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Small-molecule inhibitors of bacterial metallo-β-lactamases

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Chapter 3

Small-molecule aminocarboxylic acids as metallo-β-lactamase inhibitors; Part II.

Parts of this chapter have been published in:

Tehrani, K. H. M. E.,* Fu, H.,* Brüchle, N. C., Mashayekhi, V., Prats Luján, A., van Haren, M. J., Poelarends, G. J., and Martin, N. I. (2020) Aminocarboxylic acids related to aspergillomarasmine A (AMA) and ethylenediamine-*N*,*N*'-disuccinic acid (EDDS) are strong zinc-binders and inhibitors of the metallo-beta-lactamase NDM-1. *Chem. Comm.* **56**, 3047–3049.

1. Introduction

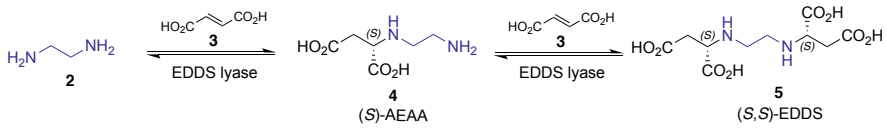
Antibiotic resistance is a global public health concern with an increasing economic burden.[1,2] Among gram-negative pathogens, β-lactam resistance due to the production of β-lactamases is a major cause of antibiotic resistance.[3] Based on their mechanism of β-lactam hydrolysis, β-lactamases can be classified as serine- or metallo-β-lactamases (SBLs and MBLs respectively). While SBLs hydrolyze β-lactams via an active site serine nucleophile, MBLs do so via a water molecule coordinated with active site zinc ion(s).[4] While there are clinically used SBL inhibitors available to counteract the infections caused by SBL-producing bacteria,[5] there are currently no approved MBL inhibitors available.

Recent screening efforts led to the identification of aspergillomarasmine A (AMA, entry **1a**, table 1) as a potent inhibitor of the clinically relevant NDM- and VIM-type MBLs.[6] This finding was followed by reports describing the chemical synthesis of AMA and its structural analogs.[7–11] Among them, synthetic routes using either a key *N*-nosyl protected aziridine intermediate[10] or a cyclic sufamidate[9] furnished AMA in relatively few steps and the highest reported yields (overall yields of 28% and 19% respectively).

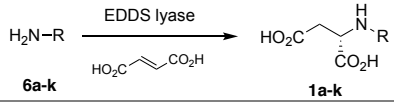
Recently, our collaborators in the Poelarends group (Groningen) reported that ethylenediamine-*N*,*N*'-disuccinic acid (EDDS) lyase naturally catalyzes a reversible two-step sequential addition of ethylenediamine (**2**) to two molecules of fumaric acid (**3**), giving (*S*)-*N*-(2-aminoethyl)aspartic acid (AEAA, **4**) as an intermediate and (*S*,*S*)-EDDS (**5**) as the final product (table 1A).[12] EDDS lyase was subsequently found to have broad substrate promiscuity,[13–15] accepting a wide range of amino acids with terminal amino groups (**6a**–**k**) for regio- and stereoselective addition to fumarate, thus providing a straightforward biocatalytic method for the asymmetric synthesis of AMA (**1a**), AMB (**1b**), and related aminocarboxylic acids (**1c**–**k**, table 1B).[13] To further explore the substrate scope of EDDS lyase, as well as to prepare a small library of EDDS derivatives as potential NDM-1 inhibitors,[16] in this chapter we describe the MBL-inhibitory activity and zinc binding affinity of AMA and AMB analogs as well as a series of EDDS analogs prepared by the Poelarends group via the EDDS-lyase catalyzed reaction of fumaric acid with various diamines containing different aliphatic linkers between the two amino functional groups (**7a**–**i**) (table 2).

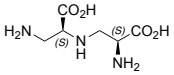
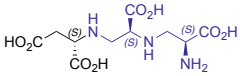
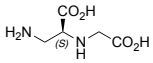
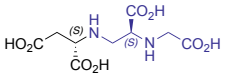
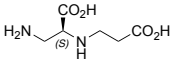
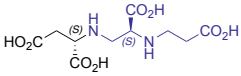
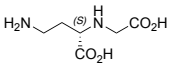
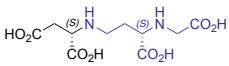
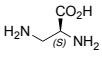
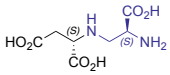
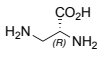
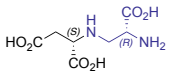
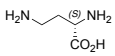
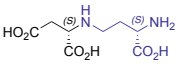
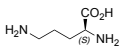
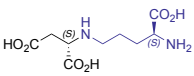
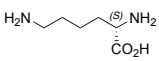
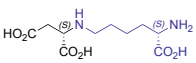
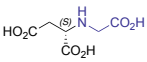
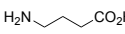
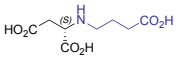
Table 1. Stereoselective C–N bond-formation reactions catalyzed by EDDS lyase

A)



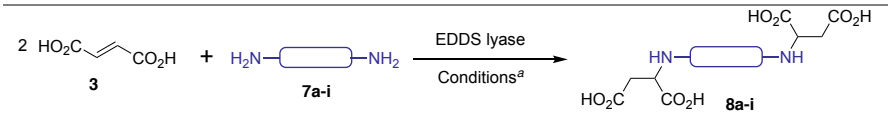
B)

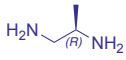
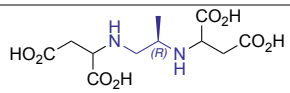
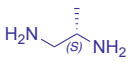
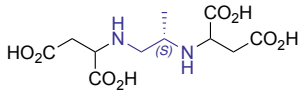
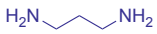
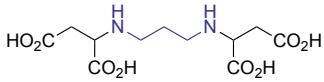
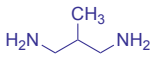
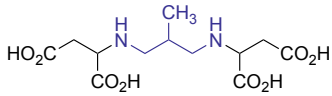
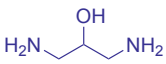
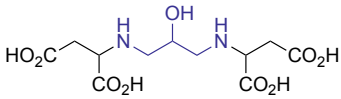
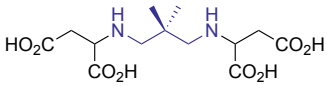
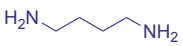
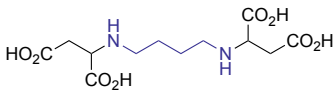
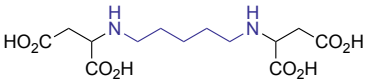
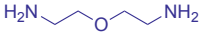
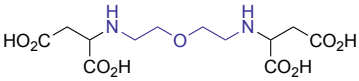


	**6a**		**1a** (AMA)
	**6b**		**1b** (AMB)
	**6c**		**1c**
	**6d**		**1d**
	**6e**		**1e** (toxin A)
	**6f**		**1f**
	**6g**		**1g**
	**6h**		**1h**
	**6i**		**1i**
	6j		**1j**
	**6k**		**1k**

(**A**) Natural reaction catalyzed by EDDS lyase. (**B**) Previously reported[12,13] analogs of AMA, AMB, and toxin A prepared using the EDDS lyase methodology and here investigated as NDM-1 inhibitors.

Table 2. Enzymatic synthesis of EDDS analogs



Entry		Diamine		Product[b]	Conv.[c] (yield[d])[%]
1	**7a**		**8a**		75 (31)
2	**7b**		**8b**		70 (21)
3	**7c**		**8c**		74 (33)
4	**7d**		**8d**		67 (31)
5	**7e**		**8e**		80 (60)
6	**7f**		**8f**		47 (26)
7	**7g**		**8g**		83 (32)
8	**7h**		**8h**		0
9	**7i**		**8i**		0

[a]Conditions and reagents: reaction mixture (15 mL) consisted of fumaric acid (3, 60 mM), a diamine substrate (**7a**–**i**, 10 mM) and purified EDDS lyase (0.05 mol% based on diamine) in 50 mM Na_2HPO_4 buffer (pH 8.5). The reaction mixture was incubated at room temperature for 48 h (**7a**–**e** and **7g**) or 96 h (**7f** and **7h**–**i**). [b]Absolute stereochemistry of products not determined. [c]Conversion yields based on comparing ^{1}H-NMR signals of substrates and corresponding products. [d]Isolated yield after ion-exchange chromatography.

2. Results and discussion

Synthetic experiments revealed that diamine substrates with two to four atoms between the two amino groups (**7a**–**g**) were well accepted as substrates by EDDS lyase, giving good conversions (47–83%) and yielding the corresponding aminocarboxylic acid products (**8a**–**g**) in 21–60% isolated yield (table 2, entries 1–7). Hence, EDDS lyase has a broad diamine scope, allowing the two-step sequential addition of appropriate diamines to fumaric acid, providing a powerful synthetic tool for the preparation of valuable aminocarboxylic acids. However, the elongated diamines with five atoms between the two amino groups (**7h**–**i**) were not accepted as substrates by EDDS lyase (table 2, entries 8 and 9).

The ability of the AMA and EDDS analogs to inhibit NDM-1 was evaluated using a fluorescence-based assay previously described by Schofield and coworkers.[17] This assay makes use of a cephalosporin substrate (known as FC5) which upon hydrolysis releases 7-hydroxycoumarin. The well characterized NDM-1 inhibitors AMA, EDTA, and dipicolinic acid (DPA) were used as positive controls. In general, most of the AMA and EDDS analogs tested showed potent activity against NDM-1 with IC_{50} values ranging from 1.3 μM to 18.3 μM (table 3).

Compared with its analogs **8a**–**g**, EDDS (**5**) proved to possess the highest activity (IC_{50} = 2.21 μM). Modifications to the central aliphatic spacer in length or steric bulk (or both) were generally tolerated. However, elongation of the linker to four methylene units (**8g**) led to a complete loss of activity. The inhibitory activity of the naturally occurring AMB (**1b**) was also promising (IC_{50} = 2.63 μM). Insertion of a methylene group (as in compounds **1c** and **1d**) maintained the activity leading to equipotent new AMB analogs.

Toxin A (**1e**) is believed to be the biosynthetic precursor of the related fungal aminocarboxylic acids AMA and AMB.[18] We found low IC_{50} values for toxin A (**1e**) and its diastereomer **1f** (IC_{50} = 2.33 μM and 2.89 μM respectively). Replacing the diaminopropionic acid moiety with the much simpler glycine unit as in **1j** led to a slight reduction of potency. Notably, elongation of the aliphatic spacers in both **1e** and **1j**, to generate compounds **1g**–**i** and **1k**, resulted in further or complete loss of NDM-1 inhibitory activity.

The majority of MBL inhibitors reported to date owe their activity to an ability to bind zinc. In general, MBL inhibitors either coordinate with zinc ions within the MBL active site or, if they are strong enough chelators, actively strip zinc from the MBL active site rendering the

Table 3. Activity of AMA and EDDS analogs against NDM-1 and an *E. coli* strain producing the same enzyme

Compound	IC_{50} (μM)[a]	RC (μM)[b,c]	FICI[c,d]
1a (AMA)	0.94 ± 0.11	50	0.063
1b (AMB)	2.63 ± 0.10	50	0.063
1c	1.35 ± 0.12	200	0.156
1d	1.37 ± 0.04	100	0.094
1e	2.33 ± 0.18	>400	>0.281
1f	2.89 ± 0.24	>400	>0.281
1g	18.34 ± 3.67	>400	>0.281
1h	>400	>400	>0.281
1i	>400	>400	>0.281
1j	7.87 ± 0.29	>400	>0.281
1k	>400	>400	>0.281
5 (EDDS.3Na)	2.21 ± 0.39	25	0.047
8a	4.33 ± 0.11	100	0.094
8b	9.65 ± 0.16	400	0.281
8c	3.11 ± 0.19	>400	>0.281
8d	2.85 ± 0.10	>400	>0.281
8e	3.50 ± 0.16	>400	>0.281
8f	2.85 ± 0.04	200	0.156
8g	>400	>400	>0.281
EDTA.2Na	1.25 ± 0.06	25	0.047
DPA	4.94 ± 0.22	100	0.094

[a]The half-maximal inhibitory concentration of the compounds tested against NDM-1 using FC5 as substrate. [b]RC (rescue concentration): the lowest concentration of the inhibitor that resensitizes the bacteria to meropenem. [c]The test microorganism was *E. coli* RC0089, an NDM-1 positive patient isolate with an MIC for meropenem of 32 mg/mL. [d]FICI: fractional inhibitory concentration index. FICI < 0.5 indicates synergy (see main text for formula used to calculate FICI).

enzyme inactive.[19,20] We have previously shown that the zinc-binding capacity of MBL inhibitors can be conveniently quantified using isothermal titration calorimetry (ITC).[21] To this end, we next measured the zinc-binding affinity of the aminocarboxylic acid analogs listed in table 3. These studies were conducted by titrating a zinc sulfate solution into the test compound with the heat of binding monitored using a microcalorimeter. The relevant thermodynamic parameters thus obtained (K_d and ΔH) are presented in table 4. For compounds **1e**–**f**, **1j**, and **8c** strong zinc binding was established with K_d values in the nM range. Notably, in the case of compounds **1b**–**d**, **5**, **8a**, **8b**, **8d**, **8e**, and **8f**, the zinc binding interactions were found to be so strong (K_d < 100 nM) that only ΔH values could be accurately determined. By comparison, for **1h**, **1i**, **1k**, and **8g** the zinc binding was too weak to allow for a reliable determination of any thermodynamic parameters. The data thus obtained reveals a clear correlation between zinc-binding affinity and

Table 4. The thermodynamic parameters of Zn^{2+} binding to the aminocarboxylic acid derivatives

Compound	K_d (μM)	ΔH (kcal/mol)
1b	ND[a]	-8.51 ± 0.06
1c	ND[a]	-6.36 ± 0.05
1d	ND[a]	-11.2 ± 0.06
1e	0.181 ± 0.013	-6.81 ± 0.03
1f	0.240 ± 0.017	-6.18 ± 0.03
1g	2.810 ± 0.088	-7.12 ± 0.04
1h	ND[b]	ND[b]
1i	ND[b]	ND[b]
1j	0.828 ± 0.035	-5.28 ± 0.03
1k	ND[b]	ND[b]
5	ND[a]	-8.04 ± 0.06
8a	ND[a]	-11.7 ± 0.04
8b	ND[a]	-5.78 ± 0.05
8c	0.334 ± 0.031	-6.49 ± 0.05
8d	ND[a]	-7.51 ± 0.04
8e	ND[a]	-7.43 ± 0.04
8f	ND[a]	-11.7 ± 0.04
8g	ND[b]	ND[b]

[a]Under the experimental conditions used, K_d values below 100 nM cannot be accurately determined. Only ΔH could be reliably measured.

[b]ND: not determinable. No binding was observed or K_d was too high to allow an accurate determination of the thermodynamic parameters.

the IC_{50} values measured against NDM-1. These findings indicate that the major mechanism of NDM-1 inhibition for the aminocarboxylic acids here studied can be attributed to their ability to bind zinc.

The compounds were also tested for their ability to resensitize an NDM-1 producing *E. coli* isolate to meropenem, a clinically important carbapenem antibiotic. This NDM-1 expressing strain of *E. coli* was found to be highly resistant to carbapenem antibiotics with a minimum inhibitory concentration (MIC) of 32 μg/mL for meropenem. As a measure of potency, we determined the "rescue concentration" of each test compound (table 3) which provides an indication of synergy.[9] Rescue concentration is defined as the lowest concentration of an MBL inhibitor that can resensitize a resistant strain to the antibiotic of interest when applied at its clinical breakpoint concentration (1 μg/mL for meropenem). In addition, the fractional inhibitory concentration index (FICI) values were determined for each compound and are provided in table 3. FICI values were established by applying the following formula where an FICI < 0.5 indicates synergy:

$$\text{FICI} = \frac{\text{MIC}_{\text{Meropenem}} \text{ in combination}}{\text{MIC}_{\text{Meropenem}} \text{ alone}} + \frac{\text{MIC}_{\text{Inhibitor}} \text{ in combination}}{\text{MIC}_{\text{Inhibitor}} \text{ alone}}$$

Among the EDDS analogs examined, **5** followed by **8a** were among the most potent synergizers. AMB (**1b**) and its related analogs **1c** and **1d** also showed potent to moderate activity. Interestingly, neither toxin A or its analogs (**1e**–**k**) demonstrated potent synergistic activity suggesting they may not be able to effectively access the enzyme target in the microorganism.

3. Conclusion

We here describe the application of a robust chemoenzymatic synthesis route in the preparation of a series of novel aminocarboxylic acids. A number of these compounds were found to be potent inhibitors of NDM-1, with inhibitory activities well correlated to their zinc binding ability. In addition, a number of the most active compounds demonstrated promising synergistic activity against an NDM-1 producing *E. coli* isolate when combined with meropenem. In the search for new agents to combat antibiotic resistance, chemoenzymatic methodologies such as those here described have the potential to provide access to novel inhibitors of metallo-β-lactamases of clinical relevance.

Acknowledgements

We would like to thank professor Gerry Wright for generously providing an authentic sample of aspergillomarasmine A, and Professor Gerrit Poelarends' team for preparing the aminocarboxylic acid molecules. The enzyme experiments were made possible thanks to the contributions of Vida Mashayekhi to express and purify NDM-1, and Matthijs van Haren to synthesize the FC5 substrate.

Experimental section

Enzyme inhibition assays

NDM-1 plasmid was a generous gift from Prof. Christopher Schofield (Oxford University). The overexpression and purification of NDM-1 has been described in chapter 2, and FC5 substrate (figure 1) was prepared according to a previously reported procedure.[17] In order to determine half-maximal inhibitory concentration (IC_{50}), the serially diluted aminocarboxylic derivatives were incubated with NDM-1 (50 pM) at 25 °C for 15 min. FC5 (0.5 μM) was then added to the wells and fluorescence was monitored immediately over 30-40 cycles (λ_{ex} 380 nm, λ_{em} 460 nm) on a Tecan Spark plate reader. The initial velocity data were used for IC_{50} curve-fitting using GraphPad prism 7 software (figure 2). Aspergillomarasmine A, EDTA and dipicolinic acid were used as positive controls. The buffer was 50 mM HEPES pH 7.2 supplemented with 0.01% Triton X-100 and 1 μM zinc sulfate. The assay microplate was μClear®, black half-area 96-well plate (Greiner Bio-one).

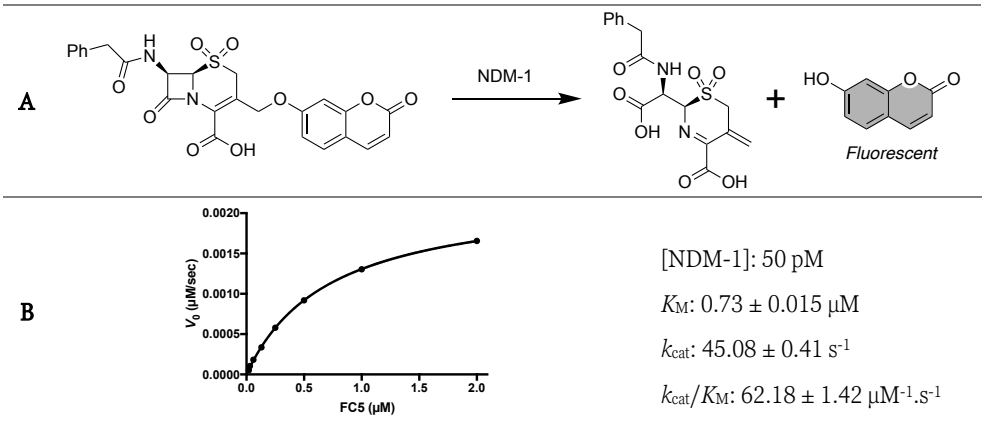


Figure 1. **A**. Hydrolysis mechanism of cephalosporin substrate known as FC5; **B**. Michaelis-Menten parameters of NDM-1 mediated hydrolysis of FC5.

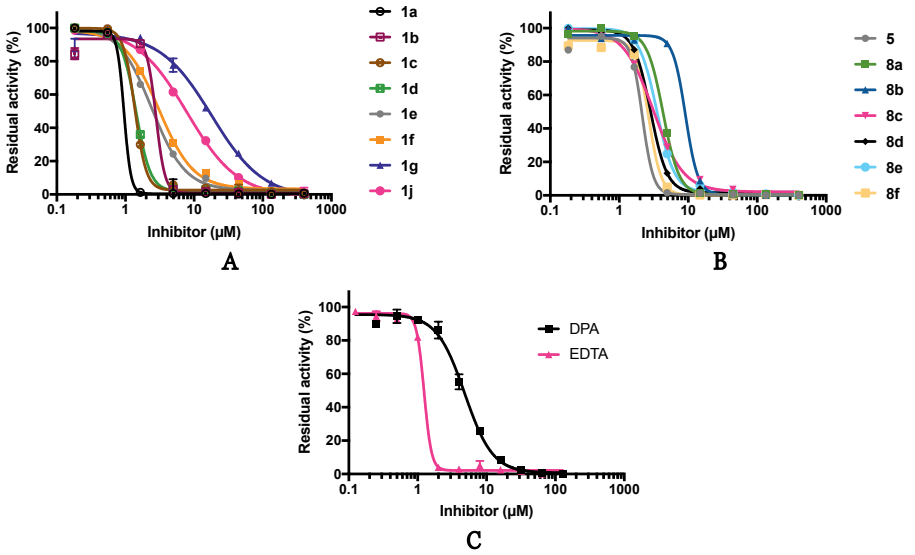
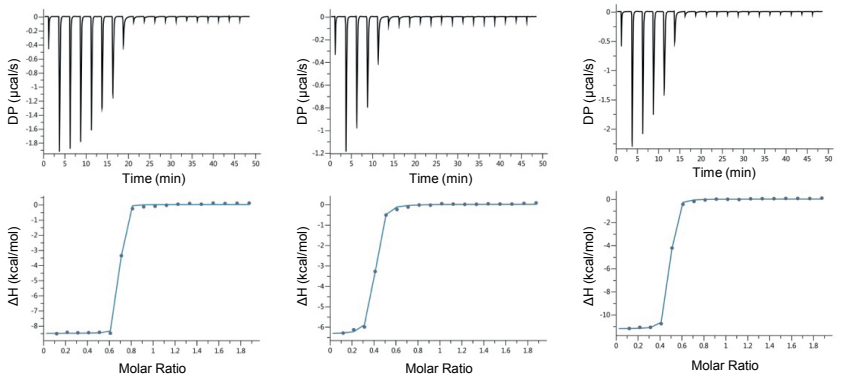


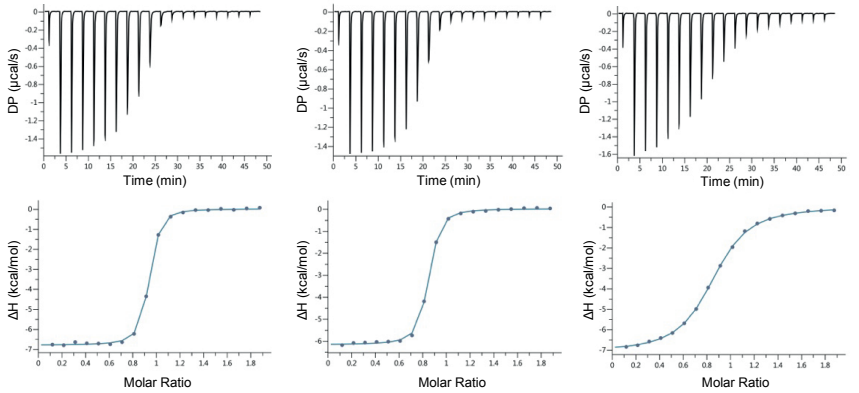
Figure 2. IC_{50} curves of **1a-j** (**A**), EDDS analogs (**B**), and control compounds (**C**) tested against NDM-1 (50 pM) using FC5 (0.5 μM) as substrate.

Isothermal titration calorimetry

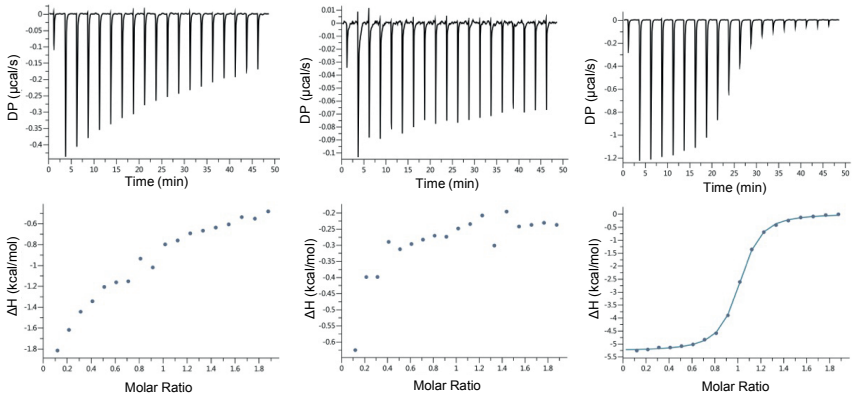
The titrations were performed on an automated PEAQ-ITC calorimeter (Malvern). Test compounds were dissolved in 20 mM tris pH 7.0. Zinc sulfate (1 mM) was titrated in the 0.1 mM solutions of the aminocarboxylic acids over 19×2 μL aliquots (first aliquot was 0.4 μL). Reference power was set at 10 μcal/sec and the assay temperature was 25 °C. The blank titrations included the titration of buffer into the test compounds and zinc sulfate into buffer all of which showed negligible heat of dilution signals (see figure 3 for the thermograms). The signals were integrated and the thermodynamic parameters were calculated using the PEAQ-ITC analysis software.



$ZnSO_4$ (1 mM) : **1b** (0.1 mM) $ZnSO_4$ (1 mM) : **1c** (0.1 mM) $ZnSO_4$ (1 mM) : **1d** (0.1 mM)

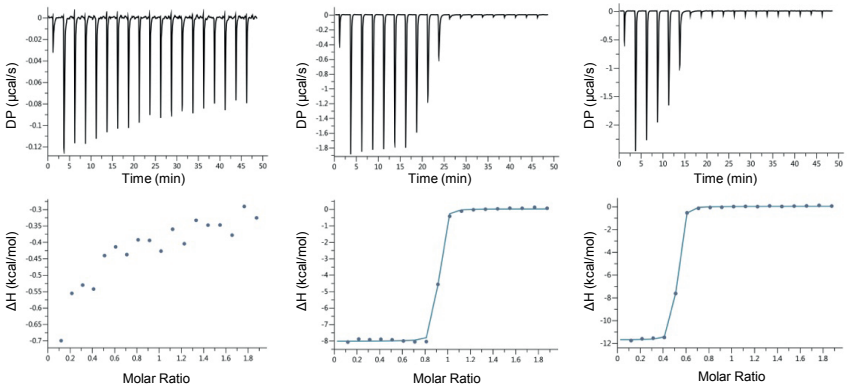


$ZnSO_4$ (1 mM) : **1e** (0.1 mM) $ZnSO_4$ (1 mM) : **1f** (0.1 mM) $ZnSO_4$ (1 mM) : **1g** (0.1 mM)

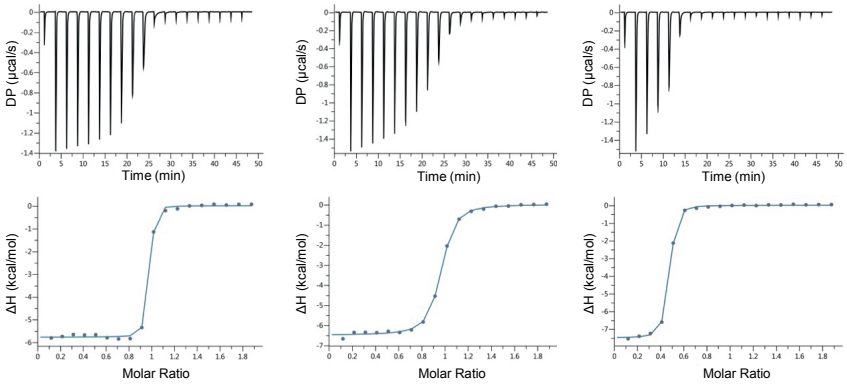


$ZnSO_4$ (1 mM) : **1h** (0.1 mM) $ZnSO_4$ (1 mM) : **1i** (0.1 mM) $ZnSO_4$ (1 mM) : **1j** (0.1 mM)

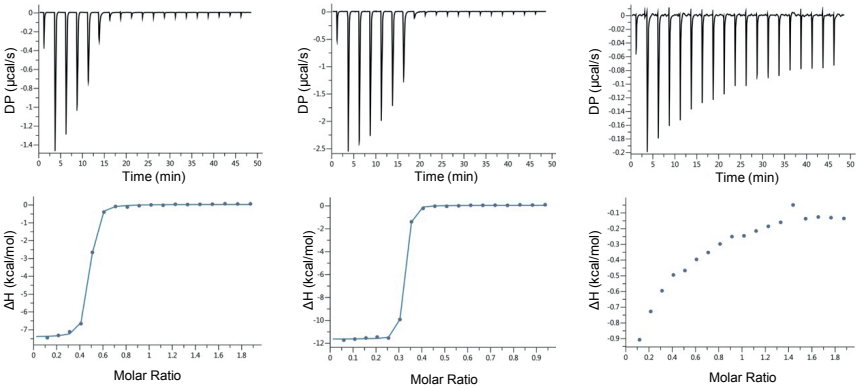
Figure 3. ITC thermograms of zinc sulfate titrated in the solutions of animocarboxylic acids.



$ZnSO_4$ (1 mM) : **1h** (0.1 mM $ZnSO_4$ (1 mM) : **1i** (0.1 mM) $ZnSO_4$ (1 mM) : **1j** (0.1 mM)

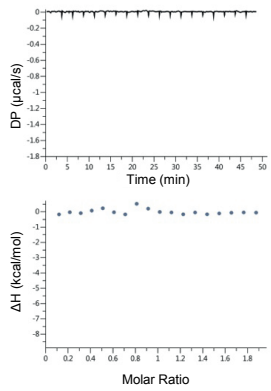


$ZnSO_4$ (1 mM) : **8b** (0.1 mM) $ZnSO_4$ (1 mM) : **8c** (0.1 mM) $ZnSO_4$ (1 mM) : **8d** (0.1 mM)

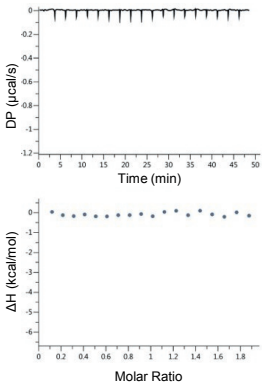


$ZnSO_4$ (1 mM) : **8e** (0.1 mM) $ZnSO_4$ (1 mM) : **8f** (0.1 mM) $ZnSO_4$ (1 mM) : **8g** (0.1 mM)

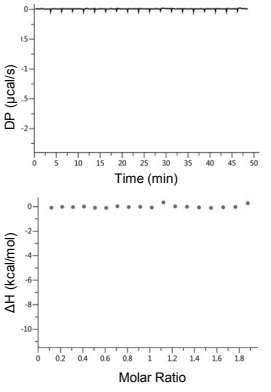
Figure 3. *Continued*



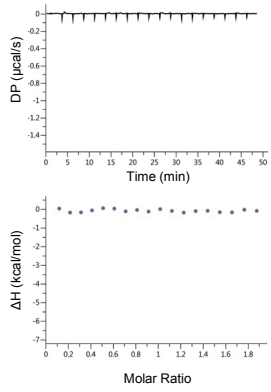
Tris buffer : **1b** (0.1 mM)



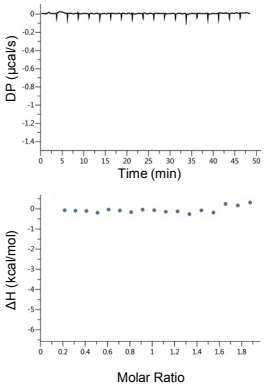
Tris buffer : **1c** (0.1 mM)



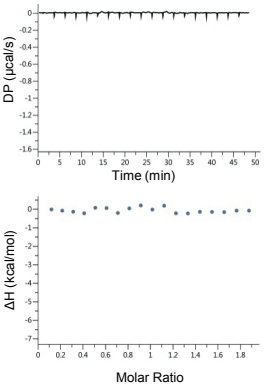
Tris buffer : **1d** (0.1 mM)



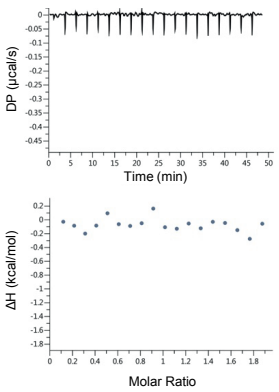
Tris buffer : **1e** (0.1 mM)



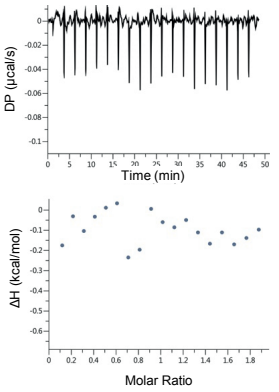
Tris buffer : **1f** (0.1 mM)



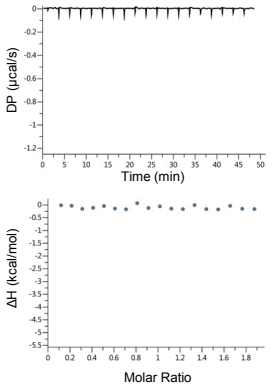
Tris buffer : **1g** (0.1 mM)



Tris buffer : **1h** (0.1 mM)

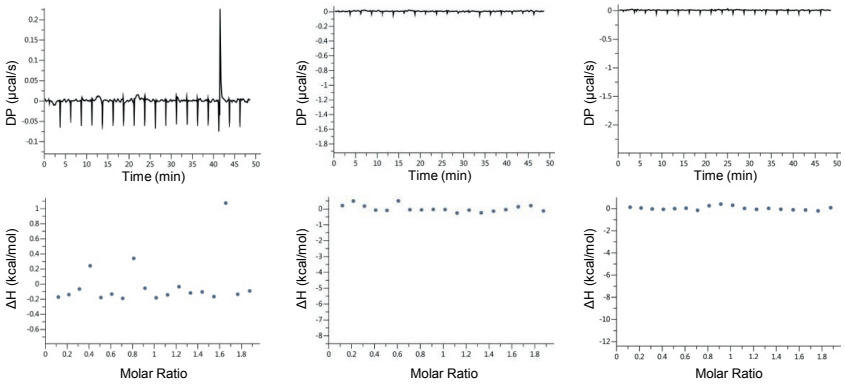


Tris buffer : **1i** (0.1 mM)

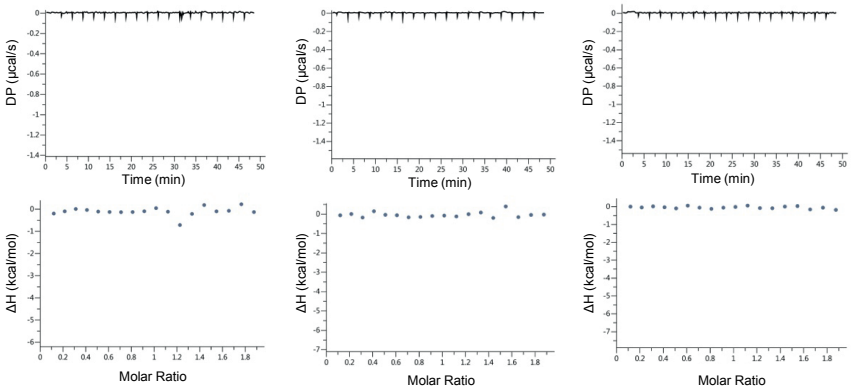


Tris buffer : **1j** (0.1 mM)

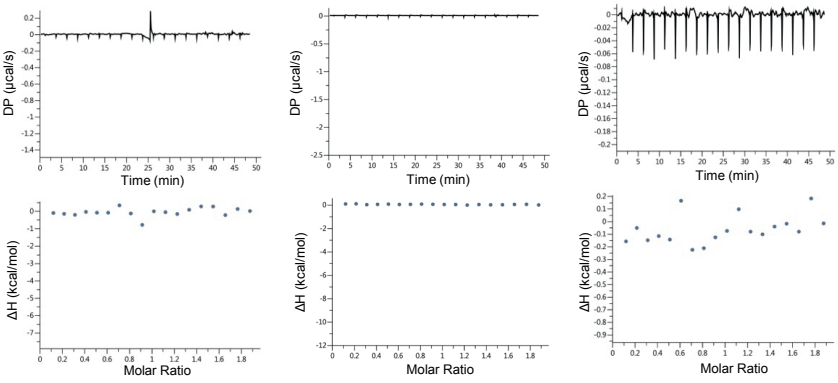
Figure 3. *Continued*



Tris buffer : **1k** (0.1 mM) Tris buffer : **5** (0.1 mM) Tris buffer : **8a** (0.1 mM)



Tris buffer : **8b** (0.1 mM) Tris buffer : **8c** (0.1 mM) Tris buffer : **8d** (0.1 mM)



Tris buffer : **8e** (0.1 mM) Tris buffer : **8f** (0.1 mM) Tris buffer : **8g** (0.1 mM)

Figure 3. *Continued*

Antibacterial activity

A. MIC assay. The antibacterial assays were performed according to the guidelines published by the clinical and laboratory standards institute (CLSI). On a polypropylene 96-well plate, the aminocarboxylic acid derivatives as well as the control compounds were serially diluted in Mueller-Hinton broth (MHB). In the same day, a few colonies of *E. coli* RC0089 (NDM-1) were suspended in tryptic soy broth (TSB) and incubated with shaking at 37 °C. When the bacteria grew to the exponential phase (OD_{600} = 0.5), the suspension was diluted in MHB to reach 10^6 CFU/mL and then added to the microplate containing the test compounds. After incubation at 37 °C for 15-20 h, the microplates were inspected for growth inhibition. MIC is defined as the lowest concentration of the compound that prevented the visible growth of the bacteria.

B. Determination of rescue concentration (RC). The test compounds were serially diluted starting from 400 μM. Meropenem was then added to the wells with the final concentration of 1 μg/mL. The bacteria were cultured and added to the microplates as described above. Rescue concentration was defined as the lowest concentration of the inhibitor that prevented the visible growth of the bacteria when combined with 1 μg/mL of meropenem.

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