Electronic Supplementary Material (ESI) for Chemical Communications.

Supporting information

Identification of bromophenol thiohydantoin as an inhibitor of DisA, a c-di-AMP synthase, from a 1000 compound library, using the coralyne assay

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a. **Additional tables and figures**

b. **Experimental section**

a. Additional tables and figures

Plate	Well	IDNUMBER	Plate	Well	IDNUMBER	Plate	Well	IDNUMBER	Plate	Well	IDNUMBER	Plate	Well	IDNUMBER	Plate	Well	IDNUMBER
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7	D10	ST030428	8	D10	ST035130	9	D10	ST040419	10	D10	ST049071	11	D10	ST054069	12	D10	ST057711
7	E10	ST030562	8	E10	ST035131	9	E10	ST040425	10	E10	ST049073	11	E10	ST054354	12	E10	ST058249
7	F10	ST030733	8	F10	ST035169	9	F10	ST040426	10	F10	ST049074	11	F10	ST054407	12	F10	ST058274
7	G10	ST031049	8	G10	ST035173	9	G10	ST040427	10	G10	ST049079	11	G10	ST054625	12	G10	ST058282
7	H10	ST031162	8	H10	ST035174	9	H10	ST040429	10	H10	ST049143	11	H10	ST054687	12	H10	ST058283
7	A11	ST031166	8	A11	ST035177	9	A11	ST040432	10	A11	ST049356	11	A11	ST054708	12	A11	ST058284
7	B11	ST031272	8	B11	ST035186	9	B11	ST040445	10	B11	ST049416	11	B11	ST054780	12	B11	ST058285
7	C11	ST031415	8	C11	ST035224	9	C11	ST040459	10	C11	ST049455	11	C11	ST054939	12	C11	ST058293
7	D11	ST031702	8	D11	ST035648	9	D11	ST040466	10	D11	ST049477	11	D11	ST054947	12	D11	ST058459
7	E11	ST031721	8	E11	ST035656	9	E11	ST040468	10	E11	ST049494	11	E11	ST055031	12	E11	ST058522
7	F11	ST031725	8	F11	ST035670	9	F11	ST040516	10	F11	ST049754	11	F11	ST055044	12	F11	ST058739
7	G11	ST031791	8	G11	ST035688	9	G11	ST040549	10	G11	ST049803	11	G11	ST055060	12	G11	ST058927
7	H11	ST031947	8	H11	ST035692	9	H11	ST040554	10	H11	ST049855	11	H11	ST055062	12	H11	ST058928
7	A12	ST032360	8	A12	ST035719	9	A12	ST040555	10	A12	ST049871	11	A12	ST055063	12	A12	ST058933
7	B12	ST032369	8	B12	ST035745	9	B12	ST040634	10	B12	ST049900	11	B12	ST055076	12	B12	ST058938
7	C12	ST032605	8	C12	ST035746	9	C12	ST040665	10	C12	ST049949	11	C12	ST055077	12	C12	ST059001
7	D12	ST032974	8	D12	ST035759	9	D12	ST040666	10	D12	ST049974	11	D12	ST055086	12	D12	ST059053
7	E12	ST033197	8	E12	ST035784	9	E12	ST040691	10	E12	ST049975	11	E12	ST055132	12	E12	ST059207
7	F12	ST033361	8	F12	ST035787	9	F12	ST040872	10	F12	ST049988	11	F12	ST055144	12	F12	ST059224
7	G12	ST033370	8	G12	ST035789	9	G12	ST040908	10	G12	ST049995	11	G12	ST055176	12	G12	ST059348
7	H12	ST033371	8	H12	ST035794	9	H12	ST041026	10	H12	ST050006	11	H12	ST055328	12	H12	ST059407

Table SI1 ID number of each compound in Timtec ActiTarg-K library.

Plate	Well	MDL number	Plate	Well	MDL number	Plate	Well	MDL number	Plate	Well	MDL number
Sigma	A1	MFCD00133539	Sigma	B1	MFCD04974495	Sigma	C1	MFCD00157720	Sigma	D1	MFCD00005607
Sigma	A2	MFCD08705319	Sigma	B2	MFCD00055790	Sigma	C2	MFCD00136466	Sigma	D2	MFCD00002497
Sigma	A3	MFCD00079619	Sigma	B3	MFCD00187724	Sigma	C3	MFCD00093078	Sigma	D3	MFCD00005183
Sigma	A4	MFCD11045307	Sigma	B4	MFCD00085853	Sigma	C4	MFCD00032240	Sigma	D4	MFCD00012780
Sigma	A5	MFCD08276917	Sigma	B5	MFCD00020684	Sigma	C5	MFCD09865273	Sigma	D5	MFCD00002339
Sigma	A6	MFCD00010555	Sigma	B6	MFCD00017824	Sigma	C6	MFCD08272809	Sigma	D6	MFCD00019481
Sigma	A7	MFCD08277040	Sigma	B7	MFCD00088782	Sigma	C7	MFCD06411391	Sigma	D7	MFCD00151027
Sigma	A8	MFCD16875436	Sigma	B8	MFCD00030041	Sigma	C8	MFCD21608518			
Sigma	A9	MFCD00864692	Sigma	B9	MFCD00030033	Sigma	C9	MFCD12828770			
Sigma	A10	MFCD00083192	Sigma	B10	MFCD00028696	Sigma	C10	MFCD00099612			
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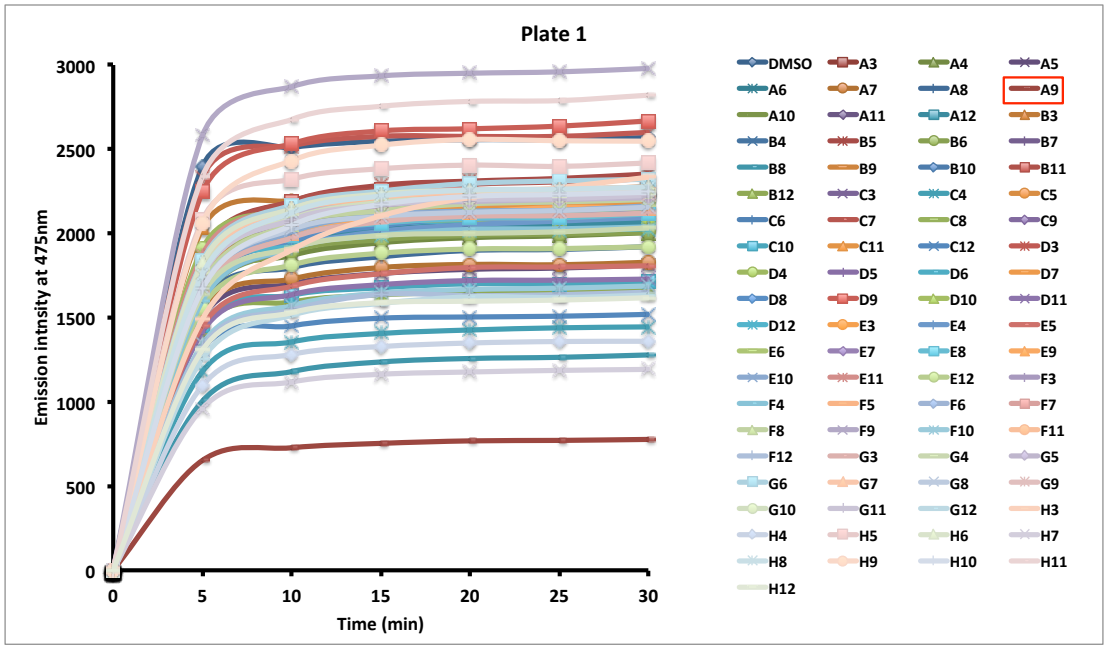
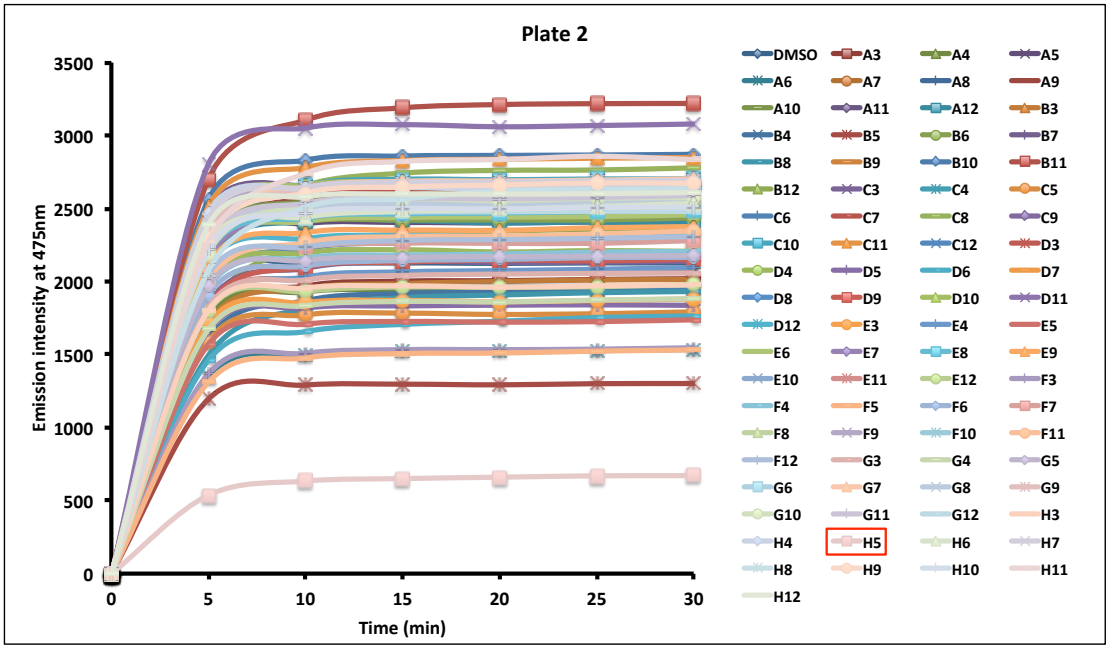
Table SI2 MDL number of each compound in Sigma-Aldrich library.





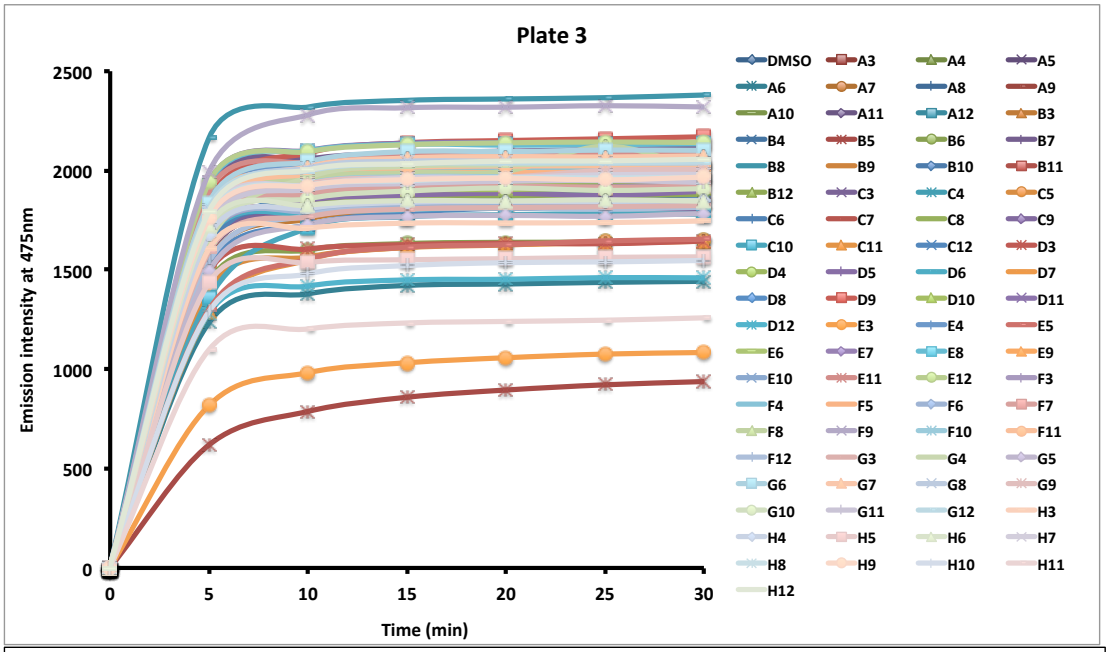


Plate 3
Emission intensity at 475nm
2500
2000
1500
1000
500
0
0
5
10
15
20
25
30
Time (min)
DMSO A3 A4 A5
A6 A7 A8 A9
A10 A11 A12 B3
B4 B5 B6 B7
B8 B9 B10 B11
B12 C3 C4 C5
C6 C7 C8 C9
C10 C11 C12 D3
D4 D5 D6 D7
D8 D9 D10 D11
D12 E3 E4 E5
E6 E7 E8 E9
E10 E11 E12 F3
F4 F5 F6 F7
F8 F9 F10 F11
F12 G3 G4 G5
G6 G7 G8 G9
G10 G11 G12 H3
H4 H5 H6 H7
H8 H9 H10 H11
H12

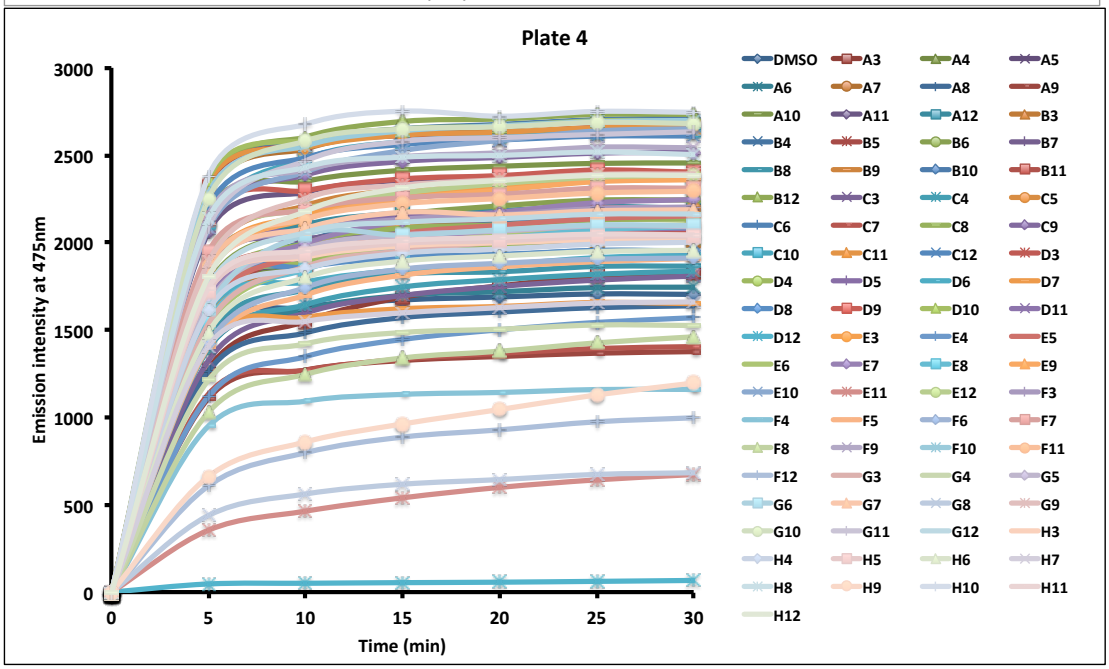


Plate 4
Emission intensity at 475nm
3000
2500
2000
1500
1000
500
0
0
5
10
15
20
25
30
Time (min)
DMSO A3 A4 A5
A6 A7 A8 A9
A10 A11 A12 B3
B4 B5 B6 B7
B8 B9 B10 B11
B12 C3 C4 C5
C6 C7 C8 C9
C10 C11 C12 D3
D4 D5 D6 D7
D8 D9 D10 D11
D12 E3 E4 E5
E6 E7 E8 E9
E10 E11 E12 F3
F4 F5 F6 F7
F8 F9 F10 F11
F12 G3 G4 G5
G6 G7 G8 G9
G10 G11 G12 H3
H4 H5 H6 H7
H8 H9 H10 H11
H12

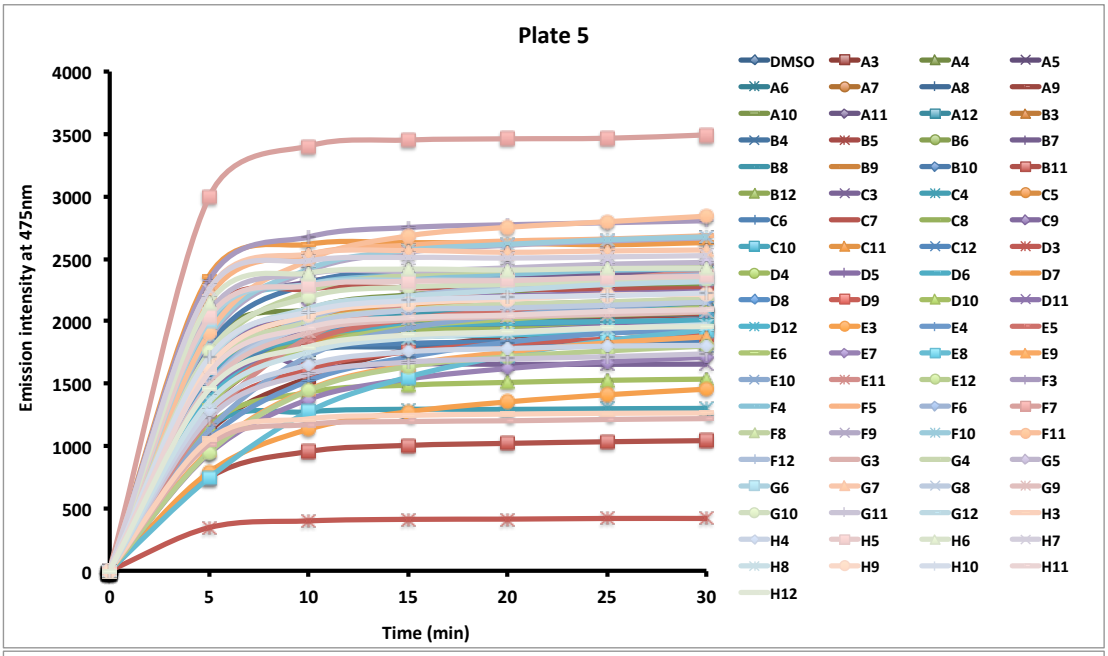


Plate 5
Emission intensity at 475nm
Time (min)
0
500
1000
1500
2000
2500
3000
3500
4000
0
5
10
15
20
25
30
DMSO A3 A4 A5
A6 A7 A8 A9
A10 A11 A12 B3
B4 B5 B6 B7
B8 B9 B10 B11
B12 C3 C4 C5
C6 C7 C8 C9
C10 C11 C12 D3
D4 D5 D6 D7
D8 D9 D10 D11
D12 E3 E4 E5
E6 E7 E8 E9
E10 E11 E12 F3
F4 F5 F6 F7
F8 F9 F10 F11
F12 G3 G4 G5
G6 G7 G8 G9
G10 G11 G12 H3
H4 H5 H6 H7
H8 H9 H10 H11
H12

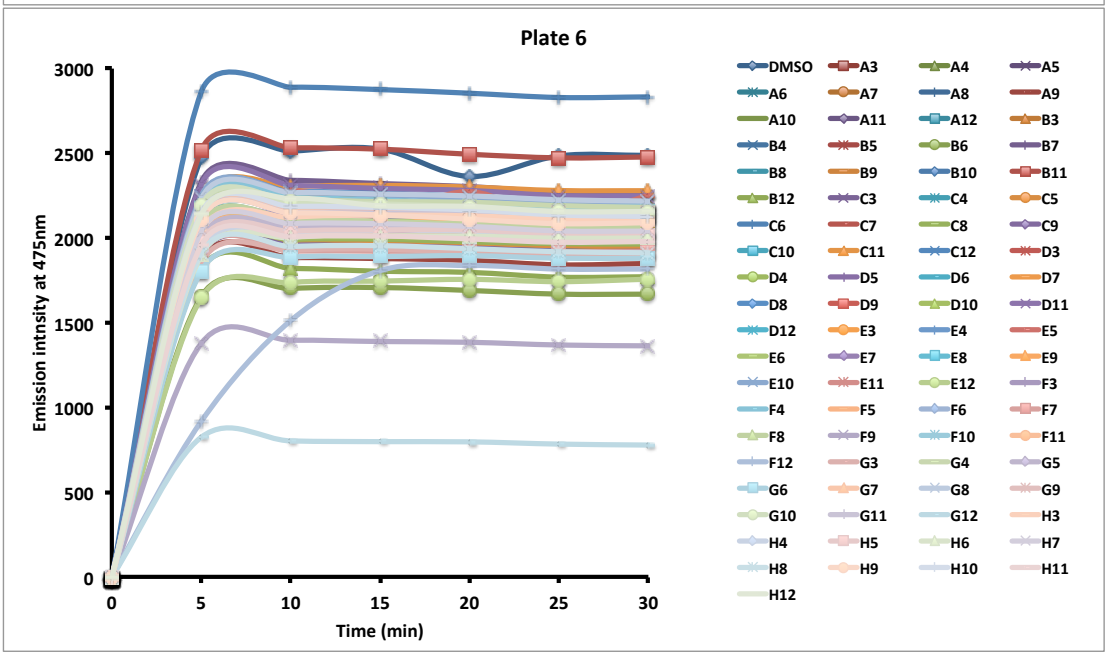


Plate 6
Emission intnsity at 475nm
Time (min)
0
500
1000
1500
2000
2500
3000
0
5
10
15
20
25
30
DMSO A3 A4 A5
A6 A7 A8 A9
A10 A11 A12 B3
B4 B5 B6 B7
B8 B9 B10 B11
B12 C3 C4 C5
C6 C7 C8 C9
C10 C11 C12 D3
D4 D5 D6 D7
D8 D9 D10 D11
D12 E3 E4 E5
E6 E7 E8 E9
E10 E11 E12 F3
F4 F5 F6 F7
F8 F9 F10 F11
F12 G3 G4 G5
G6 G7 G8 G9
G10 G11 G12 H3
H4 H5 H6 H7
H8 H9 H10 H11
H12

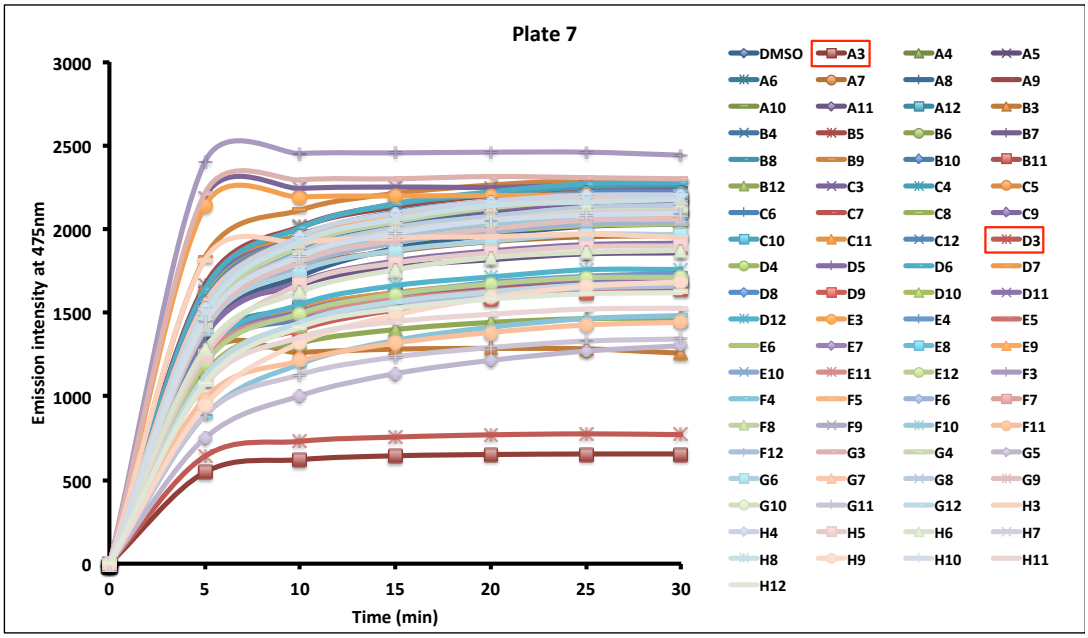


Plate 7
Emission intensity at 475nm
3000
2500
2000
1500
1000
500
0
0
5
10
15
20
25
30
Time (min)
DMSO
A3
A4
A5
A6
A7
A8
A9
A10
A11
A12
B3
B4
B5
B6
B7
B8
B9
B10
B11
B12
C3
C4
C5
C6
C7
C8
C9
C10
C11
C12
D3
D4
D5
D6
D7
D8
D9
D10
D11
D12
E3
E4
E5
E6
E7
E8
E9
E10
E11
E12
F3
F4
F5
F6
F7
F8
F9
F10
F11
F12
G3
G4
G5
G6
G7
G8
G9
G10
G11
G12
H3
H4
H5
H6
H7
H8
H9
H10
H11
H12

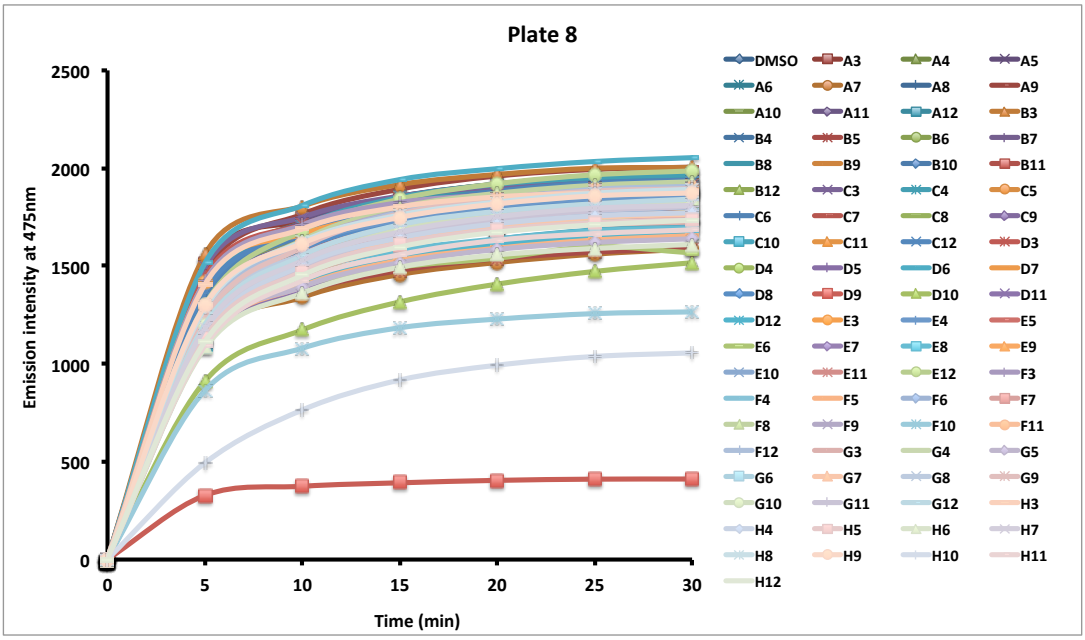


Plate 8
Emission intensity at 475nm
2500
2000
1500
1000
500
0
0
5
10
15
20
25
30
Time (min)
DMSO
A3
A4
A5
A6
A7
A8
A9
A10
A11
A12
B3
B4
B5
B6
B7
B8
B9
B10
B11
B12
C3
C4
C5
C6
C7
C8
C9
C10
C11
C12
D3
D4
D5
D6
D7
D8
D9
D10
D11
D12
E3
E4
E5
E6
E7
E8
E9
E10
E11
E12
F3
F4
F5
F6
F7
F8
F9
F10
F11
F12
G3
G4
G5
G6
G7
G8
G9
G10
G11
G12
H3
H4
H5
H6
H7
H8
H9
H10
H11
H12

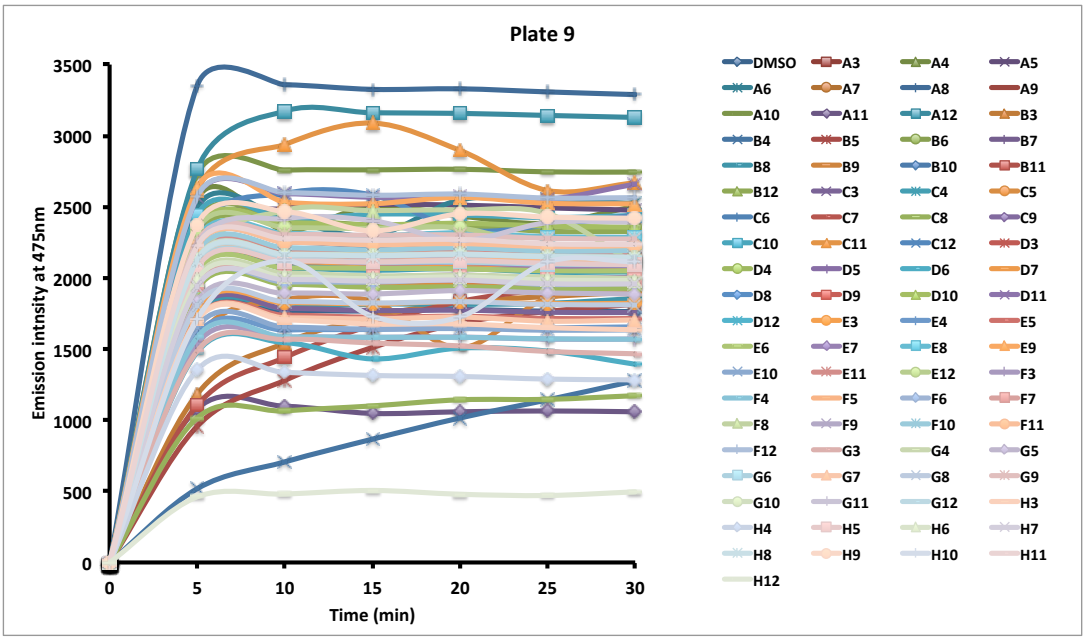


Plate 9
Emission intnsity at 475nm
Time (min)
DMSO A3 A4 A5
A6 A7 A8 A9
A10 A11 A12 B3
B4 B5 B6 B7
B8 B9 B10 B11
B12 C3 C4 C5
C6 C7 C8 C9
C10 C11 C12 D3
D4 D5 D6 D7
D8 D9 D10 D11
D12 E3 E4 E5
E6 E7 E8 E9
E10 E11 E12 F3
F4 F5 F6 F7
F8 F9 F10 F11
F12 G3 G4 G5
G6 G7 G8 G9
G10 G11 G12 H3
H4 H5 H6 H7
H8 H9 H10 H11
H12

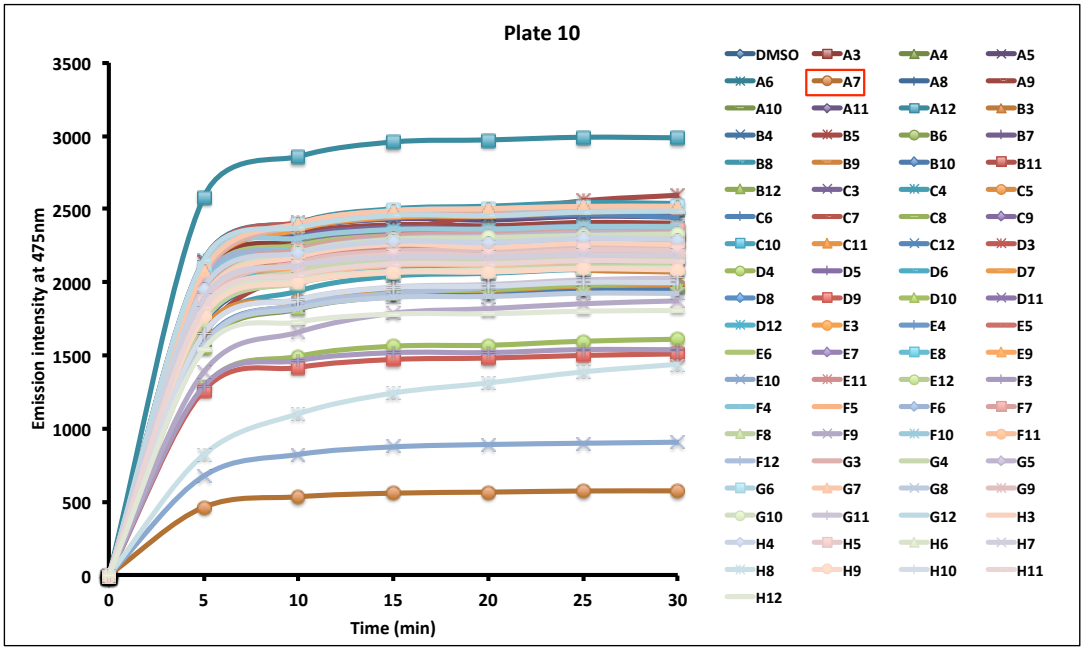


Plate 10
Emission intensity at 475nm
Time (min)
DMSO A3 A4 A5
A6 A7 A8 A9
A10 A11 A12 B3
B4 B5 B6 B7
B8 B9 B10 B11
B12 C3 C4 C5
C6 C7 C8 C9
C10 C11 C12 D3
D4 D5 D6 D7
D8 D9 D10 D11
D12 E3 E4 E5
E6 E7 E8 E9
E10 E11 E12 F3
F4 F5 F6 F7
F8 F9 F10 F11
F12 G3 G4 G5
G6 G7 G8 G9
G10 G11 G12 H3
H4 H5 H6 H7
H8 H9 H10 H11
H12

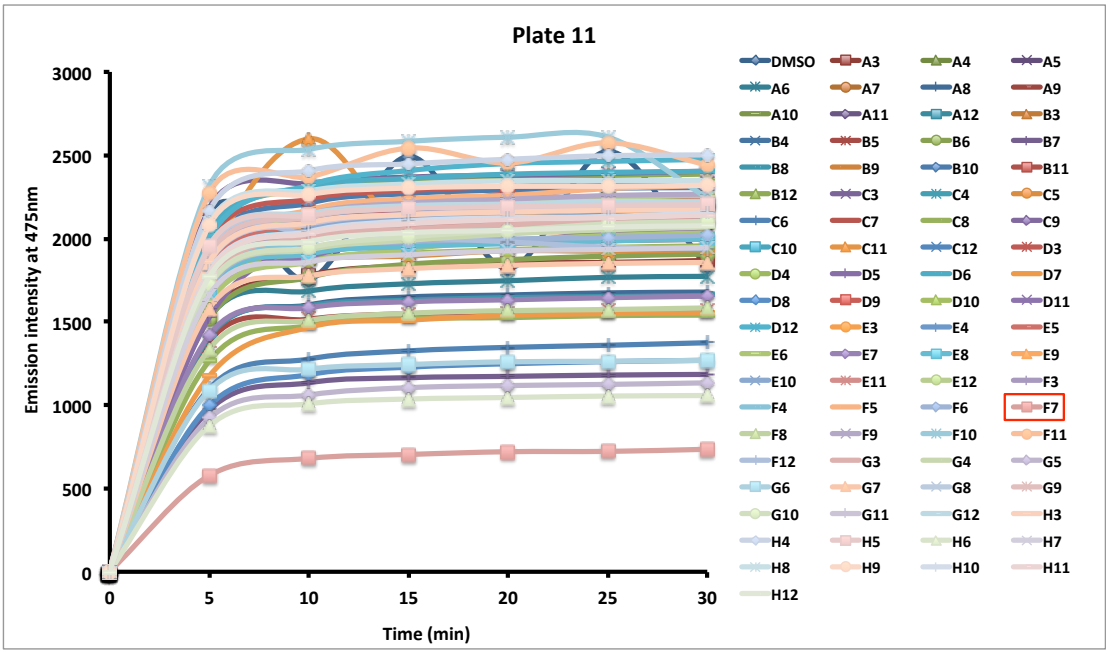


Plate 11
Emission intensity at 475nm
Time (min)
0
500
1000
1500
2000
2500
3000
0
5
10
15
20
25
30
DMSO A3 A4 A5
A6 A7 A8 A9
A10 A11 A12 B3
B4 B5 B6 B7
B8 B9 B10 B11
B12 C3 C4 C5
C6 C7 C8 C9
C10 C11 C12 D3
D4 D5 D6 D7
D8 D9 D10 D11
D12 E3 E4 E5
E6 E7 E8 E9
E10 E11 E12 F3
F4 F5 F6 F7
F8 F9 F10 F11
F12 G3 G4 G5
G6 G7 G8 G9
G10 G11 G12 H3
H4 H5 H6 H7
H8 H9 H10 H11
H12

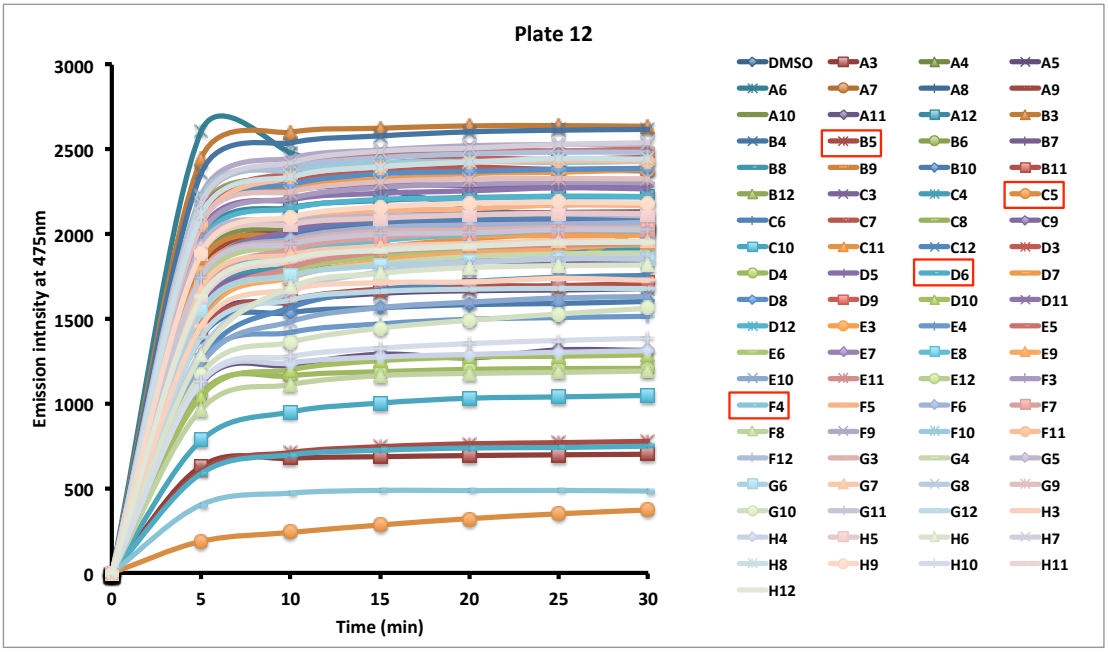


Plate 12
Emission intnsity at 475nm
Time (min)
0
500
1000
1500
2000
2500
3000
0
5
10
15
20
25
30
DMSO A3 A4 A5
A6 A7 A8 A9
A10 A11 A12 B3
B4 B5 B6 B7
B8 B9 B10 B11
B12 C3 C4 C5
C6 C7 C8 C9
C10 C11 C12 D3
D4 D5 D6 D7
D8 D9 D10 D11
D12 E3 E4 E5
E6 E7 E8 E9
E10 E11 E12 F3
F4 F5 F6 F7
F8 F9 F10 F11
F12 G3 G4 G5
G6 G7 G8 G9
G10 G11 G12 H3
H4 H5 H6 H7
H8 H9 H10 H11
H12

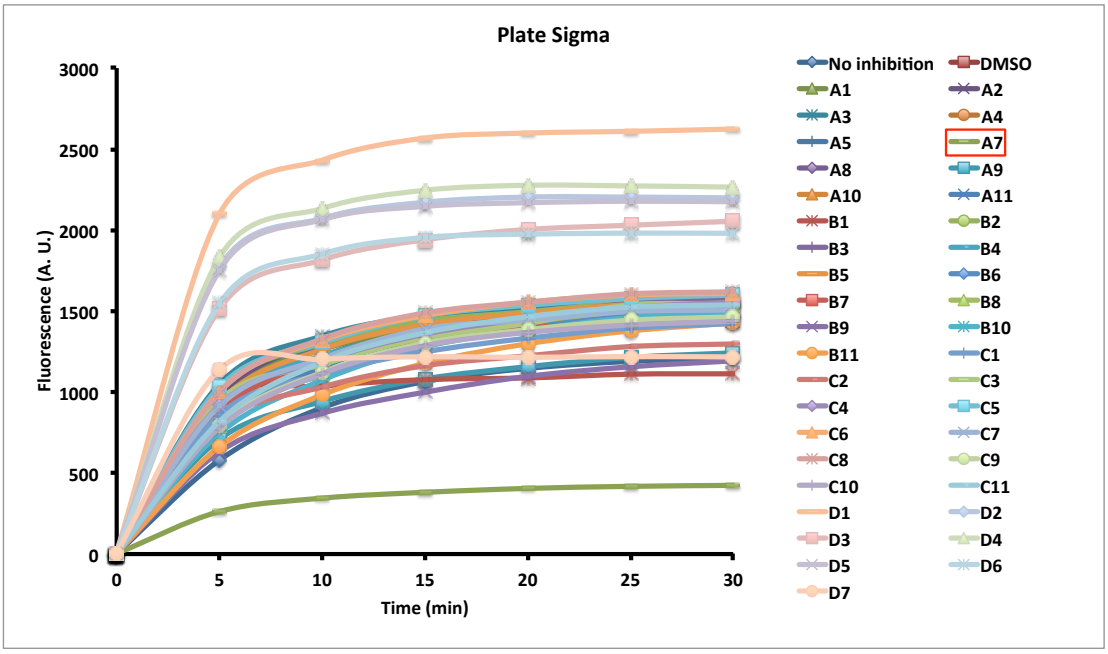


Fig. SI1. Real-time coralyne screening of Timtec ActiTarg-K library (plate 1-12) and Sigma-Aldrich library (plate Sigma). The selected hits were highlighted in red rectangle in the legend.

Plate	Well	ID number
1	A9	ST003651
2	H5	ST006494
7	A3	ST028232
7	D3	ST028249
10	A7	ST044758
11	F7	ST053308
12	B5	ST056010
12	C5	ST056083
12	D6	ST056258
12	F4	ST055987
Sigma	A7	MFCD08277040

Table SI2. ID numbers of hits selected by coralyne assay from Timtec ActiTarg-K library (plate 1-12) and Sigma-Aldrich library (plate Sigma).

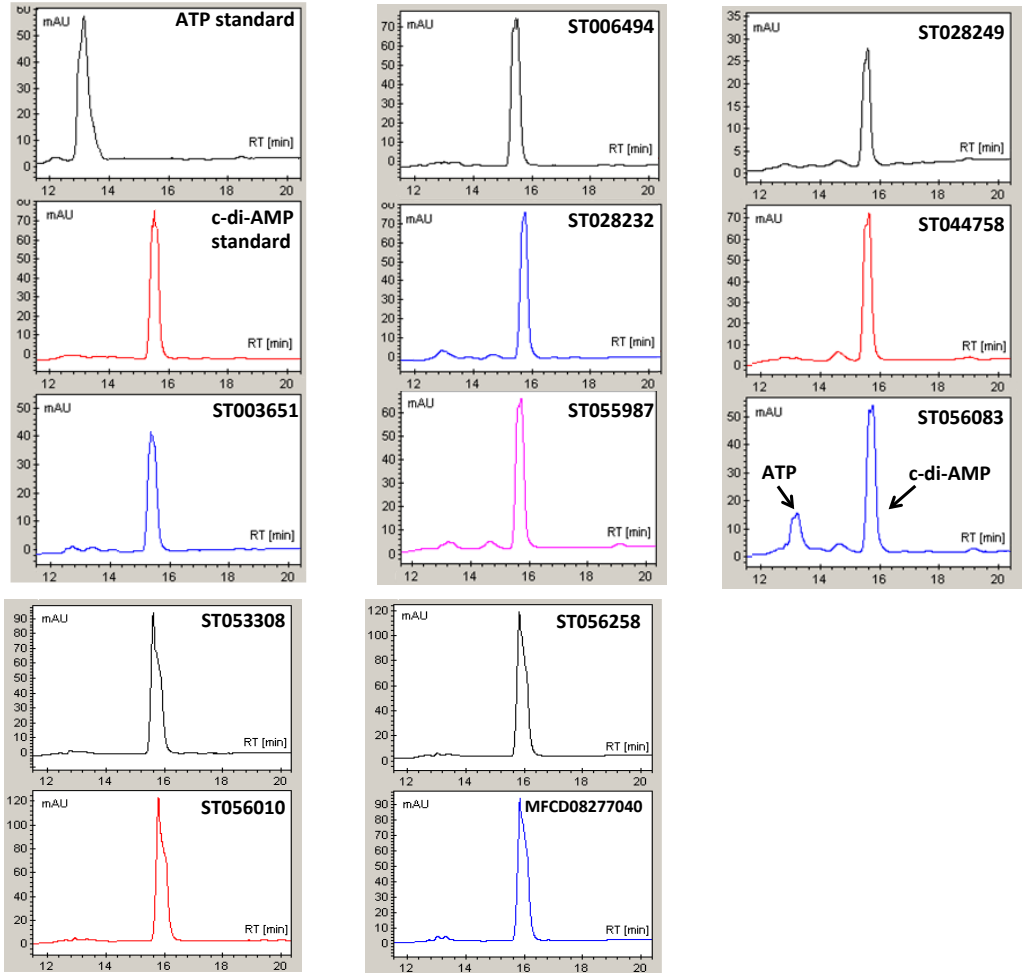


Fig. SI2. HPLC graphs of selected hits affecting DisA reactions. 50 µM hits from Timtec ActiTarg-K library and 100 µM MFCD08277040 from Sigma-Aldrich library were mixed with 100 µM ATP and 10 µM DisA. Reactions were stopped after 30 min. Enzymes were removed by filtration. ATP and c-di-AMP were used as HPLC standards. The added compound is indicated at the upper right corner of each graph.

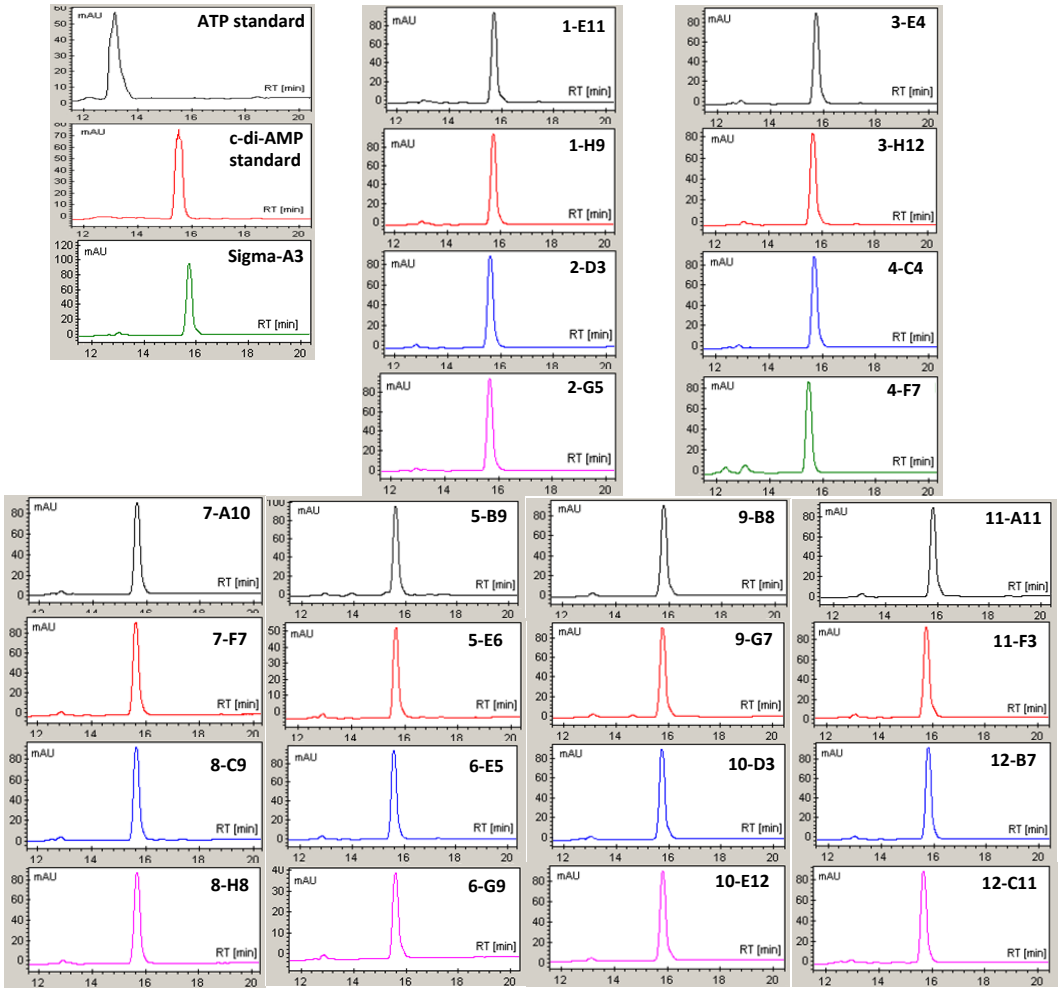


Fig. SI3. HPLC analysis of DisA reaction in the presence of 25 randomly selected library compounds that did not affect c-di-AMP synthesis, as judged by the coralyne assay. This re-testing, using HPLC analysis confirms that compounds that were judged by the coralyne assay as not inhibitors indeed do not inhibit DisA.

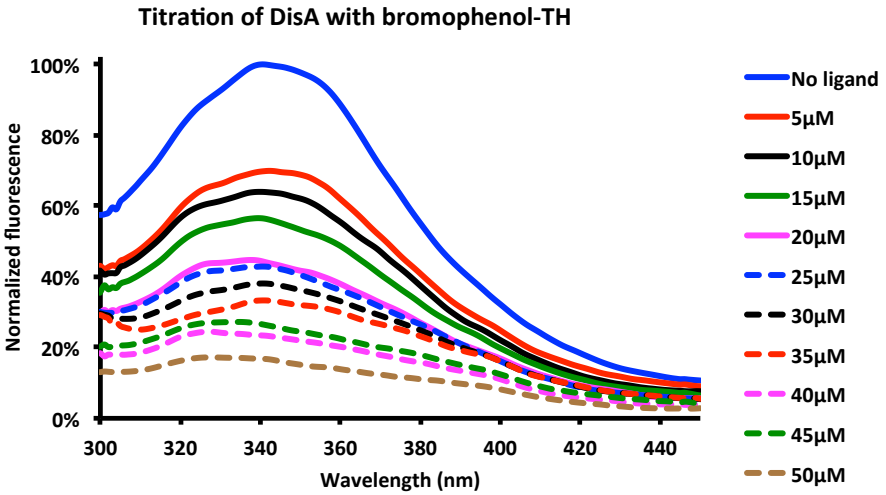


Fig. SI4. Titration of DisA with various concentrations of bromophenol-TH. The graph is normalized with respect to the tryptophan fluorescence without ligand.

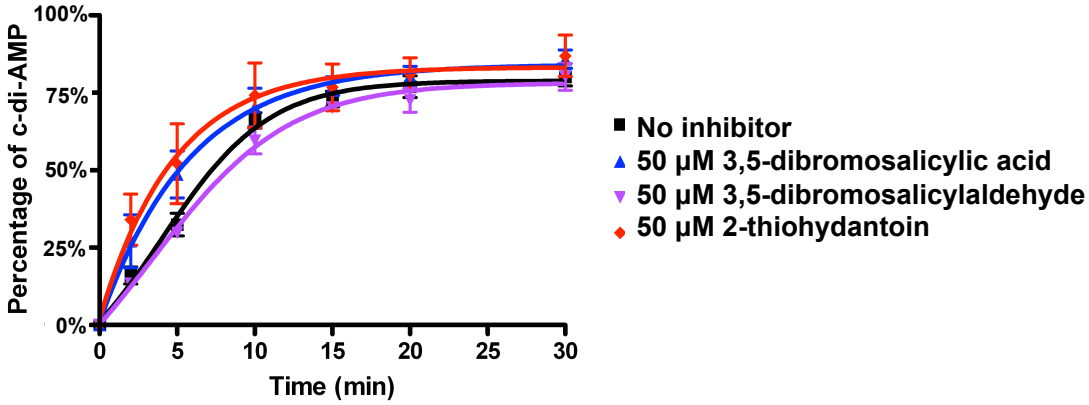


Fig. SI5. 50 µM 3,5-dibromosalicylic acid, 3,5-dibromosalicylaldehyde and 2-thiohydantoin did not inhibit DisA. Assay was performed using α-^{32}P-ATP and TLC to monitor reaction progress.

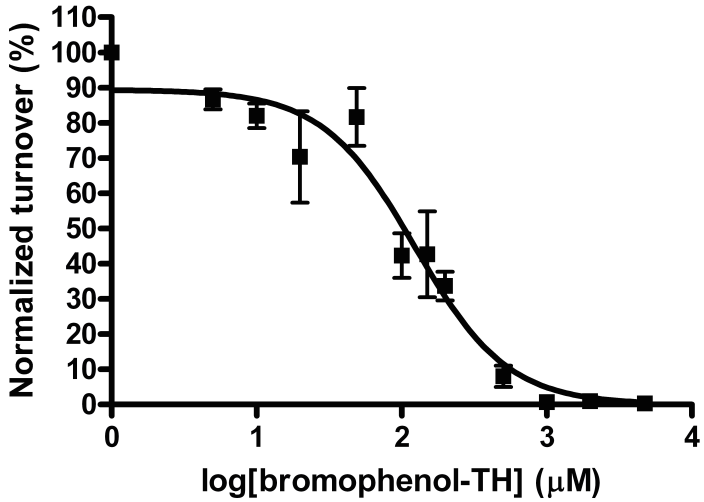


Fig. SI6. DisA inhibition by bromophenol-TH. Reactions were stopped after 5 min and percentage of c-di-AMP was calculated. Cold ATP concentrations was 500 μM. 33 nM ^{32}P-ATP was used to monitor reactions. DisA activity was normalized with respect to the percentage of c-di-AMP formation in the absence of bromophenol-TH. IC_{50} is 100 μM.

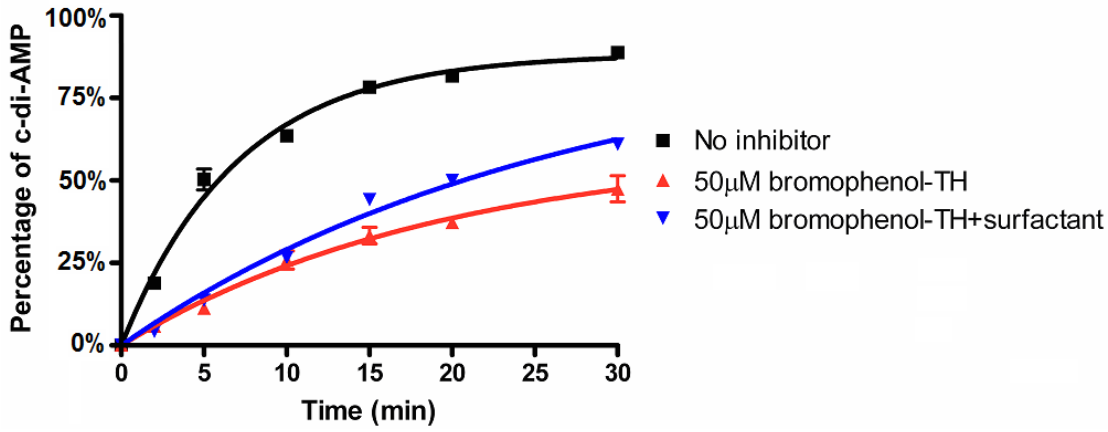


Fig. SI7. Effect of surfactant on bromophenol-TH inhibition. In the presence of 0.01% Triton X-100, 50 μM bromophenol-TH was still able to inhibit DisA. Assay was performed using α-^{32}P-ATP and TLC to monitor reaction progress.

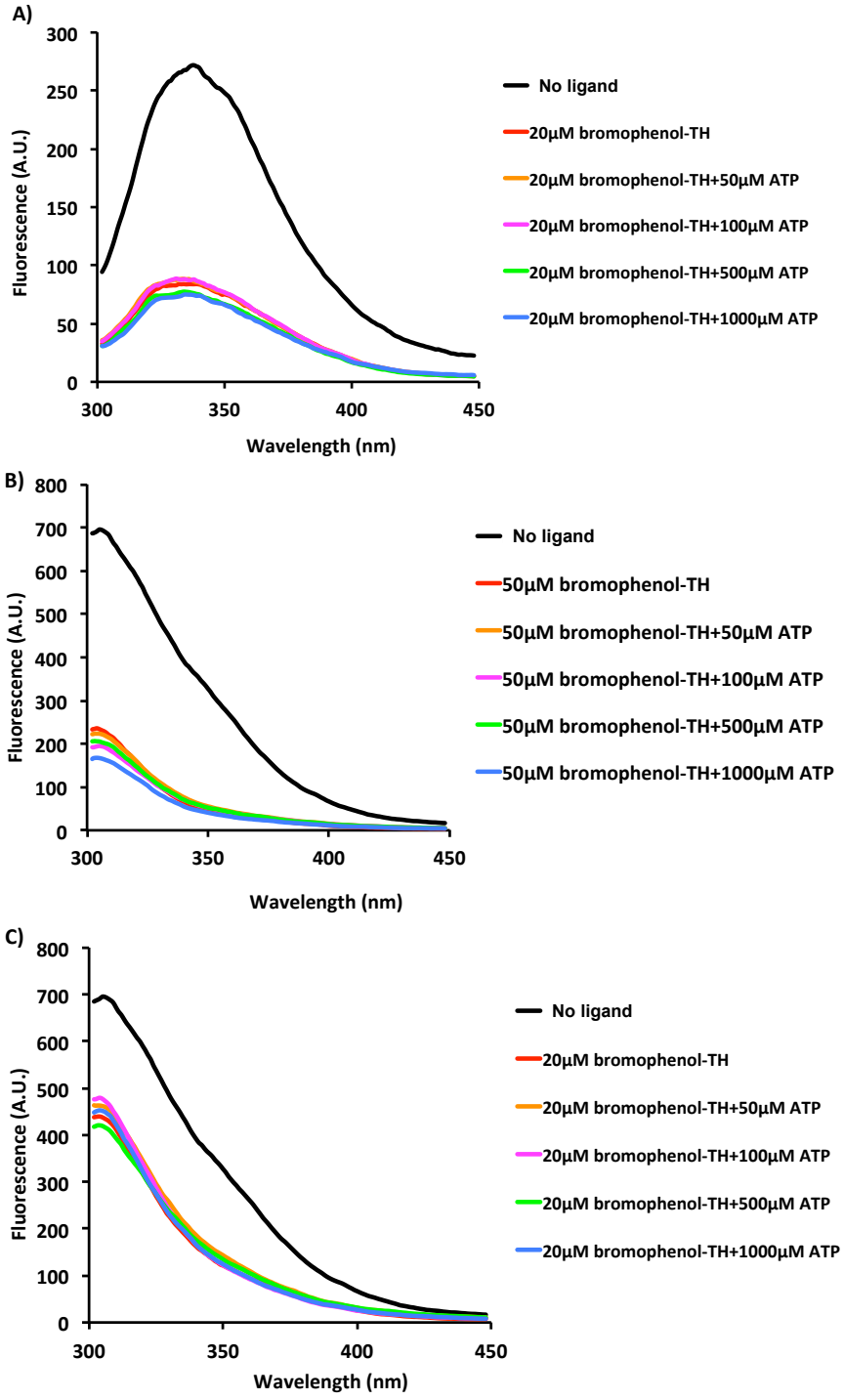


Fig. SI8. Bromophenol-TH binding to DisA in competition with ATP in the presence or absence of surfactant (tryptophan fluorescence titration). A) 20 μM bromophenol-TH competing with 50 μM, 100 μM , 500 μM or 1mM ATP to bind to DisA in the absence of surfactant, Triton-X100. 20 μM bromophenol-TH decreases DisA tryptophan fluorescence emission. ATP could not change the fluorescence emission significantly, indicating that ATP and bromophenol-TH do not bind to the same site. B) and C) 50 μM or 20 μM bromophenol-TH competing with ATP for DisA in the presence of surfactant. The presence of surfactant causes a blue-shift of tryptophan's fluorescence.

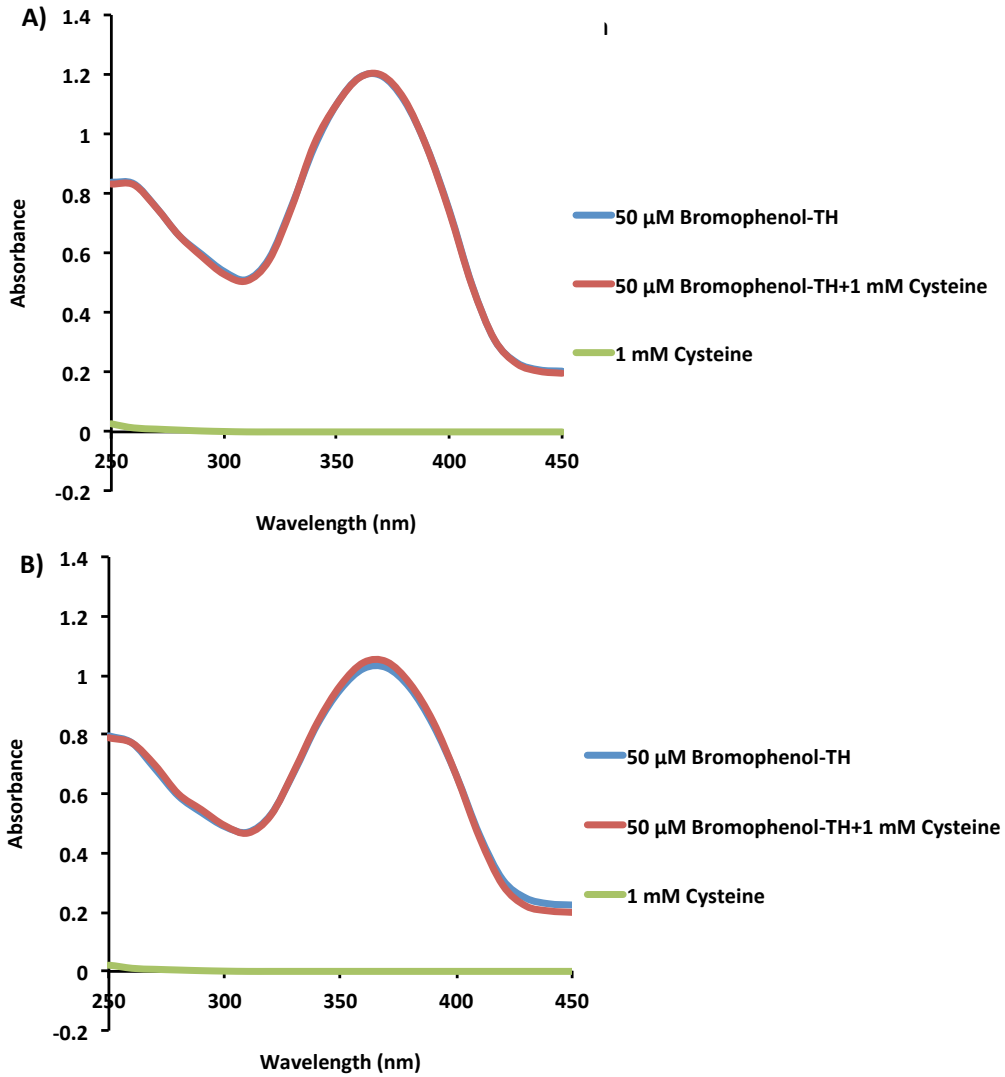
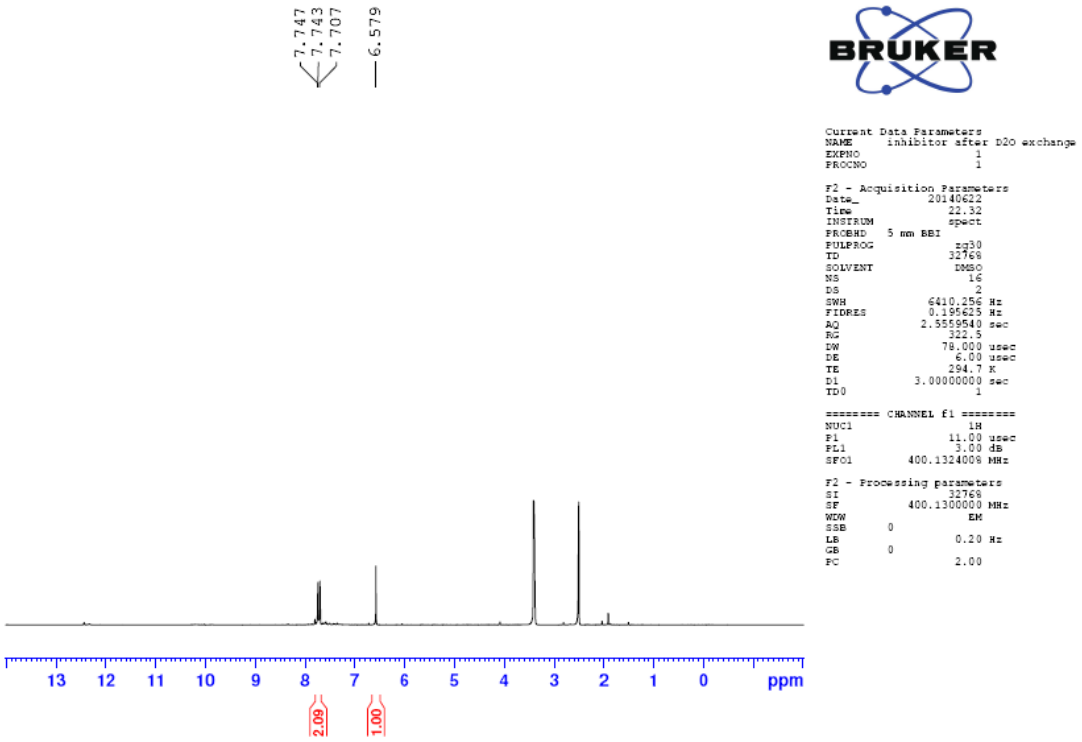


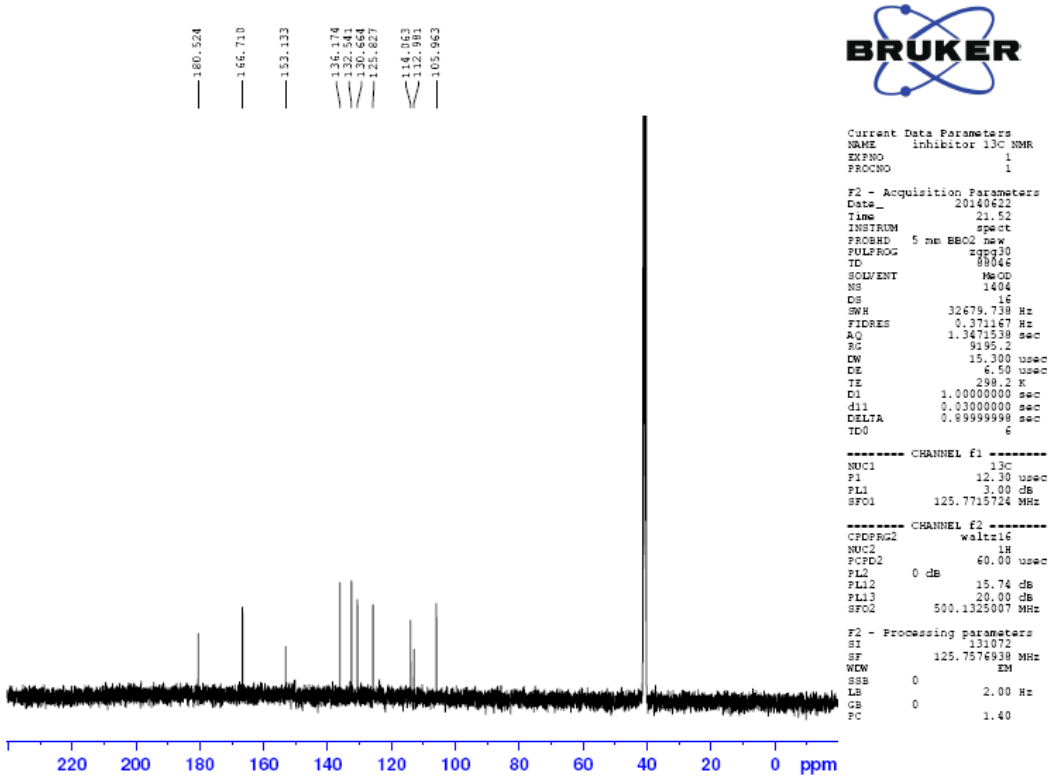
Fig SI9. Stability of bromophenol-TH in the presence of cysteine, monitored via UV absorbance. 50 µM bromophenol-TH was incubated with 1 mM cysteine. UV absorbance of bromophenol-TH (250nm~450nm) was monitored over time. A) UV absorbance before incubation. B) UV absorbance after 1-hour incubation. The UV absorbance of cysteine-bromophenol-TH mixture was constant during 1-hour incubation period, implying that bromophenol did not react with cysteine.

Confirmation of the identity of bromphenol-TH

^{1}H NMR (400 MHz, DMSO-d_6 containing 5% D_2O) δ 7.75-7.71 (m, 2H), 6.58 (s, 1H).



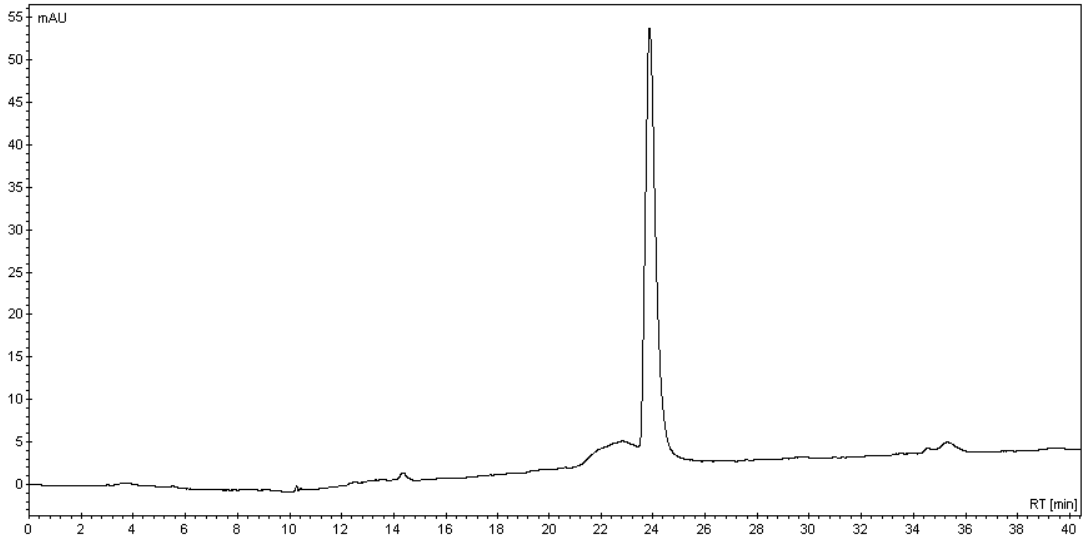
^{13}C NMR (126 MHz, DMSO-d_6) δ 180.5, 166.7, 153.1, 136.2, 132.5, 130.7, 125.8, 114.1, 112.9, 105.9.



MS of bromophenol-TH

$[C_{10}H_5{}^{79}Br_2N_2O_2S]^-$: calculated 374.8438, found 374.8401; $[C_{10}H_5{}^{79}Br^{81}BrN_2O_2S]^-$: calculated 376.8418, found 376.8412; $[C_{10}H_5{}^{81}Br_2N_2O_2S]^-$: calculated 378.8397, found 378.8396.

HPLC purity analysis of bromophenol-TH



DisA purification
BL21(DE3) cells containing DisA plasmid were grown with 50 μg/ml Kanamycin at 37 °C and expression was induced by addition of 1mM IPTG. After induction at 30 °C for 6h, cells were harvested by centrifugation at 5,000 rpm for 15 min. Cells were resuspended in lysis buffer (50mM sodium phosphate buffer, pH 8.0, 0.3M NaCl) and lysed by sonication. After centrifuge at 22,000 rpm for 25 min, DisA was purified from supernatant using a GE Hitrap Nickel column and dialyzed into lysis buffer.

Real-time detection of c-di-AMP formation by coralyne and screening of ActiTarg-K 960 compound library and Sigma-Aldrich 40 compound library
ActiTarg-K 960 compound library compounds (Timtec) and Sigma-Aldrich 40 compound library were stored as 1 mM and 2 mM stock solutions in DMSO respectively. 100 μM ATP, 10 μM coralyne, 3 mM KI and 50 μM compound from Timtec ActiTarg-K library or 100 μM compound from Sigma-Aldrich 40 compound library or 5% DMSO were mixed in reaction buffer (40 mM Tris-HCl, pH 8.0, 100 mM NaCl and 10 mM $MgCl_2$). Reaction was started by adding 10 μM DisA. Fluorescence measurements were performed on a Molecular devices SpectraMax M5e plate reader with λ_{ex}=420 nm and λ_{em}=475 nm. Reactions were monitored for 30 min (5 min interval).

Tryptophan fluorescence titration
5 μM DisA was mixed with various concentrations of ligands and incubated at 25 °C for 1 hour. Tryptophan fluorescence was measured by Varian Cary Eclipse fluorescence spectrophotometer with λ_{ex}=290 nm and λ_{em}=300~450 nm. Apparent K_d (assuming 1:1 binding ratio) was calculated using fluorescence intensity at 340 nm. The equation is as following:

$$F = F_0 + \Delta F \frac{\left(K_d^{app} + P_t + L_t\right) - \sqrt{(K_d^{app} + P_t + L_t)^2 - 4P_tL_t}}{2P_t}$$

where F is the fluorescence intensity at 340 nm. F_0 is the fluorescence intensity at 340 nm in the absence of ligand. ΔF is the fluorescence intensity change upon ligand binding. K_d^{app} is the apparent dissociation constant of binding of ligand to DisA. P_t is the total protein concentration and L_t is the total ligand concentration.[1]
In ATP competition assay, 5 μM DisA was incubated with 20 μM or 50 μM bromophenol-TH together with various concentrations of ATP. In samples indicated as 'in the presence of surfactant', 0.01% Triton X-100 was also added. After 1-hour incubation, tryptophan fluorescence was measured as previously described.

DisA inhibition by various concentrations of bromophenol-TH
50 μM or 500 μM ATP, 33 nM ^{32}P-ATP and various concentrations of bromophenol-TH or 5% DMSO were mixed in reaction buffer. 5 μM DisA was added to start reaction. Reaction was stopped at 5 min and reaction mixture was spotted on a TLC plate (EMD Millipore TLC Cellulose). TLC running buffer was saturated $(NH_4)_2SO_4$: 1.5 M KH_2PO_4 = 1:1.5.

HPLC analysis
50 μM compounds from Timtec ActiTarg-K 960 compound library and 100 μM

compounds from Sigma-Aldrich 40 compound library were mixed with 10 μM DisA and 100 μM ATP. Reactions were stopped by heating up to 95 °C for 5 min. Enzymes were removed by 10K filter and filtrate was separated by Purospher® STAR RP-18 (5 μm) LiChroCART® 250-10 column (buffer A: 0.1 M TEAA in water; buffer B: acetonitrile). Samples were eluted with 95%→80 % A at 0-16 min and 80%→10 % A at 16-18.5 min and kept 10% A at 18.5-22.5 min. Signals were detected at room temperature with a 254 nm UV detector.

For bromophenol-TH purity analysis, buffer A and B are 0.1 M TEAA and acetonitrile. Sample was eluted with 95%→67 % A at 0-33 min. Signal was detected with a 254 nm UV detector.

DisA inhibition by 3,5-dibromosalicylic acid, 3,5-dibromosalicylaldehyde and 2-thiohydantoin

50 μM ATP, 33 nM ^{32}P-ATP, 5 μM DisA and 50 μM 3,5-dibromosalicylic acid or 3,5-dibromosalicylaldehyde or 2-thiohydantoin were mixed in DisA reaction buffer. Reactions were stopped at different time points and the results were analyzed by TLC (EMD Millipore TLC Cellulose).

DisA inhibition by 50 μM bromophenol-TH in the presence and absence of surfactant

50 μM ATP, 33 nM ^{32}P-ATP, 5 μM DisA and 50 μM bromophenol-TH or 5% DMSO were mixed in DisA reaction buffer. In samples with surfactant, 0.01% Triton X-100 was added to avoid non-specific aggregation.[2] These time course experiments were analyzed by TLC (EMD Millipore TLC Cellulose).

Bromophenol-TH stability in the presence of Cysteine

50 μM bromophenol-TH was mixed with 1 mM Cysteine and UV absorbance of bromophenol-TH at 361 nm was measured by Molecular devices SpectraMax M5e plate reader every 10 min for 1 hour.

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