ARTICLE

Improving binding entropy by higher ligand symmetry? – A case study with human matriptase.

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Content

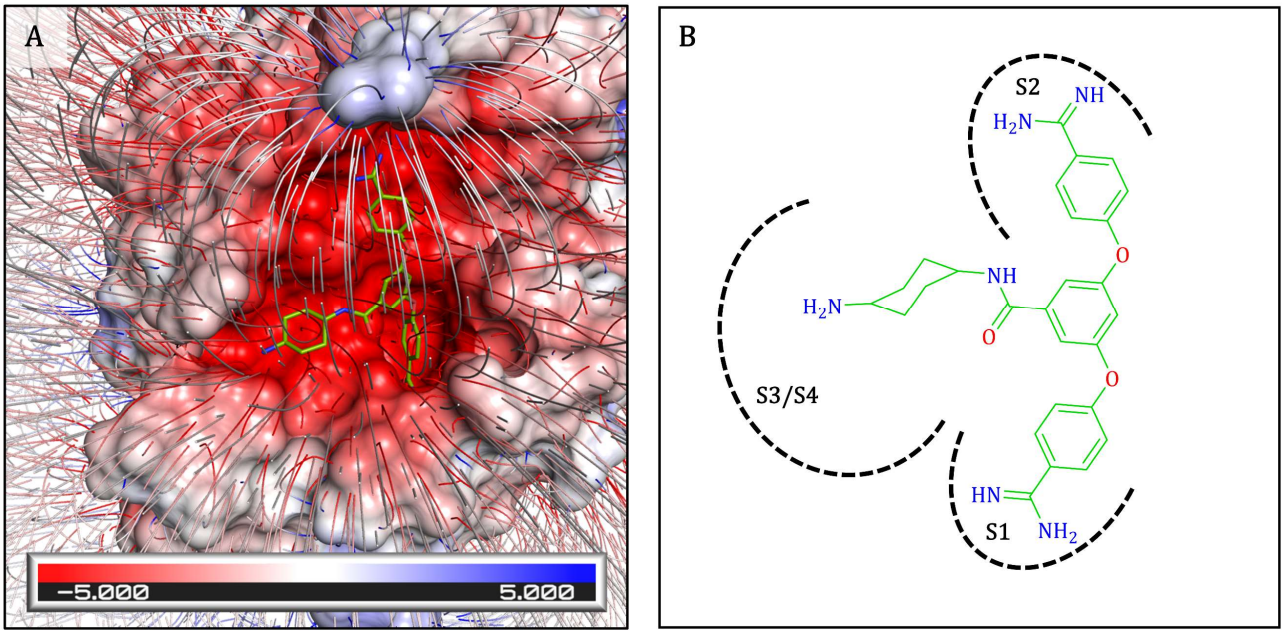


Figure S1. Electrostatic surface of the MT-SP1 binding site and 2D structure of cpd **15**. **A** The electrostatic surface and electric field lines (red to blue from –5.000 to 5.000 kBT/ec) of the protease are exemplarily shown for the complex structure (PDB ID: 4O9V)(1) with inhibitor **15** (*N*-(4-aminocyclohexyl)-3,5-bis(4-carbamimidoylphenoxy)benzamide) depicted as sticks with green carbon atoms. Calculated with the Adaptive Poisson-Boltzmann Solver (APBS)(2) plugin within PyMOL.(3) **B** 2D structure of inhibitor **15** with indicated binding sites.

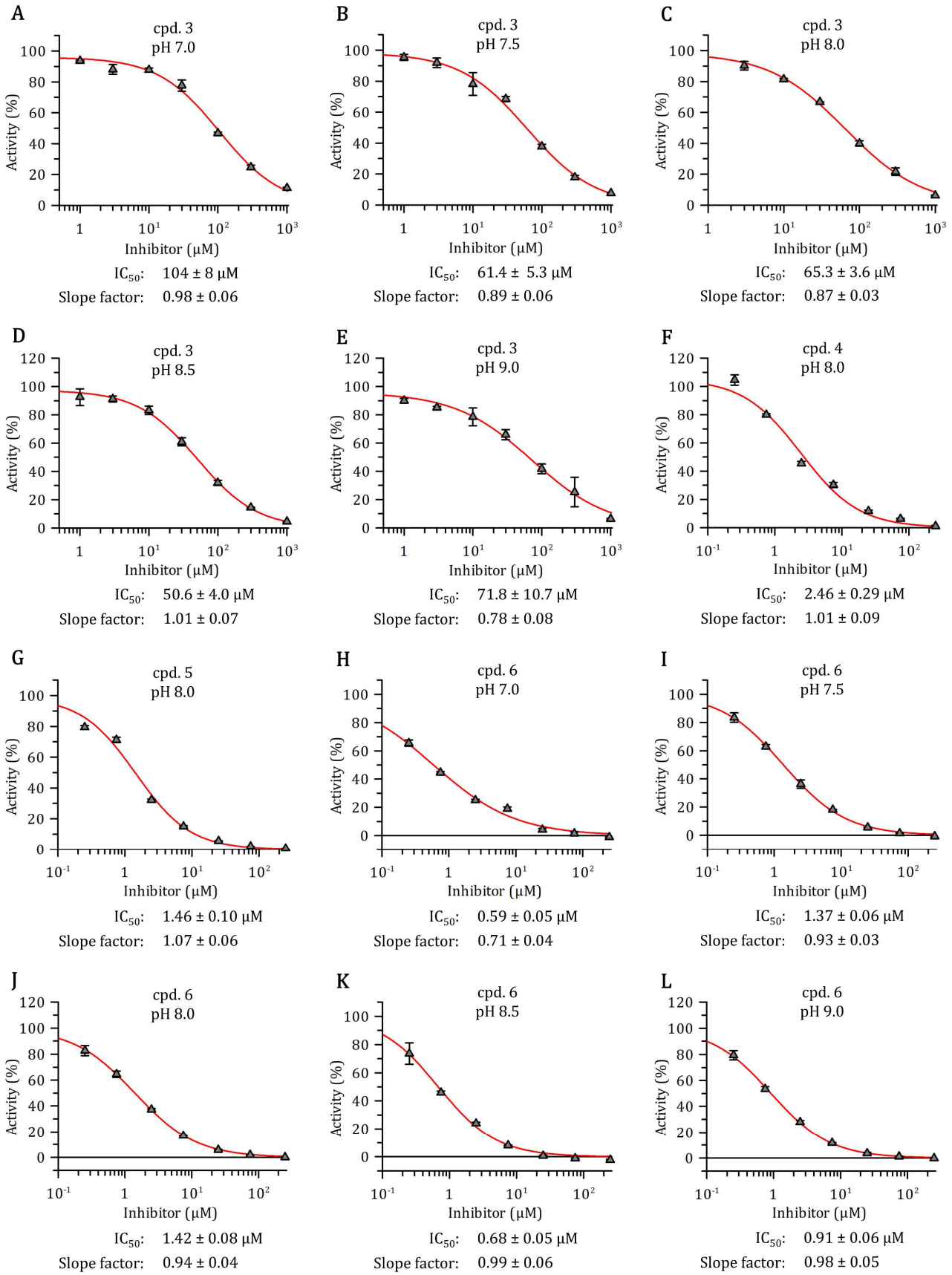


Figure S2. IC_{50} fits of enzyme inhibition assay data. **A**–**E** Cpd **3** at pH 7.0–9.0. **F**, **G** Cpds **4** and **5** at pH 8.0. **H**–**L** Cpd **6** at pH 7.0–9.0. Errors of technical triplicates are indicated as bars. Figure generated with GRAPHIT (Version 5.0.13; Erithacus Software Limited, East Grinstead, UK).(4)

Protonation states. To evaluate if the protonation states of the moieties determine the different affinities of amines and amidines, we calculated the pk_a values of ligands **1**–**6** (Figure S3A). Through the symmetry of the ligands, pk_a values for the same functional groups can be exchanged and should be understood as those of successive protonation events. The pk_a values range from 8.78 to 9.74 for amides and 11.19 to 12.15 for the protonated states of the amidines. Hence, at the experimental pH of 8.0, the great majority should be fully protonated, as even for cpd **3** with the weakest basicity, more than 80% is fully protonated (Figure S3B–F).

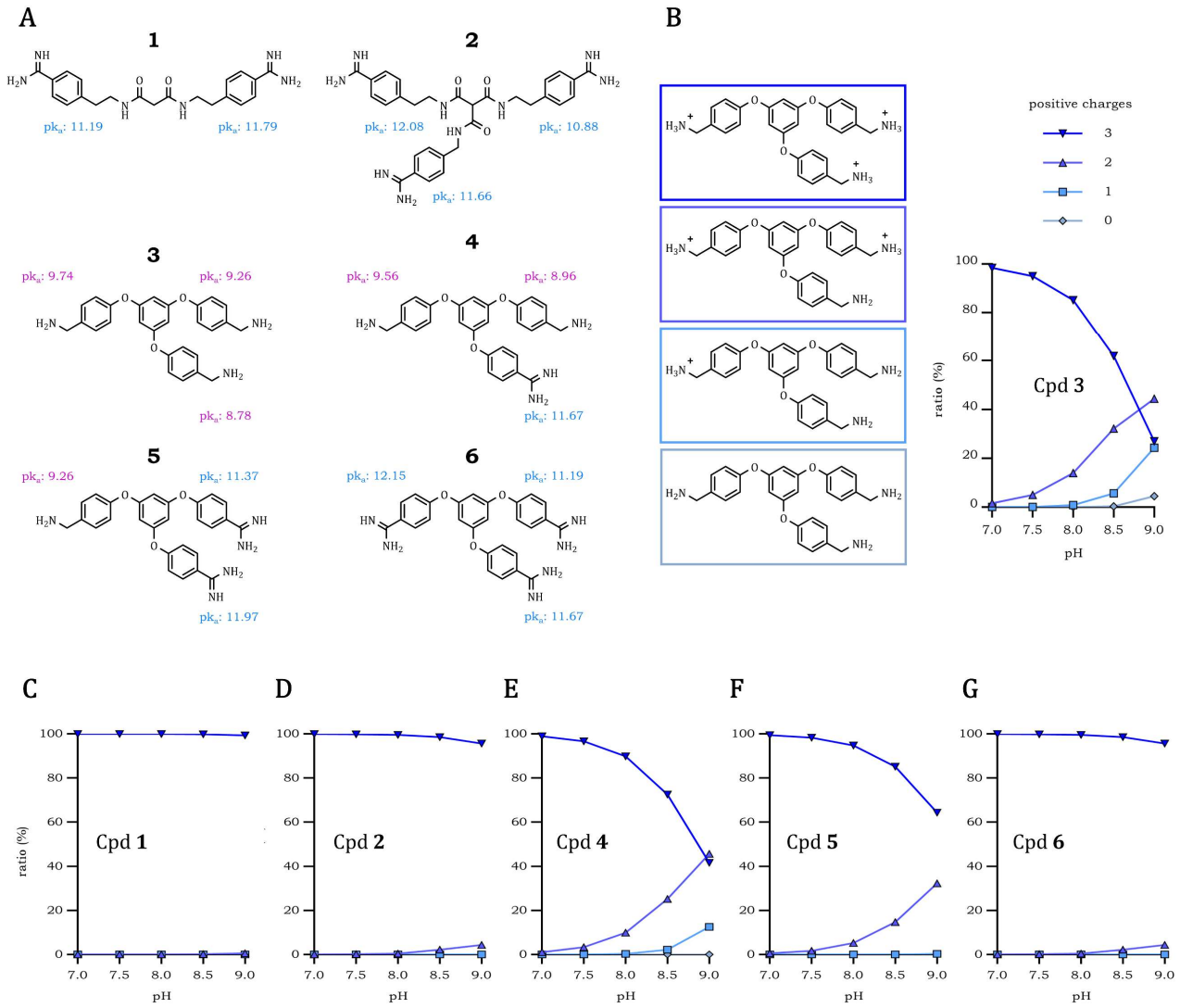


Figure S3. Evaluation of pk_a values amines and amidines used in this study. Calculated with MarvinSketch.(5) **A** pk_a of amins (purple) and amidines (blue) of inhibitors **1**–**6**. **B** Protonation states of cpd **3** and their occurrences at relevant pH values of 7.0–9.0, predicted by MarvinSketch. From dark blue to pale blue: fully protonated species to not protonated state. **C**–**G** Protonation states of cpds 1, 2, 4, 5, and 6, respectively, with only two possible positions for protonation being present in inhibitor **1** and inhibitors **2**–**6** featuring three basic sites for protonation.

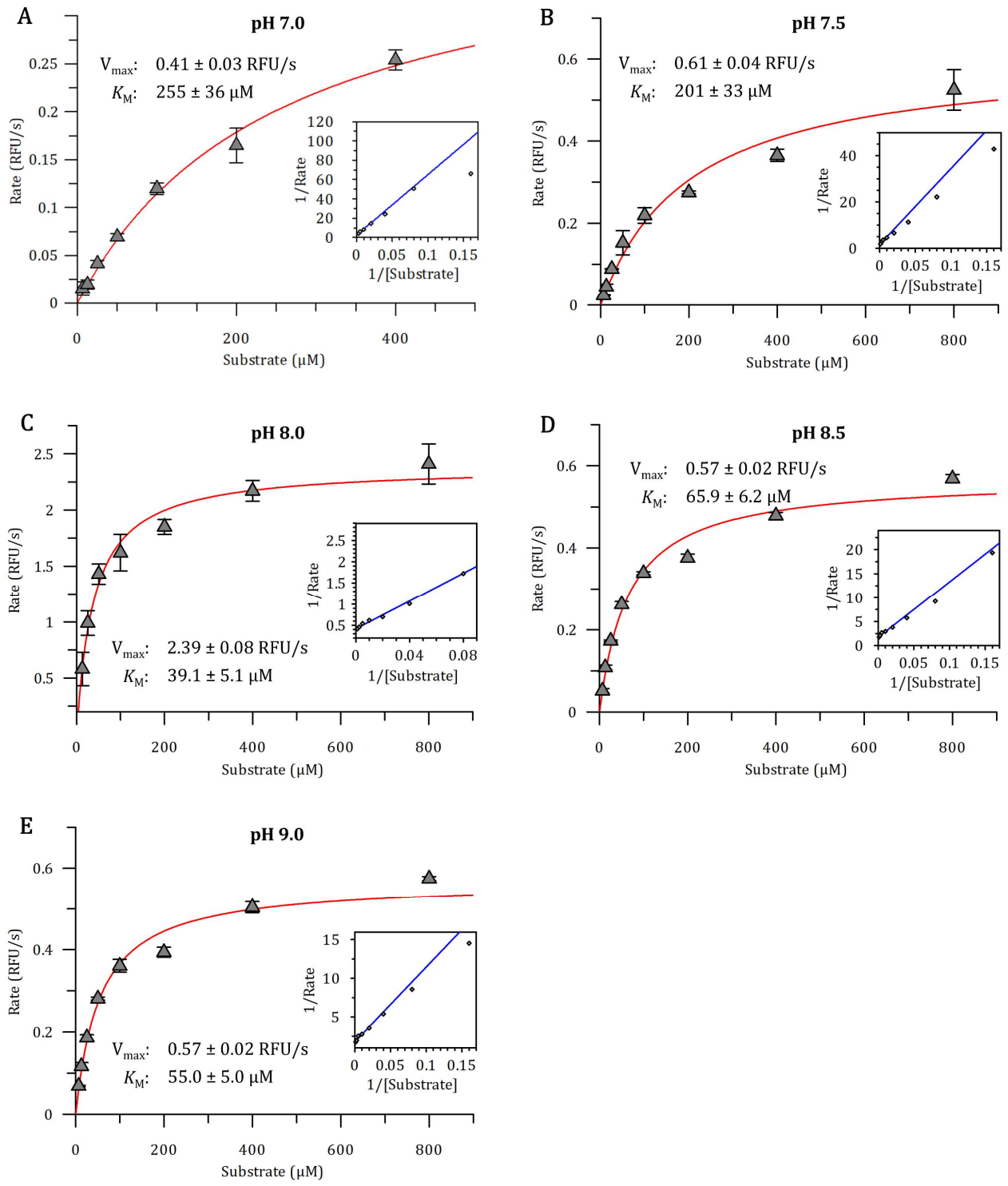
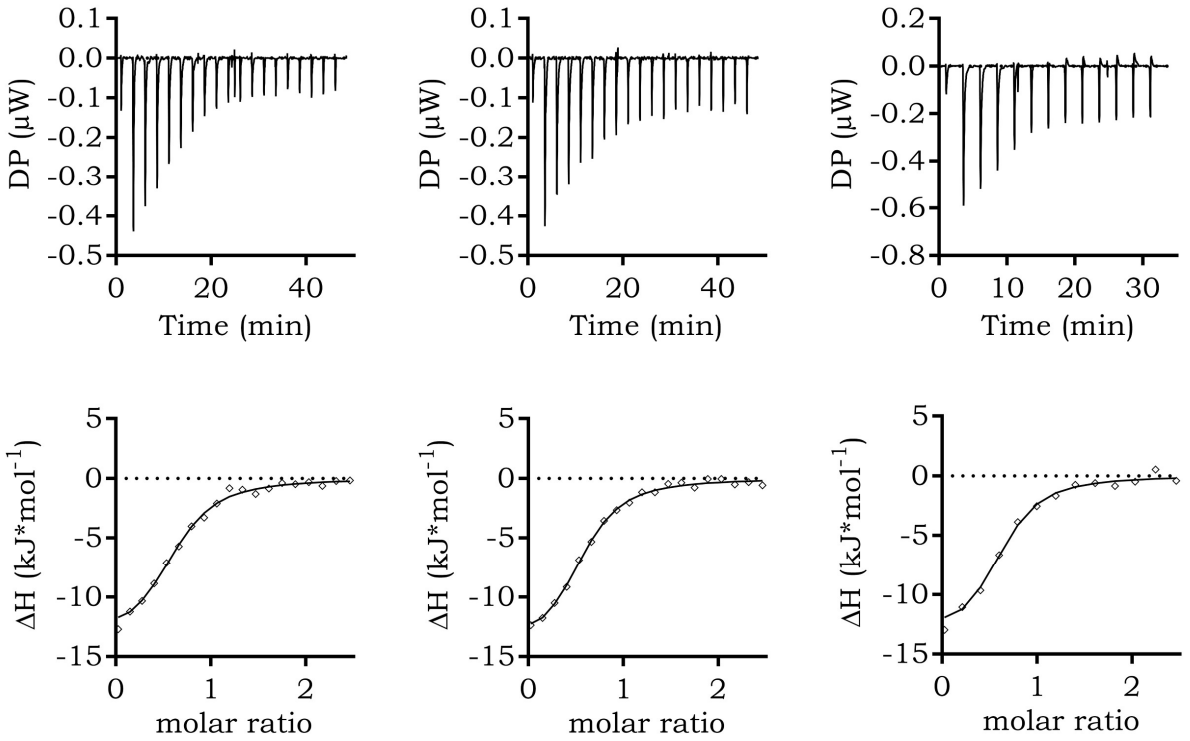


Figure S4. K_M-determinations of the MT-SP1 cleavage of Boc-LRR-AMC at different pH-Values. **A** pH 7.0; **B** pH 7.5; **C** pH 8.0; **D** pH 8.5; **E** pH 9.0. In the smaller secondary graph, the respective linearized Lineweaver-Burk plot is given. Figure generated with GRAPHIT (Version 5.0.13; Erithacus Software Limited, East Grinstead, UK).(4)

ITC experiments of cpd. 1 vs. MT-SP1 in TRIS.



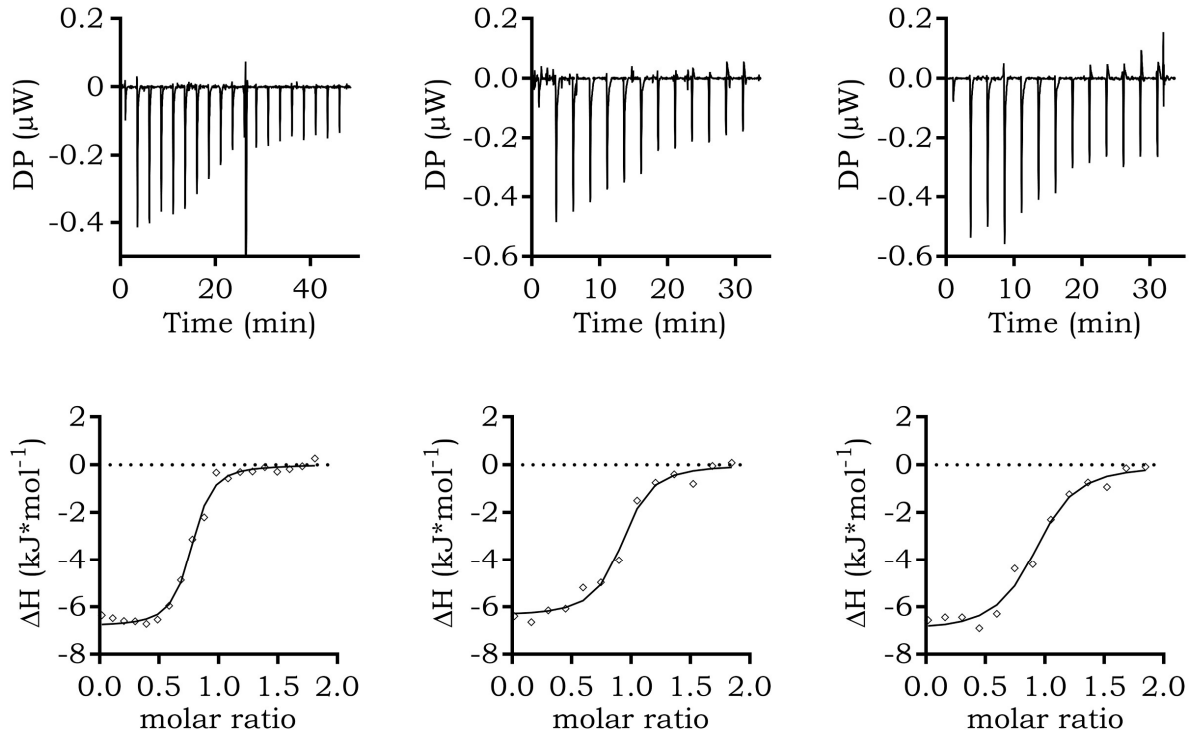
TRIS_**1**_MT-SP1_1
Model: One Set of Sites
[Cell] (M) = 15.0e-6
[Syr] (M) = 200e-6
N (sites) = 0.613 ± 1.9e-2
KD (M) = 1.56e-6 ± 309e-9
ΔH (kJ/mol) = -13.8 ± 0.826
Offset (kJ/mol) = -1.31 ± 0.152
ΔG (kJ/mol) = -33.2
-TΔS (kJ/mol) = -19.4

TRIS_**1**_MT-SP1_2
Model: One Set of Sites
[Cell] (M) = 15.0e-6
[Syr] (M) = 200e-6
N (sites) = 0.567 ± 1.5e-2
KD (M) = 1.45e-6 ± 236e-9
ΔH (kJ/mol) = -14.4 ± 0.715
Offset (kJ/mol) = -1.46 ± 0.121
ΔG (kJ/mol) = -33.4
-TΔS (kJ/mol) = -18.9

TRIS_**1**_MT-SP1_3
Model: One Set of Sites
[Cell] (M) = 15.0e-6
[Syr] (M) = 200e-6
N (sites) = 0.588 ± 2.8e-2
KD (M) = 1.34e-6 ± 416e-9
ΔH (kJ/mol) = -13.8 ± 1.23
Offset (kJ/mol) = -0.836 ± 0.222
ΔG (kJ/mol) = -33.6
-TΔS (kJ/mol) = -19.8

Figure S5A. Thermograms and isotherms of cpd **1** vs. MT-SP1 in ITC_{TRIS} buffer.

ITC experiments of cpd. **2** vs. MT-SP1 in TRIS.



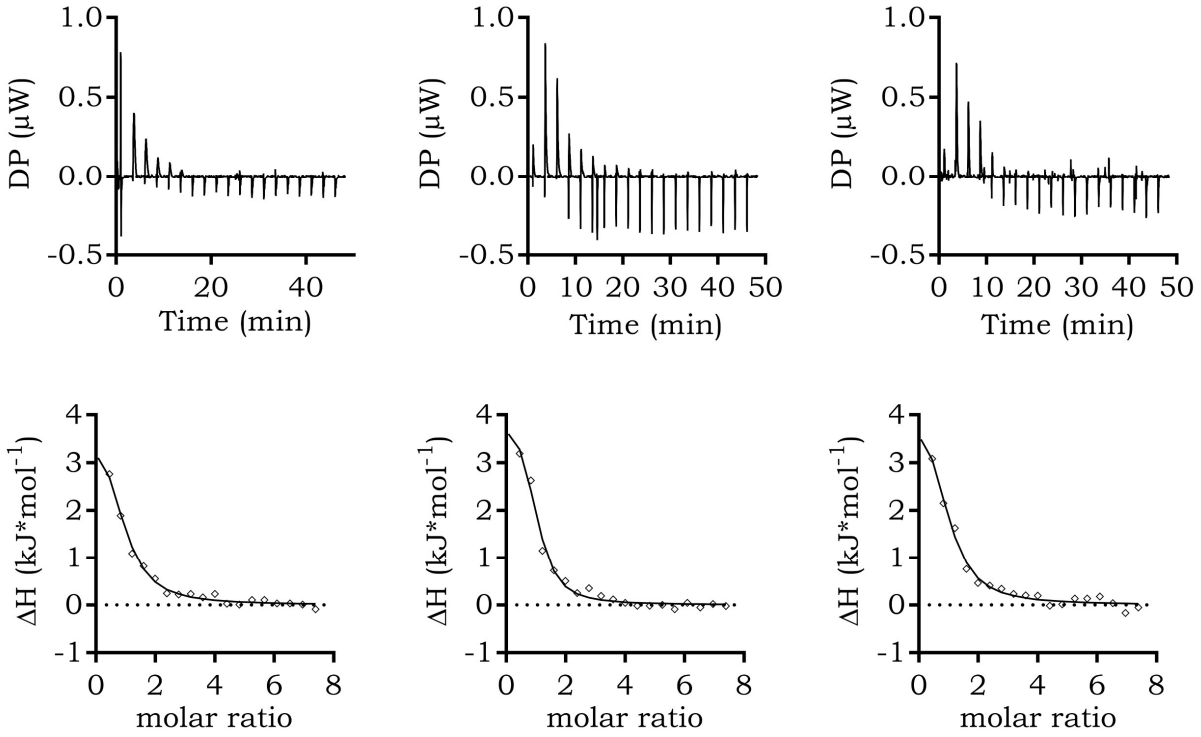
TRIS_**2**_MT-SP1_1
Model: One Set of Sites
[Cell] (M) = 20.4e-6
[Syr] (M) = 200e-6
N (sites) = 0.740 ± 1.1e-2
KD (M) = 200e-9 ± 50.0e-9
ΔH (kJ/mol) = -6.84 ± 0.197
Offset (kJ/mol) = -2.04 ± 0.104
ΔG (kJ/mol) = -38.3
-TΔS (kJ/mol) = -31.4

TRIS_**2**_MT-SP1_2
Model: One Set of Sites
[Cell] (M) = 20.0e-6
[Syr] (M) = 200e-6
N (sites) = 0.875 ± 2.8e-2
KD (M) = 332e-9 ± 155e-9
ΔH (kJ/mol) = -6.40 ± 0.419
Offset (kJ/mol) = -1.00 ± 0.237
ΔG (kJ/mol) = -37.0
-TΔS (kJ/mol) = -30.6

TRIS_**2**_MT-SP1_3
Model: One Set of Sites
[Cell] (M) = 20.0e-6
[Syr] (M) = 200e-6
N (sites) = 0.892 ± 4.1e-2
KD (M) = 645e-9 ± 326e-9
ΔH (kJ/mol) = -7.06 ± 0.644
Offset (kJ/mol) = -0.954 ± 0.346
ΔG (kJ/mol) = -35.4
-TΔS (kJ/mol) = -28.3

Figure S5B. Thermograms and isotherms of cpd **2** vs. MT-SP1 in ITC_{TRIS} buffer.

ITC experiments of cpd. **3** vs. MT-SP1 in HEPES.



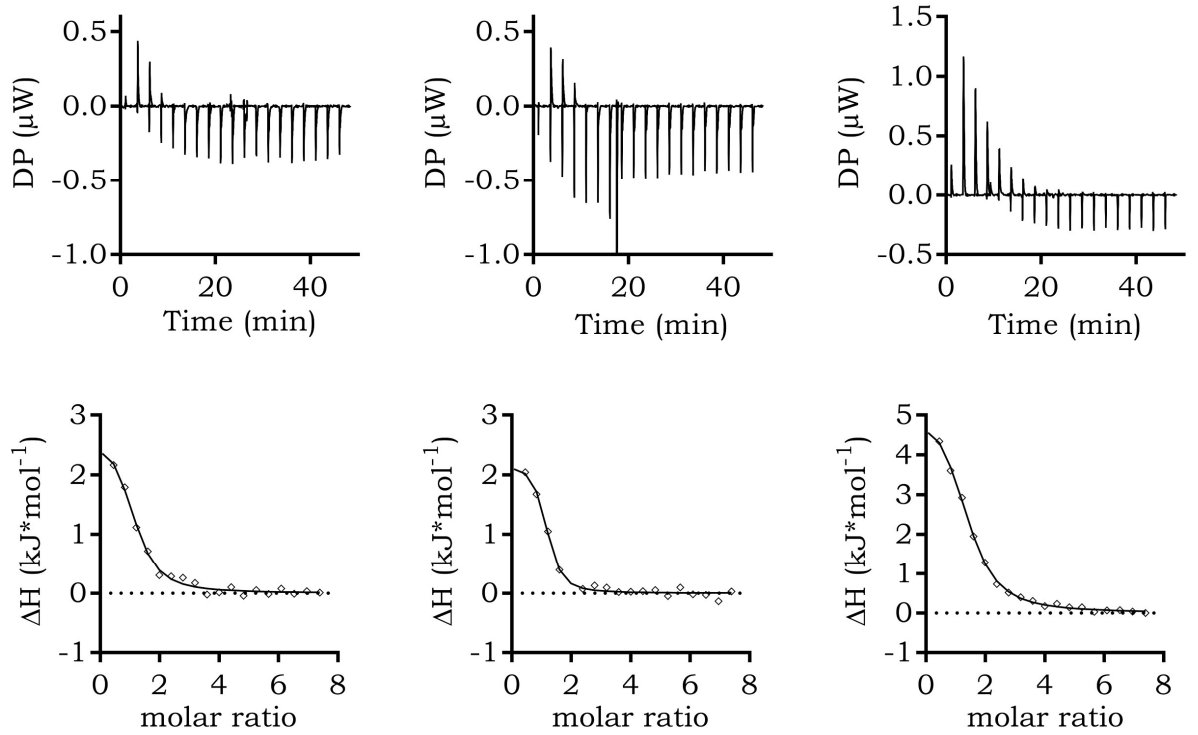
HEPES_**3**_MT-SP1_1
Model: One Set of Sites
[Cell] (M) = 50.0e-6
[Syr] (M) = 2.00e-3
N (sites) = 0.850
KD (M) = 15.6e-6 ± 2.82e-6
ΔH (kJ/mol) = 4.32 ± 0.264
Offset (kJ/mol) = 9.7e-2 ± 3.1e-2
ΔG (kJ/mol) = -27.5
-TΔS (kJ/mol) = -31.8

HEPES_**3**_MT-SP1_2
Model: One Set of Sites
[Cell] (M) = 50.0e-6
[Syr] (M) = 2.00e-3
N (sites) = 0.904 ± 6.3e-2
KD (M) = 7.38e-6 ± 2.53e-6
ΔH (kJ/mol) = 4.24 ± 0.465
Offset (kJ/mol) = 0.114 ± 4.2e-2
ΔG (kJ/mol) = -29.3
-TΔS (kJ/mol) = -33.6

HEPES_**3**_MT-SP1_3
Model: One Set of Sites
[Cell] (M) = 50.0e-6
[Syr] (M) = 2.00e-3
N (sites) = 0.891 ± 0.105
KD (M) = 15.0e-6 ± 5.46e-6
ΔH (kJ/mol) = 4.74 ± 0.857
Offset (kJ/mol) = 4.0e-2 ± 4.8e-2
ΔG (kJ/mol) = -27.6
-TΔS (kJ/mol) = -32.3

Figure S5C. Thermograms and isotherms of cpd **3** vs. MT-SP1 in ITC_{HEPES} buffer.

ITC experiments of cpd. **3** vs. MT-SP1 in TRIS.



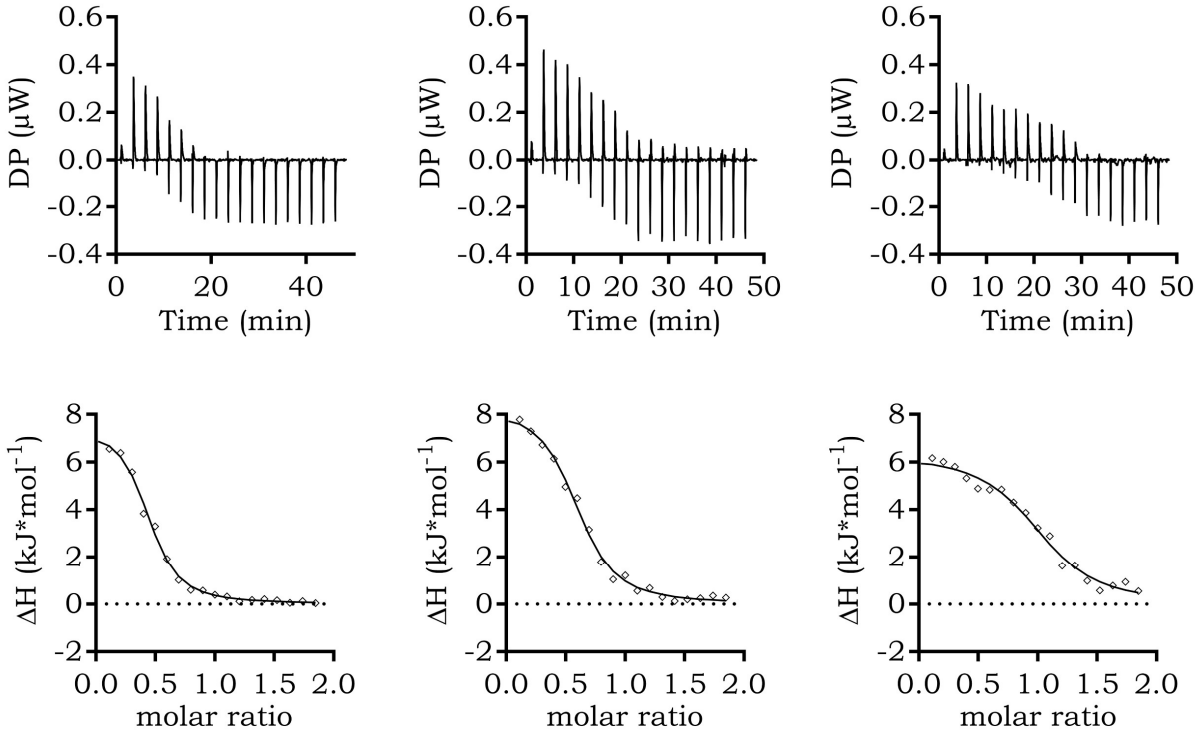
TRIS_**3**_MT-SP1_1
Model: One Set of Sites
[Cell] (M) = 50.0e-6
[Syr] (M) = 2.00e-3
N (sites) = 1.07 ± 5.6e-2
KD (M) = 9.04e-6 ± 2.40e-6
ΔH (kJ/mol) = 2.79 ± 0.236
Offset (kJ/mol) = -0.445 ± 2.5e-2
ΔG (kJ/mol) = -28.8
-TΔS (kJ/mol) = -31.6

TRIS_**3**_MT-SP1_2
Model: One Set of Sites
[Cell] (M) = 50.0e-6
[Syr] (M) = 2.00e-3
N (sites) = 1.04 ± 3.6e-2
KD (M) = 2.84e-6 ± 904e-9
ΔH (kJ/mol) = 2.22 ± 0.122
Offset (kJ/mol) = -0.540 ± 2.1e-2
ΔG (kJ/mol) = -31.7
-TΔS (kJ/mol) = -33.9

TRIS_**3**_MT-SP1_3
Model: One Set of Sites
[Cell] (M) = 50.0e-6
[Syr] (M) = 2.00e-3
N (sites) = 1.34 ± 2.4e-2
KD (M) = 11.4e-6 ± 1.14e-6
ΔH (kJ/mol) = 5.38 ± 0.168
Offset (kJ/mol) = 2.8e-3 ± 2.2e-2
ΔG (kJ/mol) = -28.2
-TΔS (kJ/mol) = -33.6

Figure S5D. Thermograms and isotherms of cpd **3** vs. MT-SP1 in ITC_{TRIS} buffer.

ITC experiments of cpd. **4** vs. MT-SP1 in HEPES.



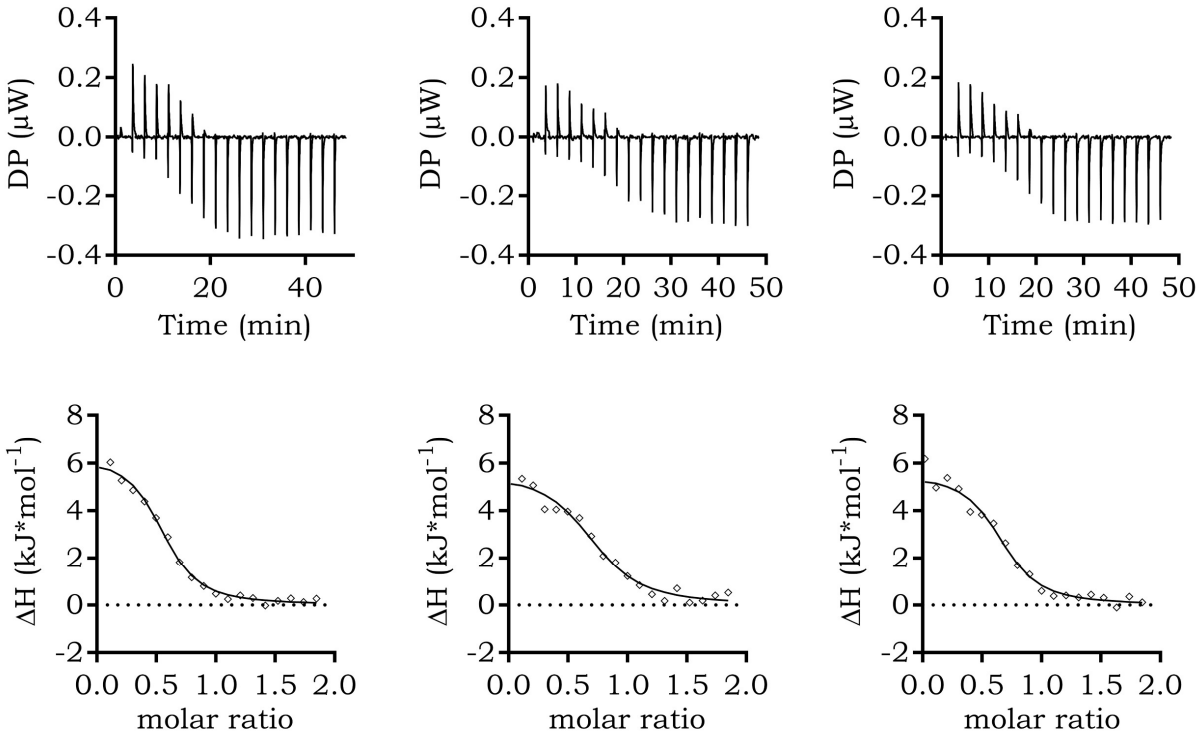
HEPES_**4**_MT-SP1_1
Model: One Set of Sites
[Cell] (M) = 50.0e-6
[Syr] (M) = 500e-6
N (sites) = 0.433 ± 1.2e-2
KD (M) = 2.00e-6 ± 411e-9
ΔH (kJ/mol) = 7.52 ± 0.350
Offset (kJ/mol) = -1.83 ± 7.7e-2
ΔG (kJ/mol) = -32.6
-TΔS (kJ/mol) = -40.1

HEPES_**4**_MT-SP1_2
Model: One Set of Sites
[Cell] (M) = 50.0e-6
[Syr] (M) = 500e-6
N (sites) = 0.595 ± 1.5e-2
KD (M) = 2.31e-6 ± 494e-9
ΔH (kJ/mol) = 8.34 ± 0.381
Offset (kJ/mol) = -1.37 ± 0.120
ΔG (kJ/mol) = -32.2
-TΔS (kJ/mol) = -40.5

HEPES_**4**_MT-SP1_3
Model: One Set of Sites
[Cell] (M) = 50.0e-6
[Syr] (M) = 500e-6
N (sites) = 1.03 ± 4.7e-2
KD (M) = 3.18e-6 ± 1.14e-6
ΔH (kJ/mol) = 6.32 ± 0.565
Offset (kJ/mol) = -2.00 ± 0.326
ΔG (kJ/mol) = -31.4
-TΔS (kJ/mol) = -37.7

Figure S5E. Thermograms and isotherms of cpd **4** vs. MT-SP1 in ITC_{HEPES} buffer.

ITC experiments of cpd. **4** vs. MT-SP1 in TRIS.



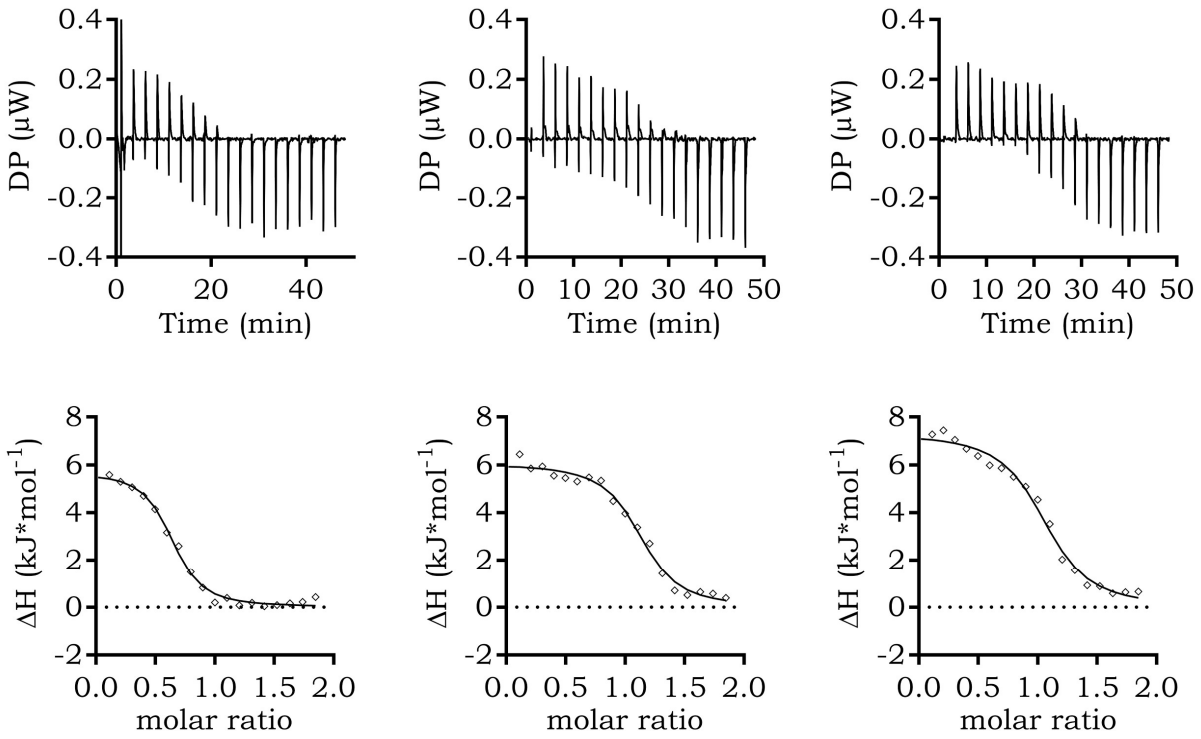
TRIS_**4**_MT-SP1_1
Model: One Set of Sites
[Cell] (M) = 50.0e-6
[Syr] (M) = 500e-6
N (sites) = 0.551 ± 1.4e-2
KD (M) = 2.24e-6 ± 482e-9
ΔH (kJ/mol) = 6.30 ± 0.293
Offset (kJ/mol) = -2.39 ± 8.4e-2
ΔG (kJ/mol) = -32.3
-TΔS (kJ/mol) = -38.6

TRIS_**4**_MT-SP1_2
Model: One Set of Sites
[Cell] (M) = 50.0e-6
[Syr] (M) = 500e-6
N (sites) = 0.724 ± 4.3e-2
KD (M) = 3.26e-6 ± 1.60e-6
ΔH (kJ/mol) = 5.60 ± 0.652
Offset (kJ/mol) = -2.51 ± 0.242
ΔG (kJ/mol) = -31.4
-TΔS (kJ/mol) = -37.0

TRIS_**4**_MT-SP1_3
Model: One Set of Sites
[Cell] (M) = 50.0e-6
[Syr] (M) = 500e-6
N (sites) = 0.659 ± 2.3e-2
KD (M) = 2.10e-6 ± 671e-9
ΔH (kJ/mol) = 5.55 ± 0.354
Offset (kJ/mol) = -2.40 ± 0.130
ΔG (kJ/mol) = -32.4
-TΔS (kJ/mol) = -38.0

Figure S5F. Thermograms and isotherms of cpd **4** vs. MT-SP1 in ITC_{TRIS} buffer.

ITC experiments of cpd. **5** vs. MT-SP1 in HEPES.



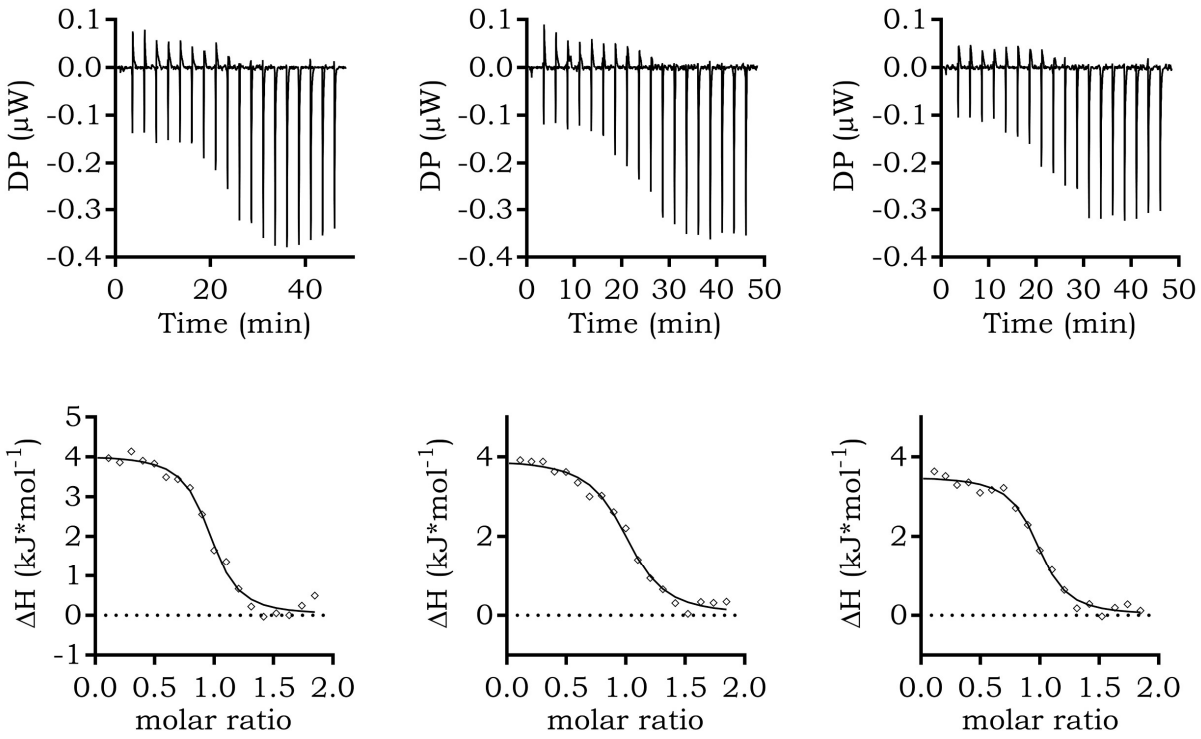
HEPES_**5**_MT-SP1_1
Model: One Set of Sites
[Cell] (M) = 50.0e-6
[Syr] (M) = 500e-6
N (sites) = 0.623 ± 1.4e-2
KD (M) = 1.33e-6 ± 322e-9
ΔH (kJ/mol) = 5.71 ± 0.232
Offset (kJ/mol) = -2.14 ± 8.8e-2
ΔG (kJ/mol) = -33.6
-TΔS (kJ/mol) = -39.3

HEPES_**5**_MT-SP1_2
Model: One Set of Sites
[Cell] (M) = 50.0e-6
[Syr] (M) = 500e-6
N (sites) = 1.11 ± 2.7e-2
KD (M) = 1.17e-6 ± 363e-9
ΔH (kJ/mol) = 6.05 ± 0.343
Offset (kJ/mol) = -3.08 ± 0.235
ΔG (kJ/mol) = -33.9
-TΔS (kJ/mol) = -39.9

HEPES_**5**_MT-SP1_3
Model: One Set of Sites
[Cell] (M) = 50.0e-6
[Syr] (M) = 500e-6
N (sites) = 1.05 ± 2.9e-2
KD (M) = 1.76e-6 ± 507e-9
ΔH (kJ/mol) = 7.32 ± 0.430
Offset (kJ/mol) = -2.94 ± 0.272
ΔG (kJ/mol) = -32.9
-TΔS (kJ/mol) = -40.2

Figure S5G. Thermograms and isotherms of cpd **5** vs. MT-SP1 in ITC_{HEPES} buffer.

ITC experiments of cpd. **5** vs. MT-SP1 in TRIS.



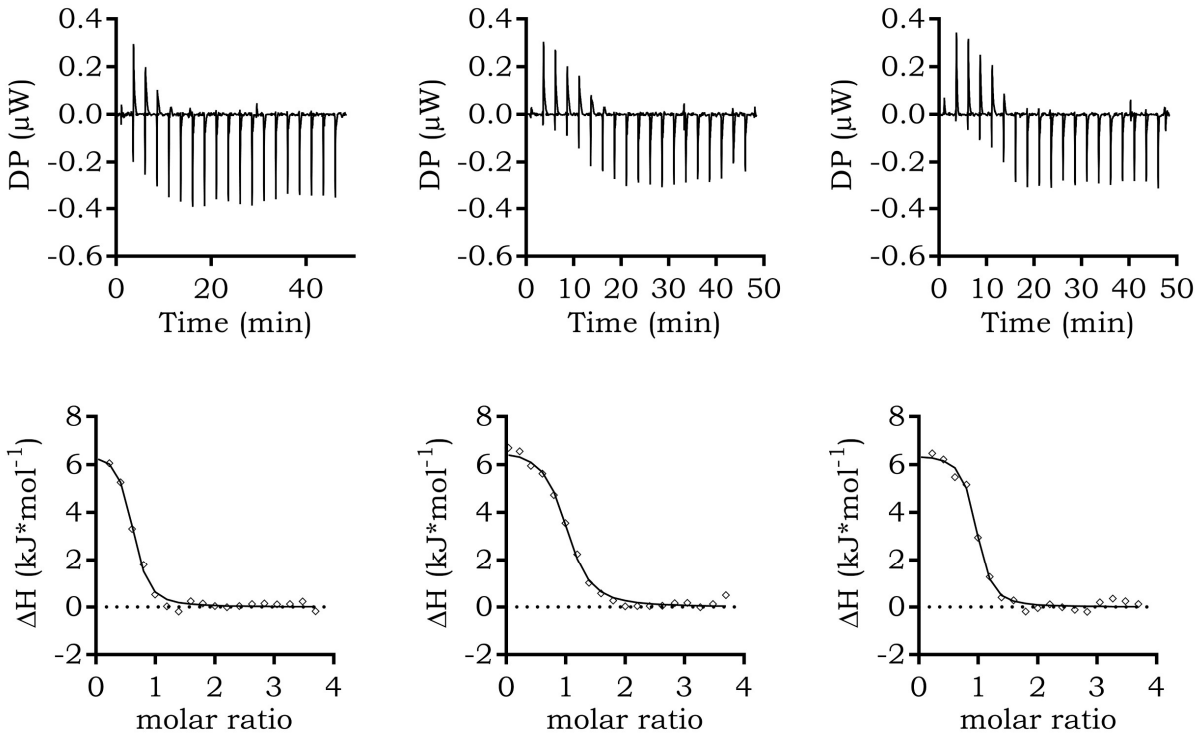
TRIS_**5**_MT-SP1_1
Model: One Set of Sites
[Cell] (M) = 50.0e-6
[Syr] (M) = 500e-6
N (sites) = 0.938 ± 2.0e-2
KD (M) = 777e-9 ± 255e-9
ΔH (kJ/mol) = 4.05 ± 0.188
Offset (kJ/mol) = -2.76 ± 0.114
ΔG (kJ/mol) = -34.9
-TΔS (kJ/mol) = -39.0

TRIS_**5**_MT-SP1_2
Model: One Set of Sites
[Cell] (M) = 50.0e-6
[Syr] (M) = 500e-6
N (sites) = 0.991 ± 2.3e-2
KD (M) = 1.35e-6 ± 378e-9
ΔH (kJ/mol) = 3.95 ± 0.197
Offset (kJ/mol) = -2.43 ± 0.120
ΔG (kJ/mol) = -33.5
-TΔS (kJ/mol) = -37.5

TRIS_**5**_MT-SP1_3
Model: One Set of Sites
[Cell] (M) = 50.0e-6
[Syr] (M) = 500e-6
N (sites) = 0.956 ± 1.7e-2
KD (M) = 801e-9 ± 212e-9
ΔH (kJ/mol) = 3.52 ± 0.135
Offset (kJ/mol) = -2.25 ± 8.3e-2
ΔG (kJ/mol) = -34.8
-TΔS (kJ/mol) = -38.3

Figure S5H. Thermograms and isotherms of cpd **5** vs. MT-SP1 in ITC_{TRIS} buffer.

ITC experiments of cpd. **6** vs. MT-SP1 in HEPES.



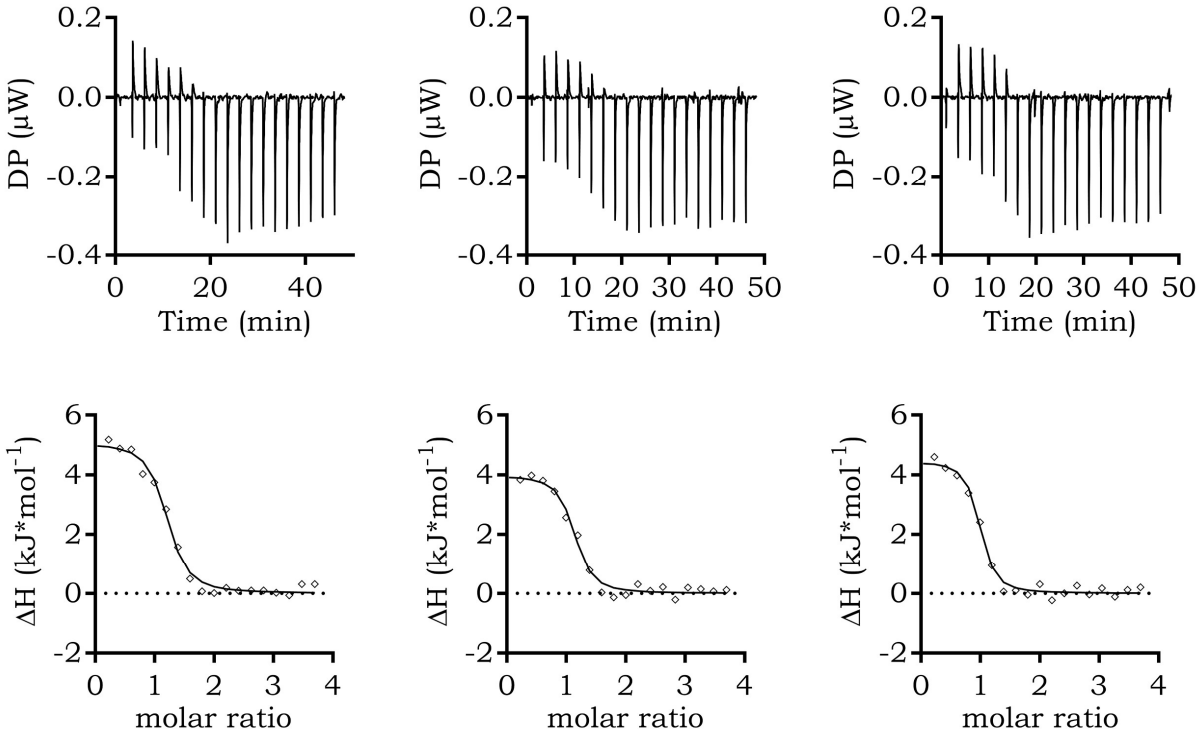
HEPES_**6**_MT-SP1_1
Model: One Set of Sites
[Cell] (M) = 25.0e-6
[Syr] (M) = 500e-6
N (sites) = 0.558 ± 1.8e-2
KD (M) = 781e-9 ± 219e-9
ΔH (kJ/mol) = 6.60 ± 0.329
Offset (kJ/mol) = -2.02 ± 5.9e-2
ΔG (kJ/mol) = -34.9
-TΔS (kJ/mol) = -41.5

HEPES_**6**_MT-SP1_2
Model: One Set of Sites
[Cell] (M) = 25.0e-6
[Syr] (M) = 500e-6
N (sites) = 0.967 ± 2.0e-2
KD (M) = 1.07e-6 ± 228e-9
ΔH (kJ/mol) = 6.69 ± 0.233
Offset (kJ/mol) = -2.04 ± 7.1e-2
ΔG (kJ/mol) = -34.1
-TΔS (kJ/mol) = -40.8

HEPES_**6**_MT-SP1_3
Model: One Set of Sites
[Cell] (M) = 25.0e-6
[Syr] (M) = 500e-6
N (sites) = 0.901 ± 1.7e-2
KD (M) = 420e-9 ± 119e-9
ΔH (kJ/mol) = 6.43 ± 0.208
Offset (kJ/mol) = -2.04 ± 7.1e-2
ΔG (kJ/mol) = -36.4
-TΔS (kJ/mol) = -42.9

Figure S5I. Thermograms and isotherms of cpd **6** vs. MT-SP1 in ITC_{HEPES} buffer.

ITC experiments of cpd. **6** vs. MT-SP1 in TRIS.



TRIS_**6**_MT-SP1_1
Model: One Set of Sites
[Cell] (M) = 25.0e-6
[Syr] (M) = 500e-6
N (sites) = 1.15 ± 2.7e-2
KD (M) = 644e-9 ± 205e-9
ΔH (kJ/mol) = 5.08 ± 0.209
Offset (kJ/mol) = -2.67 ± 8.3e-2
ΔG (kJ/mol) = -35.4
-TΔS (kJ/mol) = -40.5

TRIS_**6**_MT-SP1_2
Model: One Set of Sites
[Cell] (M) = 25.0e-6
[Syr] (M) = 500e-6
N (sites) = 1.07 ± 3.0e-2
KD (M) = 497e-9 ± 203e-9
ΔH (kJ/mol) = 3.99 ± 0.193
Offset (kJ/mol) = -2.21 ± 7.5e-2
ΔG (kJ/mol) = -36.0
-TΔS (kJ/mol) = -40.0

TRIS_**6**_MT-SP1_3
Model: One Set of Sites
[Cell] (M) = 25.0e-6
[Syr] (M) = 500e-6
N (sites) = 0.922 ± 2.2e-2
KD (M) = 409e-9 ± 151e-9
ΔH (kJ/mol) = 4.46 ± 0.185
Offset (kJ/mol) = -2.07 ± 6.5e-2
ΔG (kJ/mol) = -36.5
-TΔS (kJ/mol) = -41.0

Figure S5J. Thermograms and isotherms of cpd **6** vs. MT-SP1 in ITC_{TRIS} buffer.

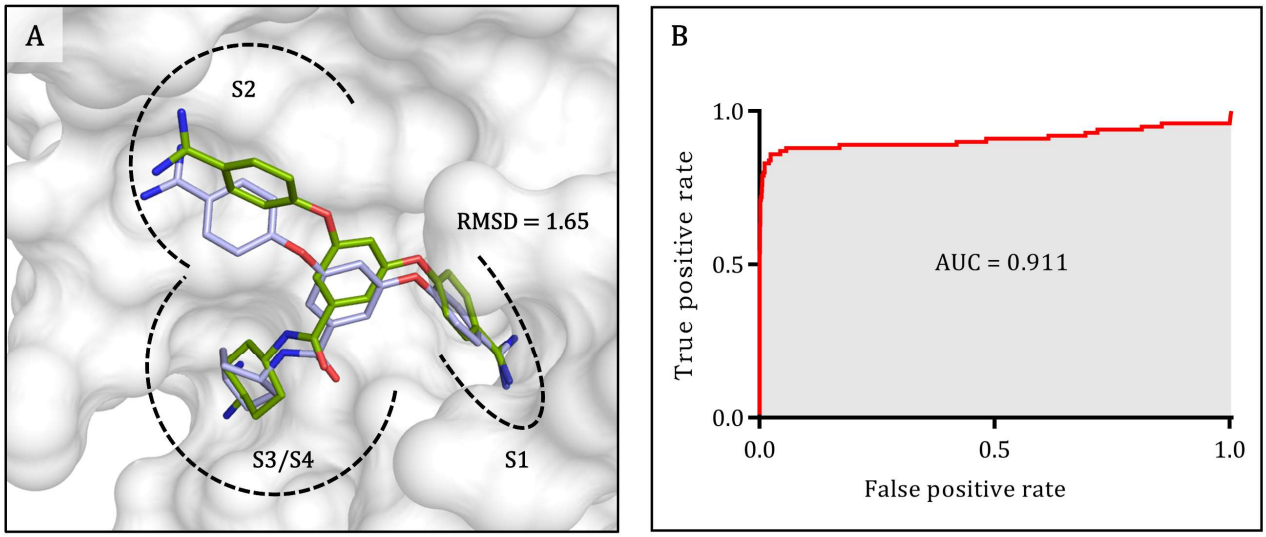


Figure S6. Docking validation. **A** redocking of cpd **15** (PDB ID 4O9V)(1) reveals an RMSD of 1.65 A (X-ray: green sticks, redocked pose: pale blue sticks, FlexX score –53.9 kJ·mol^{-1}). **B** ROC-curves of the 100 binders and 1170 decoys for validation of the molecular docking studies.

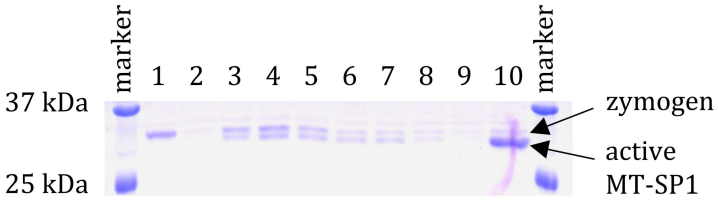


Figure S7. SDS-PAGE analysis of the MT-SP1 purification and autoactivation process. Precision Plus Protein™ Dual Xtra Prestained Protein Standards (BioRad, Hercules, CA, USA) was used as a molecular weight marker. Protein bands were stained with Coomassie brilliant blue. Lane 1 contains the MT-SP1 after Ni-NTA and subsequent dialysis, which was loaded to the AEX column. Lanes 2–9 show the respective elution fractions. Lane 10 contains a sample of the pooled and concentrated MT-SP1. In lane 1, there is mainly the zymogen, whereas autoactivation occurs during AEX. After concentrating of the eluted fractions, the active MT-SP1 is prevailing.

Table S1. Symmetry point groups of inhibitors **1**–**6**. The assignment was performed as described in Lauria *et al.*(6)

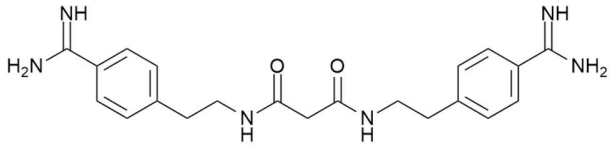
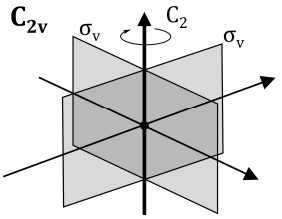
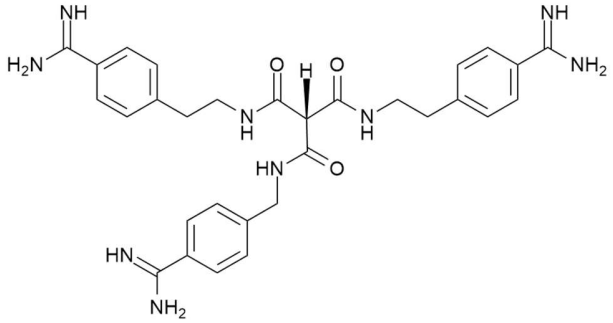
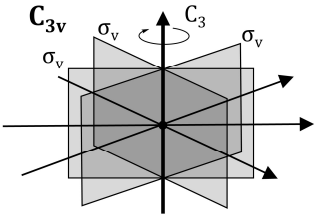
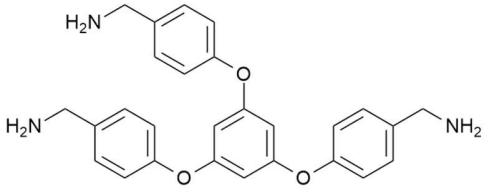
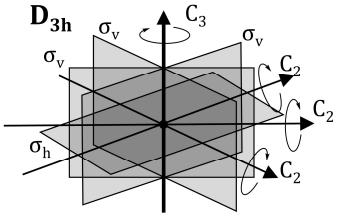
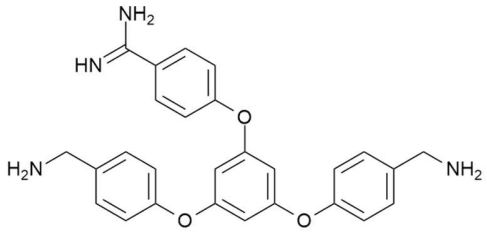
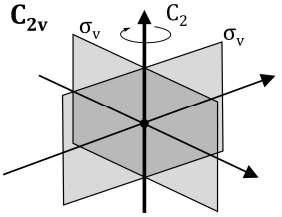
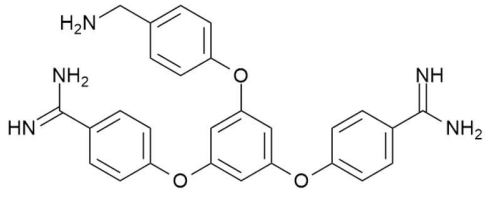
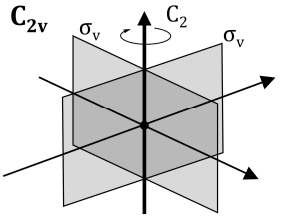
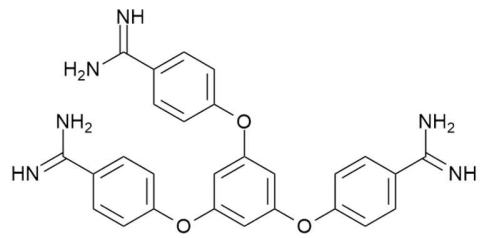
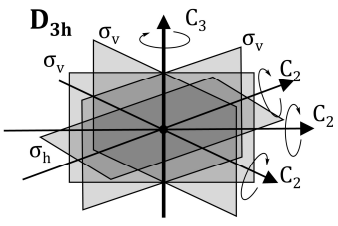
Cpd	Structure	Symmetry point group
1		
2		
3		
4		
5		
6		

Table S2. FlexX docking scores for poses resembling possible solutions. Predicted binding modes are shown in Figure 2.

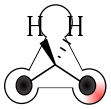
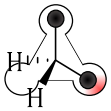
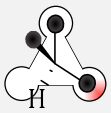
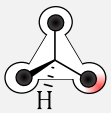
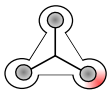
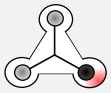
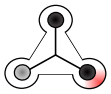
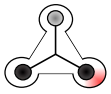
Cpd	Rank	Docking score (kJ·mol^{-1})	orientation
1	1	–43.6	
	3	–42.5	
2	1	–49.0	
			(inconclusive binding mode)
	4	–46.2	
3	1	–33.4	
4	1	–40.1	
5	1	–42.7	
	15	–41.9	
6	1	–45.5	

Table S3. K_M values of Boc-LRR-AMC, and IC_{50} and K_i values of **3**–**6** in dependence of buffer pH.

Enzyme	K_M (μM)[a]				
	pH 7.0	pH 7.5	pH 8.0	pH 8.5	pH 9.0
MT-SP1	255 ± 37	201 ± 33	39.1 ± 5.1	65.9 ± 6.2	55.0 ± 5.0
Cpd	IC_{50} (μM)				
	pH 7.0	pH 7.5	pH 8.0	pH 8.5	pH 9.0
3	104 ± 8	61.4 ± 5.3	65.3 ± 3.6	50.6 ± 4.0	71.8 ± 10.7
4	n.d.	n.d.	2.46 ± 0.29	n.d.	n.d.
5	n.d.	n.d.	1.46 ± 0.10	n.d.	n.d.
6	0.589 ± 0.048	1.37 ± 0.06	1.42 ± 0.08	0.680 ± 0.051	0.910 ± 0.057
Cpd	K_i (μM)				
	pH 7.0	pH 7.5	pH 8.0	pH 8.5	pH 9.0
3	77.9 ± 6.7	43.1 ± 4.3	18.4 ± 2.0	22.1 ± 2.1	28.2 ± 4.5
4	n.d.	n.d.	0.775 ± 0.115	n.d.	n.d.
5	n.d.	n.d.	0.459 ± 0.051	n.d.	n.d.
6	0.440 ± 0.039	0.961 ± 0.064	0.398 ± 0.043	0.297 ± 0.027	0.358 ± 0.030

a: substrate BOC-LRR-AMC. n.d.: not determined.

Table S4. ITC results including errors from direct titrations. Experiments were performed at least in triplicates.

Ligand	buffer	N	K_d (μM)	ΔG_{obs} (kJ·mol^{-1})	ΔH_{obs} (kJ·mol^{-1})	$-T\Delta S_{obs}$ (kJ·mol^{-1})
1	TRIS	0.59 ± 0.01	1.45 ± 0.19	–33.4 ± 0.2	–14.0 ± 0.5	–19.4 ± 0.4
2	TRIS	0.84 ± 0.02	0.392 ± 0.121	–36.9 ± 1.2	–6.8 ± 0.3	–30.1 ± 1.3
3	HEPES	0.88 ± 0.06	12.7 ± 2.2	–28.1 ± 0.8	4.4 ± 0.3	–32.6 ± 0.8
	TRIS	1.15 ± 0.03	7.76 ± 0.94	–29.6 ± 1.5	3.5 ± 0.1	–33.0 ± 1.0
4	HEPES	0.69 ± 0.02	2.50 ± 0.44	–32.1 ± 0.5	7.4 ± 0.6	–39.4 ± 1.2
	TRIS	0.65 ± 0.02	2.53 ± 0.60	–32.0 ± 0.5	5.8 ± 0.3	–37.9 ± 0.6
5	HEPES	0.93 ± 0.01	1.42 ± 0.23	–33.5 ± 0.4	6.4 ± 0.2	–39.8 ± 0.4
	TRIS	0.96 ± 0.01	0.976 ± 0.168	–34.4 ± 0.6	3.8 ± 0.1	–38.3 ± 0.6
6	HEPES	0.81 ± 0.01	0.757 ± 0.113	–35.1 ± 1.0	6.6 ± 0.2	–41.7 ± 0.9
	TRIS	1.05 ± 0.02	0.517 ± 0.109	–36.0 ± 0.5	4.5 ± 0.1	–40.5 ± 0.4

Table S5. Comparison of calculated ($\Delta\Delta G_{calc}$) and experimental ($\Delta\Delta G_{ITC}$) changes in ΔG, assuming equal $K_{a,inc}$ for inhibitors **4**–**6**.

Cpd	Qualitative binding mode affinity comparison	Estimated incremental affinity $K_{a,inc}$ (μM)		$\Delta\Delta G_{(calc)}$ (kJ·mol^{-1})	$\Delta\Delta G_{(ITC)}$ (kJ·mol^{-1})	$\Delta-T\Delta S_{(ITC)}$ (kJ·mol^{-1})
3	$[PL]_1 = [PL]_2 = [PL]_3 = [PL]_4 = [PL]_5 = [PL]_6$	$K_{a,app} = K_{a,1} = K_{a,2} = K_{a,3} = K_{a,4} = K_{a,5} = K_{a,6} = 6 \cdot K_{a,inc}$				
4	$[PL]_1 = [PL]_2 >> [PL]_3 = [PL]_4 \approx [PL]_5 = [PL]_6$	$K_{a,app} = K_{a,1} = K_{a,2} = 2 \cdot K_{a,inc}$	$\Delta\Delta G_{4\rightarrow5}$	$-RT\cdot\ln(2\cdot K_{a,inc}) - (-RT\cdot\ln(4\cdot K_{a,inc})) = -RT\cdot\ln(\frac{4}{2}) = -1.7$	–1.8	–1.6
5	$[PL]_1 = [PL]_2 \approx [PL]_3 = [PL]_4 >> [PL]_5 = [PL]_6$	$K_{a,app} = K_{a,1} = K_{a,2} = K_{a,3} = K_{a,4} = 4 \cdot K_{a,inc}$				
6	$[PL]_1 = [PL]_2 = [PL]_3 = [PL]_4 = [PL]_5 = [PL]_6$	$K_{a,app} = K_{a,1} = K_{a,2} = K_{a,3} = K_{a,4} = K_{a,5} = K_{a,6} = 6 \cdot K_{a,inc}$	$\Delta\Delta G_{5\rightarrow6}$	$-RT\cdot\ln(4\cdot K_{a,inc}) - (-RT\cdot\ln(6\cdot K_{a,inc})) = -RT\cdot\ln(\frac{6}{4}) = -1.0$	–1.7	–1.5

Table S6. Used concentrations for direct titrations.

Cpd	Cell (μM)	Syringe (μM)
1	15	200
2	15	200
3	50	2000
4	50	500
5	50	500
6	25	500

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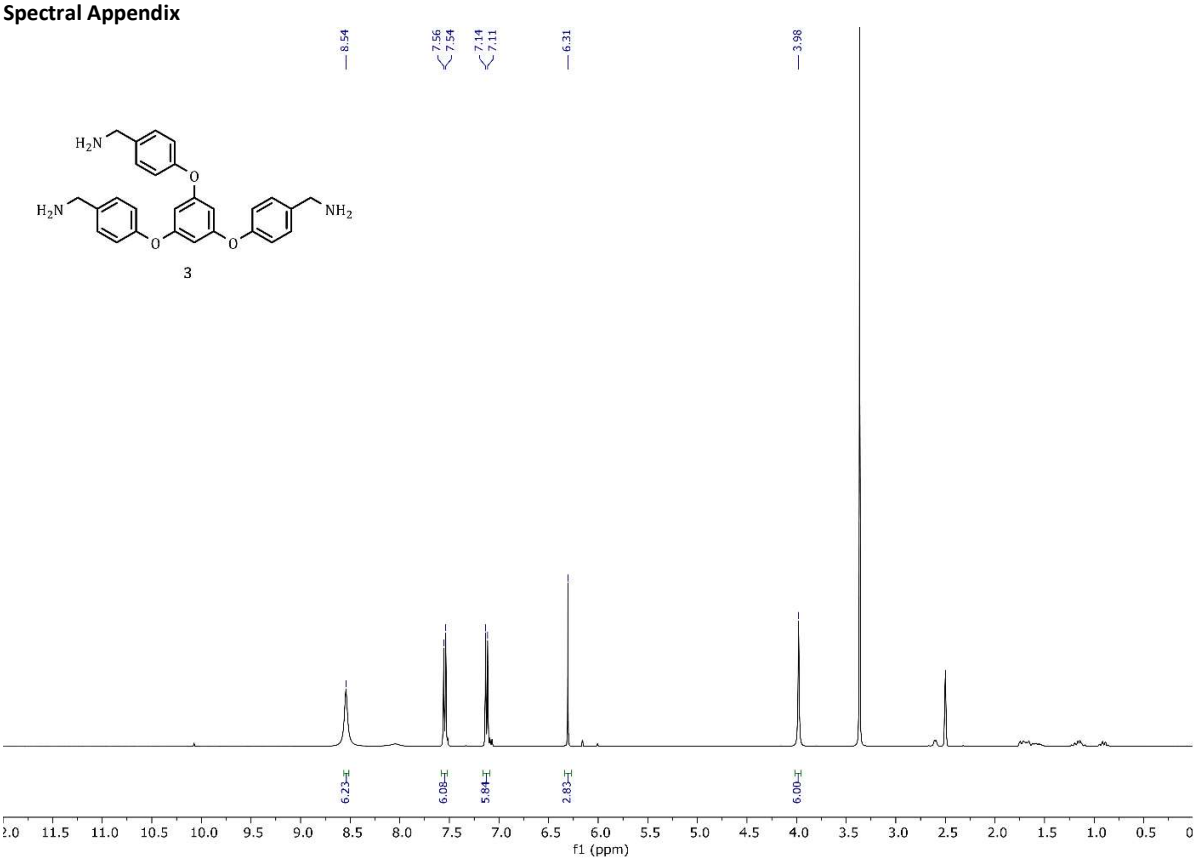
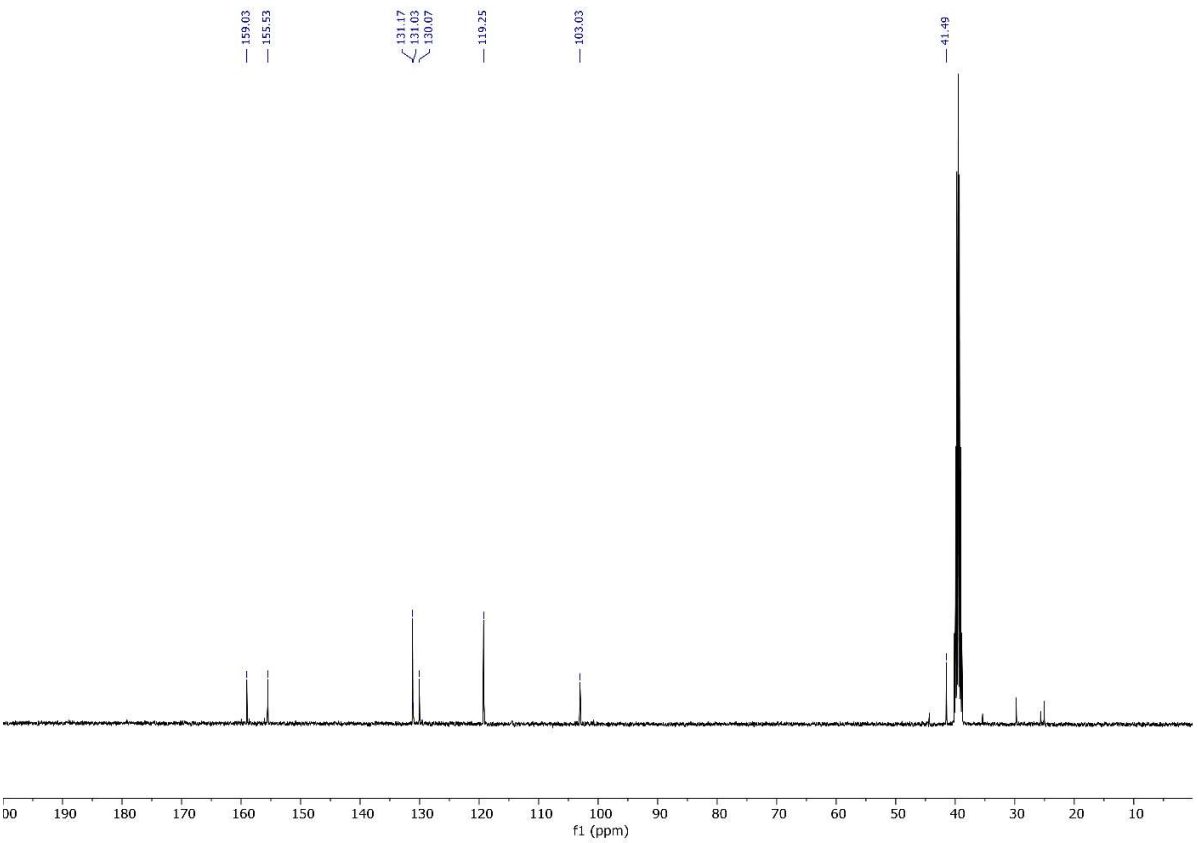
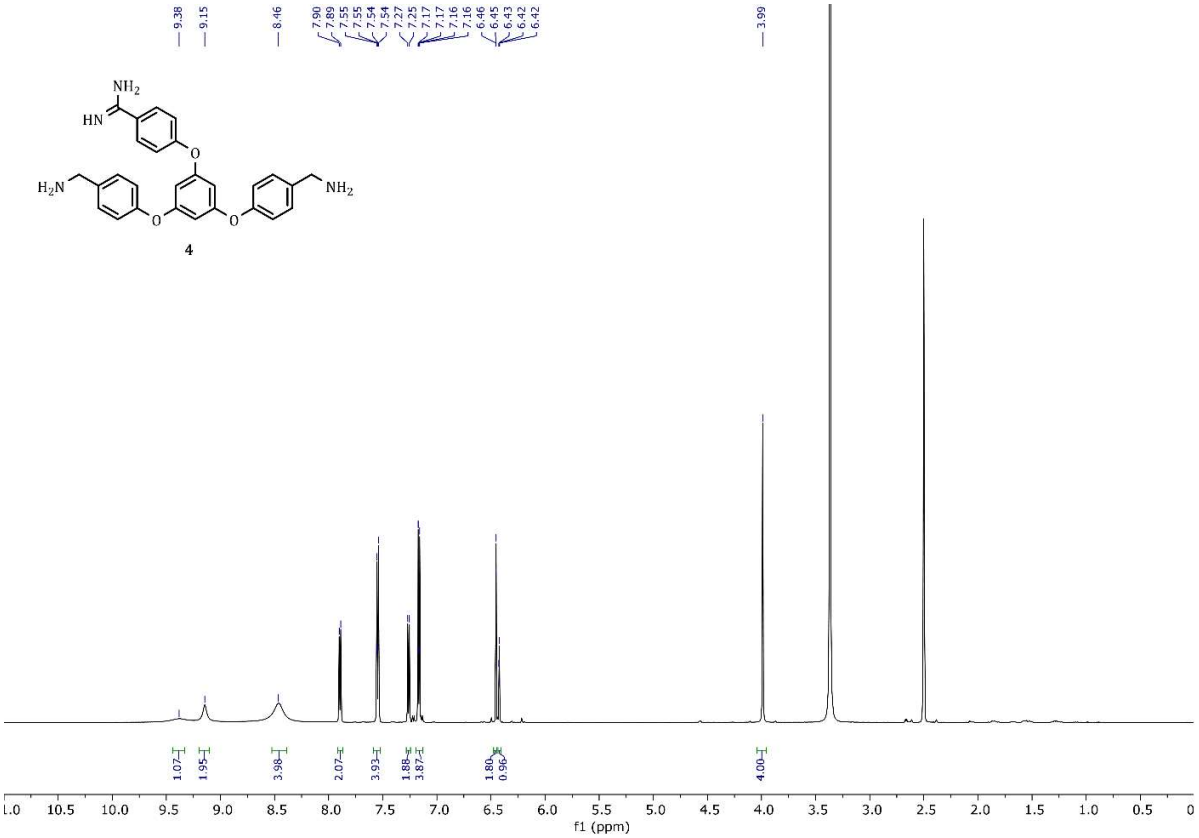
Spectral Appendix



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NH2
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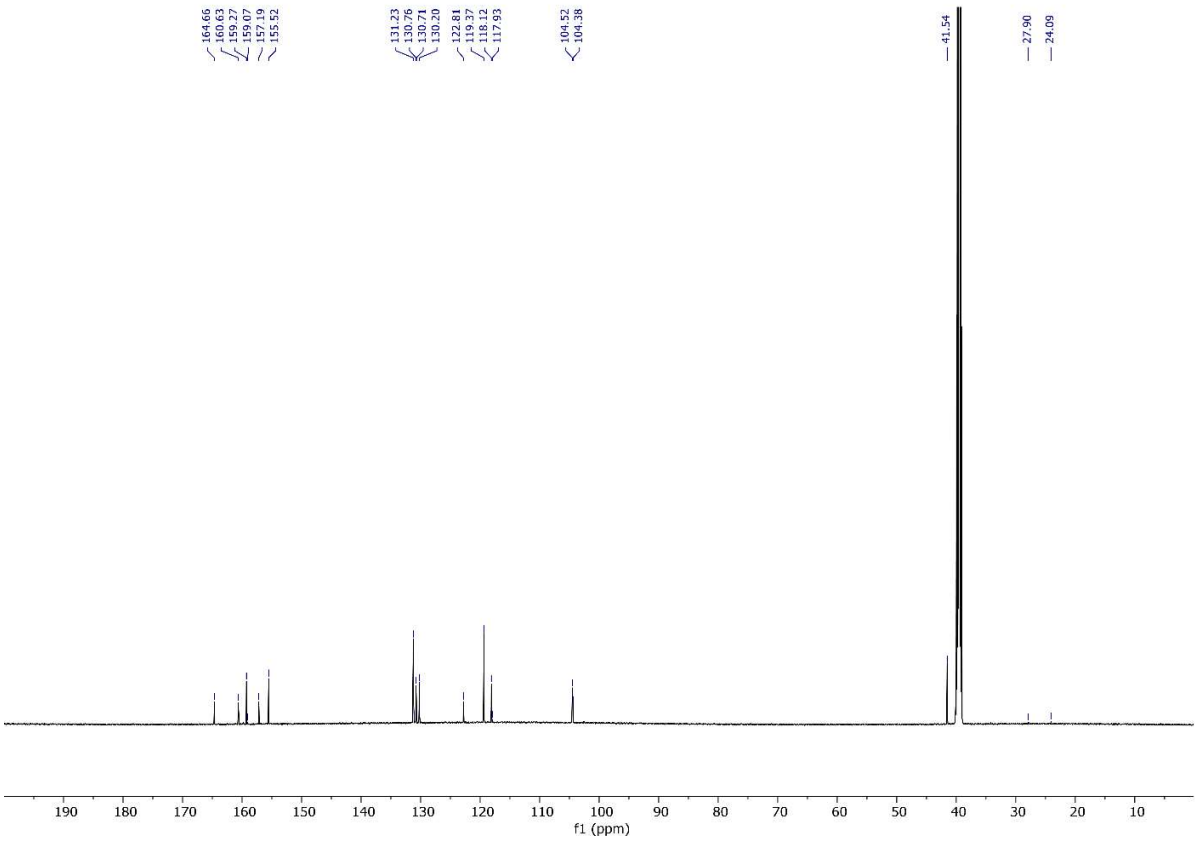
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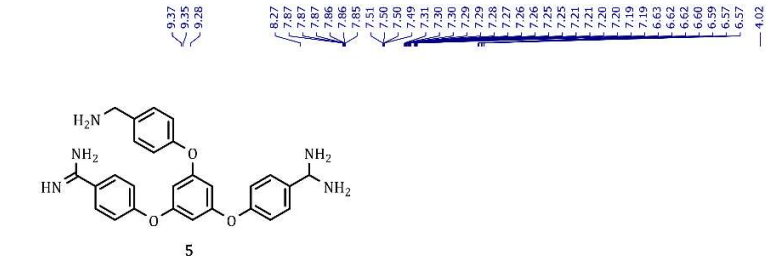
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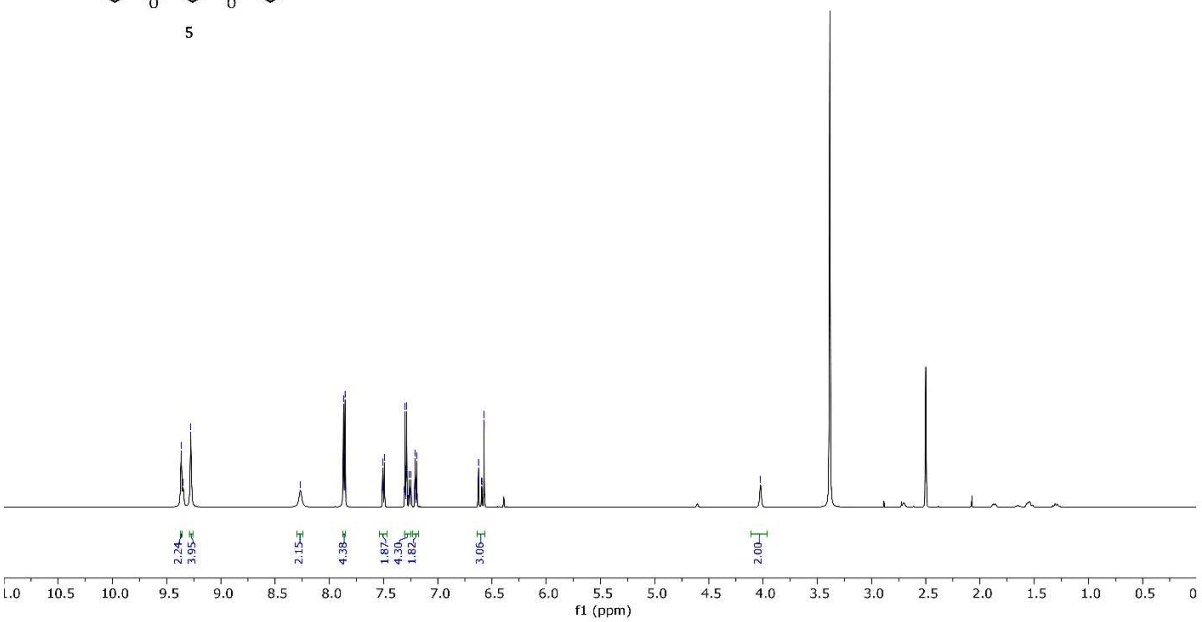
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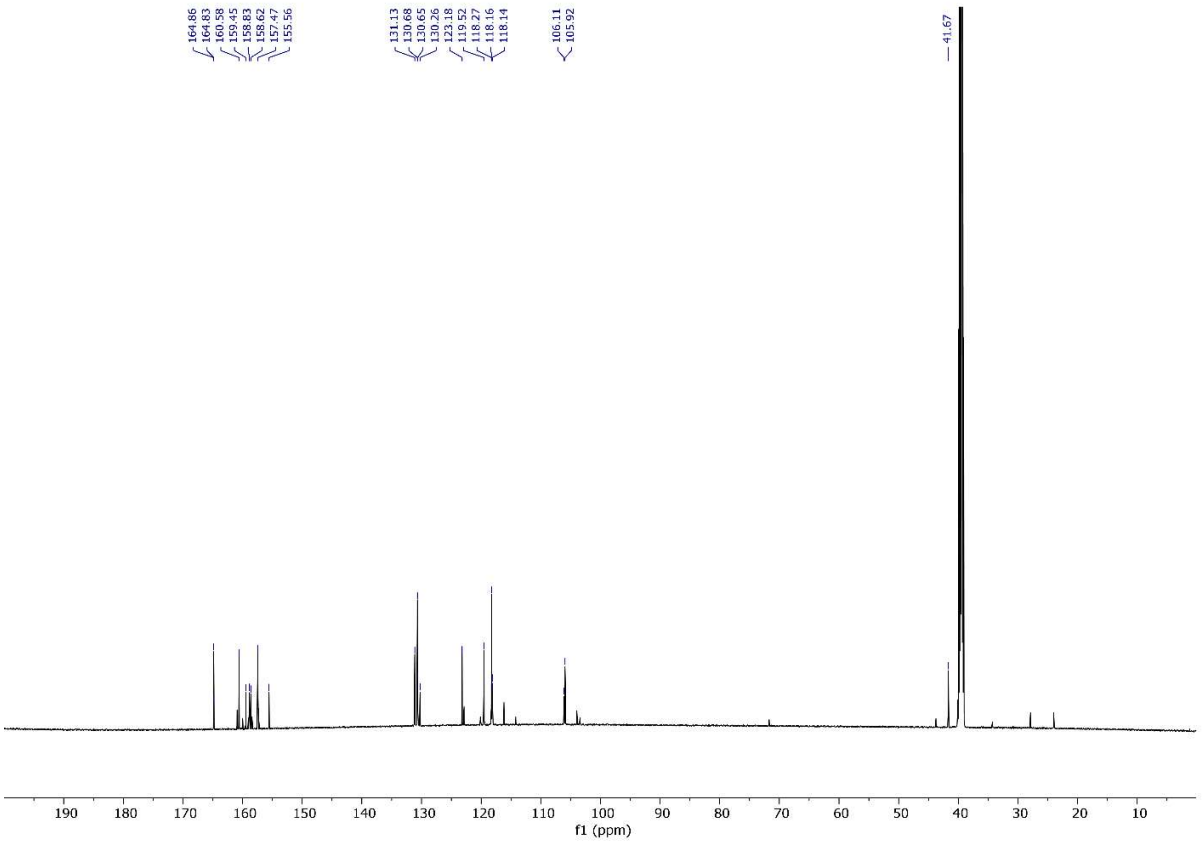
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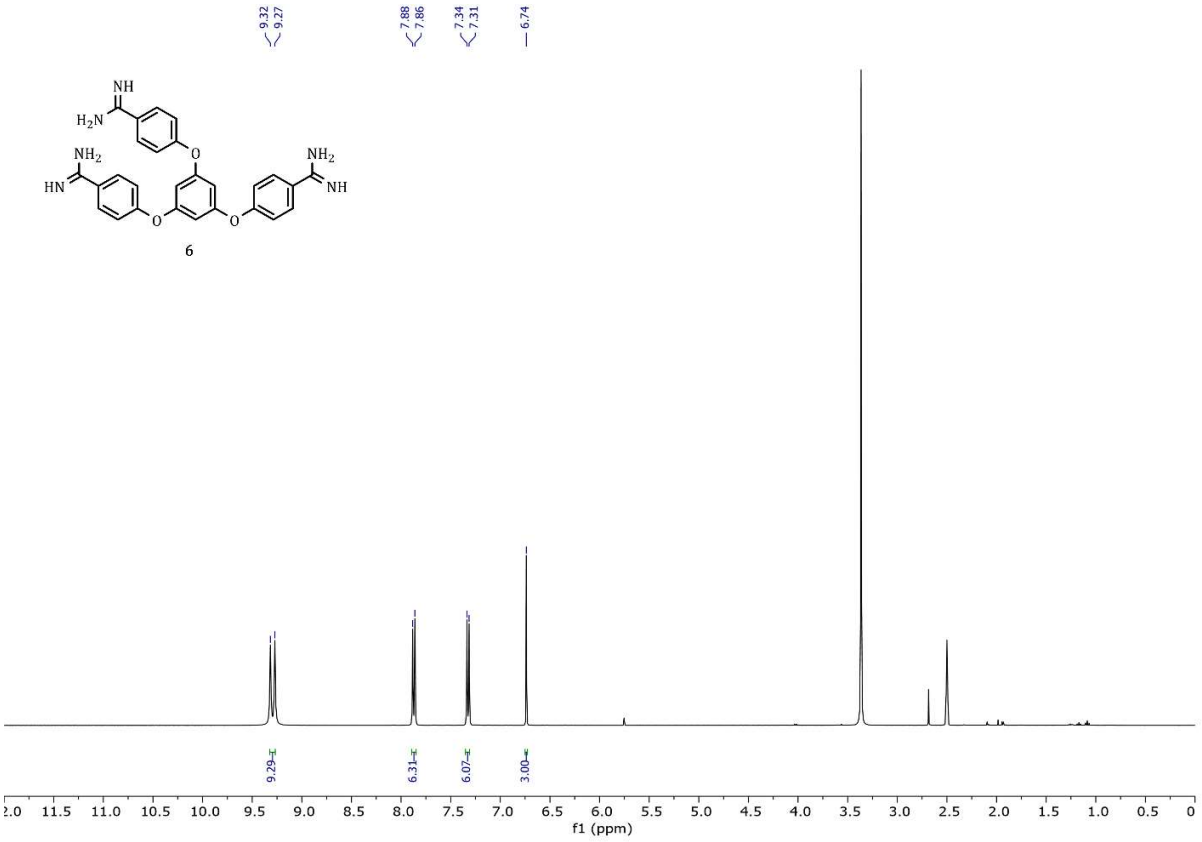
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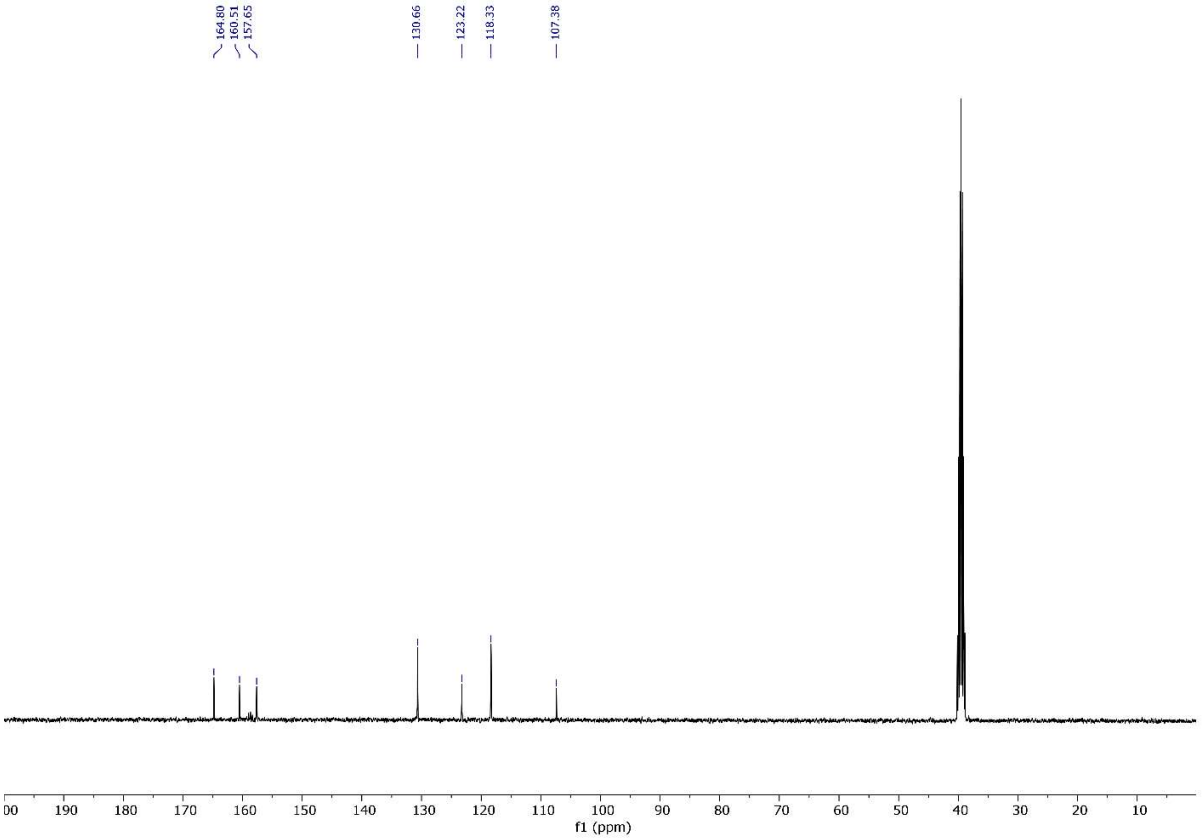
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