Electronic Supplementary Material (ESI) for RSC Advances.

Supporting Information

Synthesis and Biochemical Evaluation of Cephalosporin Analogues Equipped with Chemical Tethers

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S1. Compound Synthesis and Characterisation.

S1.1. General Experimental

Analytical thin layer chromatography (TLC) was performed with EM Science silica gel 60 F254 aluminium plates. Visualisation was carried out using a UV lamp (254 nm) and by immersion in potassium permanganate ($KMnO_4$), followed by heating using a heat gun. Organic solutions were concentrated by rotary evaporation at 40-45 °C. Purification of reaction products were generally done by flash column chromatography using Fluka Silica, pore size 60Å, 220–440 mesh, 35–75 µm.

S1.2. Materials

Unless otherwise noted, all purchased materials were used without purification. All standard solvents were purchased from Sigma Aldrich. All standard acids, bases, and drying agents were purchased from Fisher Scientific. *N*-Hydroxy succinimide (NHS) and *N*, *N*-diisopropylethylamine (DIPEA) were purchased from Acros Organics. Cephalexin monohydrate and Boc_2O were purchased from Fluorochem. Pentynoic acid, *N*,*N*'-dicyclohexylcarbodiimide (DCC), lipoic acid, glutaric anhydride, oxalyl chloride, and Et_3N were purchased from Sigma Aldrich. DMAP was purchased from TCI.

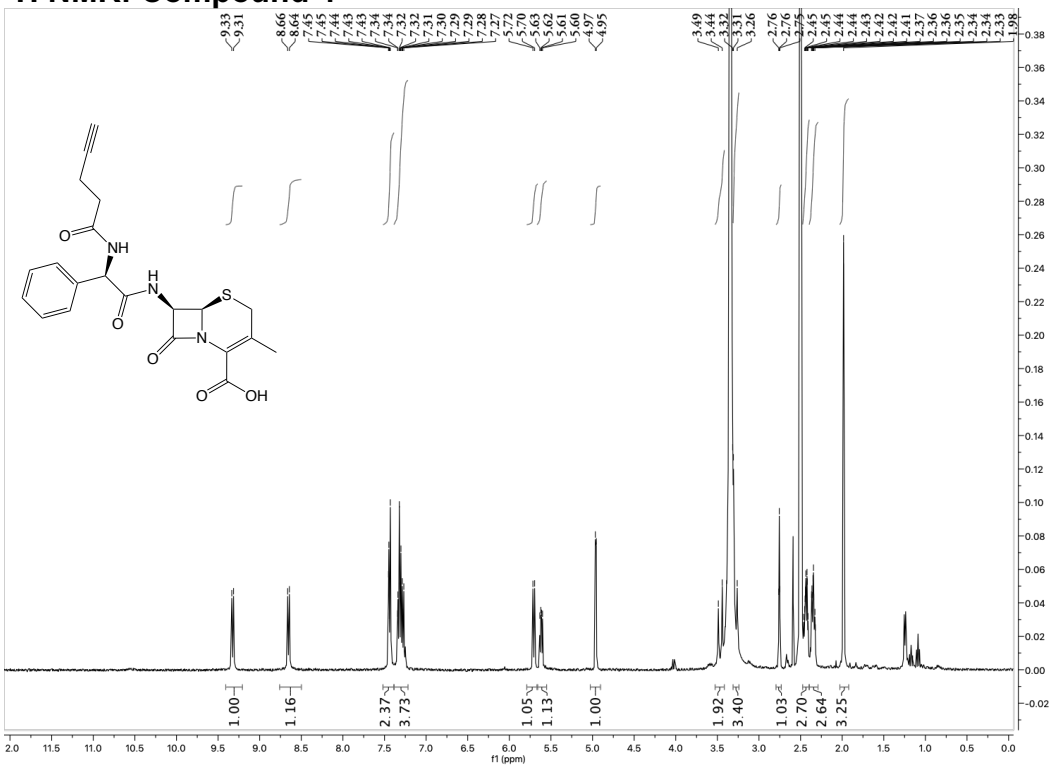
S1.3. Instrumentation

^1H and ^{13}C NMR spectra were recorded on a Jeol ECS 400 (400 MHz for ^1H, 101 MHz for ^{13}C) at ambient temperature. Chemical shifts are reported relative to residual solvent peaks and coupling constants (*J*) are given in Hertz. High-resolution ESI mass spectra were recorded on a Bruker microTOF electrospray mass spectrometer. Infrared (IR) spectra were recorded on a PerkinElmer Spectrum Two (ATIR). Analytical HPLC measurements were performed on a Shimadzu HPLC system (Prominence) equipped with a LC-20AD pump, SIL-20A autosampler, DGU-20AS degasser, CTO-20AC column oven, CBM-20A communication bus module and SPD-M20A diode array detector using a SunFire C18 column (Waters, 4.6 x 150 mm, 5 µm). Eluent gradient: 5-95% MeCN/H_2O with a 0.1% formic acid modifier, over 15 minutes.

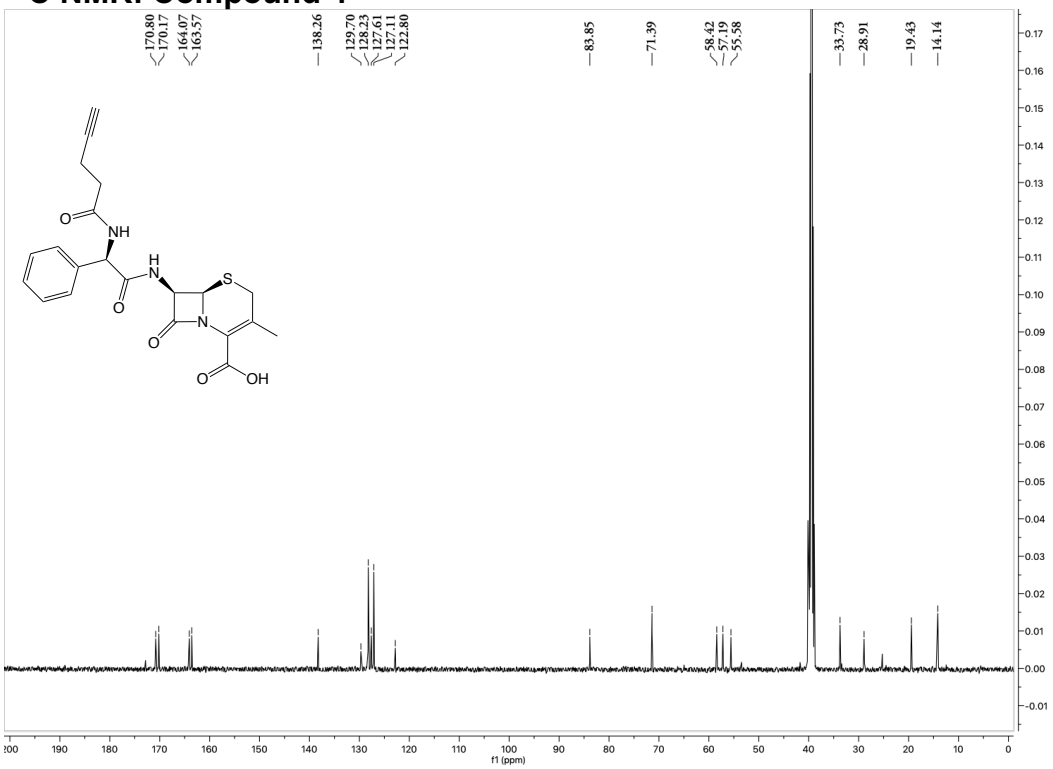
S1.4. Compound Spectra

Compound 4, (6R,7R)-3-methyl-8-oxo-7-[(2R)-2-(pent-4-ynamido)-2-phenylacetamido]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid

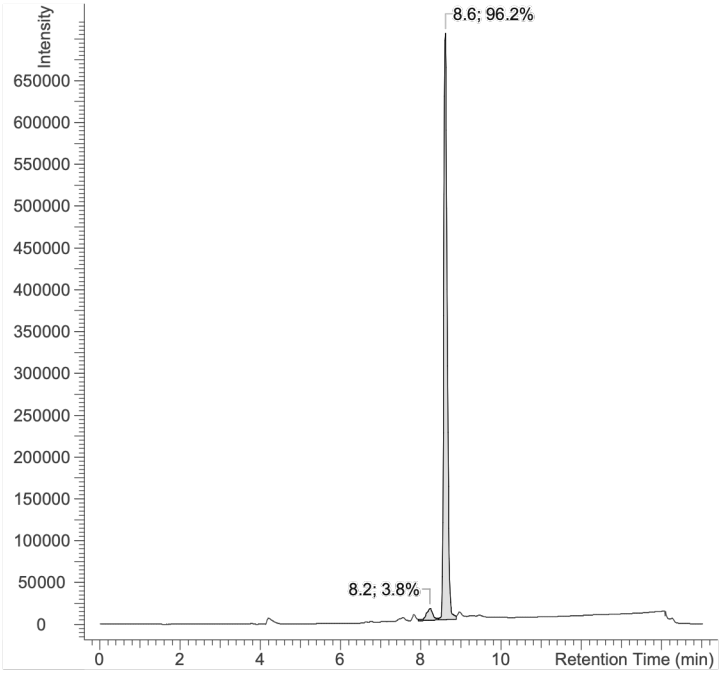
^{1}H NMR: Compound 4



^{13}C NMR: Compound 4

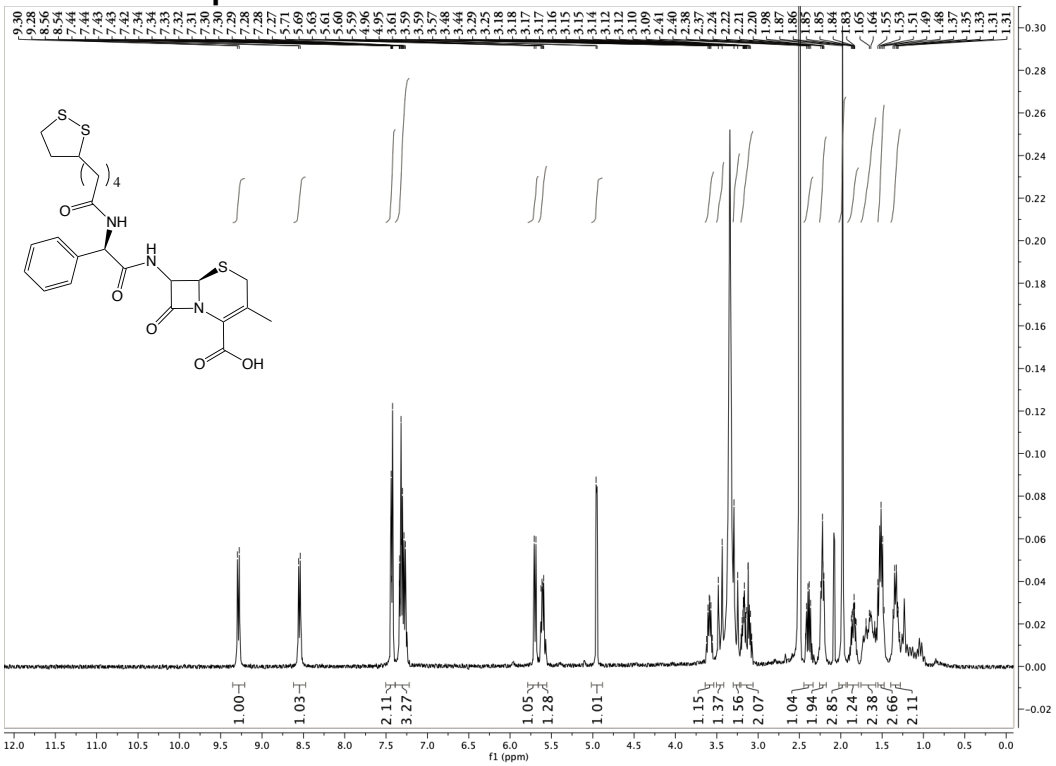


HPLC: Compound 4

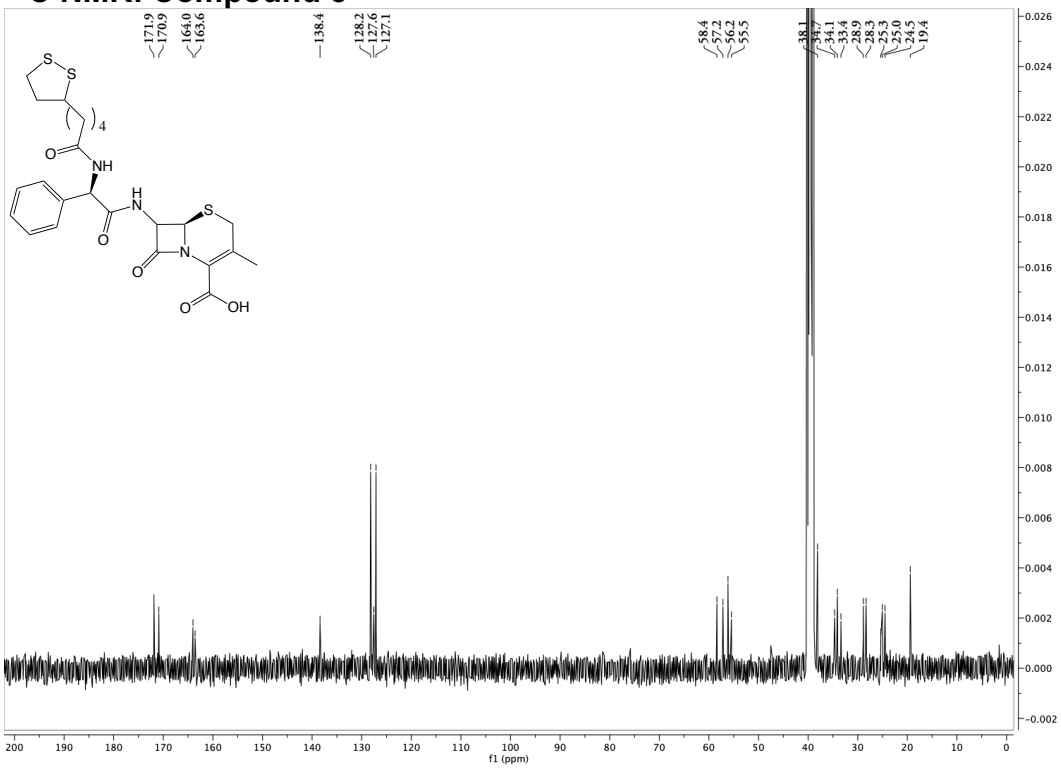


Compound 5, (6R,7R)-7-[(2R)-2-[5-(1,2-dithiolan-3-yl)pentanamido]-2-phenylacetamido]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid

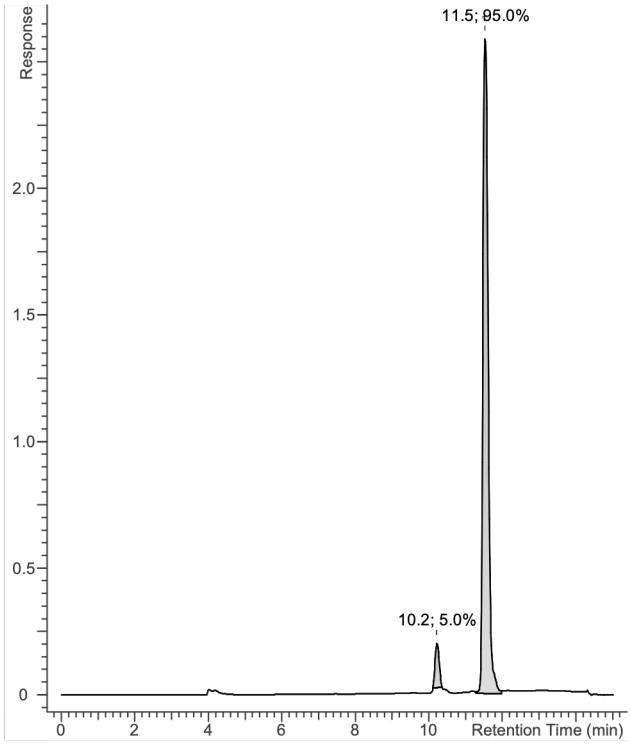
^{1}H NMR: Compound 5



^{13}C NMR: Compound 5

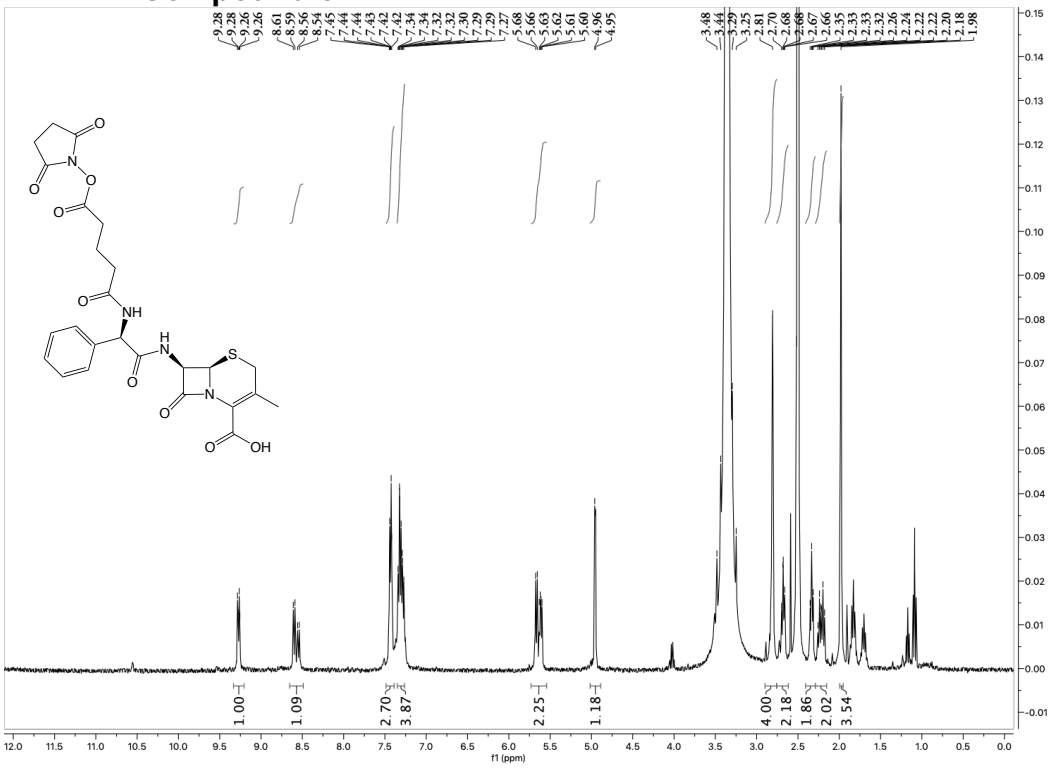


HPLC: Compound 5

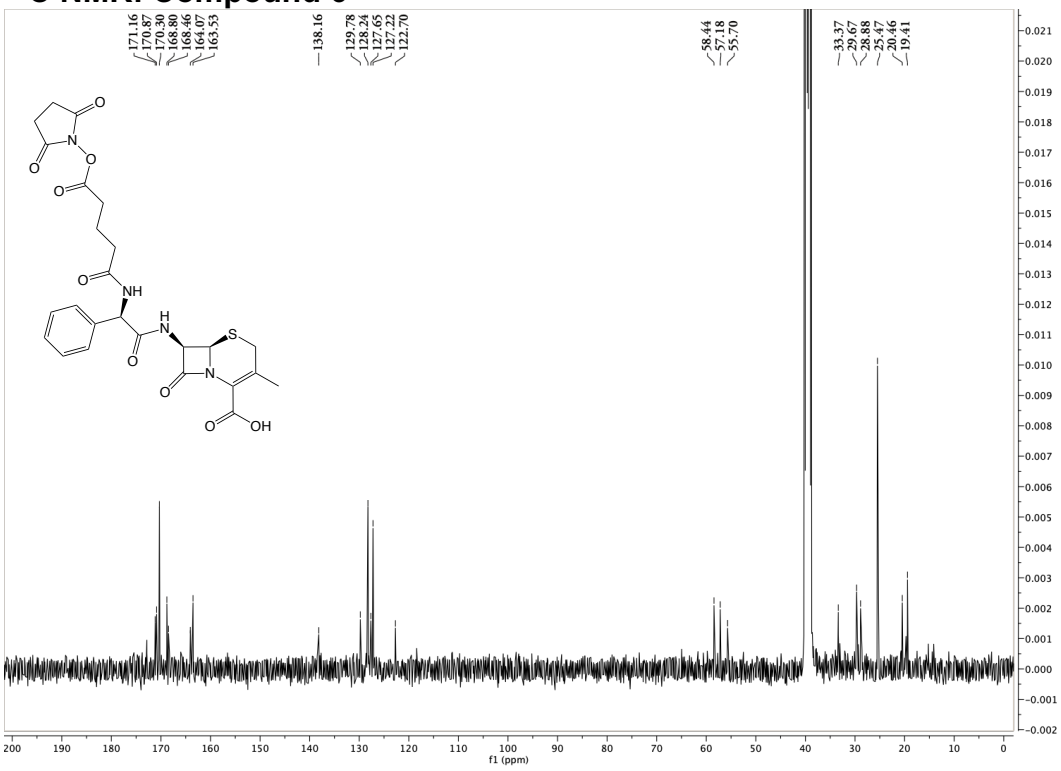


Compound 6, (6R,7R)-7-[(2R)-2-{5-[(2,5-dioxopyrrolidin-1-yl)oxy]-5-oxopentanamido}-2-phenylacetamido]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid

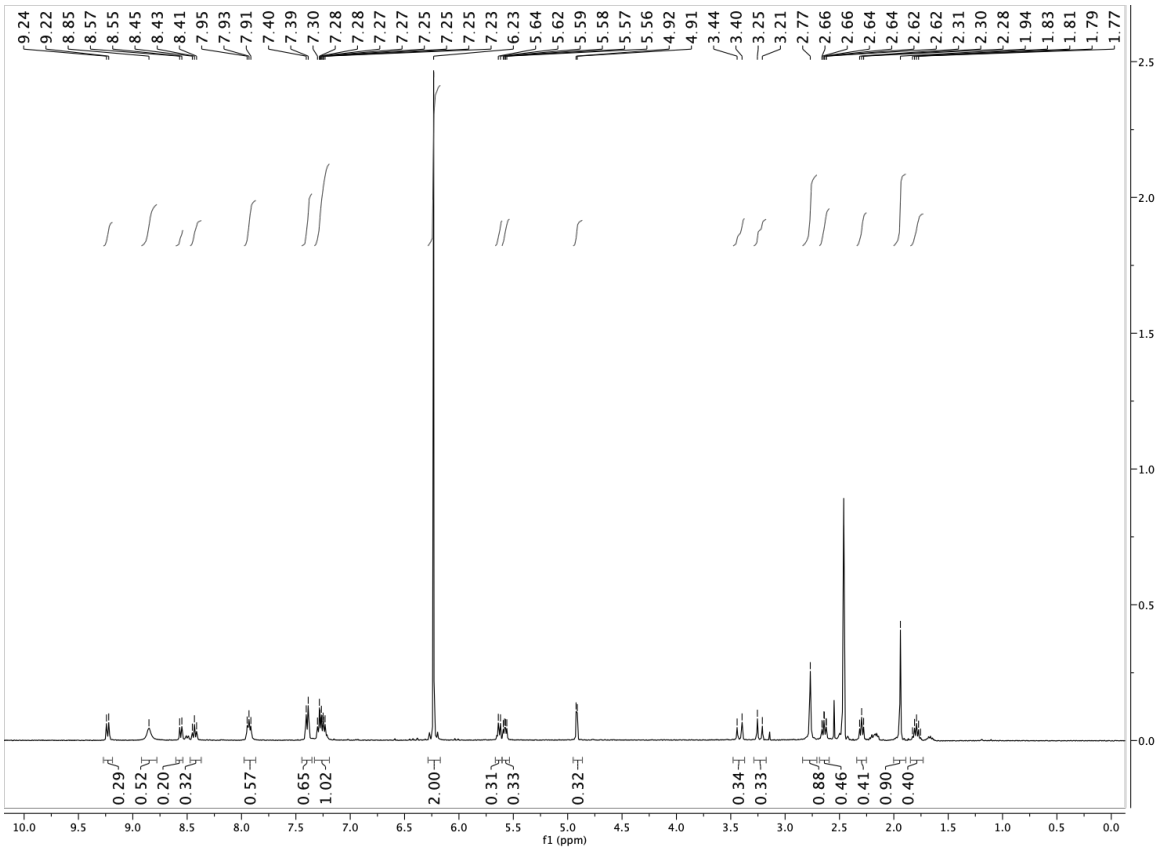
^{1}H NMR: Compound 6



^{13}C NMR: Compound 6



QNMR: Compound 6, ^{1}H NMR in DMSO-d_6 with maleic acid reference



QNMR purity determination:

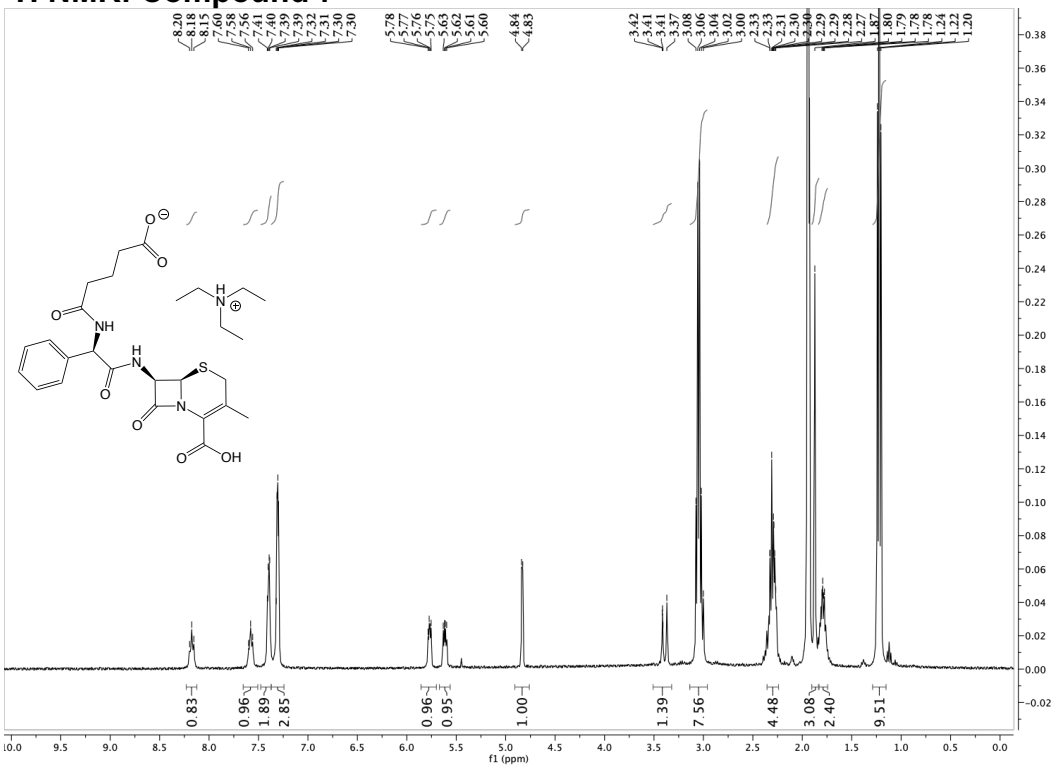
Reference: Maleic acid, MW 116, mass 2.4 mg, δ 7.23 (s, 2H)

Sample: Compound 6, MW 558, mass 4.1 mg, average of three peaks = 0.32 (δ 5.63 (d, 0.31H), 5.58 (dd, 0.33H), 4.91 (d, 0.32H))

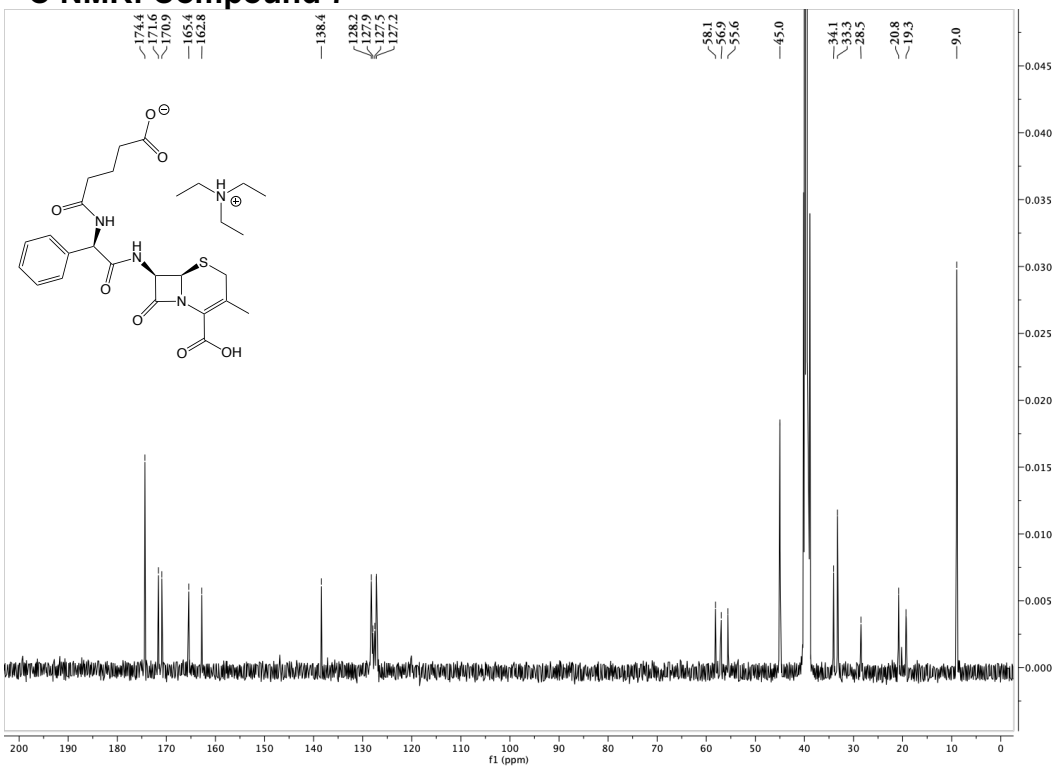
%purity = (measured mmol/predicted mmol) x 100
= (0.0066/0.0073) x 100
= 90.4%

Compound 7, (6R,7R)-7-[(2R)-2-(4-carboxybutanamido)-2-phenylacetamido]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid

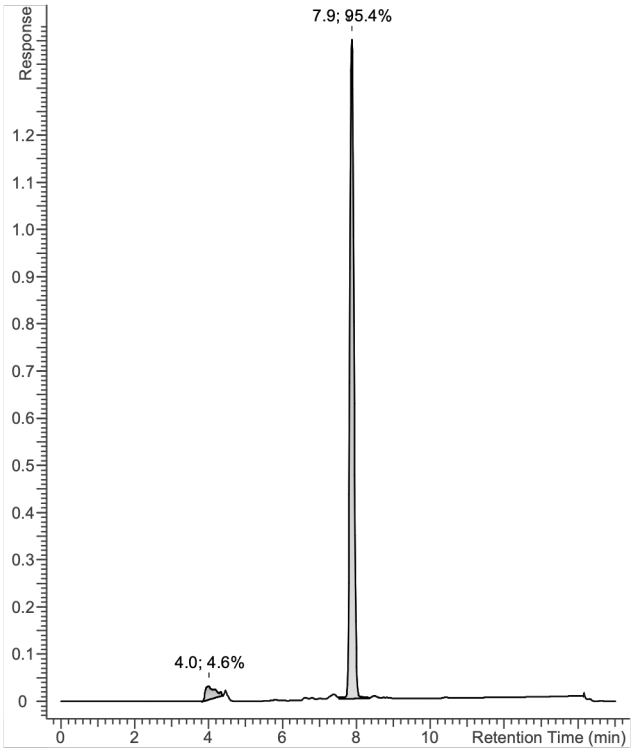
^{1}H NMR: Compound 7



^{13}C NMR: Compound 7

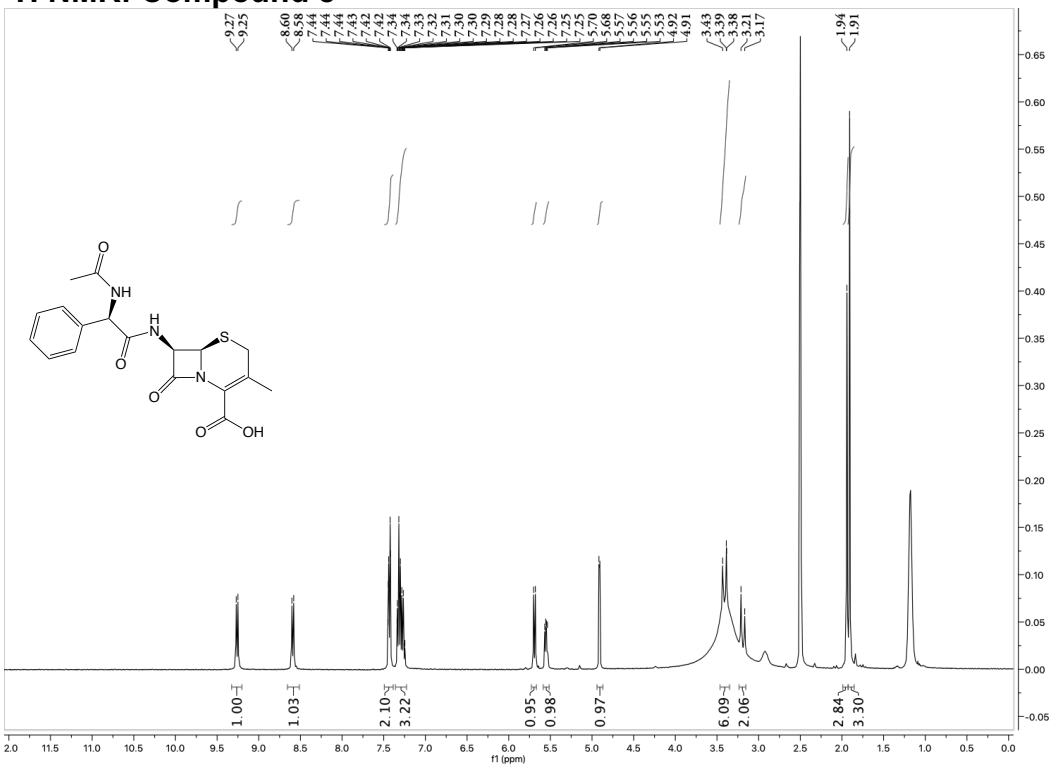


HPLC: Compound 7

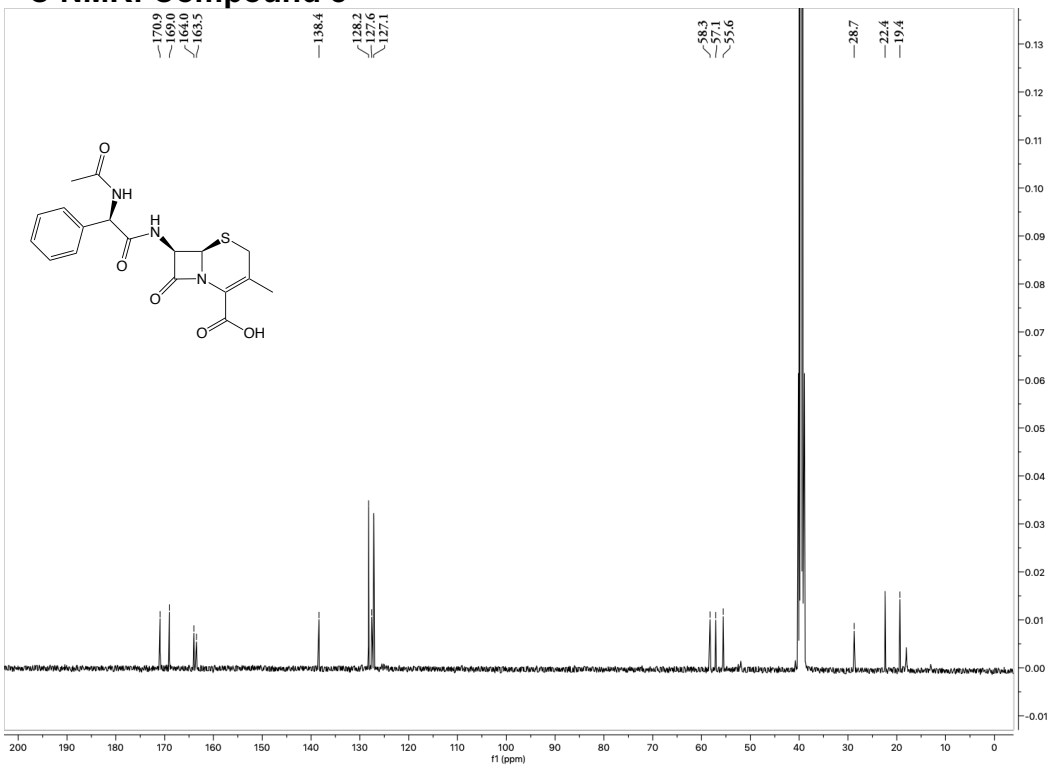


Compound 8, (6R,7R)-7-[(2R)-2-acetamido-2-phenylacetamido]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid

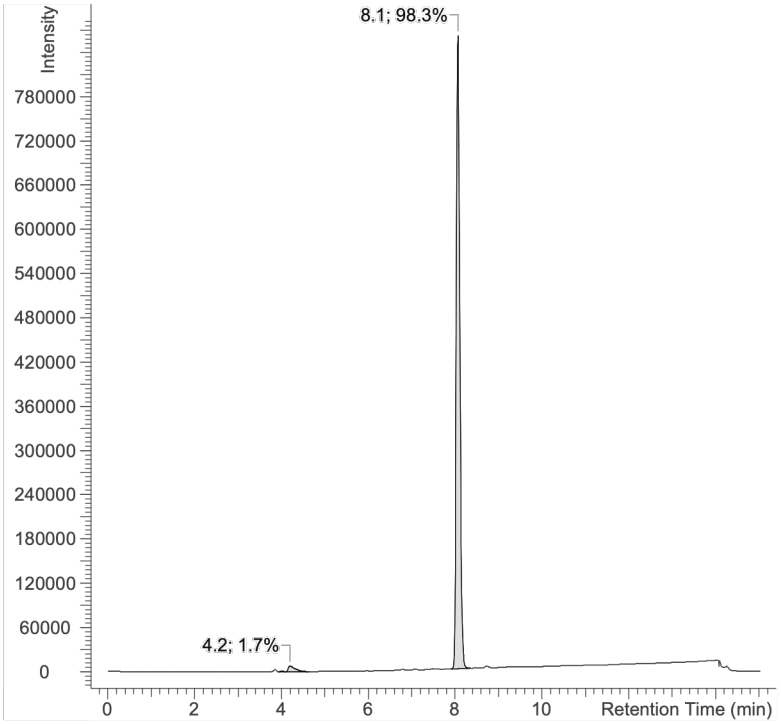
^{1}H NMR: Compound 8



^{13}C NMR: Compound 8

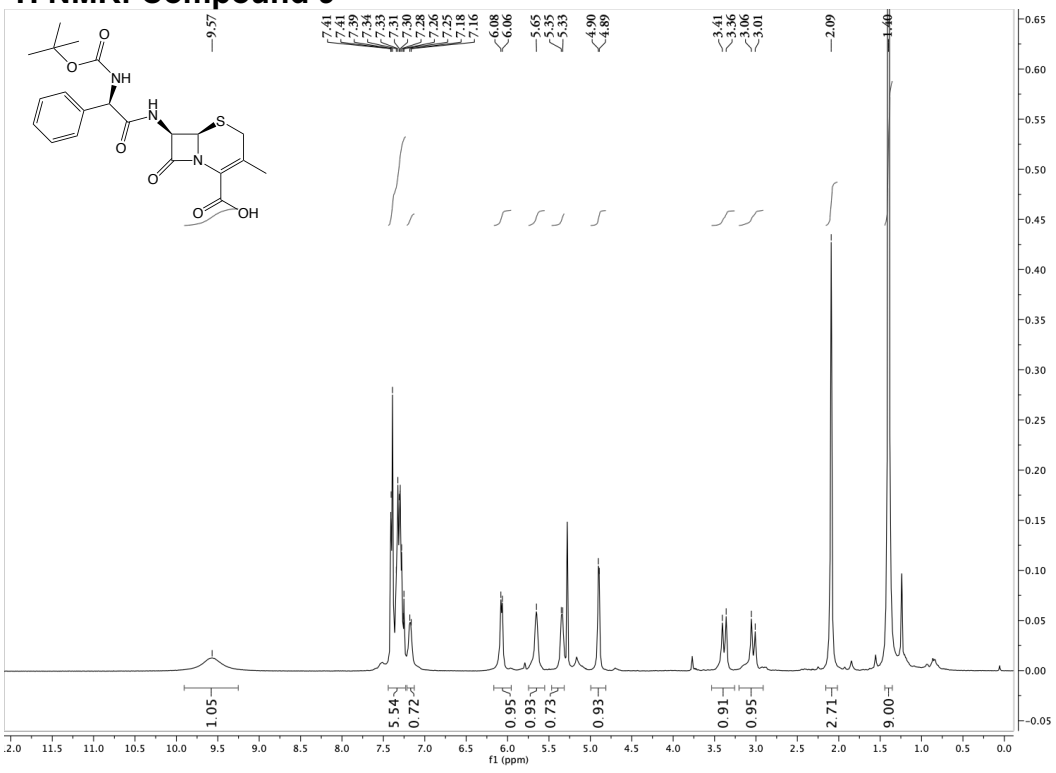


HPLC: Compound 8

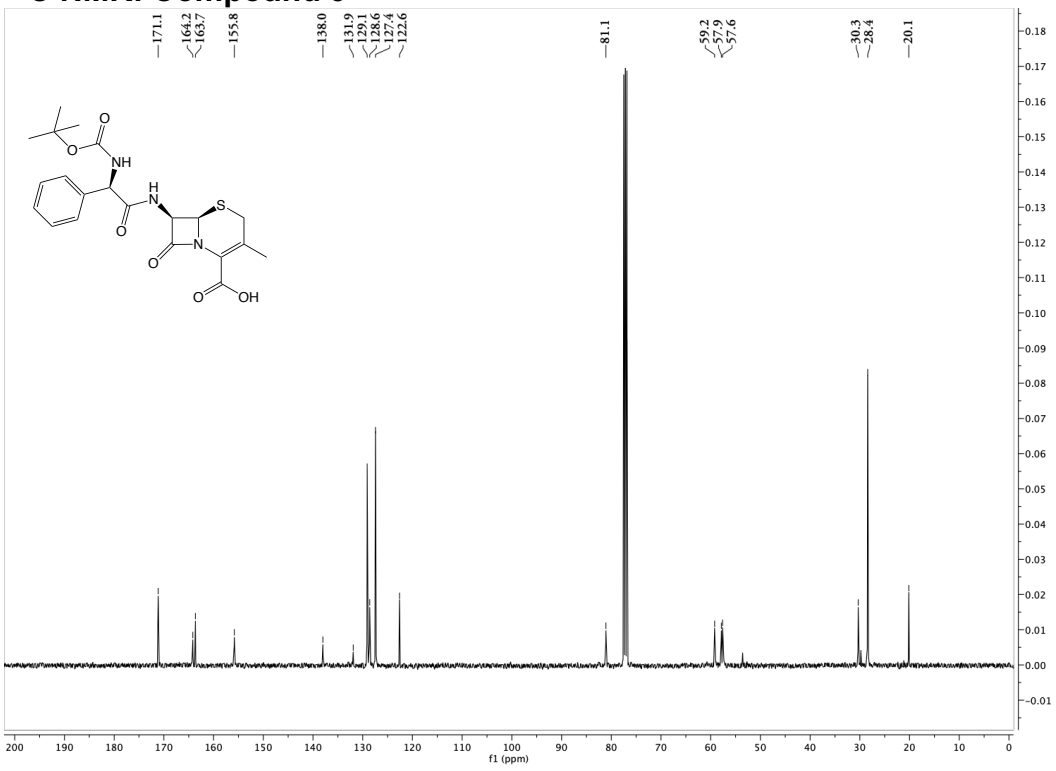


Compound 9, (6R,7R)-7-[(2R)-2-{[(tert-butoxy)carbonyl]amino}-2-phenylacetamido]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid

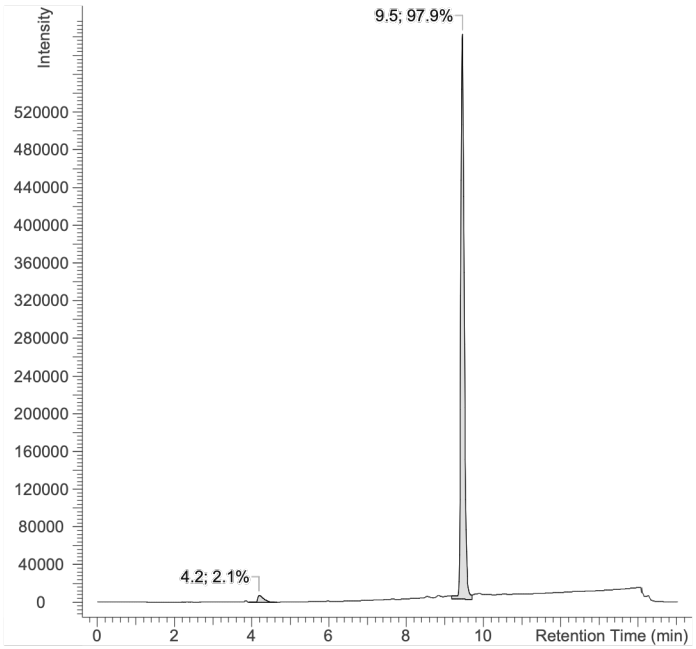
^{1}H NMR: Compound 9



^{13}C NMR: Compound 9

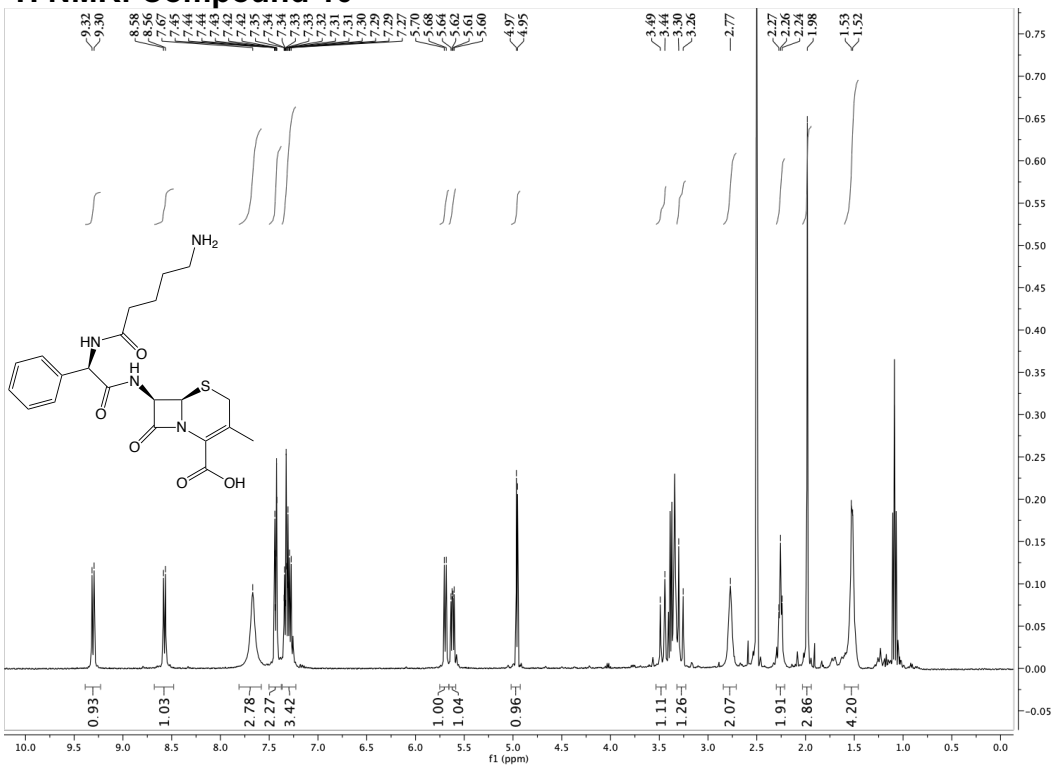


HPLC: Compound 9

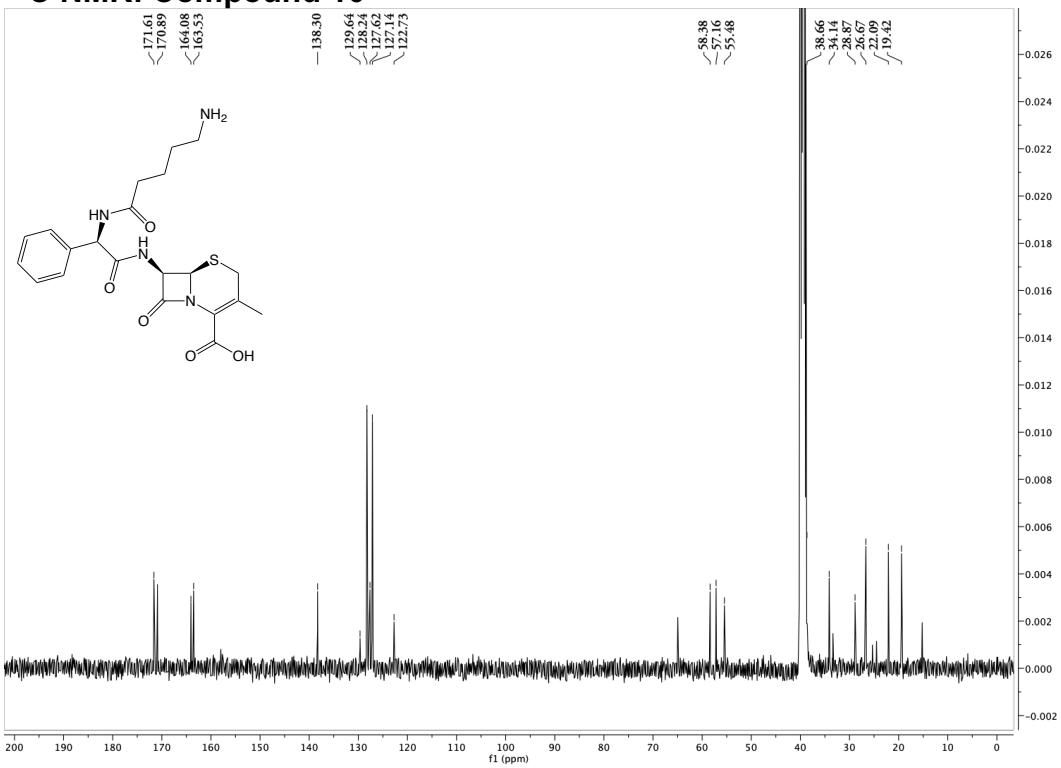


Compound 10, (6R,7R)-7-[(2R)-2-(5-aminopentanamido)-2-phenylacetamido]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid

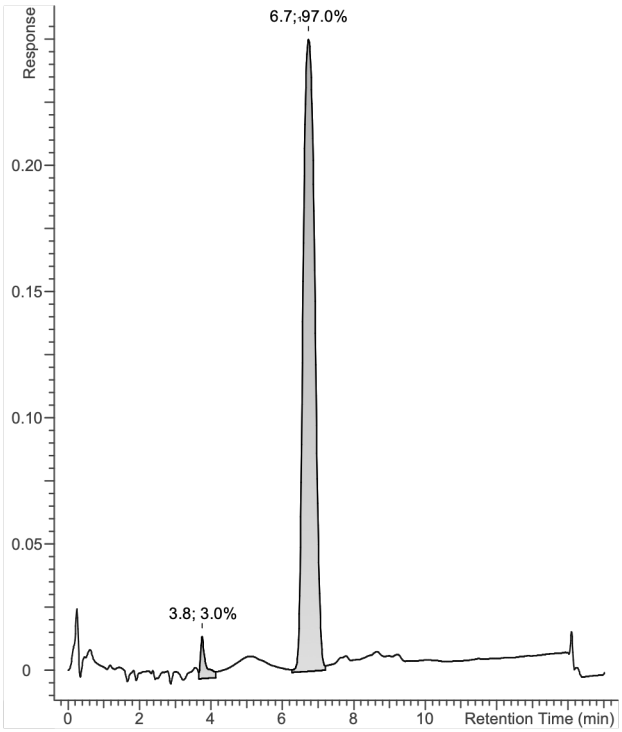
^{1}H NMR: Compound 10



^{13}C NMR: Compound 10

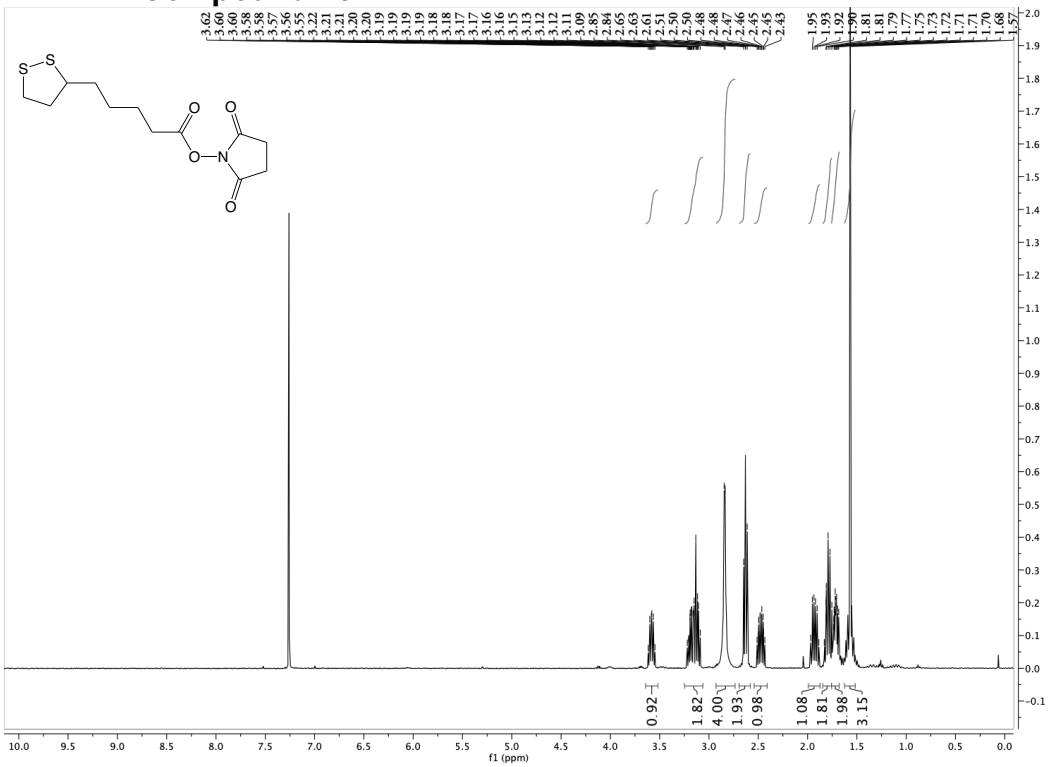


HPLC: Compound 10

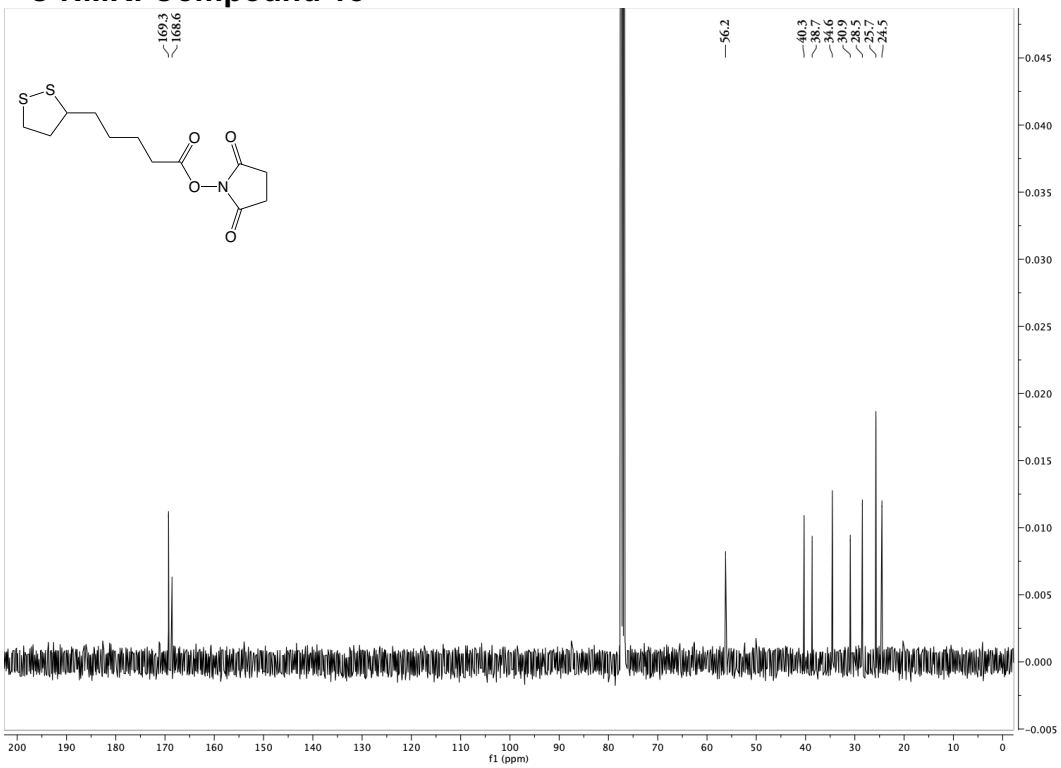


Compound 18, 2,5-dioxopyrrolidin-1-yl 5-(1,2-dithiolan-3-yl)pentanoate

^{1}H NMR: Compound 18

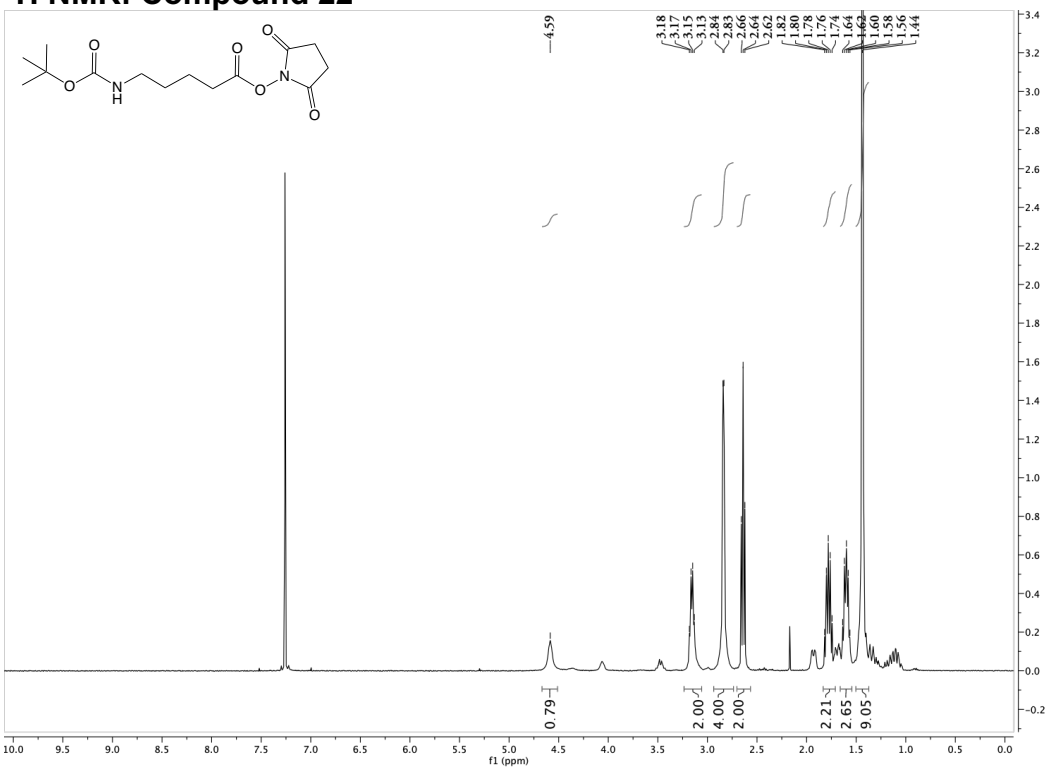


^{13}C NMR: Compound 18

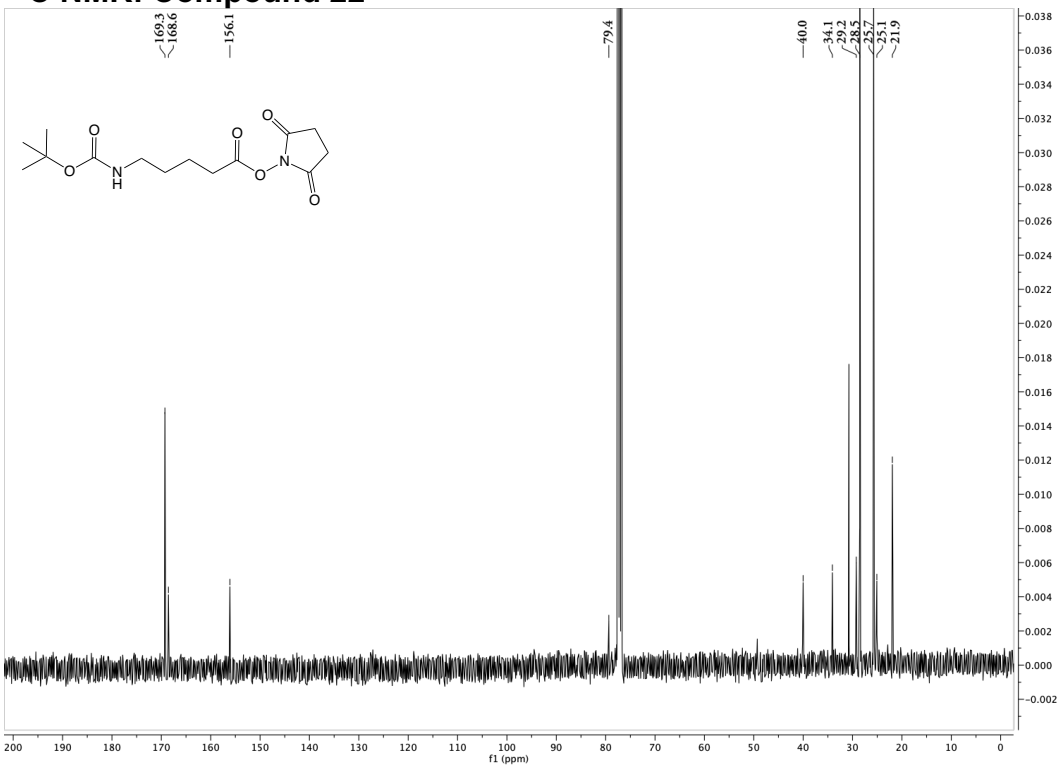


Compound 22, 2,5-dioxopyrrolidin-1-yl 5-{[(*tert*-butoxy)carbonyl]amino} pentanoate

^{1}H NMR: Compound 22

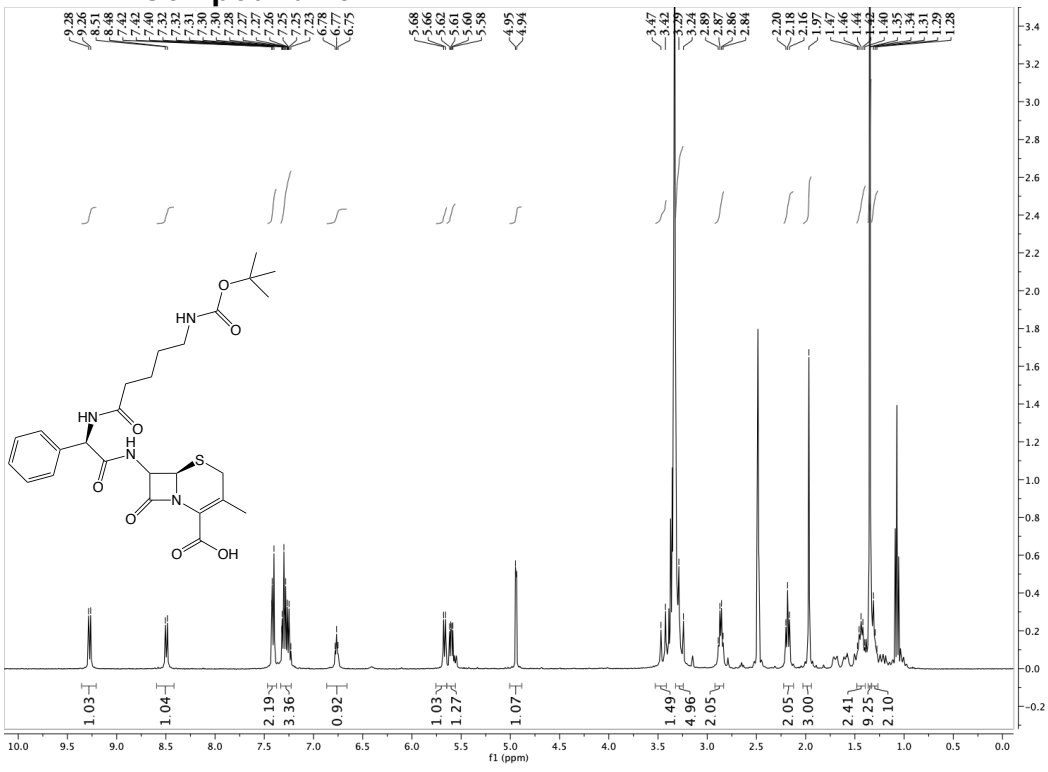


^{13}C NMR: Compound 22

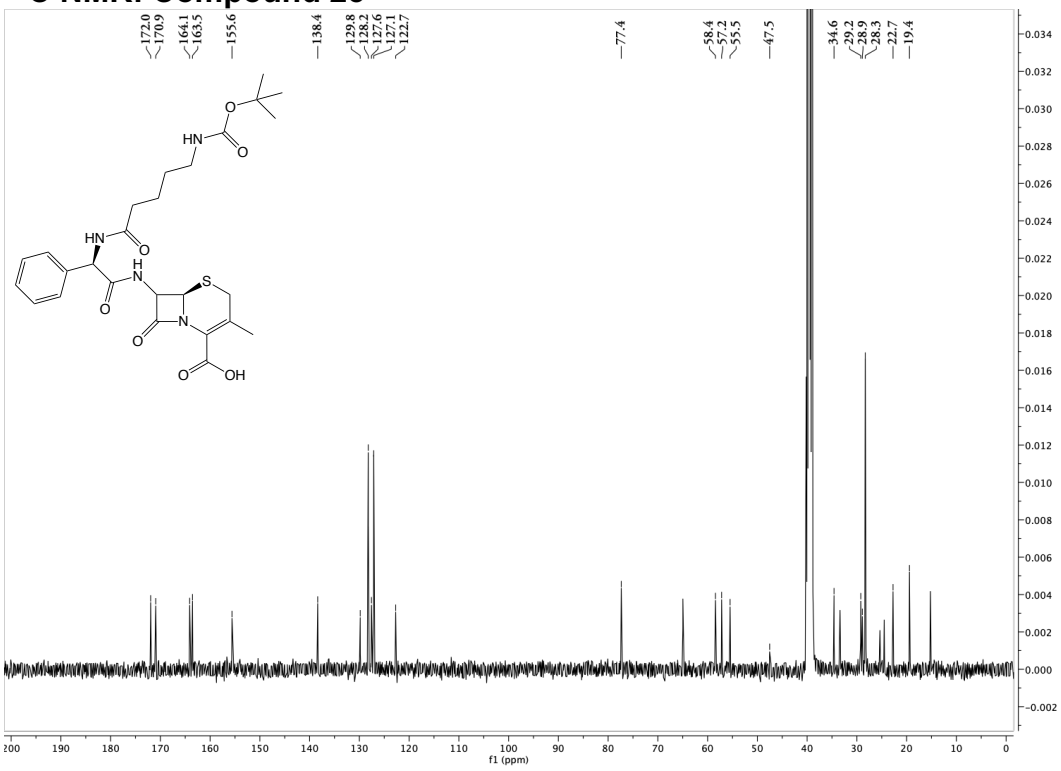


Compound 23, (6R,7R)-7-[(2R)-2-(5-{[(*tert*-butoxy)carbonyl]amino}pentanamido)-2-phenylacetamido]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid

^{1}H NMR: Compound 23



^{13}C NMR: Compound 23



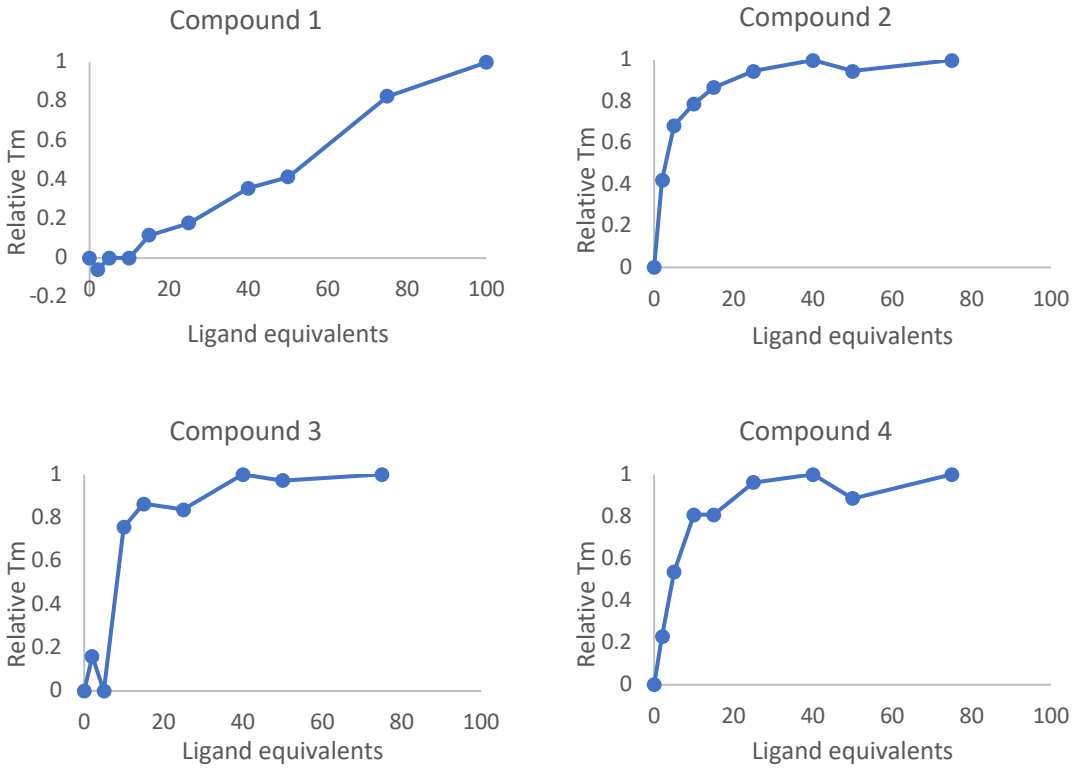
S2. PBP Thermal Shift Assay

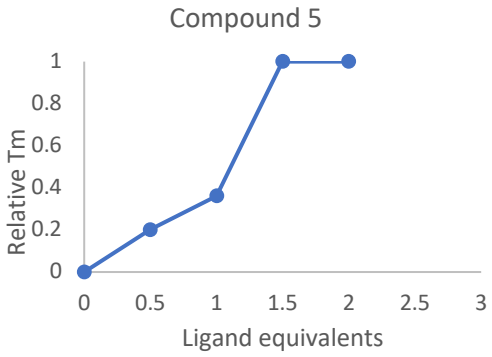
S2.1. PBP3 Data

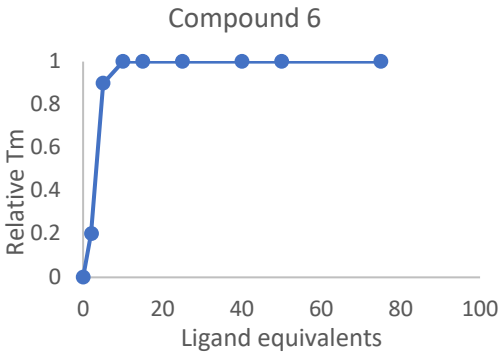
Table S1: Thermal shift assay results with PBP3

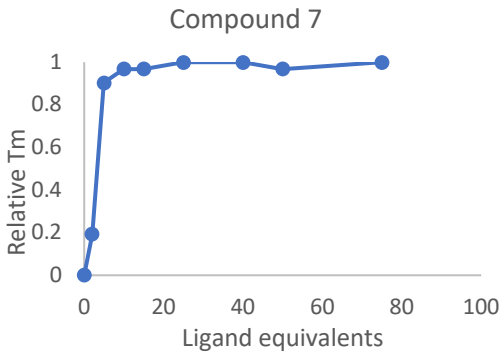
Sample	Tm 1/°C	Tm 2/°C	Tm 3/°C	Average Tm/°C	
PBP3	55.2570138	55.2570138	55.2570138	55.2570138	
	ΔTm 1/°C	**ΔTm 2/°C**	**ΔTm 3/°C**	**Average ΔTm/°C**	**ΔTm/°C, ±s.d.**
Compound 1	+1.8065	+1.8065	+1.2076	+1.6069	+1.61, ±0.35
Compound 2	+4.7994	+4.7994	+4.5585	+4.7191	+4.51, ±0.14
Compound 3	+3.5988	+3.5988	+1.8065	+3.0014	+3.00, ±1.03
Compound 4	+3.009	+3.5988	+2.4077	+3.0052	+3.01, ±0.60
Compound 5	+3.009	+3.009	+3.009	+3.009	+3.01, ±0
Compound 6	+3.5988	+3.009	+3.009	+3.2056	+3.21, ±0.34
Compound 7	+3.5988	+3.5988	+3.5988	+3.5988	+3.60, ±0
Compound 8	+3.009	+3.009	+3.009	+3.009	+3.01, ±0
Compound 9	+1.8065	+1.8065	+1.2076	+1.6069	+1.61, ±0.34
Compound 10	+3.009	+2.4077	+3.009	+2.8086	+2.81, ±0.35
Compound 11	+3.5988	+3.009	+3.5988	+3.4022	+3.40, ±0.34
Compound 12	+4.2194	+4.816	+4.2194	+4.4182	+4.42, ±0.34

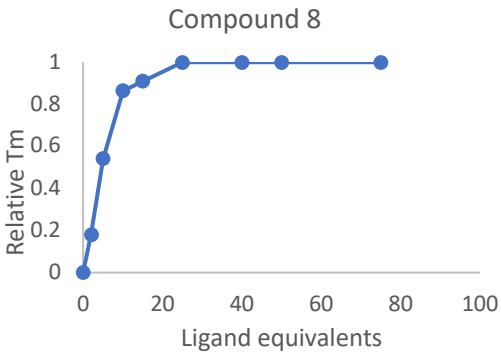
Relative Tm values vs. ligand equivalents for compounds **1**-**12** with PBP3. Relative Tm values were calculated as a ratio relative to the highest Tm value and plotted against the equivalents of ligand used. Values are an average of three runs.

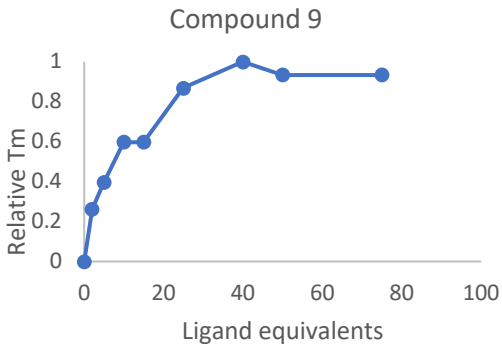


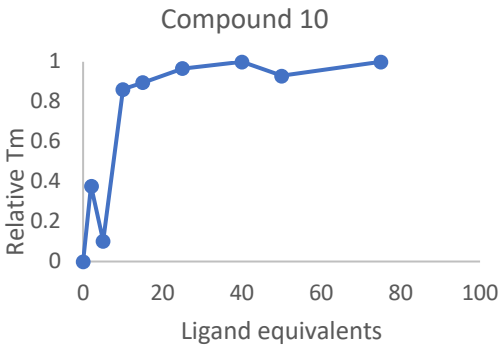

Compound 5
Relative Tm
1
0.8
0.6
0.4
0.2
0
0
0.5
1
1.5
2
2.5
3
Ligand equivalents

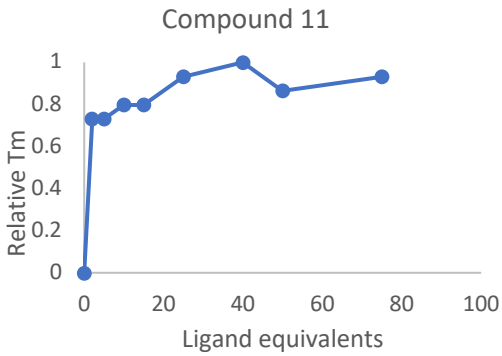

Compound 6
Relative Tm
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
Ligand equivalents

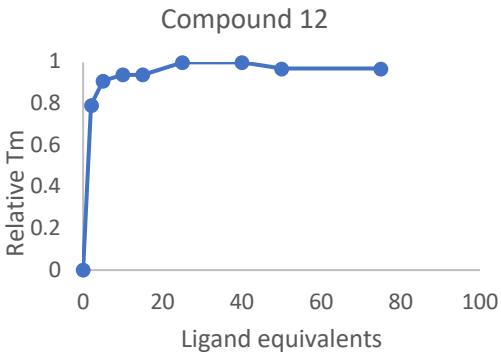

Compound 7
Relative Tm
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
Ligand equivalents


Compound 8
Relative Tm
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
Ligand equivalents


Compound 9
Relative Tm
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
Ligand equivalents


Compound 10
Relative Tm
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
Ligand equivalents


Compound 11
Relative Tm
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
Ligand equivalents


Compound 12
Relative Tm
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
Ligand equivalents

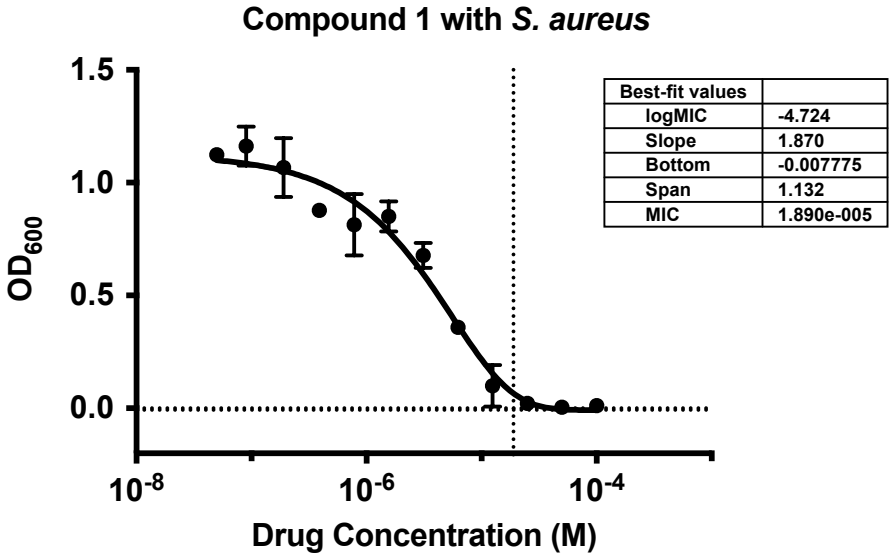
S2.2. PBP4 Data

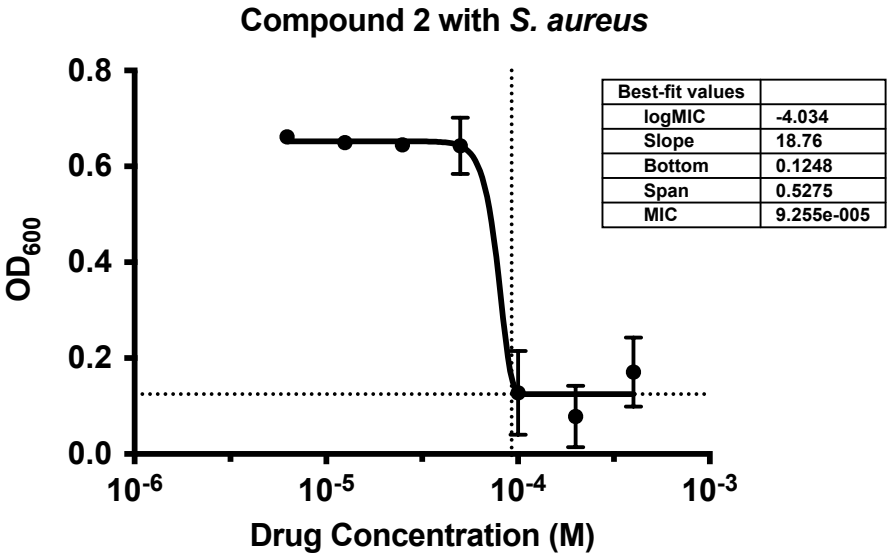
Table S3: Thermal shift assay results with PBP4

Sample	**Tm 1/°C**	**Tm 2/°C**	**Tm 3/°C**	**Average Tm/°C**	
PBP4	54.24049	54.2415771	54.4431267	54.3083979	
	ΔTm 1/°C	**ΔTm 2/°C**	**ΔTm 3/°C**	**Average ΔTm/°C**	**ΔTm/°C, ±s.d.**
Compound 1	-1.0801036	-0.8823153	-0.6821696	-0.8815295	-0.88, ±0.20
Compound 2	-2.3243078	-2.1986516	-1.33793	-1.9536298	-1.95, ±0.54
Compound 3	-0.2168897	-0.0951398	-0.82245	-0.3781598	-0.38, ±0.39
Compound 4	-0.8124097	-0.6918615	-1.0553525	-0.8532079	-0.85, ±0.19
Compound 5	-0.0615412	-1.2663256	-0.0632731	-0.4637133	-0.46, ±0.70
Compound 6	-0.8645109	-0.5057653	-0.7545522	-0.7082761	-0.71, ±0.18
Compound 7	-0.2618154	-0.5028661	-0.502477	-0.4223862	-0.42, ±0.14
Compound 8	0.24304072	-0.4932111	-0.4936002	-0.2479235	-0.25, ±0.43
Compound 9	-0.7306188	-0.7299665	-0.9672788	-0.809288	-0.81, ±0.14
Compound 10	0.25836054	-0.4749921	0.13492076	-0.0272369	-0.03, ±0.39
Compound 11	-0.7074216	-0.5856679	-0.9453786	-0.7461561	-0.75, ±0.18
Compound 12	-0.6960424	-0.5755933	-0.8143819	-0.6953392	-0.70, ±0.12

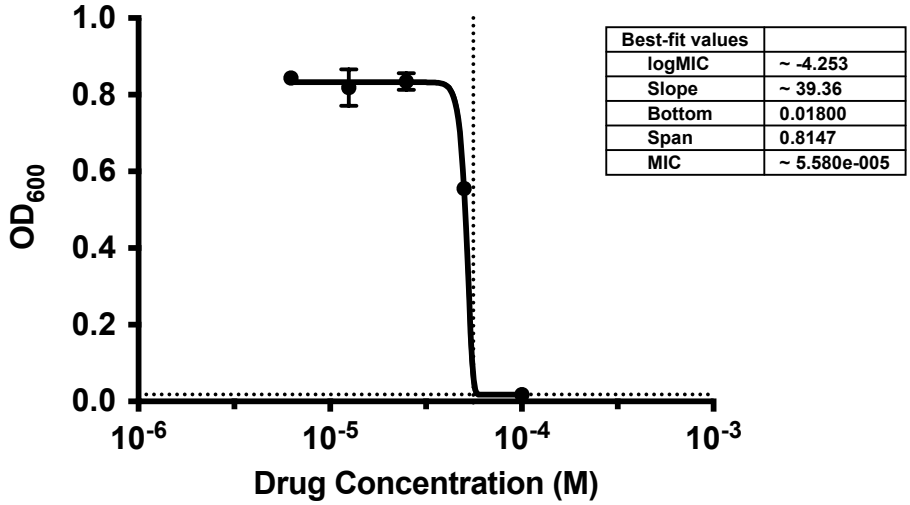
S3. MIC Assay Results

S3.1. *S. aureus* MIC curves



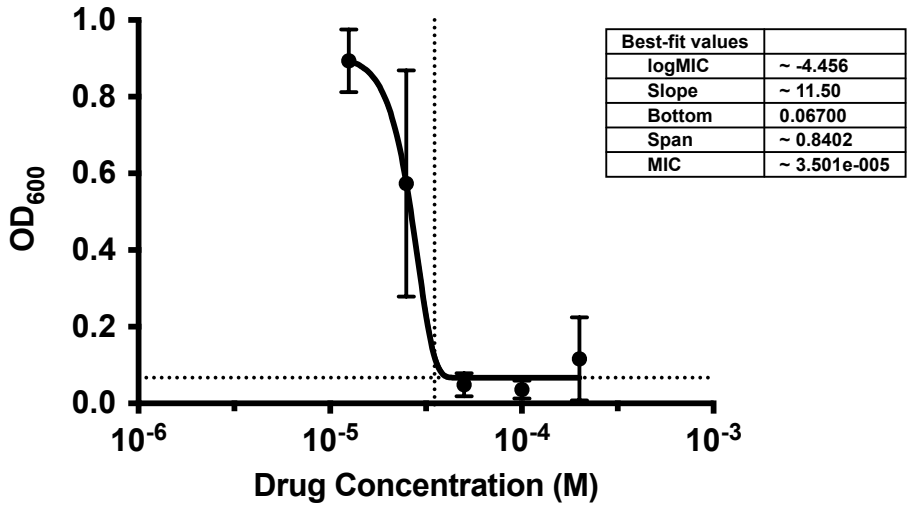


Compound 3 with *S. aureus*



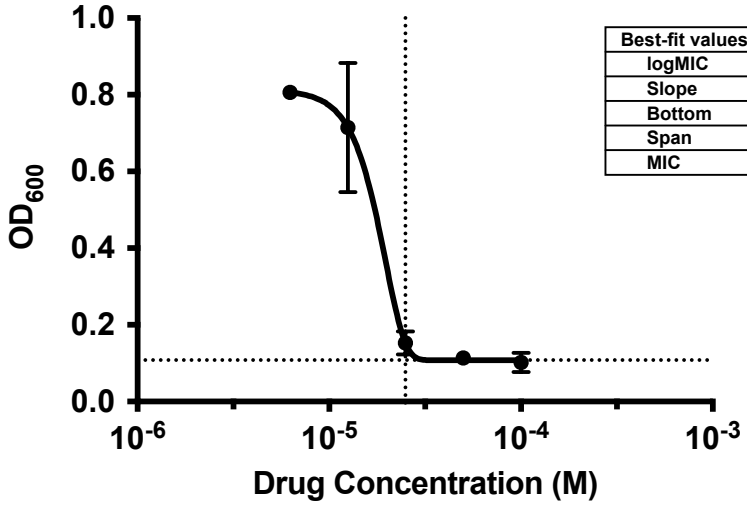
Best-fit values	
logMIC	~ -4.253
Slope	~ 39.36
Bottom	0.01800
Span	0.8147
MIC	~ 5.580e-005

Compound 4 with *S. aureus*



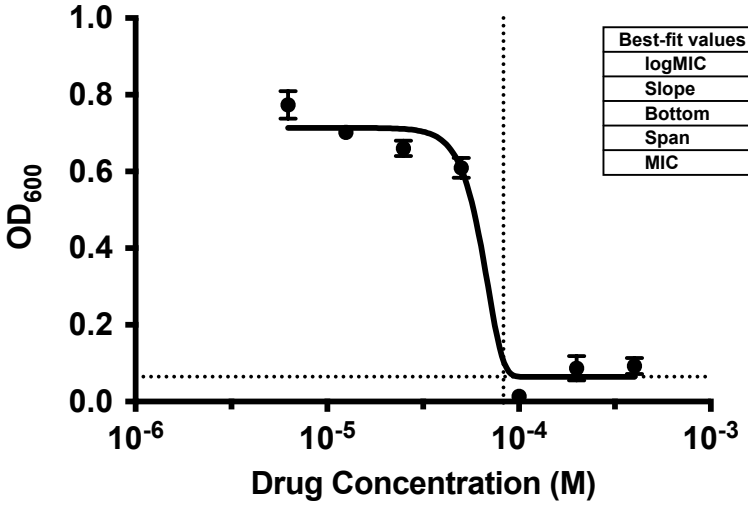
Best-fit values	
logMIC	~ -4.456
Slope	~ 11.50
Bottom	0.06700
Span	~ 0.8402
MIC	~ 3.501e-005

Compound 5 with *S. aureus*



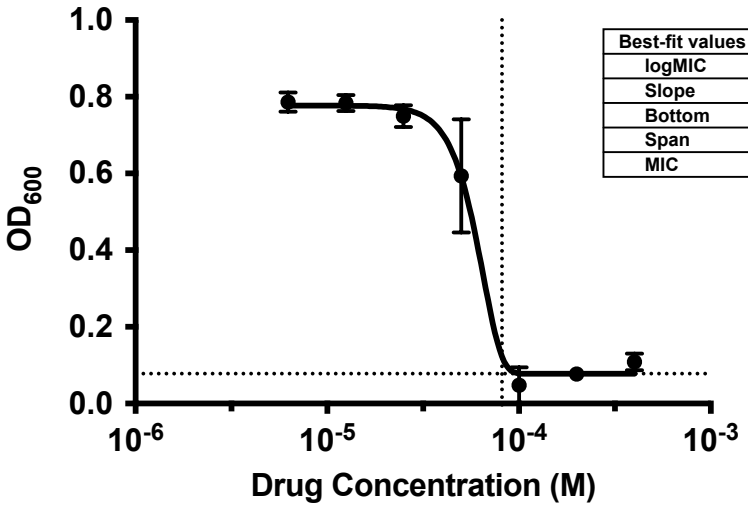
Best-fit values	
logMIC	-4.603
Slope	9.715
Bottom	0.1080
Span	0.7036
MIC	2.493e-005

Compound 6 with *S. aureus*



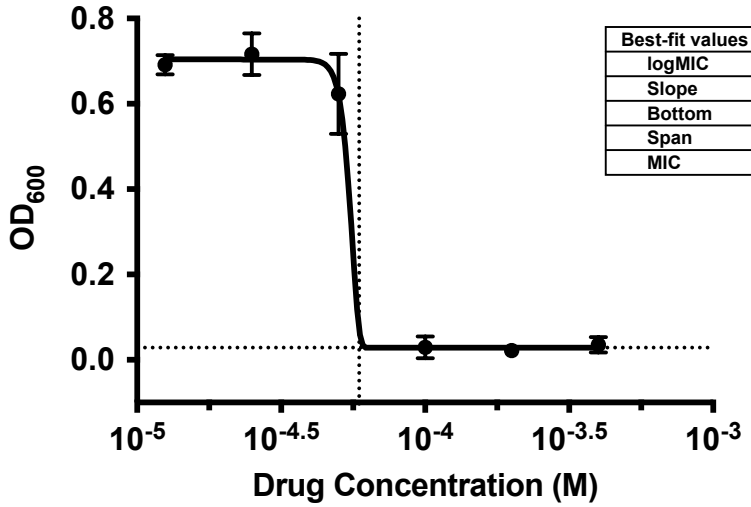
Best-fit values	
logMIC	-4.081
Slope	12.37
Bottom	0.06475
Span	0.6491
MIC	8.290e-005

Compound 7 with *S. aureus*



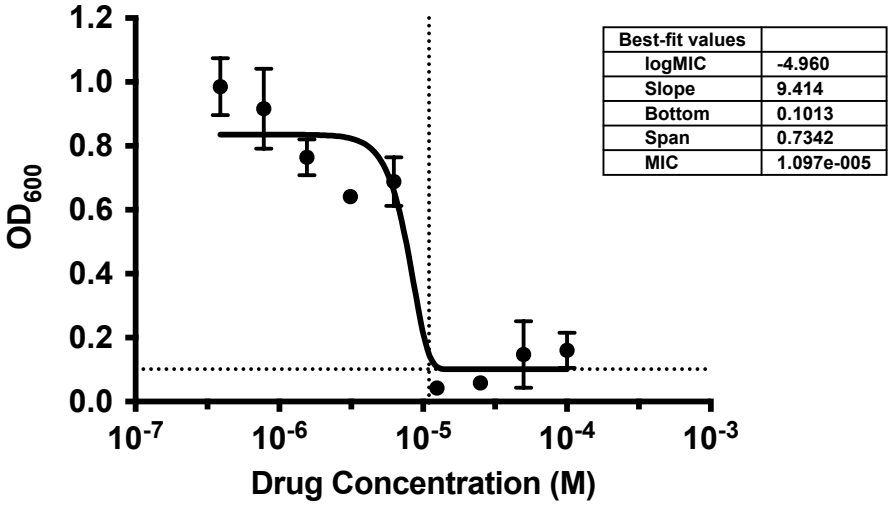
Best-fit values	
logMIC	-4.089
Slope	10.28
Bottom	0.07785
Span	0.6994
MIC	8.144e-005

Compound 8 with *S. aureus*



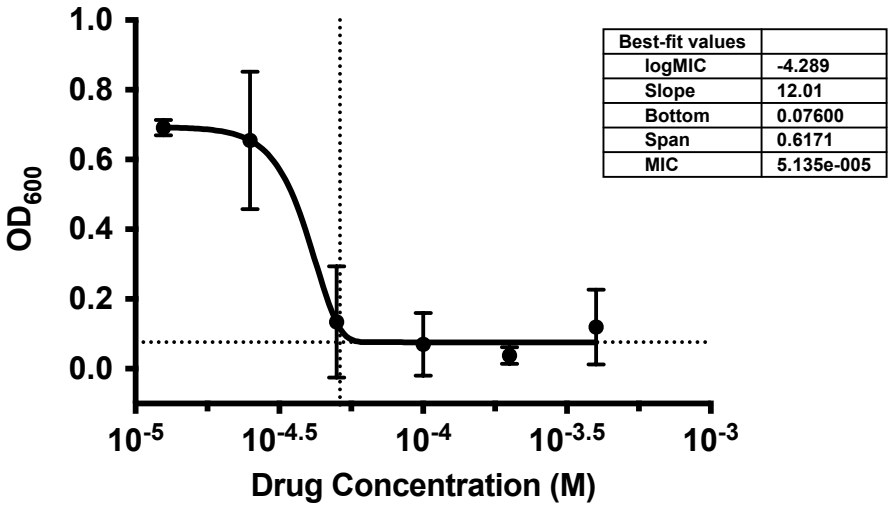
Best-fit values	
logMIC	~ -4.229
Slope	~ 42.68
Bottom	0.02850
Span	0.6755
MIC	~ 5.897e-005

Compound 9 with *S. aureus*



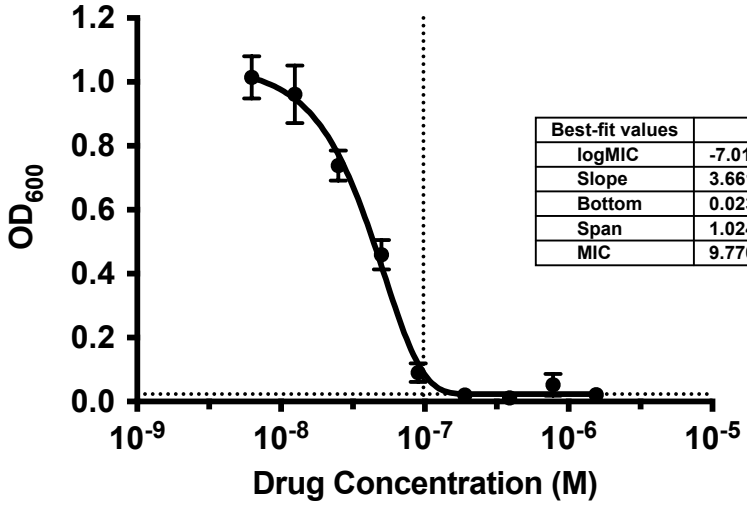
Best-fit values	
logMIC	-4.960
Slope	9.414
Bottom	0.1013
Span	0.7342
MIC	1.097e-005

Compound 10 with *S. aureus*

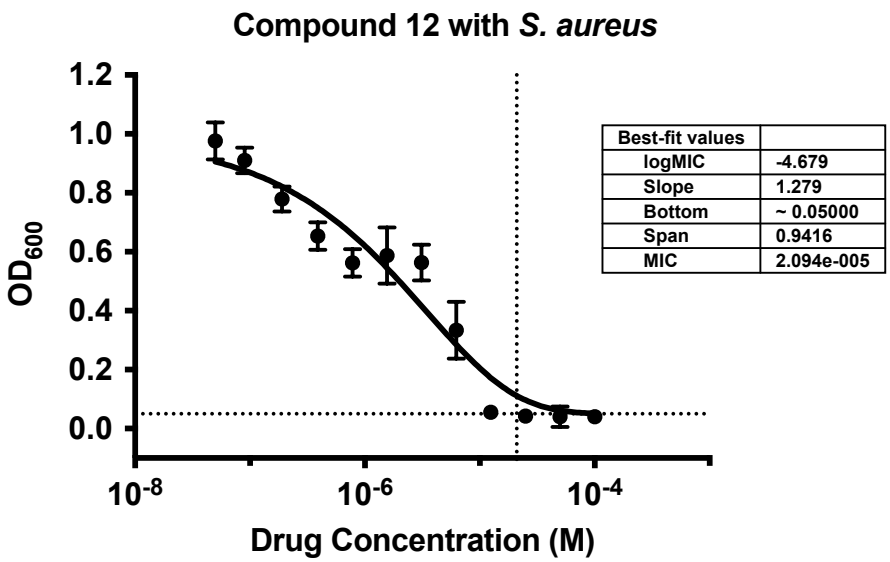


Best-fit values	
logMIC	-4.289
Slope	12.01
Bottom	0.07600
Span	0.6171
MIC	5.135e-005

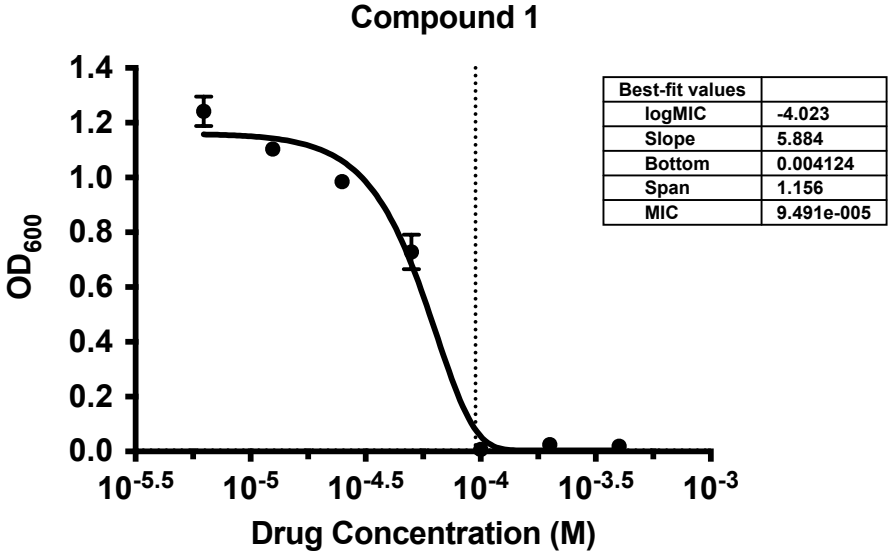
Compound 11 with *S. aureus*

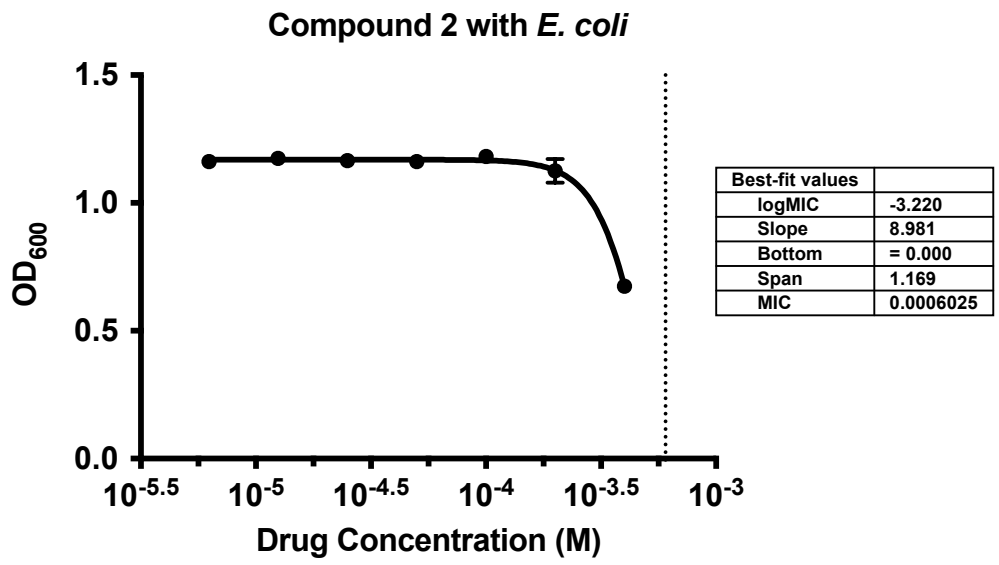


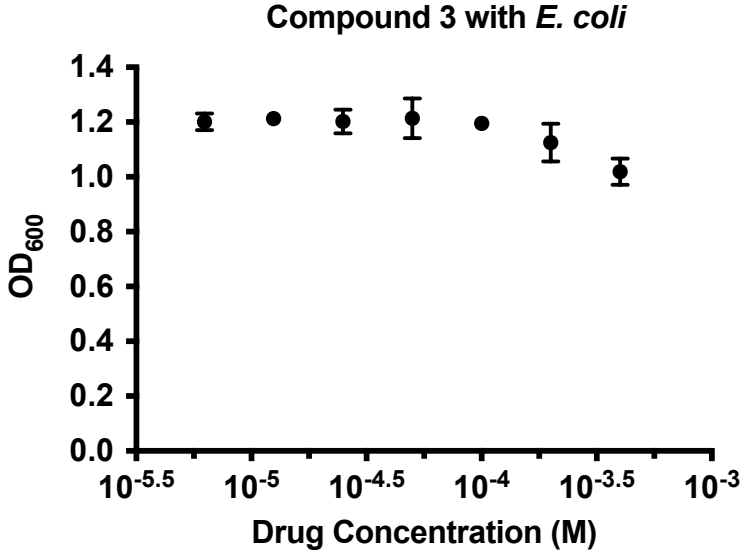
Best-fit values	
logMIC	-7.010
Slope	3.661
Bottom	0.02317
Span	1.024
MIC	9.770e-008

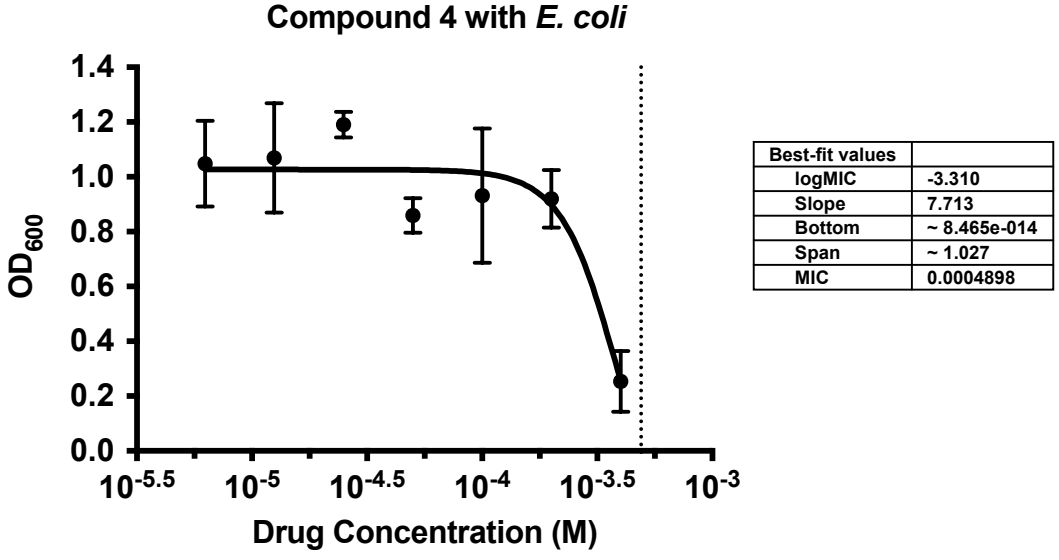


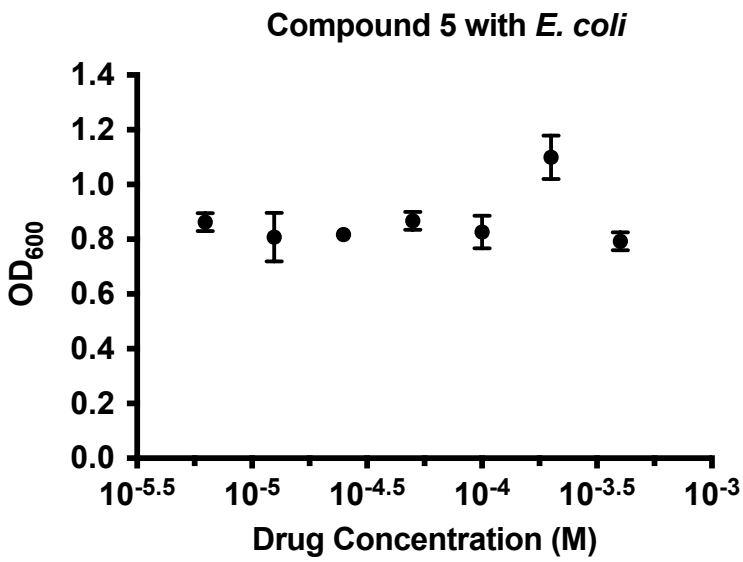
S3.2. *E. coli* MIC curves

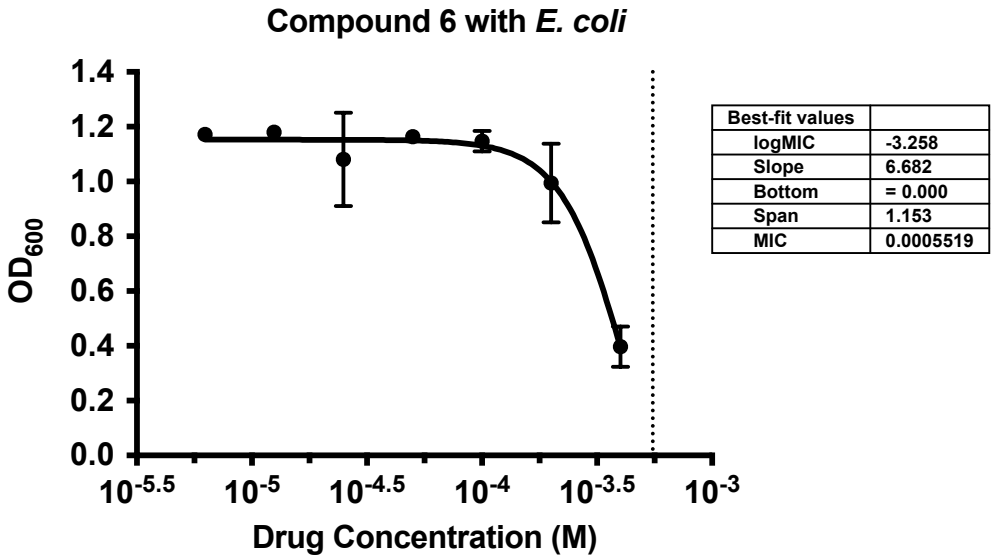


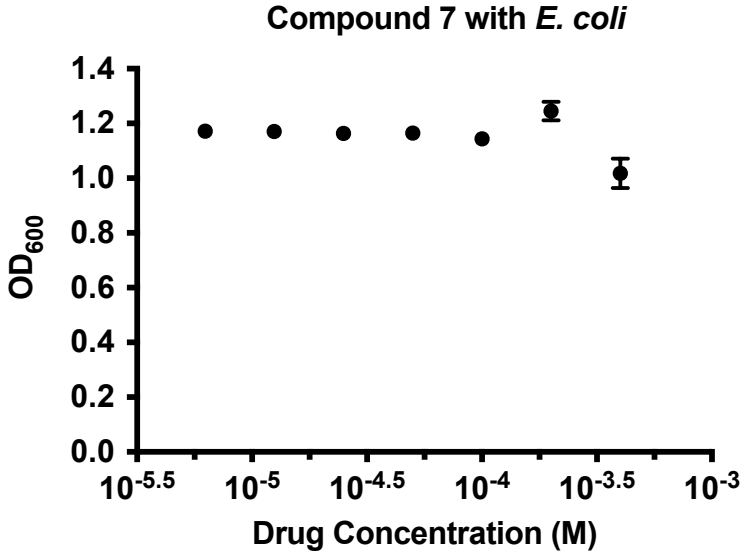

Compound 2 with E. coli
1.5
1.0
0.5
0.0
OD600
10-5.5
10-5
10-4.5
10-4
10-3.5
10-3
Drug Concentration (M)
Best-fit values
logMIC -3.220
Slope 8.981
Bottom = 0.000
Span 1.169
MIC 0.0006025


Compound 3 with E. coli
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD600
10-5.5
10-5
10-4.5
10-4
10-3.5
10-3
Drug Concentration (M)

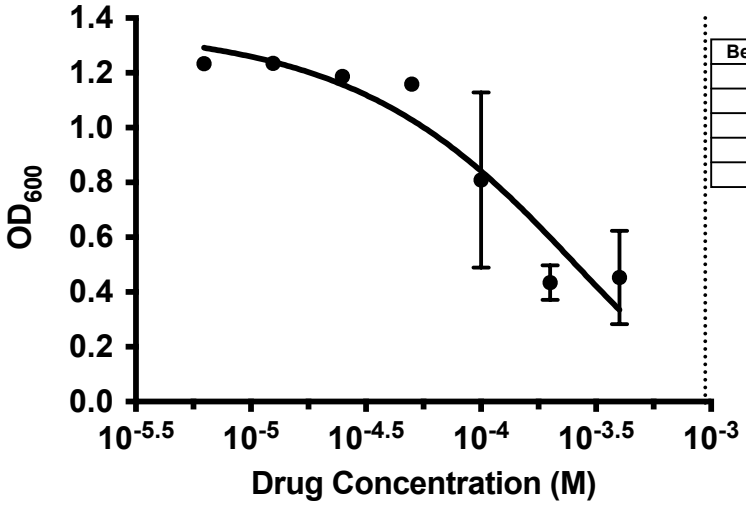

Compound 4 with E. coli
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD600
10-5.5
10-5
10-4.5
10-4
10-3.5
10-3
Drug Concentration (M)
Best-fit values
logMIC -3.310
Slope 7.713
Bottom ~ 8.465e-014
Span ~ 1.027
MIC 0.0004898


Compound 5 with E. coli
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD600
10-5.5
10-5
10-4.5
10-4
10-3.5
10-3
Drug Concentration (M)


Compound 6 with E. coli
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD600
10-5.5
10-5
10-4.5
10-4
10-3.5
10-3
Drug Concentration (M)
Best-fit values
logMIC -3.258
Slope 6.682
Bottom = 0.000
Span 1.153
MIC 0.0005519

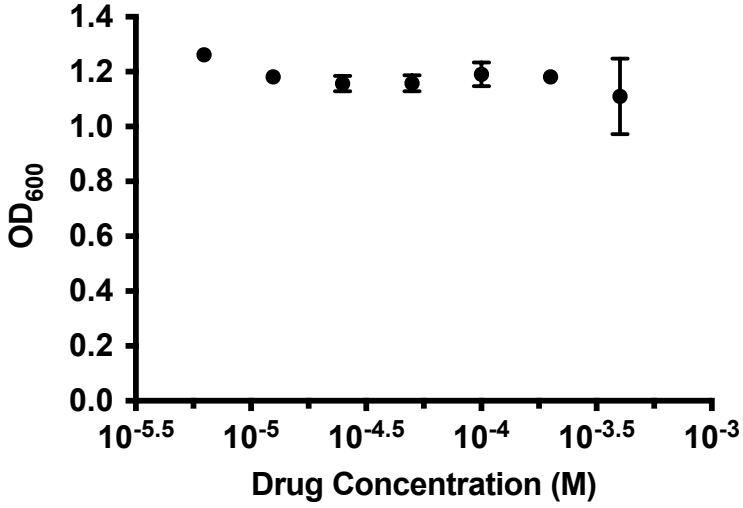

Compound 7 with E. coli
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD600
10-5.5
10-5
10-4.5
10-4
10-3.5
10-3
Drug Concentration (M)

Compound 8 with *E. coli*

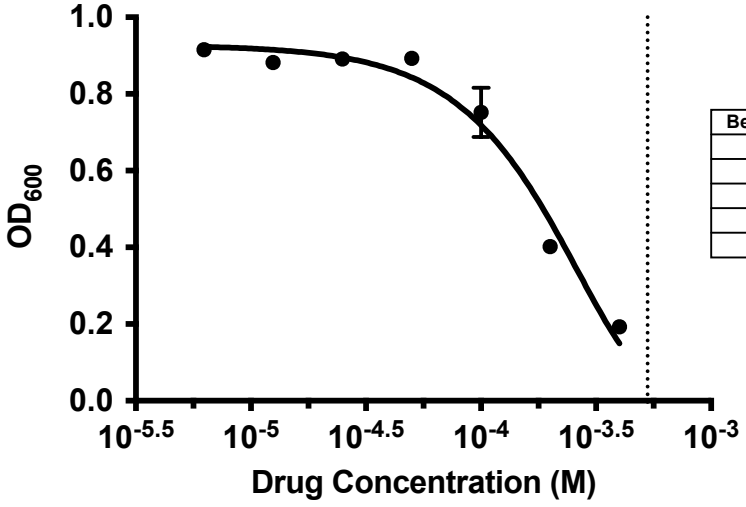


Best-fit values	
logMIC	-3.026
Slope	1.769
Bottom	= 0.000
Span	1.368
MIC	0.0009423

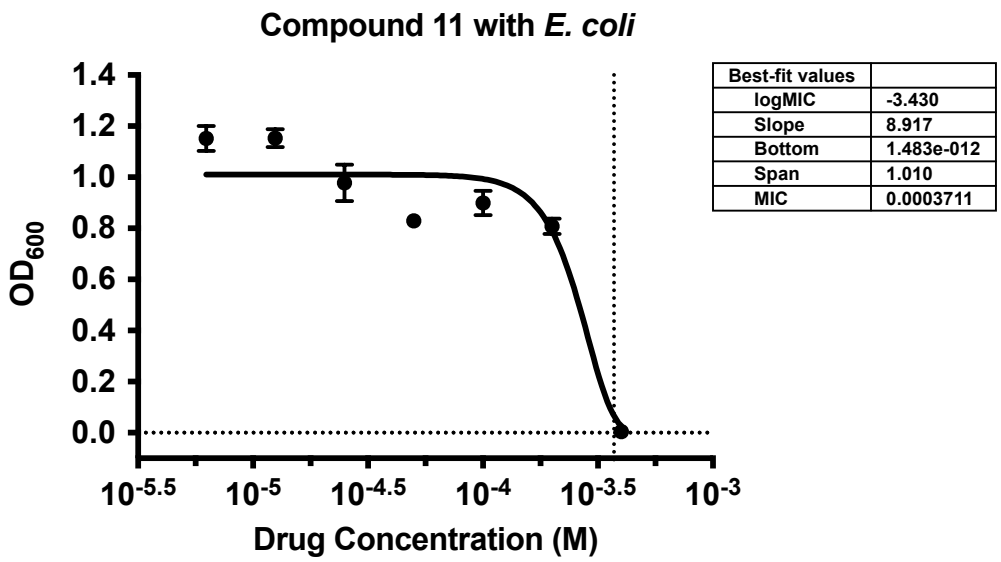
Compound 9 with *E. coli*

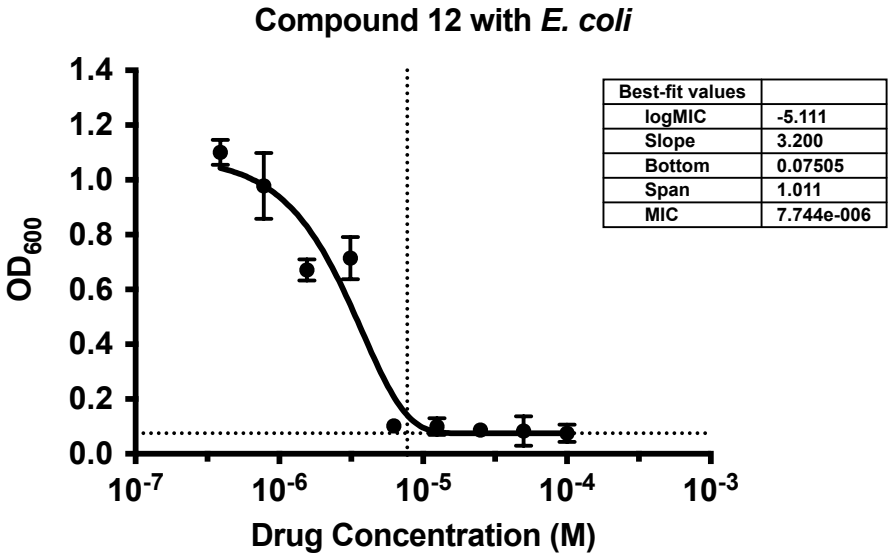


Compound 10 with *E. coli*



Best-fit values	
logMIC	-3.276
Slope	3.272
Bottom	= 0.000
Span	0.9275
MIC	0.0005292


Compound 11 with E. coli
Best-fit values
logMIC -3.430
Slope 8.917
Bottom 1.483e-012
Span 1.010
MIC 0.0003711
OD600
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
10-5.5
10-5
10-4.5
10-4
10-3.5
10-3
Drug Concentration (M)


Compound 12 with E. coli
Best-fit values
logMIC -5.111
Slope 3.200
Bottom 0.07505
Span 1.011
MIC 7.744e-006
OD600
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
10-7
10-6
10-5
10-4
10-3
Drug Concentration (M)

S3.3. MBC_{50} data

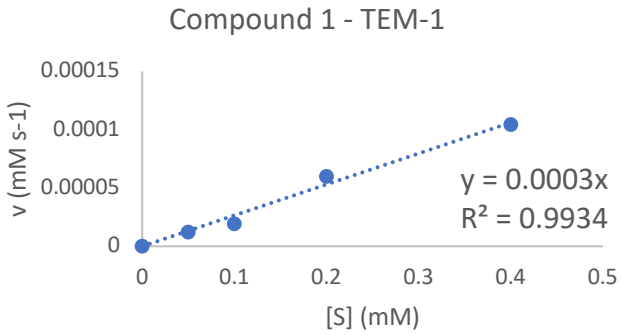
Protocol: After 16 h of incubation with compounds at 37 °C as per the MIC assay protocol, a 1 μL aliquot was taken from wells with no visible bacterial growth and transferred to the surface of the agar plate. Plates were incubated for a further 24 h at 37 °C. MBC_{50} values were the lowest concentration of compound required to kill 50%, plates with no antibiotics were used as references for 100% growth. Experiments were performed in triplicate.

Compound	MBC_{50} *S. aureus* (NCTC 6571)	MBC_{50} *E. coli* (BW25113)
1	400 μM	>400 μM[a]
2	200 μM	>400 μM[a]
3	200 μM	>400 μM[a]
4	50 μM	>400 μM[a]
5	50 μM	>400 μM[a]
6	200 μM	>400 μM[a]
7	200 μM	>400 μM[a]
8	200 μM	>400 μM[a]
9	50 μM	>400 μM[a]
10	50 μM	>400 μM[a]
11	10 μM	400 μM
12	100 μM	100 μM

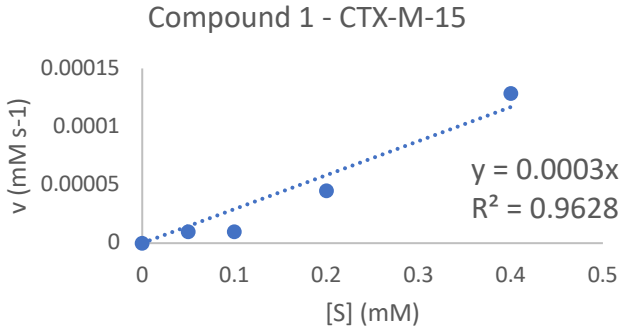
[a] MBC_{50} not determined in concentration range tested (up to 400 μM).

S4. UV Kinetics Assay

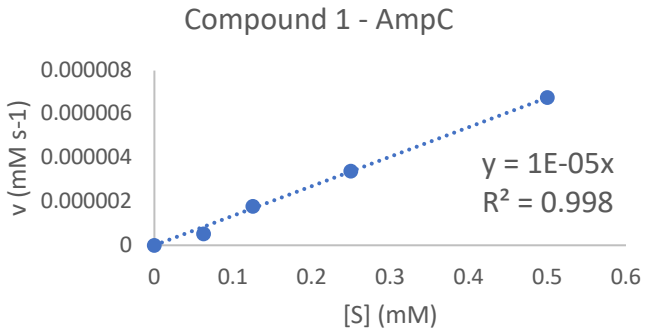
S4.1. Compound 1



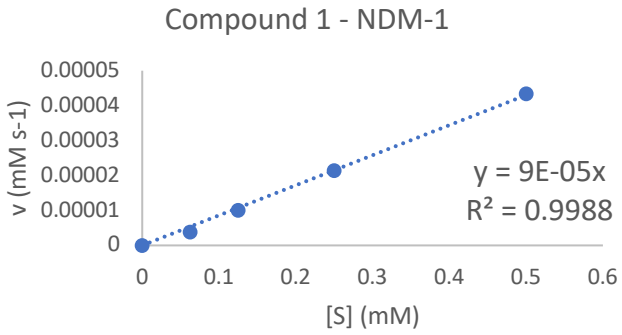
Compound 1 – TEM-1	
[E]	500 nM
Kcat/K_M (mM^{-1} s^{-1})	0.530
Relative k_{cat}/K_M	1.0



Compound 1 – CTX-M-15	
[E]	100 nM
Kcat/K_M (mM^{-1} s^{-1})	2.922
Relative k_{cat}/K_M	1.0

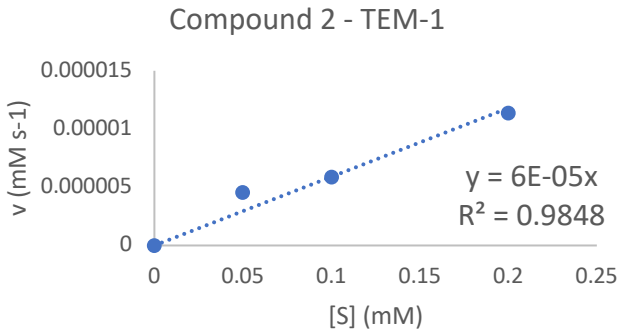


Compound 1 – AmpC	
[E]	50 nM
Kcat/K_M (mM^{-1} s^{-1})	0.270
Relative k_{cat}/K_M	1.0

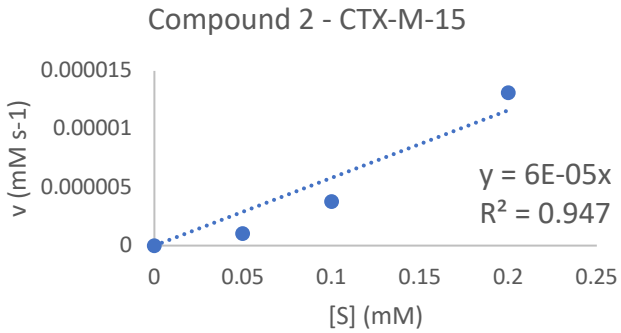


Compound 1 – NDM-1	
[E]	50 nM
Kcat/K_M (mM^{-1} s^{-1})	1.721
Relative k_{cat}/K_M	1.0

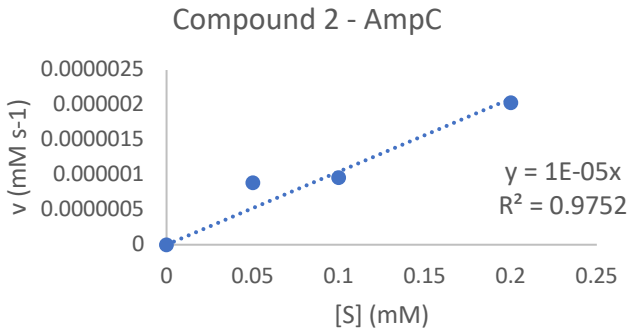
S4.2. Compound 2



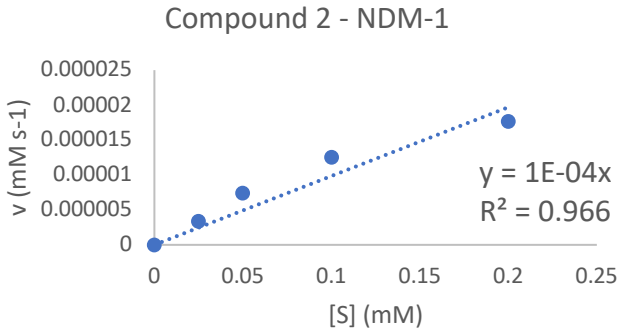
Compound 2 – TEM-1	
[E]	500 nM
Kcat/K_M (mM^{-1} s^{-1})	0.118
Relative k_{cat}/K_M	0.2



Compound 2 – CTX-M-15	
[E]	100 nM
Kcat/K_M (mM^{-1} s^{-1})	0.581
Relative k_{cat}/K_M	0.2

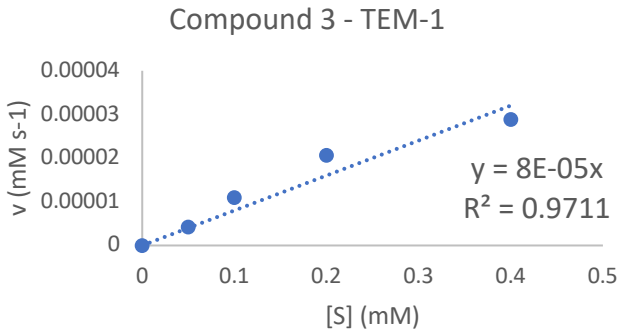


Compound 2 – AmpC	
[E]	50 nM
Kcat/K_M (mM^{-1} s^{-1})	0.209
Relative k_{cat}/K_M	0.7

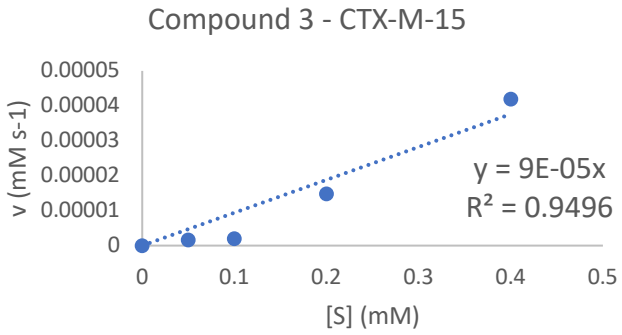


Compound 2 – NDM-1	
[E]	50 nM
Kcat/K_M (mM^{-1} s^{-1})	1.969
Relative k_{cat}/K_M	1.1

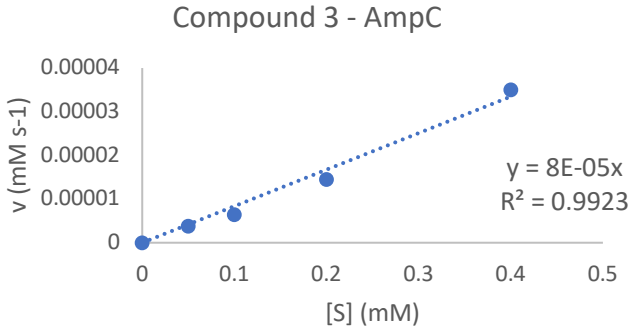
S4.3. Compound 3



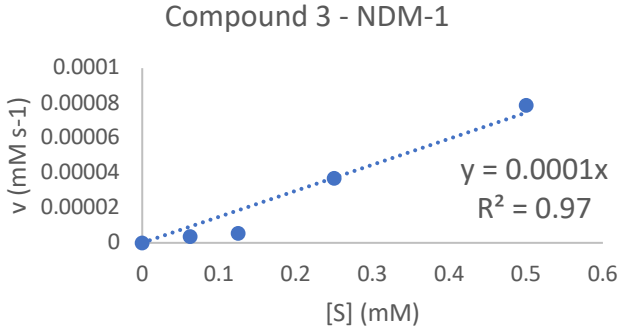
Compound 3 – TEM-1	
[E]	100 nM
K_{cat}/K_M (mM^{-1} s^{-1})	0.800
Relative k_{cat}/K_M	1.5



Compound 3 – CTX-M-15	
[E]	100 nM
K_{cat}/K_M (mM^{-1} s^{-1})	0.941
Relative k_{cat}/K_M	0.3

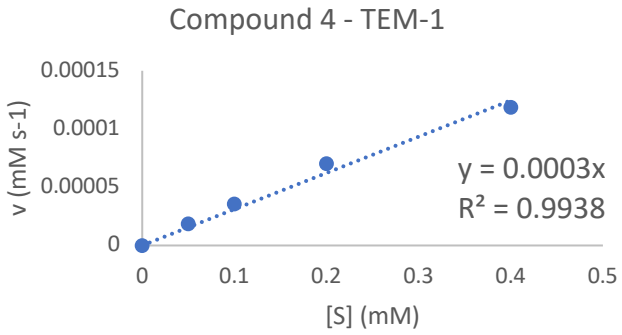


Compound 3 – AmpC	
[E]	50 nM
K_{cat}/K_M (mM^{-1} s^{-1})	1.673
Relative k_{cat}/K_M	5.7

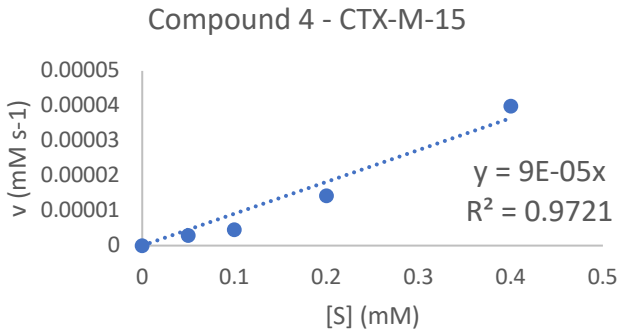


Compound 3 – NDM-1	
[E]	50 nM
K_{cat}/K_M (mM^{-1} s^{-1})	2.981
Relative k_{cat}/K_M	1.7

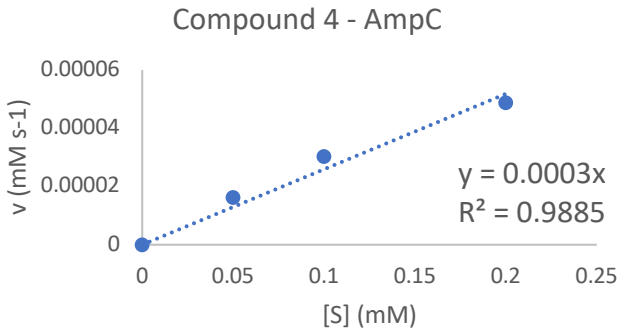
S4.4. Compound 4



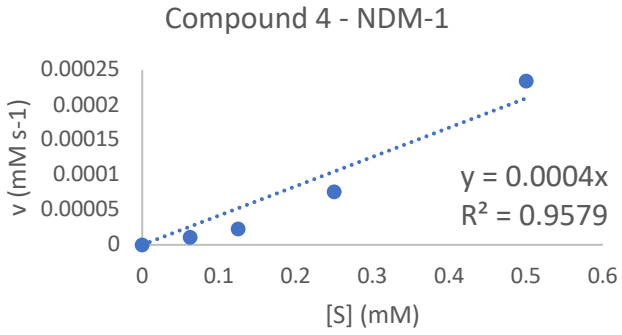
Compound 4 – TEM-1	
[E]	500 nM
Kcat/K_M (mM^{-1} s^{-1})	0.623
Relative k_{cat}/K_M	1.2



Compound 4 – CTX-M-15	
[E]	100 nM
Kcat/K_M (mM^{-1} s^{-1})	0.912
Relative k_{cat}/K_M	0.3

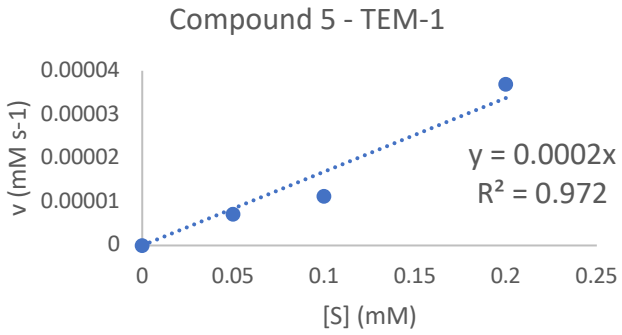


Compound 4 – AmpC	
[E]	50 nM
Kcat/K_M (mM^{-1} s^{-1})	5.182
Relative k_{cat}/K_M	17.3

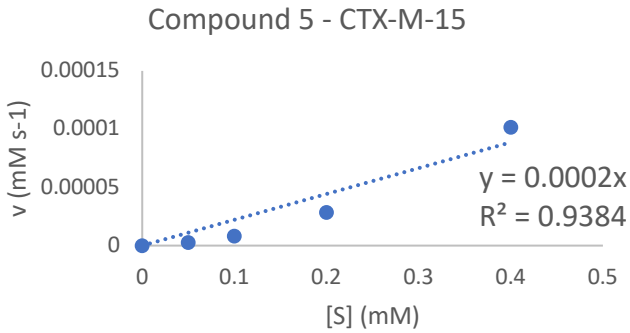


Compound 4 – NDM-1	
[E]	50 nM
Kcat/K_M (mM^{-1} s^{-1})	8.391
Relative k_{cat}/K_M	4.9

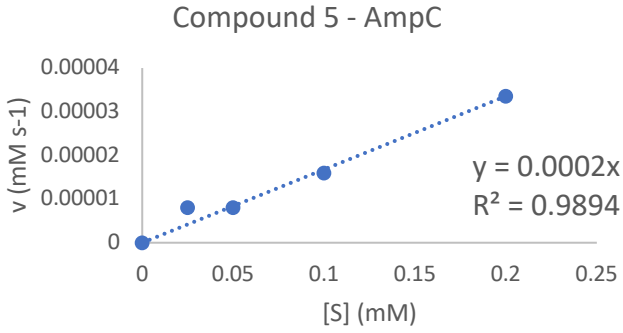
S4.5. Compound 5



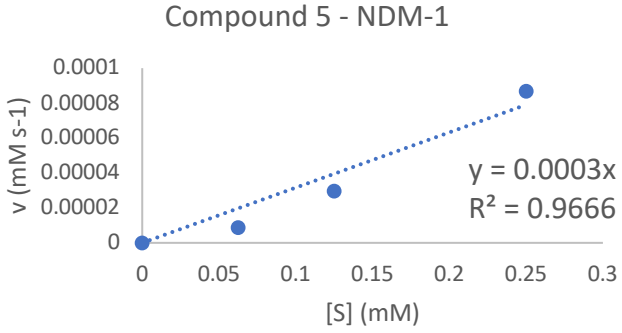
Compound 5 – TEM-1	
[E]	500 nM
Kcat/K_M (mM^{-1} s^{-1})	0.338
Relative k_{cat}/K_M	0.6



Compound 5 – CTX-M-15	
[E]	100 nM
Kcat/K_M (mM^{-1} s^{-1})	2.216
Relative k_{cat}/K_M	0.8

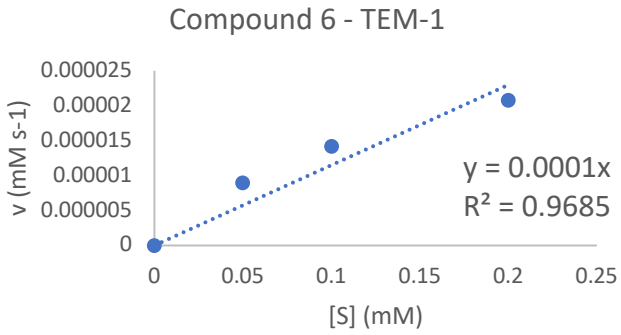


Compound 5 – AmpC	
[E]	50 nM
Kcat/K_M (mM^{-1} s^{-1})	3.355
Relative k_{cat}/K_M	11.3

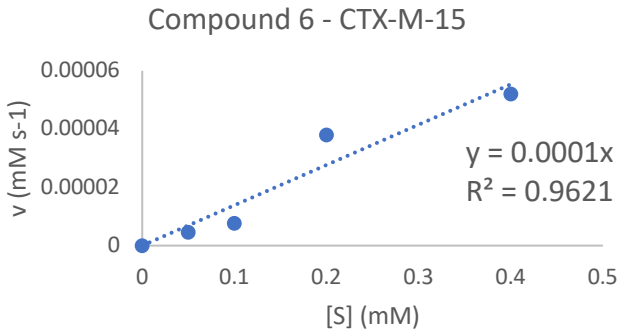


Compound 5 – NDM-1	
[E]	50 nM
Kcat/K_M (mM^{-1} s^{-1})	6.323
Relative k_{cat}/K_M	3.7

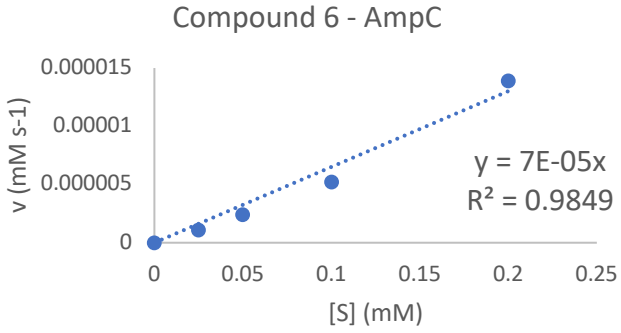
S4.6. Compound 6



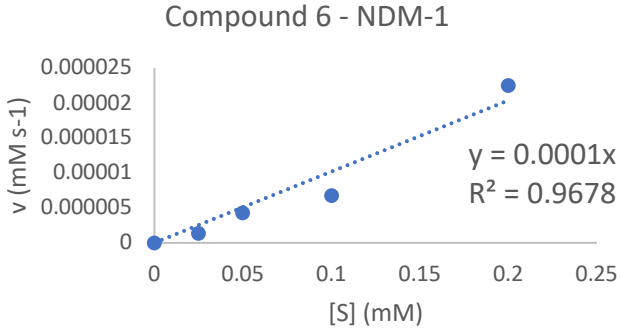
Compound 6 – TEM-1	
[E]	500 nM
Kcat/K_M (mM^{-1} s^{-1})	0.230
Relative k_{cat}/K_M	0.4



Compound 6 – CTX-M-15	
[E]	100 nM
Kcat/K_M (mM^{-1} s^{-1})	1.385
Relative k_{cat}/K_M	0.5

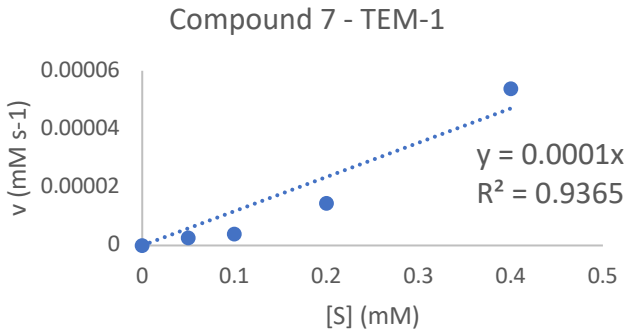


Compound 6 – AmpC	
[E]	50 nM
Kcat/K_M (mM^{-1} s^{-1})	1.301
Relative k_{cat}/K_M	4.3

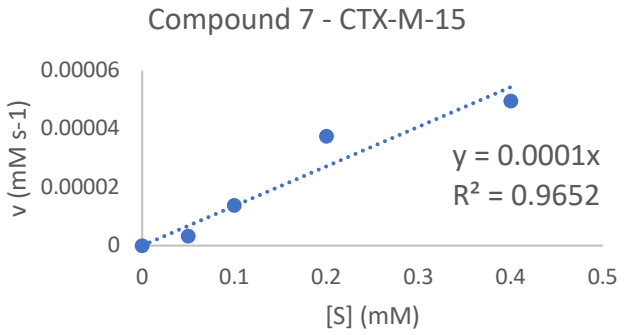


Compound 6 – NDM-1	
[E]	50 nM
Kcat/K_M (mM^{-1} s^{-1})	2.038
Relative k_{cat}/K_M	1.2

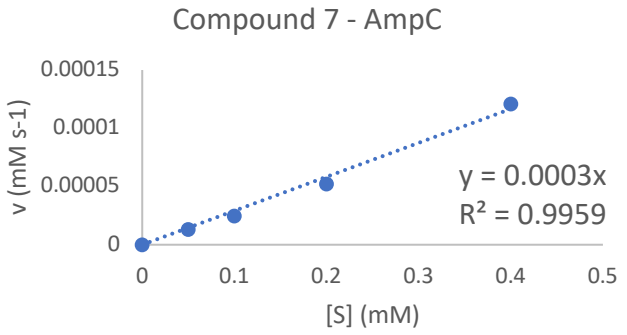
S4.7. Compound 7



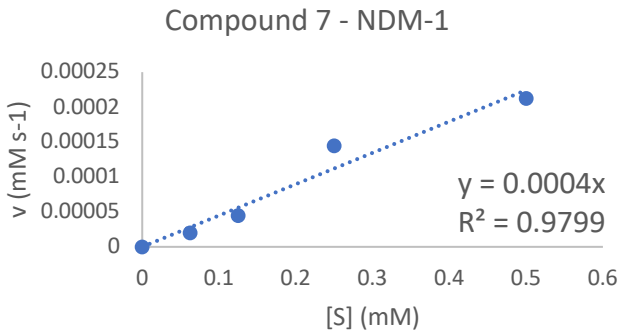
Compound 7 – TEM-1	
[E]	100 nM
Kcat/K_M (mM^{-1} s^{-1})	1.176
Relative k_{cat}/K_M	2.2



Compound 7 – CTX-M-15	
[E]	100 nM
Kcat/K_M (mM^{-1} s^{-1})	1.361
Relative k_{cat}/K_M	0.5

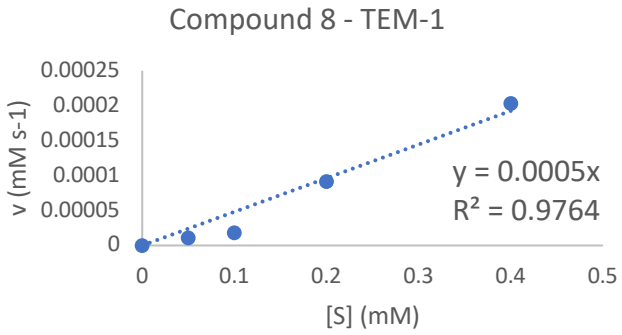


Compound 7 – AmpC	
[E]	50 nM
Kcat/K_M (mM^{-1} s^{-1})	5.830
Relative k_{cat}/K_M	19.3

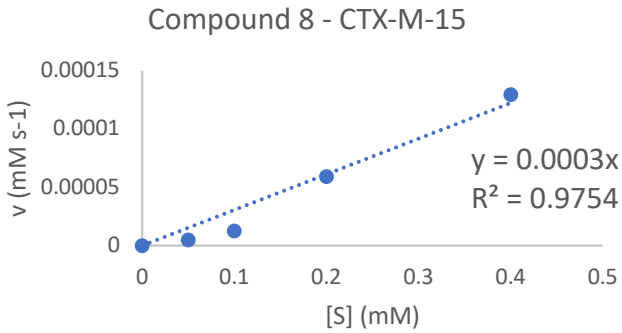


Compound 7 – NDM-1	
[E]	50 nM
Kcat/K_M (mM^{-1} s^{-1})	8.969
Relative k_{cat}/K_M	5.2

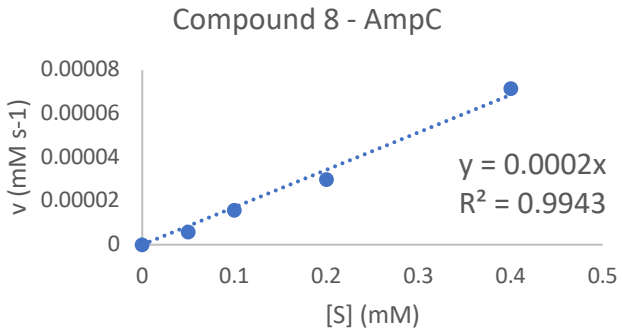
S4.8. Compound 8



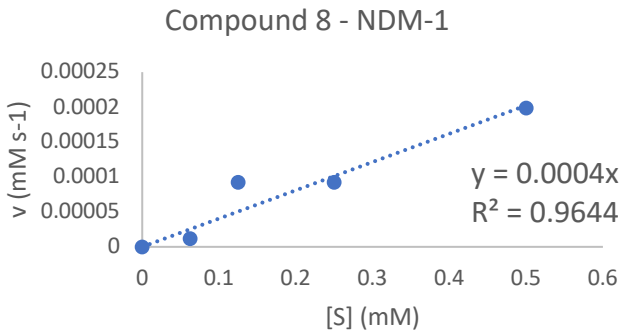
Compound 8 – TEM-1	
[E]	500 nM
Kcat/K_M (mM^{-1} s^{-1})	0.963
Relative k_{cat}/K_M	1.8



Compound 8 – CTX-M-15	
[E]	100 nM
Kcat/K_M (mM^{-1} s^{-1})	3.061
Relative k_{cat}/K_M	1.0

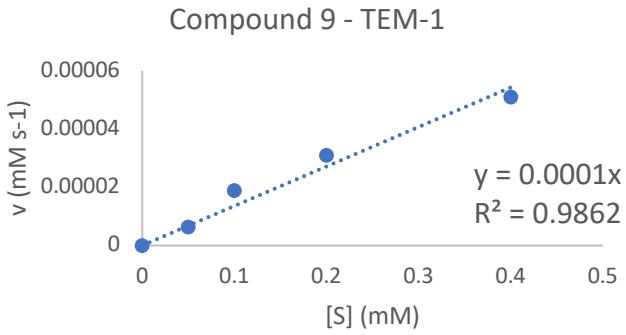


Compound 8 – AmpC	
[E]	50 nM
Kcat/K_M (mM^{-1} s^{-1})	3.432
Relative k_{cat}/K_M	11.3

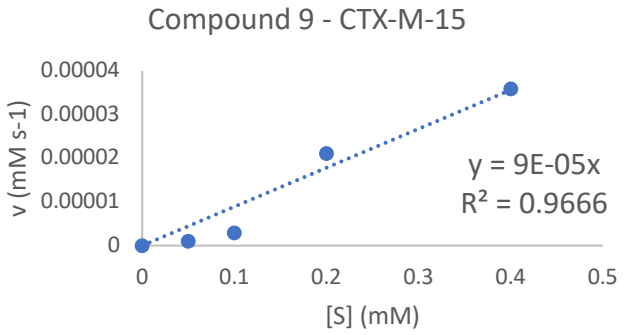


Compound 8 – NDM-1	
[E]	50 nM
Kcat/K_M (mM^{-1} s^{-1})	8.092
Relative k_{cat}/K_M	4.7

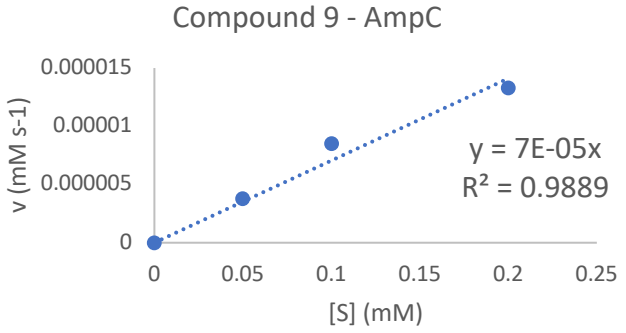
S4.9. Compound 9



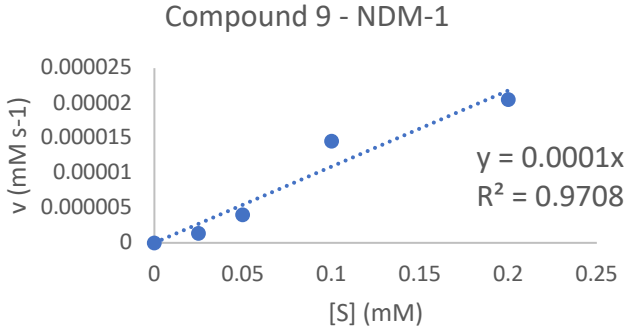
Compound 9 – TEM-1	
[E]	100 nM
Kcat/K_M (mM^{-1} s^{-1})	1.357
Relative k_{cat}/K_M	2.6



Compound 9 – CTX-M-15	
[E]	100 nM
Kcat/K_M (mM^{-1} s^{-1})	0.891
Relative k_{cat}/K_M	0.3

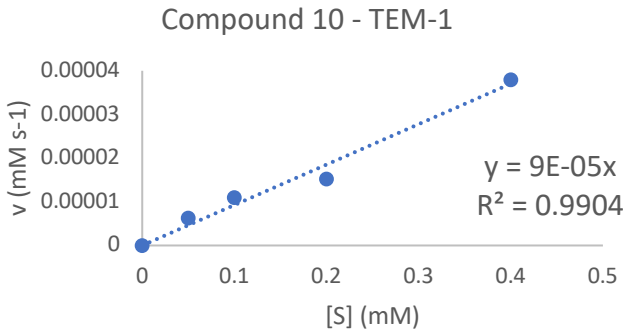


Compound 9 – AmpC	
[E]	50 nM
Kcat/K_M (mM^{-1} s^{-1})	1.411
Relative k_{cat}/K_M	4.7

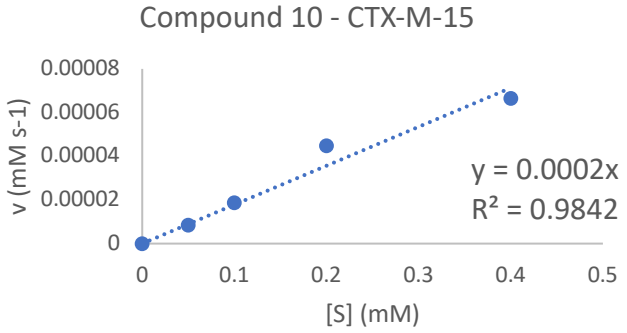


Compound 9 – NDM-1	
[E]	50 nM
Kcat/K_M (mM^{-1} s^{-1})	2.181
Relative k_{cat}/K_M	1.3

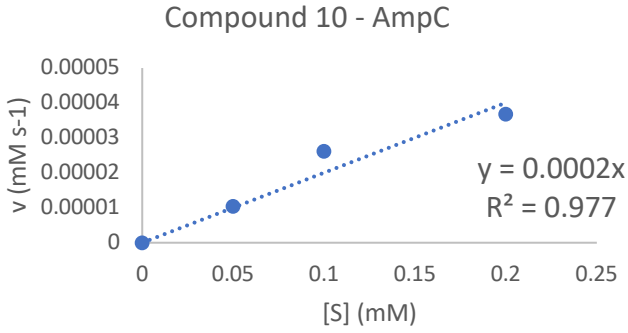
S4.10. Compound 10



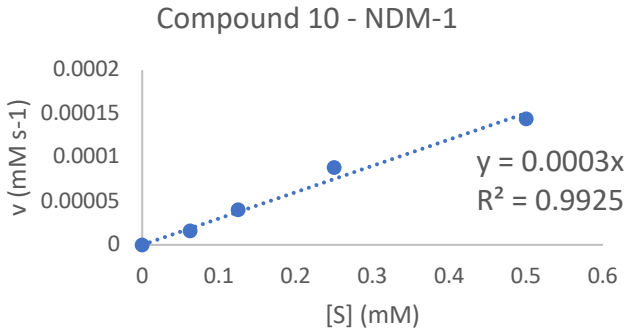
Compound 10 – TEM-1	
[E]	500 nM
Kcat/K_M (mM^{-1} s^{-1})	0.185
Relative k_{cat}/K_M	0.3



Compound 10 – CTX-M-15	
[E]	100 nM
Kcat/K_M (mM^{-1} s^{-1})	1.783
Relative k_{cat}/K_M	0.6



Compound 10 – AmpC	
[E]	50 nM
Kcat/K_M (mM^{-1} s^{-1})	4.007
Relative k_{cat}/K_M	13.3



Compound 10 – NDM-1	
[E]	50 nM
Kcat/K_M (mM^{-1} s^{-1})	6.031
Relative k_{cat}/K_M	3.5