signals generated from 100 individual laser shots (337 nm) were signal averaged to generate the resulting MALDI-TOF MS. MALDI-TOF MS were externally mass calibrated by the use of the flight times and known masses of the singly and doubly protonated ions produced by MALDI-TOF MS from a sample of *EcMetAP-I*. Co(II)-loaded *EcMetAP-I* after reaction with DTNB or **1** in HEPES buffer, pH 7.5, and 150 mM KCl were run down a C4 ZipTip (Millipore Corp., Bedford, MA) prior to MALDI-TOF analysis to remove excess buffer and salts. The ZipTip was first treated with 30 mL of a 75% acetonitrile/25% distilled, deionized (DDI) water solution followed by 40 mL of a 0.1% trifluoroacetic acid (TFA) in DDI water solution.

MS fingerprints of trypsin digested WT, C59A, and C70A *EcMetAP-I* samples before and after reaction with **1** or DTNB were obtained on a Waters Micromass MALDI-TOF MS (TofSpec 2E). Reflectron mode was used with a 20.00 kV operating voltage, 5000 V source voltage, 2400 V pulse voltage, and 2 GHz sampling rate. The samples for MS analysis were prepared in water by dialyzing them in a Slide-A-Lyzer mini dialysis unit. Catalytic amounts of trypsin were then added in situ before MS data were obtained. The buffer used for the trypsin in situ digest was  $\text{NH}_4\text{HCO}_3$  (pH = 8). The samples were then diluted 10-fold with acetonitrile: $\text{H}_2\text{O}$  (50:50). Masses for predicted peptides after trypsin digestion were determined using Peptide Mass software available at ExPASy Proteomics tools (<http://us.expasy.org/tools/peptide-mass.html>). Masses for up to three uncleaved sites were



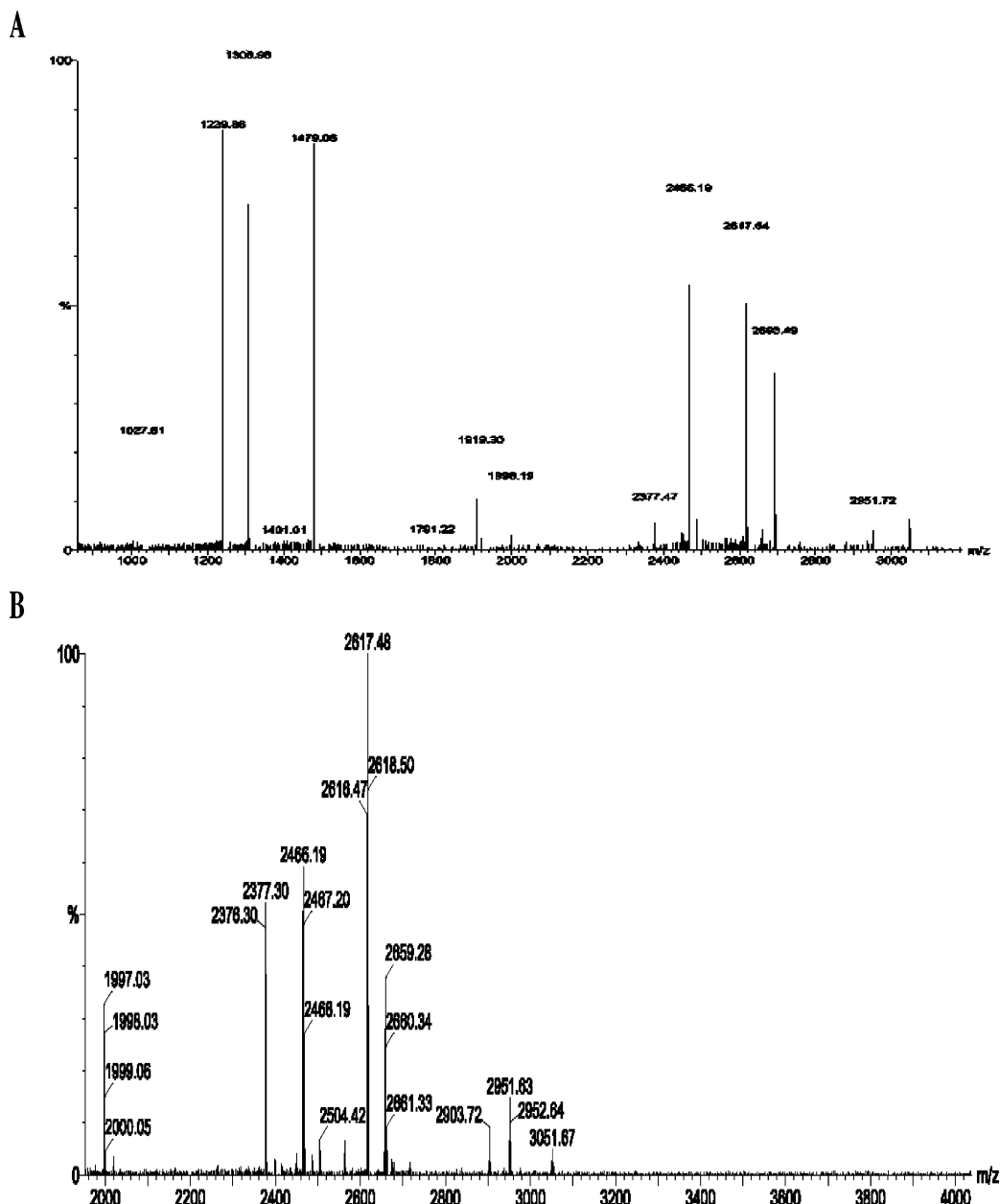


FIGURE 4: MALDI-TOF MS of tryptic digested samples of (A) Co(II)-loaded WT *EcMetAP-I* and (B) Co(II)-loaded WT *EcMetAP-I* + DTNB.

determined and compared to the experimentally observed masses.

**Spectroscopic Measurements.** All spectrophotometric measurements were performed on a Shimadzu UV-3101PC spectrophotometer equipped with a constant-temperature holder and a Haake (Type 423) constant-temperature circulating bath. The use of 200 mL, 1 cm path-length microcuvettes (QS, Hellma) stoppered with rubber septa facilitated the recording of the optical spectra under anaerobic conditions.

## RESULTS

**Inactivation of MetAP's by DTNB.** In order to determine if the covalent attachment of a reagent to C59 or C70 inactivates *EcMetAP-I*, the cysteine-specific chemical modification reagent, DTNB, was examined. A 200  $\mu$ M solution of DTNB was prepared in 50 mM HEPES buffer, pH 7.5, 150 mM KCl and added to a 5  $\mu$ M Co(II)-loaded *EcMetAP-I*

solution under anaerobic conditions. Upon the addition of DTNB to *EcMetAP-I*, the coupled prolidase hydrolysis of MP-*p*-NA by *EcMetAP-I* quickly decreased, and *EcMetAP-I* was completely inactive after only  $\sim 50$  min (Figure 3). Identical experiments, performed on *PfMetAP-II*, which does not contain any cysteine residues, revealed no inactivation due to DTNB even after several hours (Figure 3). Similarly, no loss in catalytic activity was observed for *HsMetAP-II* in the presence of 1 mM DTNB.

Reaction of DTNB with Cys residues on *EcMetAP-I* was also monitored at 410 nm. A 5  $\mu$ M Co(II)-loaded *EcMetAP-I* sample was reacted with 200  $\mu$ M DTNB (50 mM HEPES, pH 7.5, 150 mM KCl), and the change in absorbance at 410 nm was monitored as a function of time. After reaction for 200 min, no additional absorbance was observed, indicating that all exposed Cys residues had reacted within this time period. Using the known molar absorptivity for DTNB (13 600  $\text{M}^{-1} \text{cm}^{-1}$ ) the number of solvent exposed Cys

Table 1: Predicted and Observed  $m/z$  for Trypsin Digested *EcMetAP-I* in the Absence and Presence of DTNB

predicted $m/z$ for <i>EcMetAP-I</i>	position	#MC <sup>a</sup>	peptide sequence <sup>b</sup>	obsd $m/z$	
				<i>EcMetAP-I</i>	<i>EcMetAP-I</i> + DTNB <sup>c</sup>
3060.63	139–166	2	MVKPGINLREIGAAIQKFVEAEGFSVVR	3055.95	3051.67
2963.34	252–227	2	DRSLSAQYEHTIVVTDNGCEILTLRK	2951.72	2951.66
2893.03	44–67	0	ICNDYIVNEQHAVSACLGYHGYPK + <b>DTNB</b>		<b>2903.72</b> (C45 or C59)
2696.03	44–67	0	ICNDYIVNEQHAVSACLGYHGYPK	2693.49	2694.13
2659.85	167–189	0	EYCGHGIGQGFEHPQVLHYDSR	2659.12	2659.28
2618.02	20–43	0	LAAEVLEMIPEYVKPGVSTGELDR	2617.64	2617.48
2563.95	229–251	0	SLSAQYEHTIVVTDNGCEILTLR	2563.78	
2424.80	130–124	1	DGFHGDTSKMFIVGKPTIMGER	2455.10	2466.19
2377.82	190–211	0	ETNVVLKPGMTFTIEPMVNAGK	2377.47	2377.3
2197.29	68–86	0	SVCISINEVVCHGIPDDAK + <b>DTNB</b>		
2000.29	68–86	0	SVCISINEVVCHGIPDDAK	1998.19	1997.03
				1919.30	1919.30
1479.84	112–124	0	MFIVGKPTIMGER	1479.05	1479.80

<sup>a</sup> The MC number indicates the number of uncleaved trypsin cleavage sites within the peptide of the given mass. <sup>b</sup> Predicted peptides after trypsin digestion were determined using the Peptide Mass software available at ExPASy Proteomics tools (<http://us.expasy.org/tools/peptide-mass.html>).

<sup>c</sup> The boldface peptides indicate those with Cys residues which are provided in parentheses.

residues was calculated using the maximum absorption (0.13) observed at 410 nm (27). The number of modified Cys residues in *EcMetAP-I* is 1.2. Since seven total Cys residues exist in *EcMetAP-I*, another surface accessible Cys residue may react with DTNB, so the inactivity due to reaction with DTNB and Cys residues besides C59 or C70 cannot be ruled out. In order to clarify the roles of C59 and C70 in DTNB inactivation, MALDI-TOF MS data were obtained on trypsin digests of WT *EcMetAP-I* in the absence and presence of 200  $\mu$ M DTNB (Figure 4; Table 1). The only new mass peak observed in the *EcMetAP-I*-DTNB complex occurred at 2900  $m/z$ , consistent with the peptide fragment containing 23 amino acids (44 to 67) including C45 and C59, indicating that one of these two Cys residues reacted with DTNB.

***EcMetAP-I* Specific Inhibitors.** Since *EcMetAP-I* is inactivated by the cysteine-specific chemical modification reagent DTNB, two small molecules 5-iodopentaphosphonic acid (**1**) and 6-phosphonohexanoic acid (**2**) were designed and synthesized (Figure 5). Reaction of WT *EcMetAP-I* with a 1 mM sample of **1** for ~12 h resulted in the complete and irreversible loss of catalytic activity (Figure 5). As expected, **2** did not significantly alter the catalytic activity of *EcMetAP-I* but functions as a simple, weak competitive inhibitor ( $K_i = 4.9$  mM). Significantly, no inhibition was observed for *PfMetAP-II* upon the addition of either **1** or **2**. MALDI-TOF MS spectrometric analysis performed on *EcMetAP-I* in the absence of **1** provided a molecular mass of  $29\,630 \pm 10$ , whereas, upon reaction of *EcMetAP-I* with **1**, a small shift in the mass peak was observed, suggesting the addition of ~149 mass units. These data match well with the calculated mass of **1** after reaction with *EcMetAP-I*. Therefore, **1** is covalently bound to *EcMetAP-I*. Additional MS data were obtained on trypsin digests of *EcMetAP-I* in the absence and presence of **1**. These data revealed that **1** binds only to the C45/C59 containing peptide (Table 2; mass 2904).

***C59A* and *C70A* Altered *EcMetAP-I* Enzymes.** In order to determine if C59 is the target for **1**, we prepared and purified both the C59A and C70A *EcMetAP-I* altered enzymes. Both the C59A and C70A *EcMetAP-I* altered enzymes bind one equivalent of Co(II) tightly, based on ICP-AES analysis. Their kinetic parameters are listed in Table 3. Reaction of a 10 mM solution of **1** with C70A *EcMetAP-I* results in the

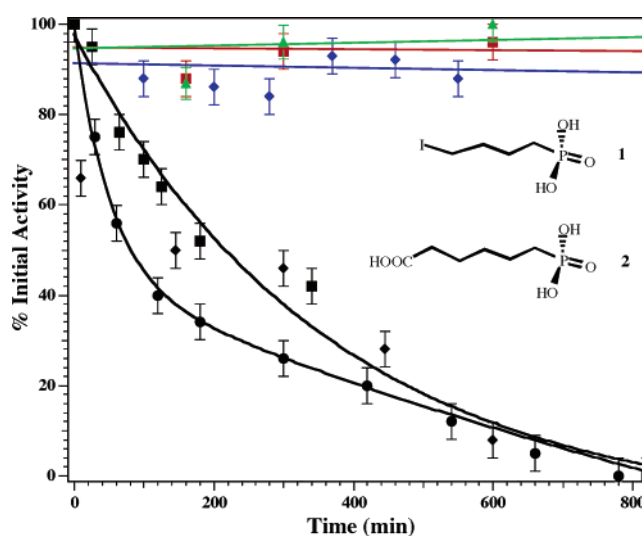


FIGURE 5: Reaction of **1** with WT *EcMetAP-I* (black squares 30 mM, black diamonds 10 mM), WT *PfMetAP-II* (green triangles, 10 mM), C59A *EcMetAP-I* (red squares, 10 mM), and C70A *EcMetAP-I* (black circles, 10 mM). Reaction of a 30 mM solution of **2** with WT *EcMetAP-I* (blue diamonds). Reaction conditions: 1  $\mu$ M Co(II)-loaded MetAP in 50 mM HEPES buffer, pH 7.5, 150 mM KCl under anaerobic conditions.

complete loss of catalytic activity after ~12 h; however, **1** does not decrease the catalytic activity observed for C59A *EcMetAP-I* even after more than 8 h (Figure 5). These data suggest that **1** covalently modifies C59. Additional proof comes from MS data obtained on trypsin digests of C59A and C70A *EcMetAP-I* that were reacted with 1 mM **1** for 12 h. These data indicated that **1** does not covalently bind to the C59A *EcMetAP-I* altered enzyme but does covalently modify the C70A *EcMetAP-I* enzyme (mass 2903). Taken together, these data establish that **1** covalently binds C59, which is located in the *EcMetAP-I* substrate-binding pocket.

## DISCUSSION

Bacterial infections (such as tuberculosis, gonorrhea, malaria, and childhood meningitis, to name a few) are a significant and growing medical problem in the United States and throughout the world. In order to combat this problem, new enzymatic targets must be identified and novel inhibitors that function as antibiotics need to be designed and synthesized.

Table 2: Predicted and Observed  $m/z$  for Trypsin Digested *EcMetAP-I* and the C59A and C70A Altered *EcMetAP-I* Enzymes in the Presence and Absence of **1**

predicted $m/z$ for <i>EcMetAP-I</i>	position	#MC <sup>a</sup>	peptide sequence <sup>b</sup>	obsd $m/z$		
				MetAP + <b>1</b> <sup>c</sup>	C59A MetAP + <b>1</b> <sup>c</sup>	C70A MetAP + <b>1</b> <sup>c</sup>
3060.63	139–166	2	MVKPGINLREIGAAIQKFVEAEGFSVVR	3051.40	3051.36	3047.25
2963.34	252–227	2	DRSLSAQYEHTIVVTDNCGCEILTLRK	2963.25	2963.15	2951.41
2904.03	44–67	0	ICNDYIVNEQHAVSACLGYPK + <b>Co</b> + <b>1</b>	<b>2903.49</b> (C59)		<b>2903.51</b> (C59)
2696.03	44–67	0	ICNDYIVNEQHAVSACLGYPK	2696.25	<b>2660.46</b> (C59A)	2700.35
2659.85	167–189	0	EYCGHGIGQGFEHPQVLHYDSR	2658.18	2659.42	2659.23
2618.02	20–43	0	LAAEVLEMIEPYVKPGVSTGELDR	2617.34	2616.60	2617.34
2505.99	190–212	1	ETNVVLKPGMTFTIEPMVNAGKK	2509.34	2509.45	2505.28
2424.80	130–124	1	DGFHGDTSKMFIVGKPTIMGER	2465.15	2466.20	2460.25
2377.82	190–211	0	ETNVVLKPGMTFTIEPMVNAGK	2377.23	2377.16	2377.16
2346.56	90–111	1	DGDIVNIDVTVIKDGFGDTSK	2346.17		2356.16
2149.29	68–86	0	SVCISINEVVCHGIPDDAK + <b>1</b>			
2000.29	68–86	0	SVCISINEVVCHGIPDDAK	1997.99	1999.19	<b>1968.31</b> (C70A)
				1919.06	1919.31	1920.04
1479.84	112–124	0	MFIVGKPTIMGER	1478.85	1479.04	1478.84

<sup>a</sup> The MC number indicates the number of uncleaved trypsin cleavage sites within the peptide of the given mass. <sup>b</sup> Predicted peptides after trypsin digestion were determined using the Peptide Mass software available at ExPASy Proteomics tools (<http://us.expasy.org/tools/peptide-mass.html>).

<sup>c</sup> The boldface peptides indicate those with Cys residues which are provided in parentheses.

Table 3: Kinetic Constants for Co(II)-Loaded Wild-Type C59A and C70A *EcMetAP-I* toward MGMM at 30 °C and pH 7.5

kinetic constants	wild-type	C59A	C70A
$K_m$ (mM)	$3.2 \pm 0.2$	$3.0 \pm 0.2$	$6.3 \pm 0.2$
$k_{cat}$ (s <sup>-1</sup> )	18	22	1.8
$k_{cat}/K_m$ (M <sup>-1</sup> s <sup>-1</sup> )	5630	7270	290
SA (units/mg)	$37 \pm 3$	$32 \pm 0.5$	$3.6 \pm 0.2$

Bacteria and fungi contain only type-I MetAPs while eukaryotic cells contain both type-I and -II MetAPs; therefore, type-I MetAPs represent a potential antibiotic target. Although type-I and -II MetAPs are nearly structurally identical, two cysteine residues that are strictly conserved in type-I MetAPs but are absent in all type-II MetAPs (*EcMetAP-I*: C59 and C70) reside at the back of the putative substrate-binding pocket (Figure 1) (13–16, 19–21, 28–30). These Cys residues are 4.4 Å apart and, therefore, do not form a disulfide bond. In order to determine if either of these Cys residues (C59 or C70) can be specifically targeted by small molecule inhibitors, *EcMetAP-I* was reacted with the cysteine-specific chemical modification reagent DTNB. Upon reaction with DTNB, *EcMetAP-I* quickly loses catalytic activity and is completely inactive after ~50 min. Identical experiments, performed on *PfMetAP-II*, which does not contain cysteine residues in the substrate recognition pocket, revealed no inactivation due to DTNB even after several hours. Similarly, no loss in catalytic activity was observed for the human type-II MetAP even in the presence of 1 mM DTNB. These data indicate that type-I MetAPs can be specifically inhibited by covalent modification of Cys residues and support the hypothesis that the conserved Cys residues in type-I MetAPs can be exploited as targets for antibacterial agents.

Several potential pitfalls exist if chemical modification studies are overinterpreted. First, a specific reagent may not only modify residues in the active site of the enzyme but also react with other amino acid groups on the enzyme surface. Since five additional Cys residues exist in *EcMetAP-I* (C45, C78, C126, C169, C245), reaction of DTNB with other Cys residues that potentially can inhibit catalytic activity cannot be ruled out. However, the number of

covalently modified Cys residues was calculated to be 1.2 based on the absorbance of DTNB observed at 410 nm (27). These data suggest that, under the reaction conditions used, DTNB reacts with only one Cys residue in *EcMetAP-I*. In order to clarify which Cys residue in *EcMetAP-I* was covalently modified after reaction with DTNB, MS data were obtained on trypsin digests of WT *EcMetAP-I* in the absence and presence of DTNB. These data indicated that DTNB binds only to the peptide fragment of the WT enzyme containing C45 and C59 (mass 2900). Interestingly, MS data reveals that the *EcMetAP-I* peptide fragment containing C70 is not covalently bound by DTNB. In addition, the MS data indicated that, under the reaction conditions used, DTNB does not covalently modify any of the other Cys residues present in the polypeptide chain of *EcMetAP-I*. Combination of these data with the kinetic results suggests that C59 may be the primary target for cysteine-specific chemical modification reagents.

In order to unequivocally show that C59 is the target for DTNB and not C45, we prepared and purified both the C59A and C70A *EcMetAP-I* altered enzymes. Both the C59A and C70A *EcMetAP-I* altered enzymes bind one equivalent of Co(II) tightly, based on ICP-AES analysis, which is identical to WT *EcMetAP-I*. Interestingly,  $k_{cat}$  does not change significantly for C59A *EcMetAP-I* but decreases 10-fold for the C70A *EcMetAP-I* enzyme. Moreover, the  $K_m$  value obtained for C59A *EcMetAP-I* does not change; however,  $K_m$  doubles for the C70A altered *EcMetAP-I* enzyme. These data suggest that C70 may play a role in substrate recognition and binding. Reaction of DTNB with C59A *EcMetAP-I* under strict anaerobic conditions results in the loss of catalytic activity. Interestingly, the observed inactivation of C70A *EcMetAP-I* is identical to wild-type enzyme. Based on MS data, the expected mass shift was observed for DTNB binding to C59 but not C70. These data confirm the covalent modification of C59 and not C70 or C45.

Since *EcMetAP-I* is inactivated by DTNB binding to C59, we designed small a molecule inhibitor that potentially targets C59 based on previously reported kinetic, spectroscopic, and X-ray crystallographic data. First, phosphonic acid derivatives



of L-methionine have been shown to be weak competitive inhibitors of *EcMetAP-I*; therefore, the molecules listed in Figure 5 will bind to the active site metal ion in *EcMetAP-I* (13, 14, 16, 28). Second, based on the X-ray crystallographic data of Lowther et al. (13, 14, 16, 28) on various inhibited forms of *EcMetAP-I*, side-chain lengths of 4 and 5 appear to fit most efficiently in the substrate-binding pocket (13, 14, 16, 28). Third, both C59 and C70 must be reduced for *EcMetAP-I* to be fully active, suggesting that these Cys residues must be reasonable nucleophiles (31). The test compound, 5-iodopentaphosphonic acid (**1**), was designed not only to bind the divalent metal ion in the active site of MetAPs but also to covalently modify C59 or C70 via an electrophilic carbon center on **1**, that is presumably positioned adjacent to C59 or C70 allowing nucleophilic attack to occur. As a control, we synthesized a derivative of **1** in which the iodide was replaced with a carboxylate residue (6-phosphonohexanoic acid; **2**). This molecule was designed to bind to the active site divalent metal ion but not to covalently modify either C59 or C70.

Reaction of WT *EcMetAP-I* with **1** for ~12 h resulted in the complete and irreversible loss of catalytic activity. As expected, **2** did not significantly alter the catalytic activity of *EcMetAP-I* but functioned as a simple, weak competitive inhibitor ( $K_i = 4.9$  mM). Significantly, *PfMetAP-II* was not inhibited by either **1** or **2**. MALDI-TOF analysis was also performed on *EcMetAP-I* in the presence and absence of **1**. In the absence of **1**, a molecular mass of  $29\,630 \pm 10$  was obtained, whereas, upon reaction of *EcMetAP-I* with **1**, a small shift in the mass peak was observed, suggesting the addition of ~149 mass units. These data match well with the calculated mass of **1** after reaction with *EcMetAP-I*. Additional MS data were obtained on trypsin digests of *EcMetAP-I* in the absence and presence of **1**, which showed that **1** binds only to the peptide containing C45 and C59 ( $m/z$  2904). Therefore, **1** is covalently bound to *EcMetAP-I*. The chloro and bromo derivatives of **1** were also prepared but did not covalently modify *EcMetAP-I*. The lack of reactivity by the chloro and bromo derivatives of **1** presumably occurs because the carbon center to which they are attached is not electrophilic enough to react with C59.

In order to unequivocally show that **1** binds only to C59, **1** was reacted with C70A *EcMetAP-I*, resulting in the complete loss of catalytic activity after ~12 h, similar to WT *EcMetAP-I*. However, **1** did not decrease the observed activity of C59A *EcMetAP-I* even after ~8 h. These data are consistent with the covalent modification of *only* C59. Additional proof comes from MS data obtained on trypsin digests of C59A and C70A *EcMetAP-I* that were reacted with **1** for ~12 h. These data revealed that **1** does not covalently modify the C59A *EcMetAP-I* altered enzyme but does covalently modify the C70A *EcMetAP-I* altered enzyme (mass 2903). Taken together, these data establish that **1** covalently binds *only* to C59, which is located in the *EcMetAP-I* substrate-binding pocket.

In conclusion, the data presented herein provide "proof-of-concept" that type-I MetAPs can be selectively inhibited over type-II MetAPs. Since bacteria contain only type-I MetAPs while all eukaryotic cells contain both type-I and type-II MetAPs, compounds that selectively inhibit the Cys residue at position C59 (*E. coli* numbering) represent a novel class of antibiotics. The inhibitor described herein (**1**)

covalently modifies the *EcMetAP-I* enzyme but does not inhibit type-II MetAPs by selectively binding to C59. It is anticipated that compounds that target C59 will not inhibit *HsMetAP-I* or *HsMetAP-II* since neither enzyme contains this active site Cys residue. Therefore, such compounds as **1** represent a new and novel class of antibiotic.

## ACKNOWLEDGMENT

We would like to thank Professor Robert Brown for obtaining the MALDI-TOF data.

## SUPPORTING INFORMATION AVAILABLE

MALDI-TOF MS of WT *EcMetAP-I* + **1**, C59A *EcMetAP-I* + **1**, and C70A *EcMetAP-I* + **1**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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BI047752G