Supplementary Information for:

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

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General information

Fumaric acid and diamines (**7a-i**) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO), TCI Europe N.V., or Thermo Fisher Scientific (Geel, Belgium). Solvents were purchased from Biosolve (Valkenswaard, The Netherlands) or Sigma-Aldrich Chemical Co. Ingredients for buffers and media were obtained from Duchefa Biochemie (Haarlem, The Netherlands) or Merck (Darmstadt, Germany). Dowex 50W X8 resin (hydrogen form, 100-200 mesh) was purchased from Sigma-Aldrich Chemical Co. and AG 1X8 resin (acetate form, 100-200 mesh) was purchased from Bio-Rad Laboratories Inc. Ni sepharose 6 fast flow resin was purchased from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). Proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions on precast gels (NuPAGE™ 4–12% Bis-Tris protein gels). The gels were stained with Coomassie brilliant blue. NMR analysis was performed on a Brucker 500 MHz machine at the Drug Design laboratory of the University of Groningen. Chemical shifts (δ) are reported in parts per million (ppm). Electrospray ionization orbitrap high resolution mass spectrometry (HRMS) was performed by the Mass Spectrometry core facility of the University of Groningen.

Screening diamines (7a-i) as non-natural substrates for EDDS lyase

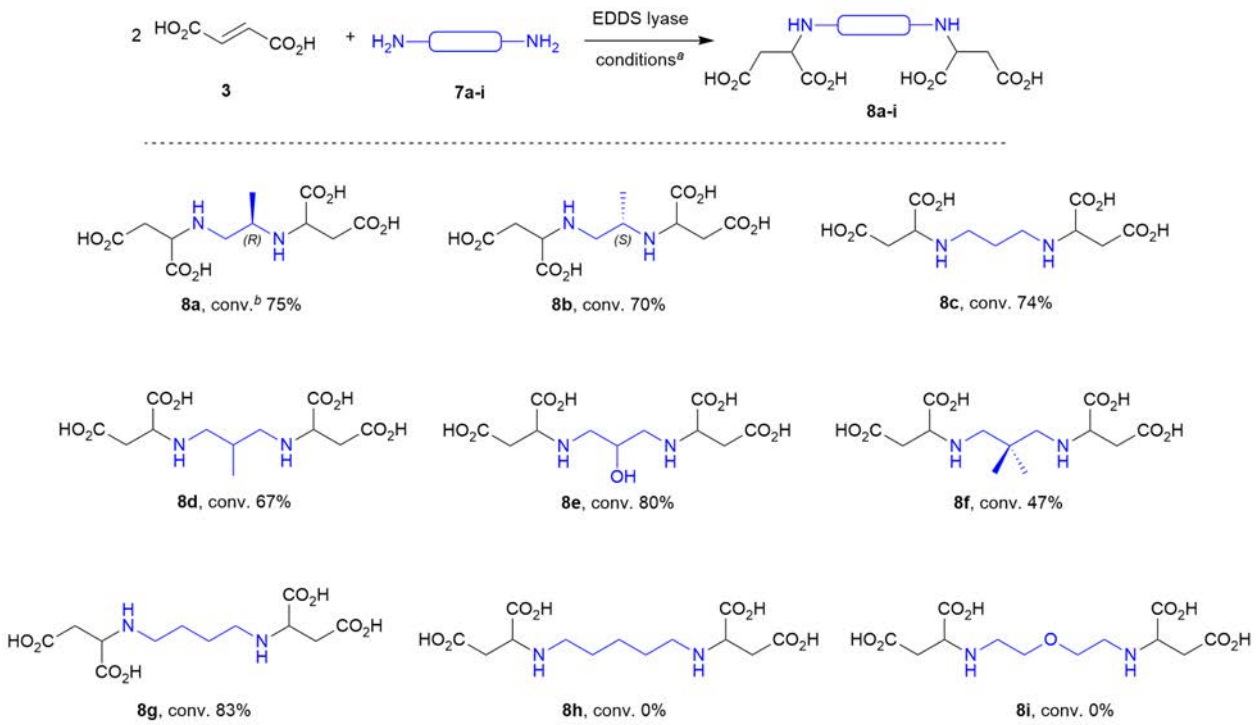
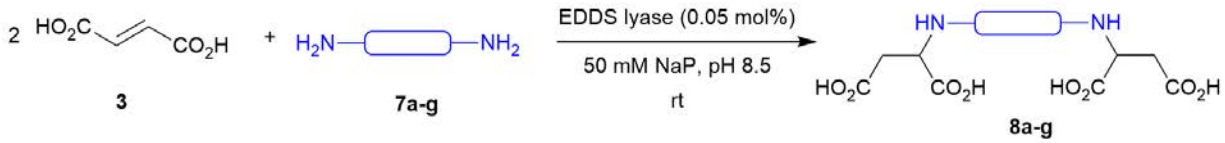


Figure S1. Screening diamines (**7a-i**) as non-natural substrates for EDDS lyase. [a]Conditions and reagents: fumaric acid (**3**, 60 mM), diamine substrates **7a-i** (10 mM) and purified EDDS lyase (0.05 mol% based on amine) in buffer (50 mM Na_2HPO_4, pH 8.5), at room temperature, 48-96 h. [b]Conversions were determined by comparing ^{1}H NMR signals of substrates and corresponding products.

General procedure: The enzyme EDDS lyase was overproduced and purified to homogeneity by following previously described protocols.[1,2] The initial reaction mixture (1.2 mL) consisted of fumaric acid (0.12 mmol) and a diamine (see above, 0.02 mmol) in 50 mM Na_2HPO_4 buffer (pH 8.5), and the pH of the reaction mixture was adjusted to pH 8.5. The enzymatic reaction was started by addition of freshly purified EDDS lyase (0.05 mol%) and the final volume of the reaction mixture was adjusted immediately to 2 mL with the same buffer. The reaction mixture was incubated at room temperature for 48 h (**7a-e** and **7g**) or 96 h (**7f** and **7h-i**). The enzyme was inactivated by heating at 70 °C for 10 min. A sample (0.5 mL) was taken from the reaction mixture, filtered and the filtrate was evaporated under vacuum. The resulting residue was dissolved in 0.5 mL of D_2O for ^{1}H NMR measurement. The conversion was estimated by comparing the signals of substrate and corresponding product in ^{1}H NMR spectra.

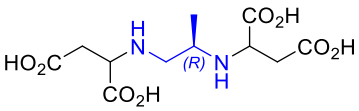
Enzymatic synthesis of EDDS derivatives (8a-g).



General procedure: The reaction mixture (15 mL) consisted of fumaric acid (**3**, 60 mM) and a diamine substrate (**7a-g**, 10 mM) in 50 mM Na_2HPO_4 buffer (pH 8.5). The pH of the reaction mixture was adjusted to pH 8.5. The enzymatic reaction was started by addition of freshly purified EDDS lyase (0.05 mol%). The reaction mixture was then incubated at room temperature for 48 h (**7a-e** and **7g**) or 96 h (**7f**). After completion of the reaction, the enzyme was inactivated by heating to 70 °C for 10 min. The progress of the enzymatic reaction was monitored by ^{1}H NMR spectroscopy by comparing signals of substrates and corresponding products.

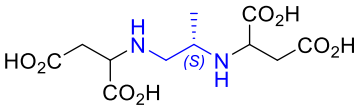
The enzymatic products were purified by two steps of ion-exchange chromatography. For a typical purification procedure, the precipitated enzyme was removed by filtration (diameter 0.45 μm). The filtrate was loaded slowly onto an anion-exchange column (10 g of AG 1-X8 resin, acetate form, 100-200 mesh), which was pretreated with 5 column volumes of 1 M acetic acid aqueous solution and then water (until the pH was near neutral). The column was washed with water (3 column volumes) and then 1 M acetic acid (3 column volumes) until all the unreacted diamine (**7a-g**) was removed. The product was eluted with 1 M HCl (4 column volumes). The ninhydrin positive fractions were collected and loaded onto a cation-exchange column (10 g of Dowex 50W X8 resin, hydrogen form, 100-200 mesh), which was pretreated with 2 M aqueous ammonia (5 column volumes), 1 M HCl (3 column volumes) and water (until the pH was near neutral). After product loading, the column was washed with water (3 column volumes) to remove the remaining fumaric acid and subsequently eluted with 2 M aqueous ammonia until the desired product was collected. The ninhydrin-positive fractions were collected, concentrated under vacuum and lyophilized to provide the desired products **8a-g** as ammonium salts, which were characterized by ^{1}H NMR, ^{13}C NMR and HRMS.

2,2'-{[(*R*)-propane-1,2-diyl]bis(azanediyl)}disuccinic acid (**8a**)



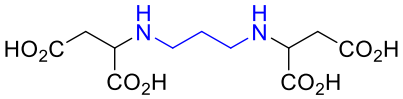
White solid. 14 mg (isolated yield 31%). ^{1}H NMR (500 MHz, D_2O) δ 3.50 (dd, J = 8.0, 5.8 Hz, 1H), 3.33 (dd, J = 8.4, 5.3 Hz, 1H), 2.60 (q, J = 6.2 Hz, 1H), 2.50 – 2.37 (m, 4H), 2.34 – 2.27 (m, 2H), 0.99 (d, J = 6.3 Hz, 3H); ^{13}C NMR (126 MHz, D_2O) δ 182.1, 181.6, 179.9, 179.6, 61.5, 59.0, 53.5, 51.0, 41.8, 41.0, 16.7. HRMS (ESI$^+$): calcd. for $C_{11}H_{19}N_2O_8$ $[M+H]^+$: 307.1136, found: 307.1136.

2,2'-{[(*S*)-propane-1,2-diyl]bis(azanediyl)}disuccinic acid (**8b**)



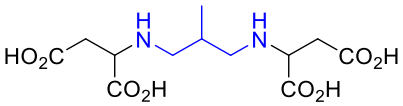
White solid. 9 mg (isolated yield 21%). ^{1}H NMR (500 MHz, D_2O) δ 3.41 (dd, J = 8.0, 5.4 Hz, 1H), 3.28 (dd, J = 8.0, 5.8 Hz, 1H), 2.65 – 2.61 (m, 1H), 2.54 (dd, J = 11.4, 4.5 Hz, 1H), 2.44 – 2.36 (m, 2H), 2.30 – 2.22 (m, 3H), 0.99 (d, J = 6.3 Hz, 3H); ^{13}C NMR (126 MHz, D_2O) δ 182.1, 181.6, 179.8, 179.7, 61.6, 59.0, 52.1, 50.8, 41.6, 41.3, 18.2. HRMS (ESI$^+$): calcd. for $C_{11}H_{19}N_2O_8$ $[M+H]^+$: 307.1136, found: 307.1136.

2,2'-[propane-1,3-diylbis(azanediyl)]disuccinic acid (**8c**)



White solid. 15 mg (isolated yield 33%). ^{1}H NMR (500 MHz, D_2O) δ 3.80 (dd, J = 8.6, 3.9 Hz, 2H), 3.19 – 3.16 (m, 4H), 2.79 (dd, J = 17.4, 4.0 Hz, 2H), 2.65 (dd, J = 17.5, 8.5 Hz, 2H), 2.17 (p, J = 7.9 Hz, 2H); ^{13}C NMR (126 MHz, D_2O) δ 177.3 (2C), 173.4 (2C), 59.7, 59.6, 43.6 (2C), 35.8 (2C), 23.2. HRMS (ESI$^+$): calcd. for $C_{11}H_{19}N_2O_8$ $[M+H]^+$: 307.1136, found: 307.1137.

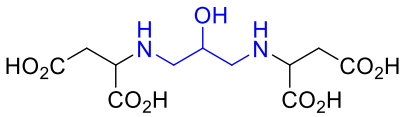
2,2'-[(2-methylpropane-1,3-diyl)bis(azanediyl)]disuccinic acid (**8d**)



White solid. 16 mg, (isolated yield 31%). ^{1}H NMR (500 MHz, D_2O) δ 3.32 (dd, J = 7.9, 5.8 Hz, 2H), 2.51 – 2.41 (m, 4H), 2.33 – 2.20 (m, 4H), 1.80 – 1.73 (m, 1H), 0.89 (d, J = 6.6 Hz, 3H); ^{13}C

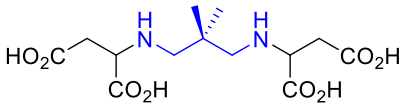
NMR (126 MHz, D_2O) δ 181.6, 181.5, 179.9, 179.8, 61.7, 61.6, 52.1, 51.9, 41.4, 41.1, 32.6, 15.7. HRMS (ESI^+): calcd. for $C_{12}H_{21}N_2O_8$ $[M+H]^+$: 321.1292, found: 321.1291.

2,2'-[(2-hydroxypropane-1,3-diyl)bis(azanediyl)]disuccinic acid (**8e**)



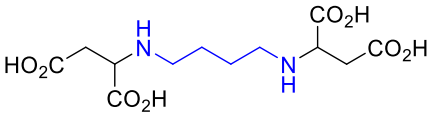
White solid. 30 mg, (isolated yield 60%). ^1H NMR (500 MHz, D_2O) δ 3.77 – 3.74 (m, 1H), 3.36 – 3.32 (m, 2H), 2.56 (ddd, J = 27.0, 12.3, 3.5 Hz, 2H), 2.51 – 2.40 (m, 4H), 2.28 (ddd, J = 15.2, 8.7, 2.7 Hz, 2H); ^{13}C NMR (126 MHz, D_2O) δ 181.5, 181.4, 179.9, 179.8, 69.8, 61.6, 61.3, 51.3, 51.2, 41.5, 41.2. HRMS (ESI^+): calcd. for $C_{11}H_{18}N_2O_9$ $[M+H]^+$: 323.1085, found: 323.1085.

2,2'-[(2,2-dimethylpropane-1,3-diyl)bis(azanediyl)]disuccinic acid (**8f**)



White solid. 13 mg, (isolated yield 26%). ^1H NMR (500 MHz, D_2O) δ 3.31 (dd, J = 8.2, 5.8 Hz, 2H), 2.51 (dd, J = 15.3, 5.6 Hz, 2H), 2.40 – 2.21 (m, 6H), 0.90 (s, 6H); ^{13}C NMR (126 MHz, D_2O) δ 181.2 (2C), 179.8 (2C), 62.0 (2C), 56.9 (2C), 40.5 (2C), 33.6, 23.4 (2C). HRMS (ESI^+): calcd. for $C_{13}H_{23}N_2O_8$ $[M+H]^+$: 335.1449, found: 335.1447.

2,2'-[butane-1,4-diylbis(azanediyl)]disuccinic acid (**8g**)



White solid. 16 mg, (isolated yield 32%). ^1H NMR (500 MHz, D_2O) δ 3.30 (dd, J = 8.1, 5.7 Hz, 2H), 2.45 – 2.38 (m, 6H), 2.24 (dd, J = 15.1, 8.1 Hz, 2H), 1.43 – 1.36 (m, 4H); ^{13}C NMR (126 MHz, D_2O) δ 181.7 (2C), 179.8 (2C), 61.4 (2C), 47.1 (2C), 41.4 (2C), 26.6 (2C). HRMS (ESI^+): calcd. for $C_{12}H_{21}N_2O_8$ $[M+H]^+$: 321.1292, found: 321.1293.

NMR spectra

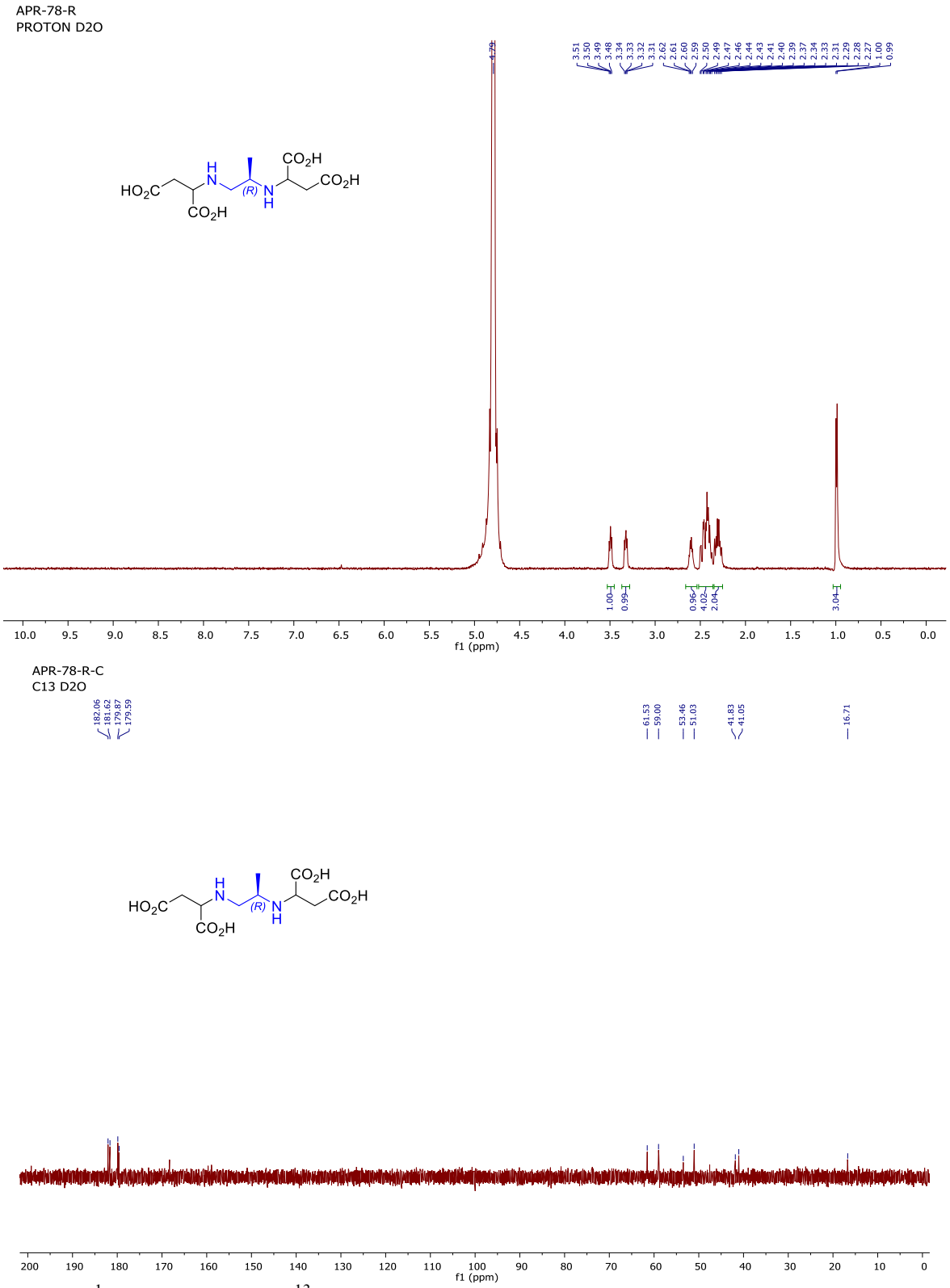


Figure S2. ^{1}H NMR (top) and ^{13}C NMR (bottom) of compound **8a**.

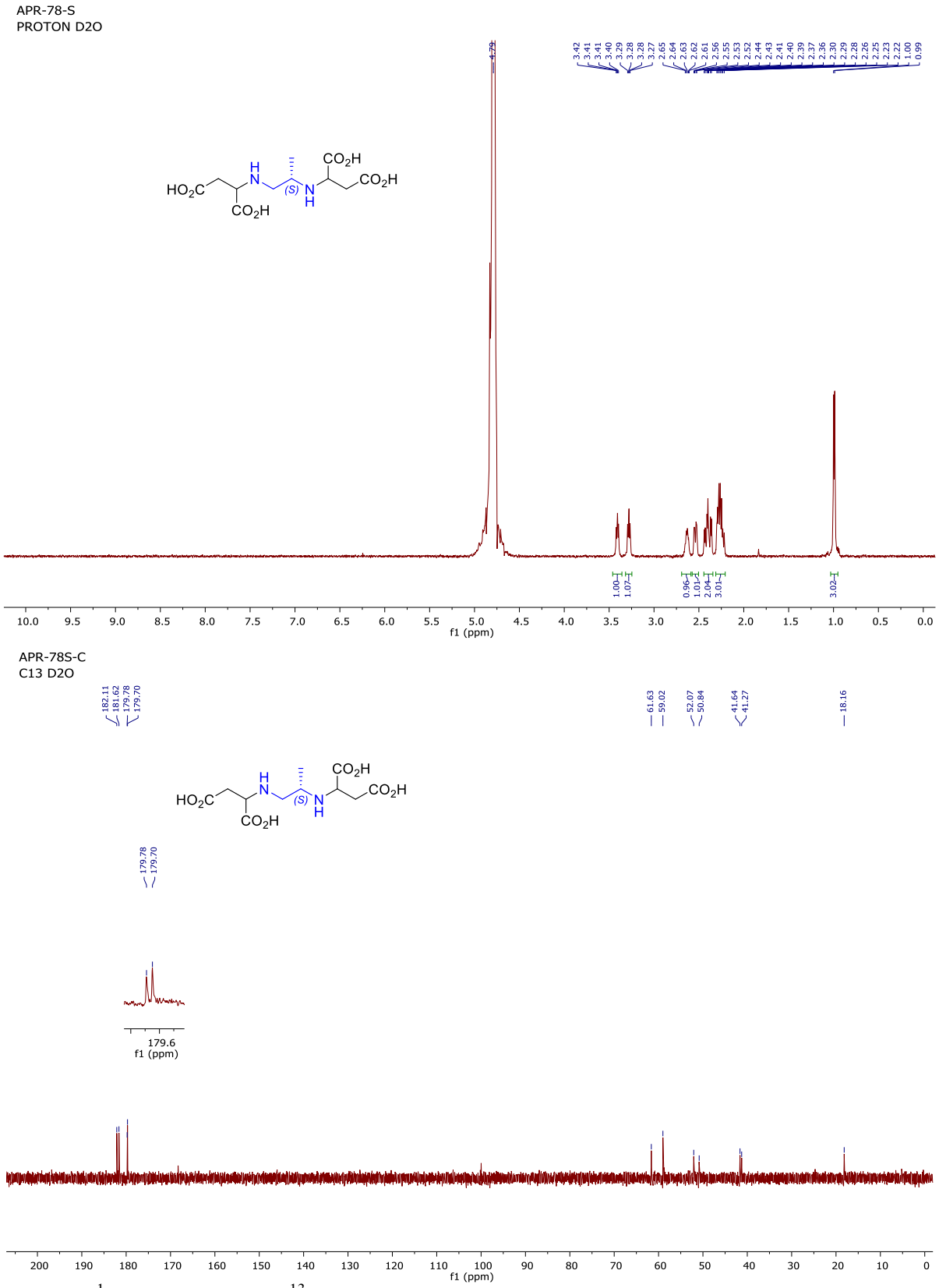


Figure S3. ^{1}H NMR (top) and ^{13}C NMR (bottom) of compound **8b**.

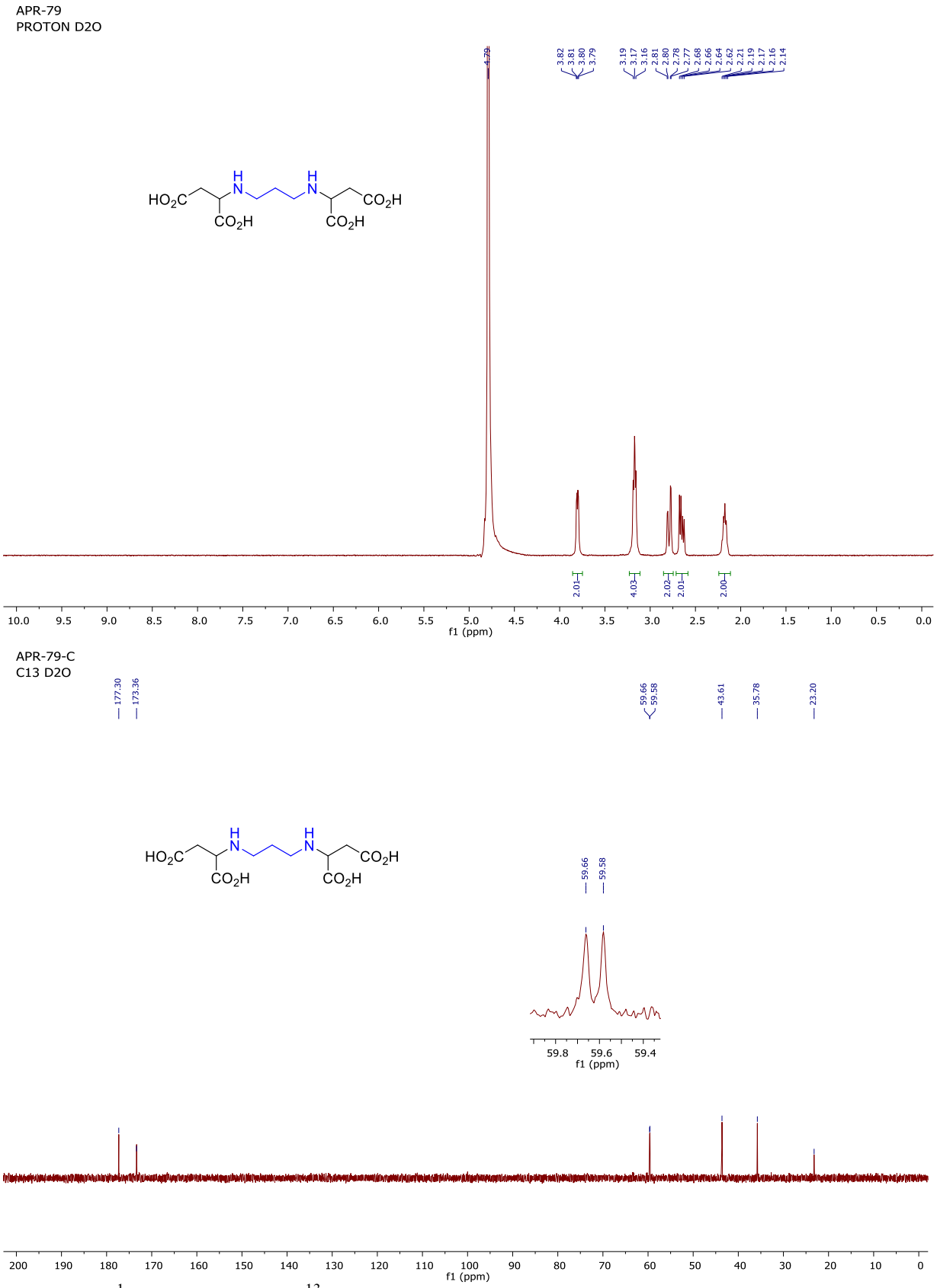


Figure S4. ^{1}H NMR (top) and ^{13}C NMR (bottom) of compound **8c**.

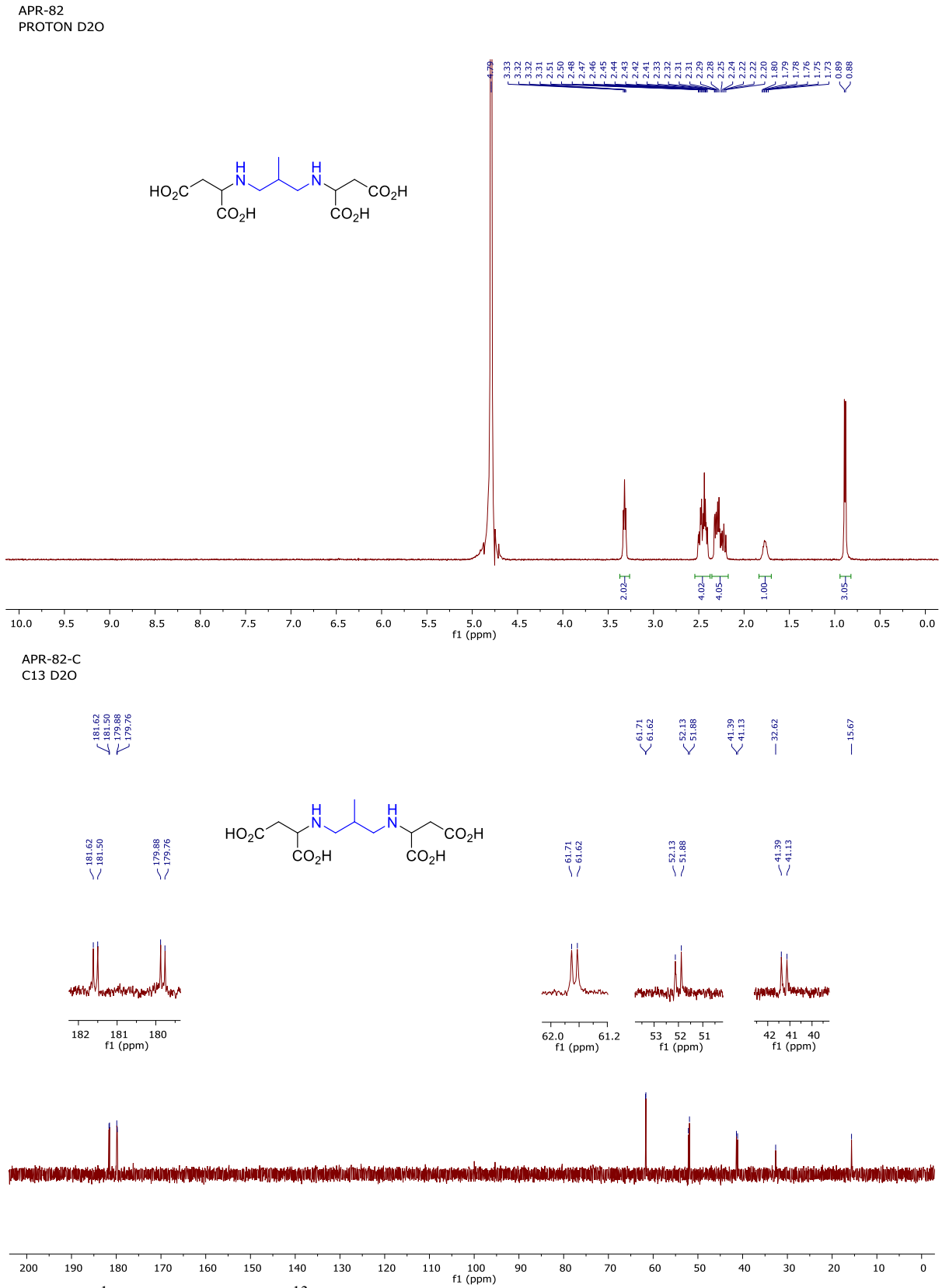


Figure S5. ^{1}H NMR (top) and ^{13}C NMR (bottom) of compound **8d**.

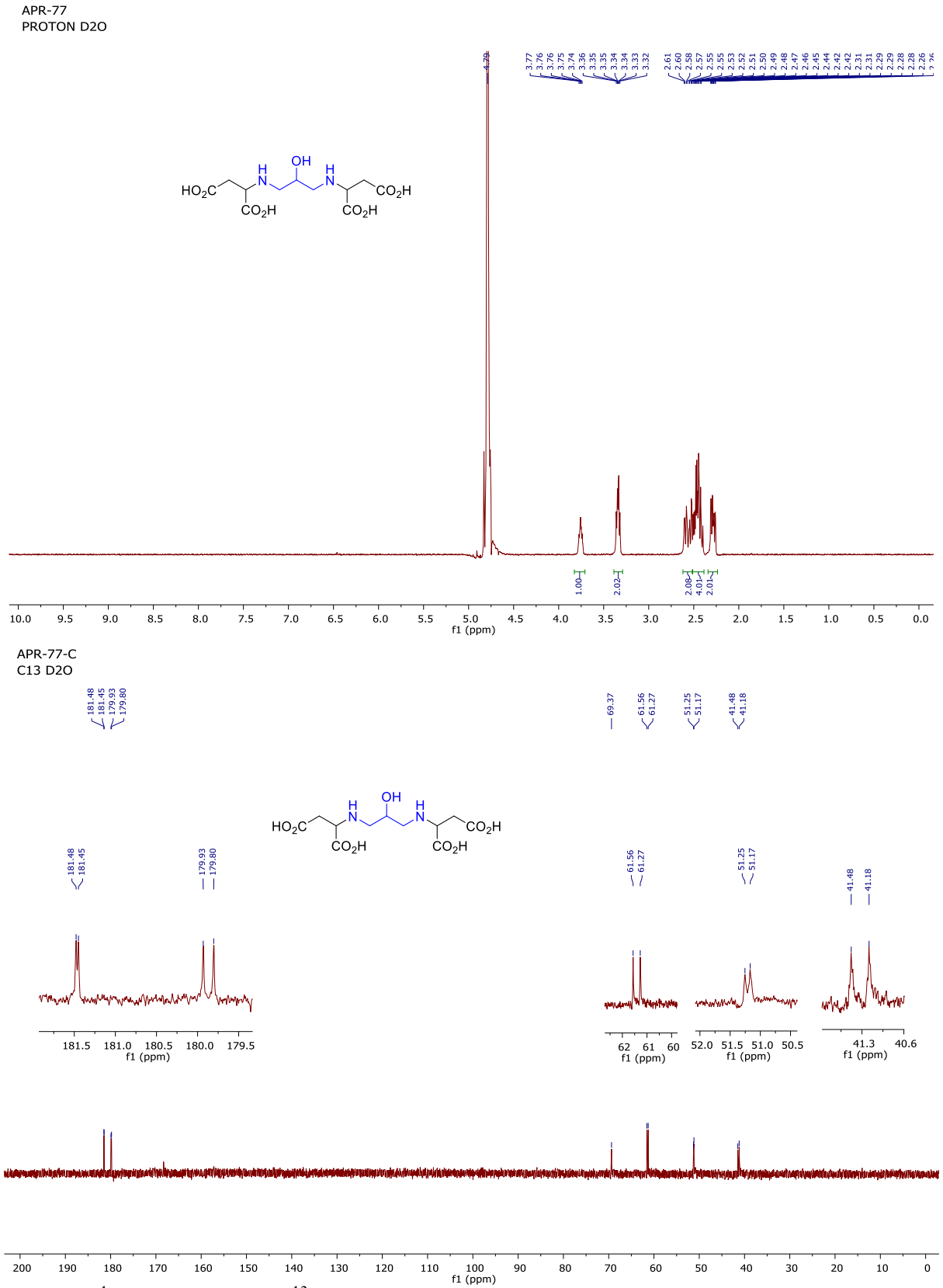


Figure S6. ^{1}H NMR (top) and ^{13}C NMR (bottom) of compound **8e**.

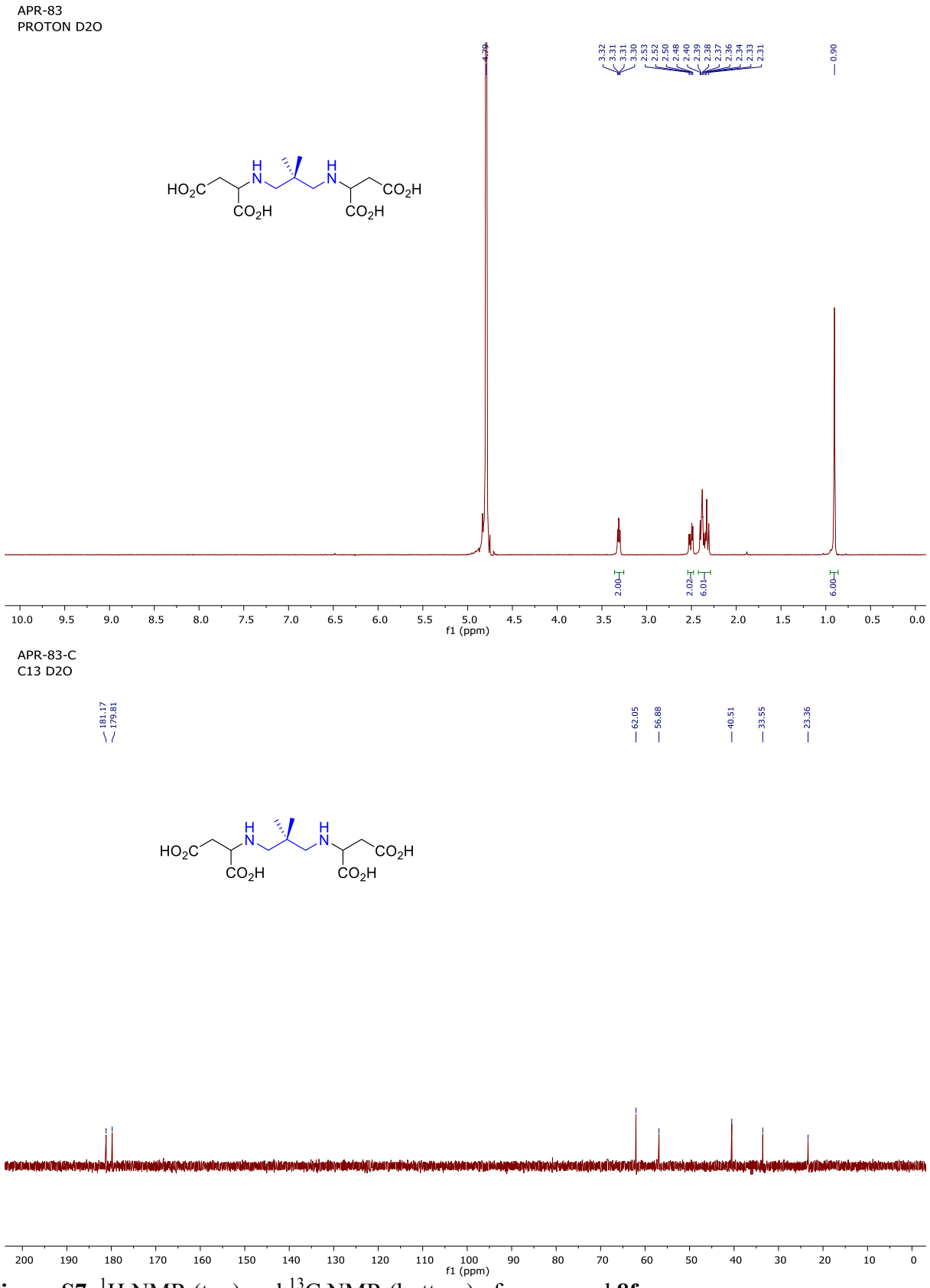


Figure S7. ^{1}H NMR (top) and ^{13}C NMR (bottom) of compound **8f**.

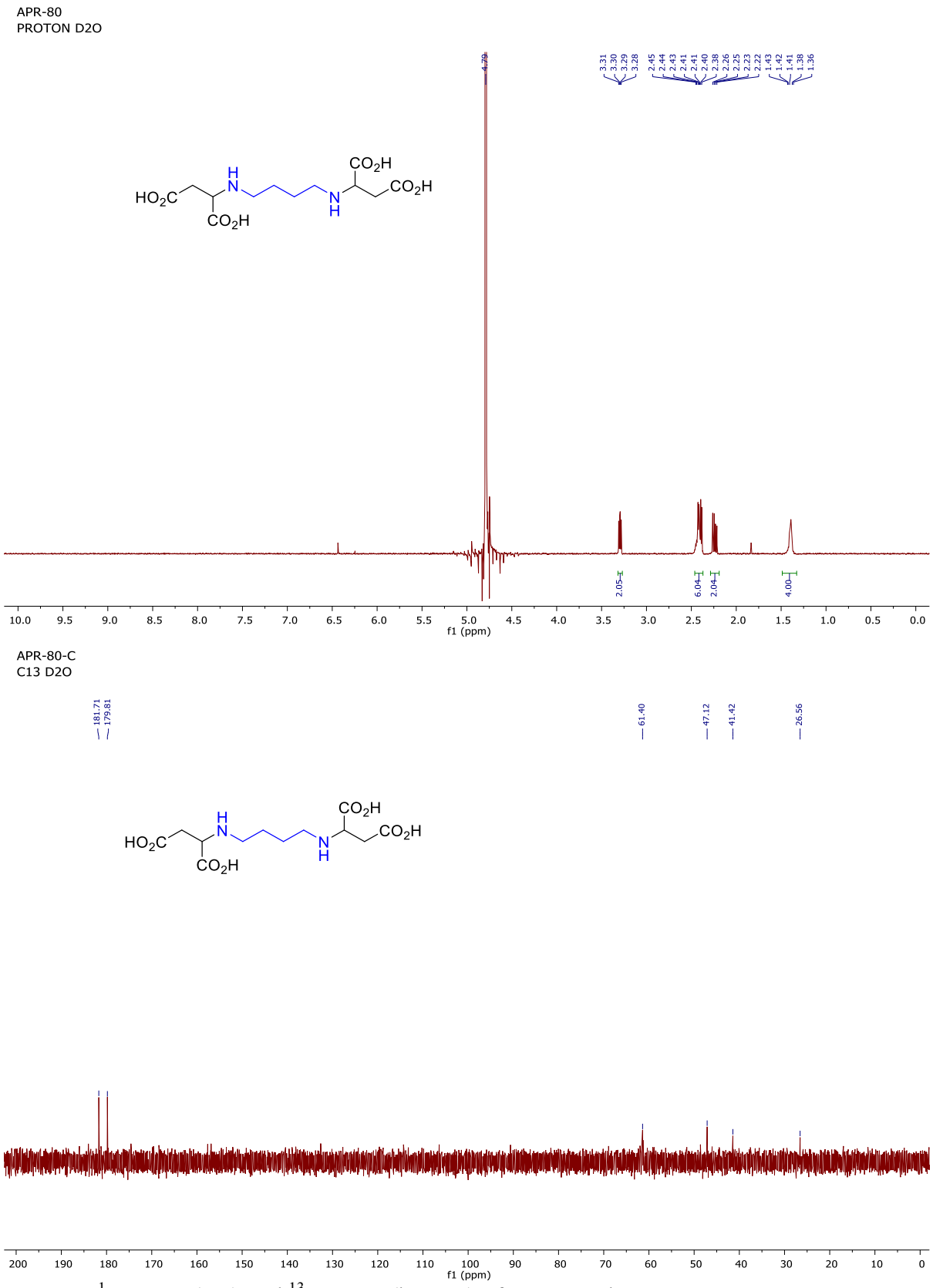


Figure S8. ^{1}H NMR (top) and ^{13}C NMR (bottom) of compound **8g**.

Enzyme inhibition assays

NDM-1 plasmid was a generous gift from Prof. Christopher Schofield (Oxford University). The over-expression and purification of NDM-1 as well as the synthesis of FC5 substrate was performed according to the previously reported procedures.[3]

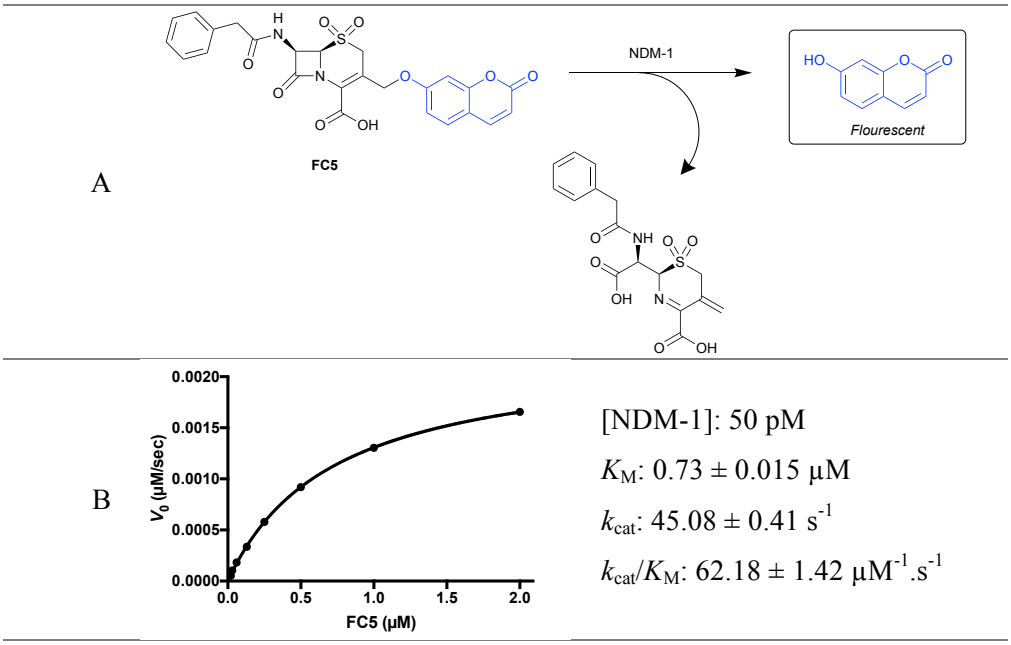


Figure S9. **A**. Hydrolysis mechanism of cephalosporin substrate known as FC5; **B**. Michaelis-Menten parameters of NDM-1 mediated hydrolysis of FC5.

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. 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).

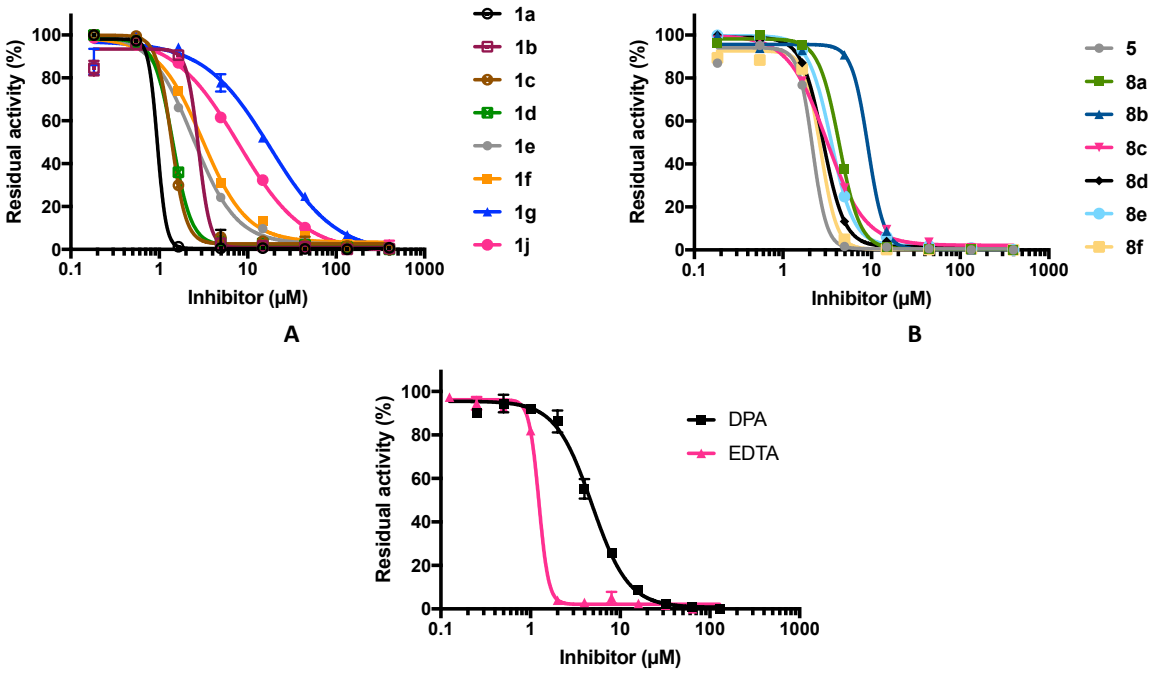


Figure S10. IC_{50} curves of **1a-j** (**A**), EDDS analogs (**B**), and control compounds (**C**) tested against NDM-1 (50 pM) using FC5 (0.5 μM) as substrate.

Isothermal titration calorimetry

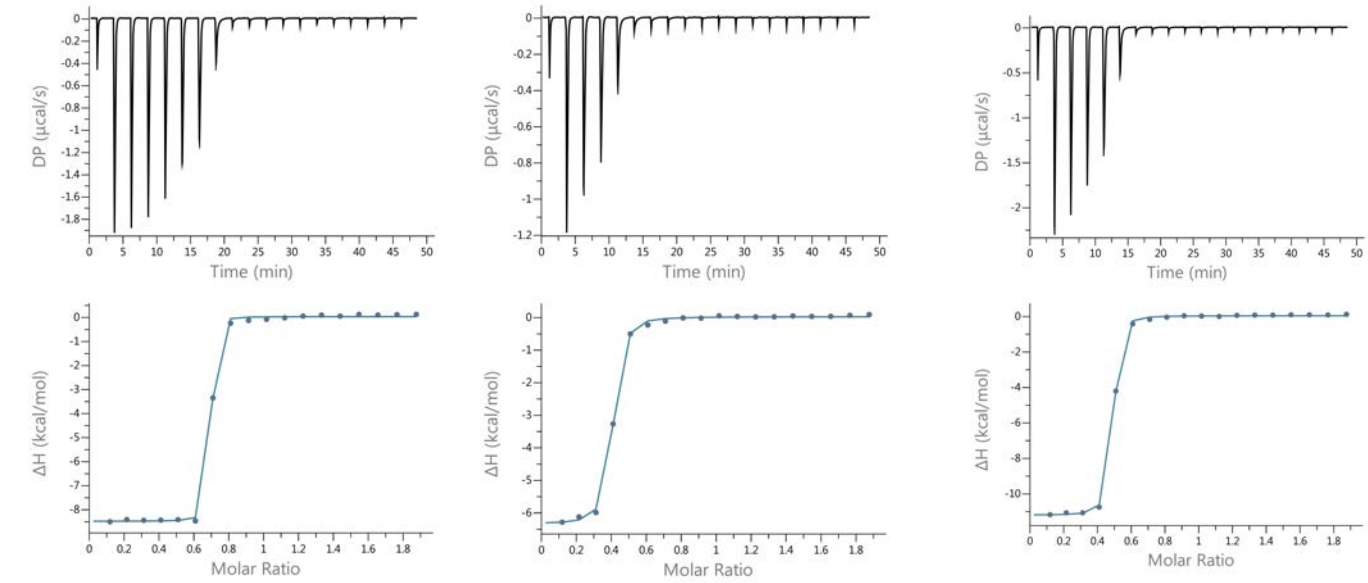
The titrations were performed on an automated PEAG-ITC calorimeter (Malvern). Test compounds were dissolved in 20 mM tris pH 7.0. Zinc sulfate (1 mM) was titrated in the 0.1 mM solutions of the aminocarboxylic acids over 19 x 2µL aliquots (first aliquot was 0.4 µL). Reference power was set at 10 µcal/sec 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 the thermograms below). The signals were integrated and the thermodynamic parameters were calculated using the PEAQ-ITC analysis software.

Table S1. The thermodynamic parameters of zinc^{2+} binding to the aminocarboxylic acid derivatives

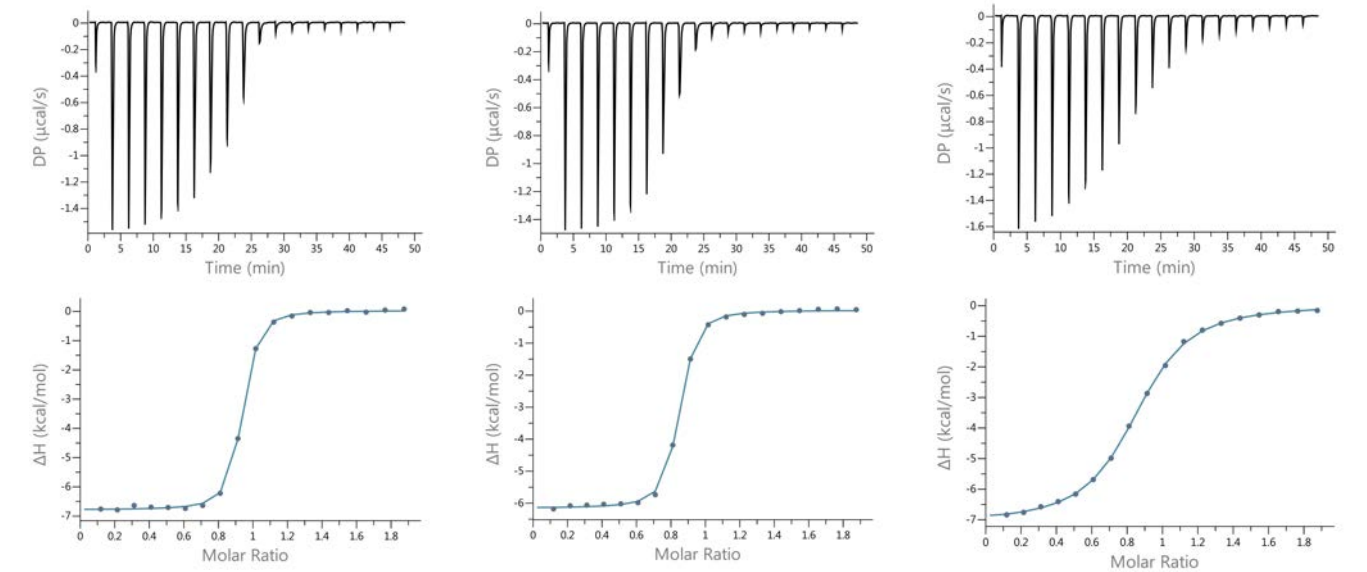
Compound	K_d (nM)	ΔH (kcal/mol)
1b	<100[a]	-8.51 ± 0.06
1c	<100[a]	-6.36 ± 0.05
1d	<100[a]	-11.2 ± 0.06
1e	181 ± 13	-6.81 ± 0.03
1f	240 ± 17	-6.18 ± 0.03
1g	2810 ± 88	-7.12 ± 0.04
1h	ND[b]	ND[b]
1i	ND[b]	ND[b]
1j	828 ± 35	-5.28 ± 0.03
1k	ND[b]	ND[b]
5	<100[a]	-8.04 ± 0.06
8a	<100[a]	-11.7 ± 0.04
8b	<100[a]	-5.78 ± 0.05
8c	334 ± 31	-6.49 ± 0.05
8d	<100[a]	-7.51 ± 0.04
8e	<100[a]	-7.43 ± 0.04
8f	<100[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.

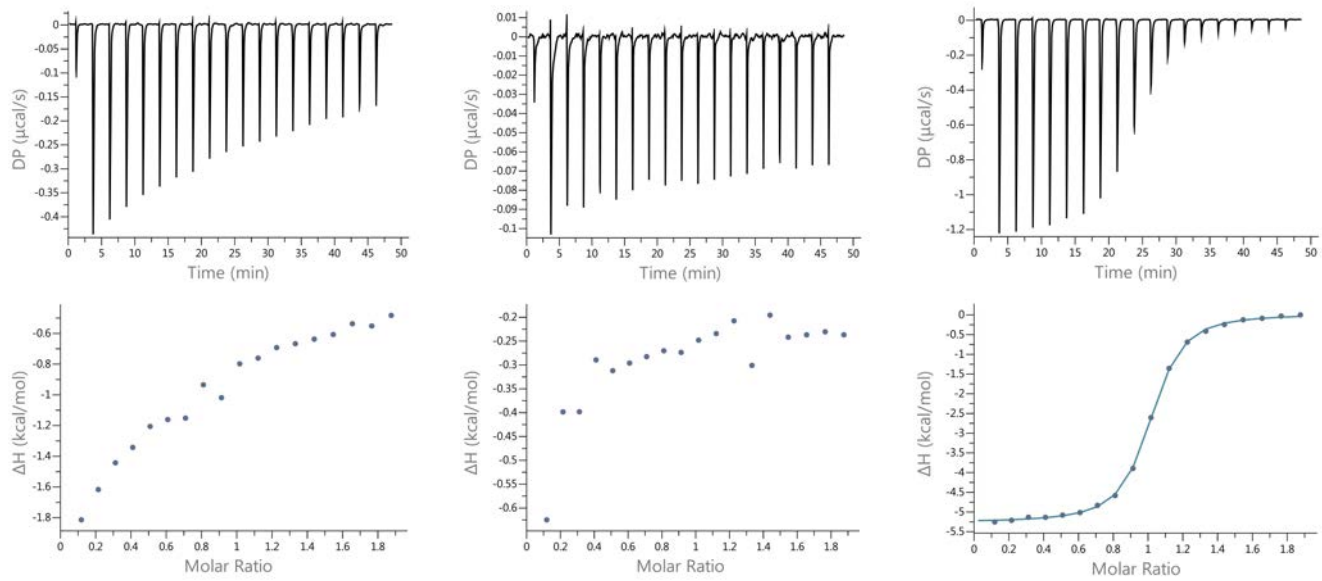
Figure S11. ITC thermograms of zinc sulfate titrated in the solutions of animocarboxylic acid derivatives.



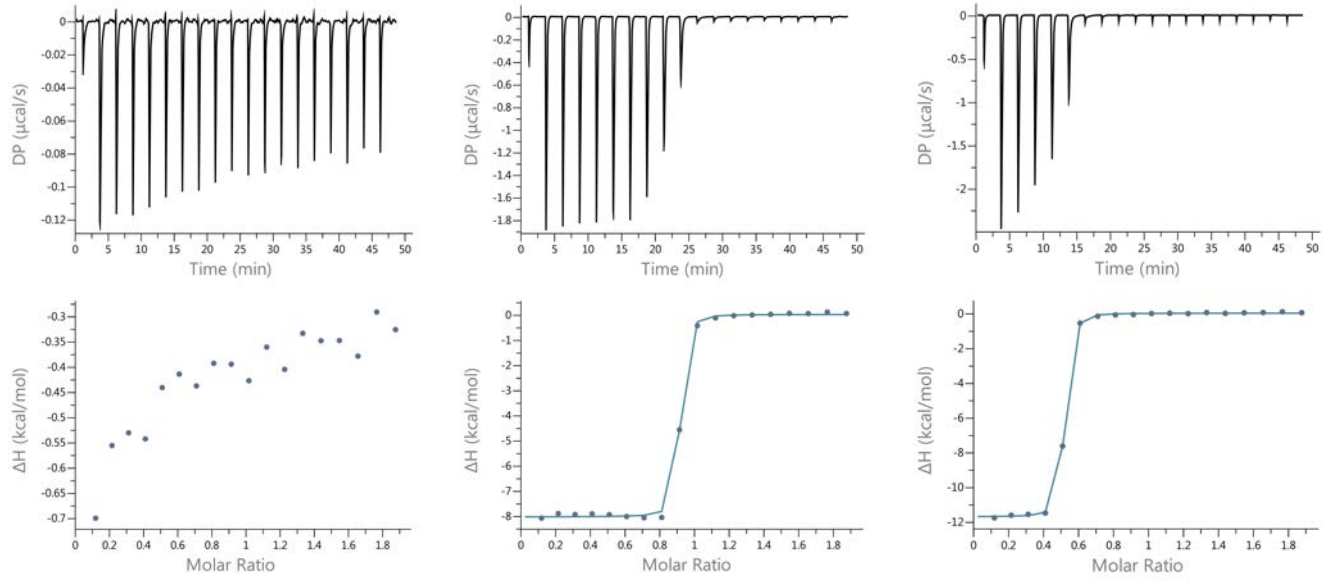
$ZnSO_4$ (1 mM) : **1b** (0.1 mM) $ZnSO_4$ (1 mM) : **1c** (0.1 mM) $ZnSO_4$ (1 mM) : **1d** (0.1 mM)



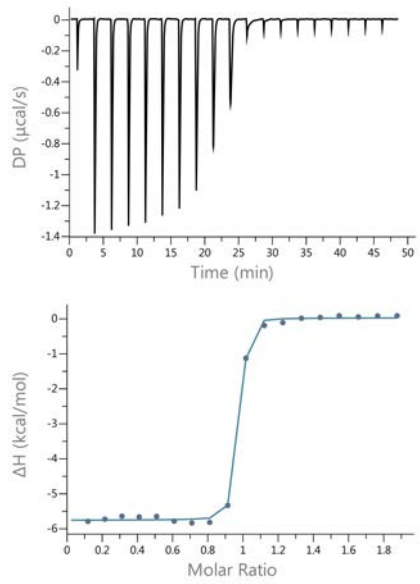
$ZnSO_4$ (1 mM) : **1e** (0.1 mM) $ZnSO_4$ (1 mM) : **1f** (0.1 mM) $ZnSO_4$ (1 mM) : **1g** (0.1 mM)



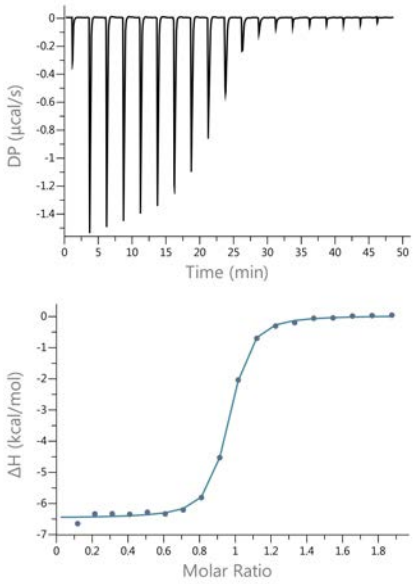
$ZnSO_4$ (1 mM) : **1h** (0.1 mM) | $ZnSO_4$ (1 mM) : **1i** (0.1 mM) | $ZnSO_4$ (1 mM) : **1j** (0.1 mM)



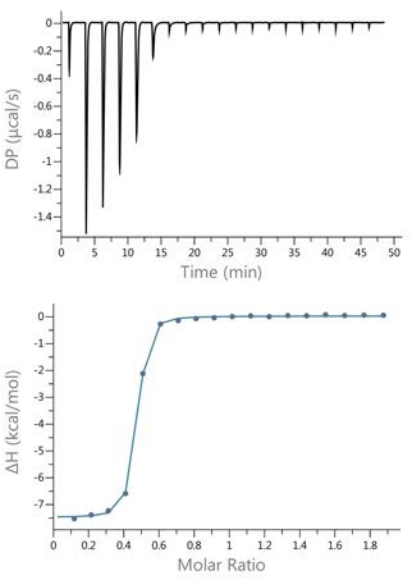
$ZnSO_4$ (1 mM) : **1k** (0.1 mM) | $ZnSO_4$ (1 mM) : **5** (0.1 mM) | $ZnSO_4$ (1 mM) : **8a** (0.1 mM)



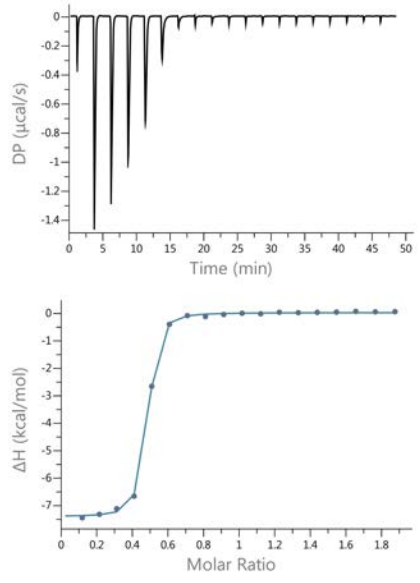
$ZnSO_4$ (1 mM) : **8b** (0.1 mM)



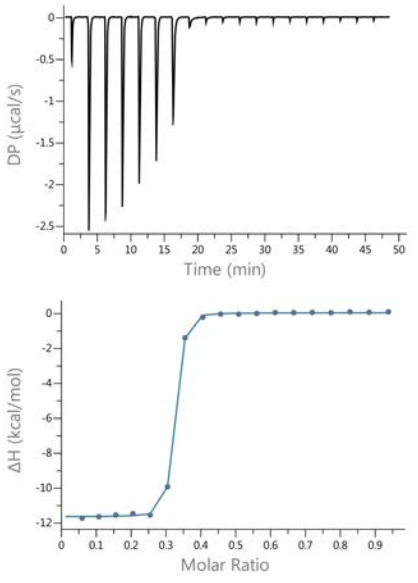
$ZnSO_4$ (1 mM) : **8c** (0.1 mM)



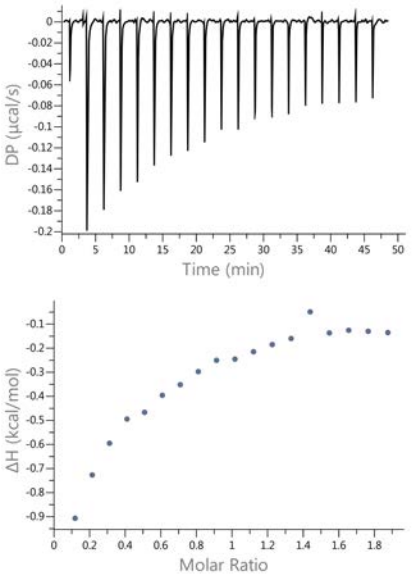
$ZnSO_4$ (1 mM) : **8d** (0.1 mM)



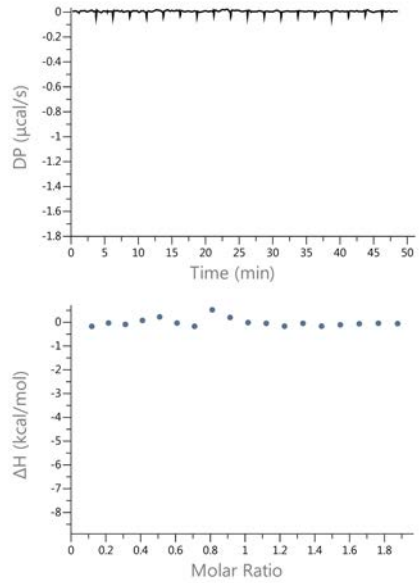
$ZnSO_4$ (1 mM) : **8e** (0.1 mM)



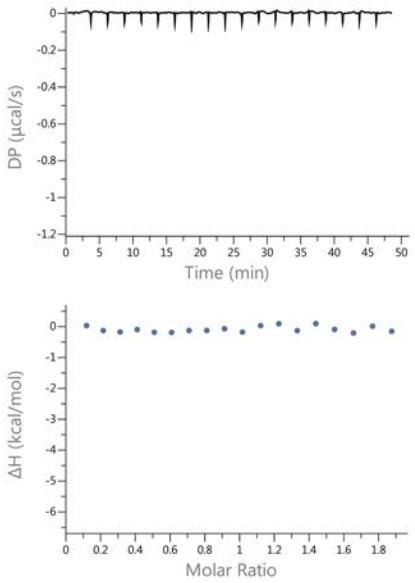
$ZnSO_4$ (1 mM) : **8f** (0.1 mM)



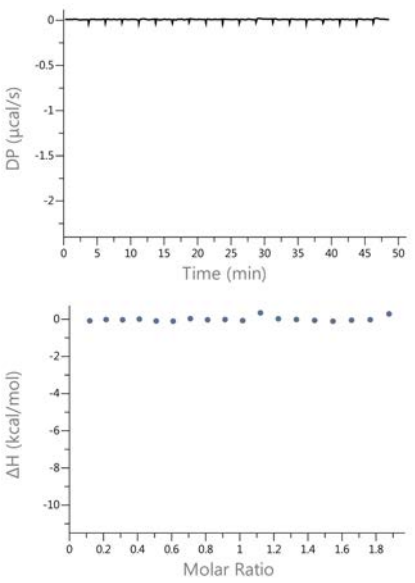
$ZnSO_4$ (1 mM) : **8g** (0.1 mM)



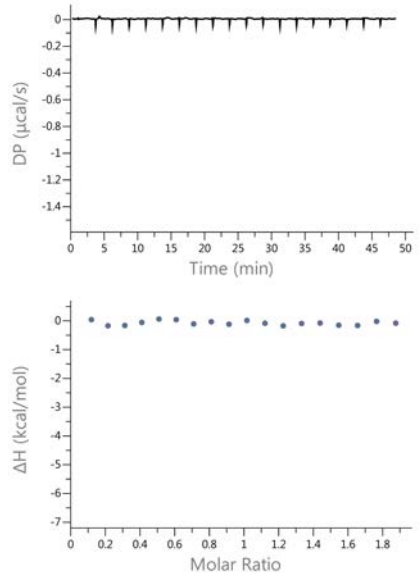
Tris buffer : **1b** (0.1 mM)



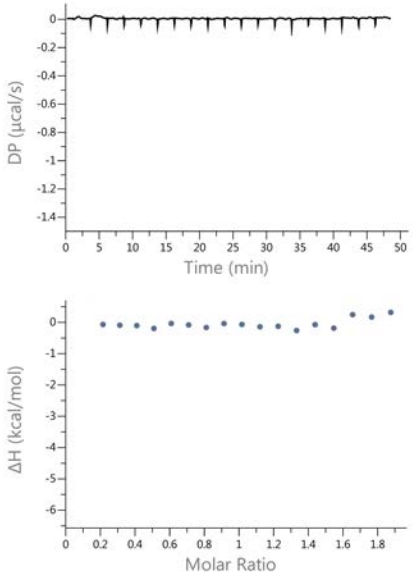
Tris buffer : **1c** (0.1 mM)



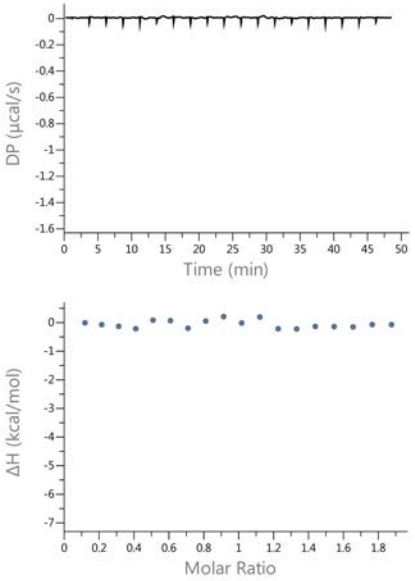
Tris buffer : **1d** (0.1 mM)



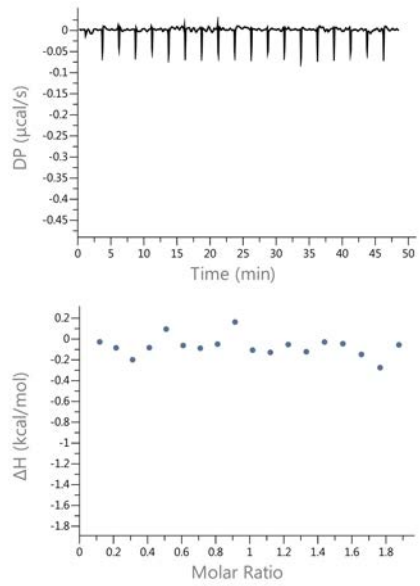
Tris buffer : **1e** (0.1 mM)



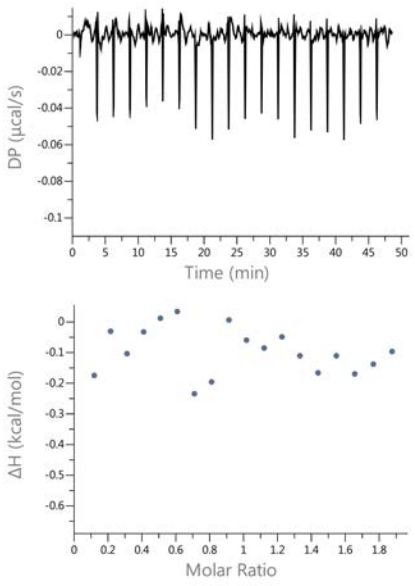
Tris buffer : **1f** (0.1 mM)



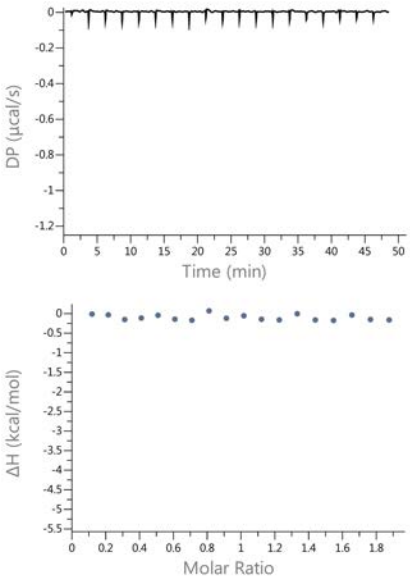
Tris buffer : **1g** (0.1 mM)



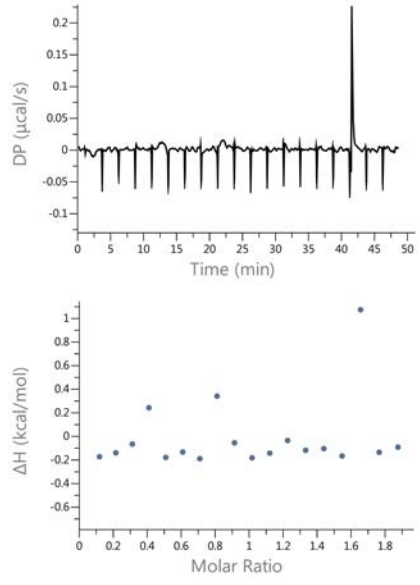
Tris buffer : **1h** (0.1 mM)



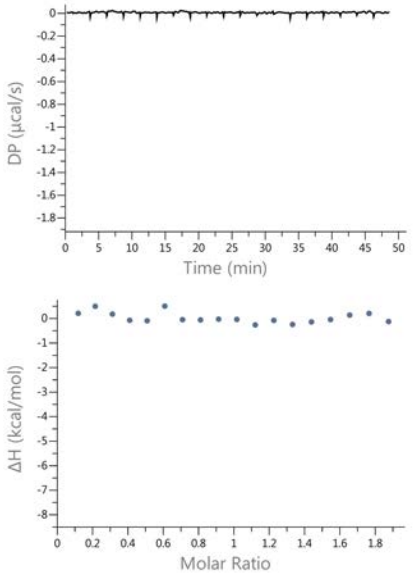
Tris buffer : **1i** (0.1 mM)



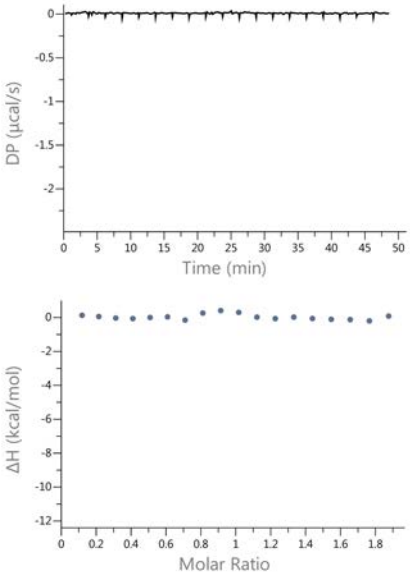
Tris buffer : **1j** (0.1 mM)



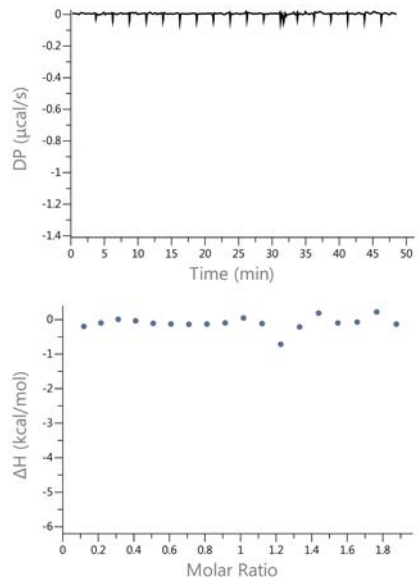
Tris buffer : **1k** (0.1 mM)



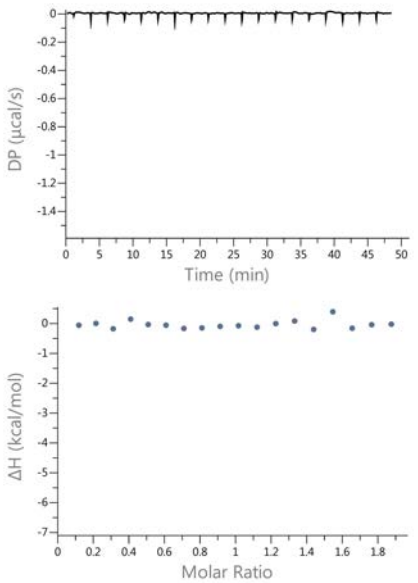
Tris buffer : **5** (0.1 mM)



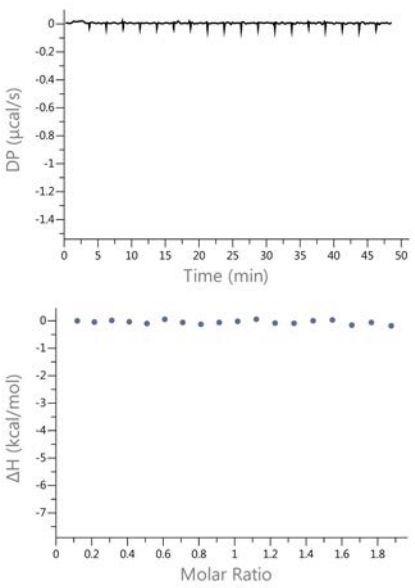
Tris buffer : **8a** (0.1 mM)



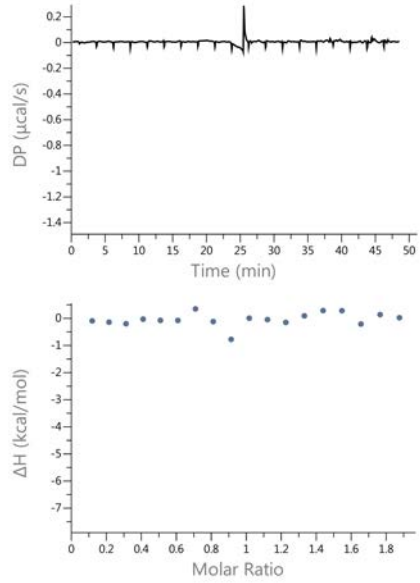
Tris buffer : **8b** (0.1 mM)



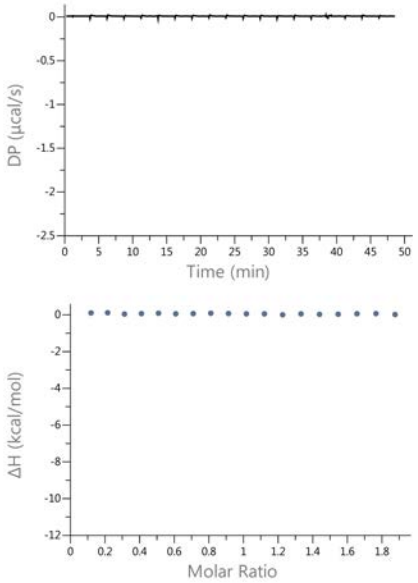
Tris buffer : **8c** (0.1 mM)



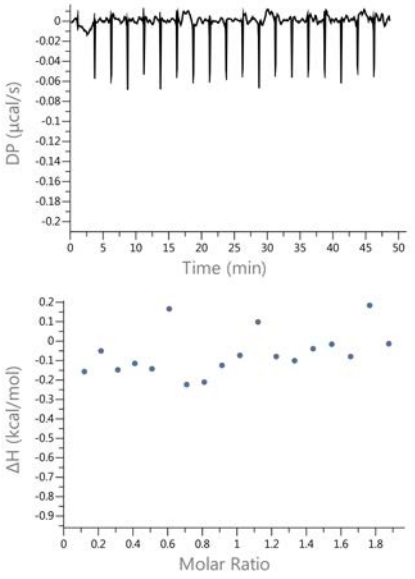
Tris buffer : **8d** (0.1 mM)



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



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



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

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 was 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.

Table S2. The activity of the analogs against NDM-1 and an *E. coli* strain producing the same enzyme[a]

Compound	RC (μM)[b,c,d]	FICI[f]
1a (AMA)	50[e]	0.063
1b (AMB)	50	0.063
1c	200	0.156
1d	100	0.094
1e	>400	>0.281
1f	>400	>0.281
1g	>400	>0.281
1h	>400	>0.281
1i	>400	>0.281
1j	>400	>0.281
1k	>400	>0.281
5 (EDDS.3Na)	25	0.047
8a	100	0.094
8b	400	0.281
8c	>400	>0.281
8d	>400	>0.281
8e	>400	>0.281
8f	200	0.156
8g	>400	>0.281
EDTA.2Na	25	0.047
DPA	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*, a patient isolate producing NDM-1.
[d] The MIC of meropenem against the strain was 32 μg/mL.
[e] None of the test compounds were toxic at 800 μM.
[f] FICI: fractional inhibitory concentration index. See the main text for definition.

References

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2 H. Poddar, J. de Villiers, J. Zhang, V. Puthan Veetil, H. Raj, A.-M. W. H. Thunnissen and G. J. Poelarends, *Biochemistry*, 2018, **57**, 3752–3763.

3 S. S. van Berkel, J. Brem, A. M. Rydzik, R. Salimraj, R. Cain, A. Verma, R. J. Owens, C. W. G. Fishwick, J. Spencer and C. J. Schofield, *J. Med. Chem.*, 2013, **56**, 6945–6953.