

## Supporting Information

### Allosteric Competitive Inhibitors of the Glucose-1-Phosphate Thymidyltransferase (RmlA) from *Pseudomonas aeruginosa*.

Magnus S. Alphey<sup>a+</sup>, Lisa Pirrie<sup>a,b+</sup>, Leah S. Torrie<sup>c</sup>, Wassila Abdelli Boulkeroua<sup>a</sup>, Mary Gardiner<sup>c</sup>, Aurijit Sarkar<sup>c</sup>, Marko Maringer<sup>d</sup>, Wulf Oehlmann<sup>e</sup>, Ruth Brenk<sup>c</sup>, Michael Scherman<sup>f</sup>, Michael McNeil<sup>f</sup>, Martin Rejzek<sup>g</sup>, Robert A. Field<sup>g</sup>, Mahavir Singh<sup>e</sup>, David Gray<sup>c</sup>, Nicholas J. Westwood<sup>a,b,\*</sup>, James H. Naismith<sup>a\*</sup>

<sup>a</sup> Biomedical Sciences Research Complex, University of St. Andrews, St. Andrews, KY16 9ST, UK; <sup>b</sup> School of Chemistry, University of St Andrews and EaStCHEM, St. Andrews, KY16 9ST, UK; <sup>c</sup> Biological Chemistry and Drug Discovery, College of Life Sciences, University of Dundee, Dundee, DD1 5EH, UK; <sup>d</sup> mfd Diagnostics GmbH, Mikroforum Ring 5, 55234 Wendelsheim, Germany; <sup>e</sup> Lionex GmbH, Salzdahlumer Str. 196, 38126 Braunschweig, Germany; <sup>f</sup> Dept. of Microbiology, Immunology and Pathology, Colorado State University, 1682 Campus Delivery, Ft. Collins, CO 80523-1682, U.S.A. <sup>g</sup> Department of Biological Chemistry, John Innes Centre, Norwich NR4 7UH

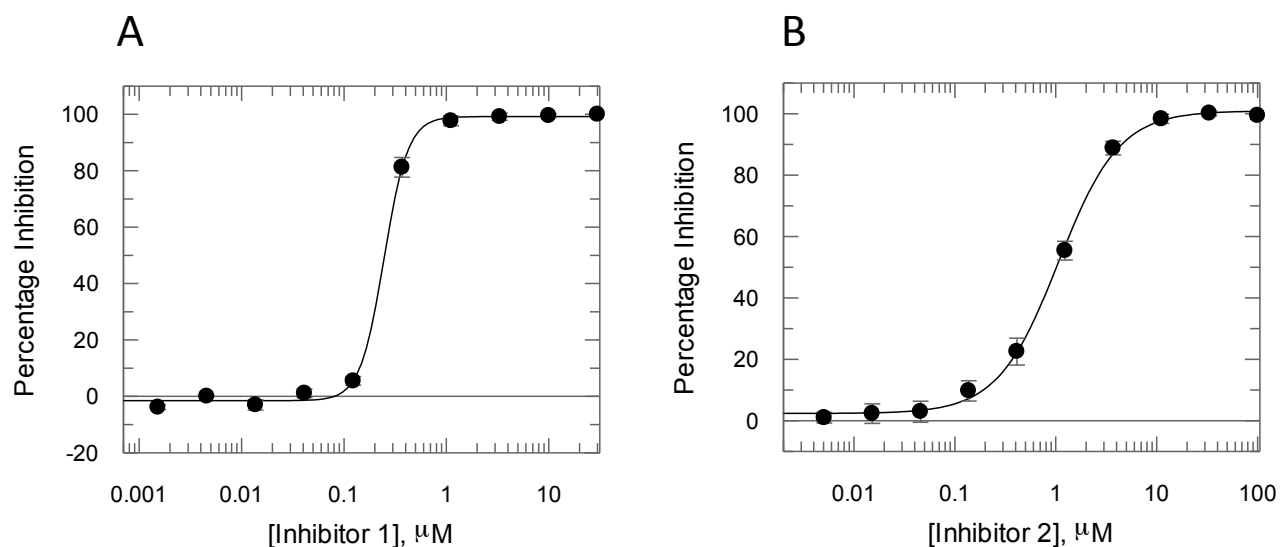
+ These authors have contributed equally to this work

\* Correspondence: [naismith@st-andrews.ac.uk](mailto:naismith@st-andrews.ac.uk) (J.H.N) and [njw3@st-andrews.ac.uk](mailto:njw3@st-andrews.ac.uk) (N.J.W).

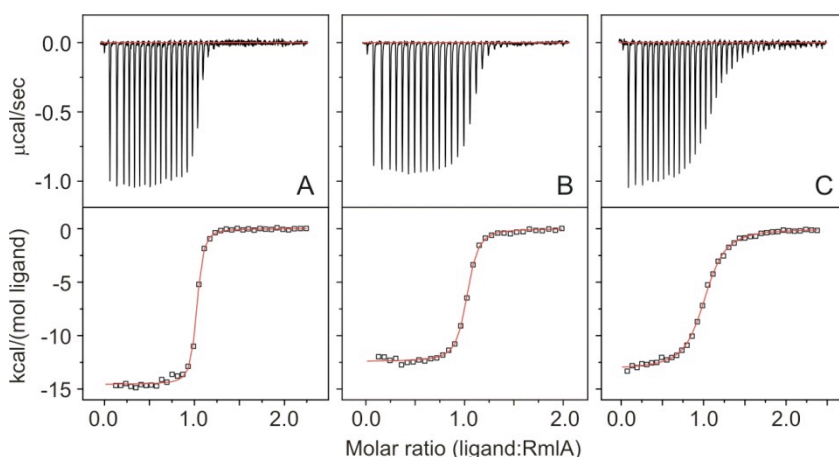
#### Contents

Page S3	IC <sub>50</sub> and Hill Coefficient determination for <b>1</b> and <b>2</b> .
Page S4	Isothermal Calorimetry Analysis of the binding of RmlA to <b>1</b> , <b>2</b> and <b>8a</b> .
Page S5	Data collection and model refinement statistics for the complexes of RmlA with inhibitors <b>1</b> and <b>2</b> .
Page S6	Schematic representation of the interactions between <b>1</b> and RmlA and <b>2</b> and RmlA in the allosteric site.
Page S7	Crystal complex structures of <b>2</b> showing the position of the glycerol.
Page S8	Schematic of the interactions of <b>1</b> and <b>2</b> with RmlA that inspired the selection of parameters for the 2D similarity search.
Page S9	Inhibition data against RmlA for commercially available compounds.
Pages S10	Data collection and model refinement statistics for the complexes of RmlA with <b>3-7</b> .
Page S11	Overlay of crystal complex structures of N1-substituted analogues of <b>2</b> with the structure of the RmlA- <b>2</b> complex.
Page S12	Inhibition data of other synthesised analogues against RmlA.
Page S13	Overlay of the structures of RmlA- <b>1</b> and RmlA- <b>8a</b> complexes.
Page S14-15	Data collection and model refinement statistics for the complexes of RmlA with <b>8a</b> , <b>8f</b> , <b>8j</b> , <b>8k</b> , <b>8p</b> , <b>15a</b> and <b>12b</b> .
Page S16	Overlay of the structures of the RmlA- <b>8a</b> and RmlA- <b>8f</b> complexes.
Page S17	Comparison of complex structures of RmlA- <b>1</b> and RmlA- <b>3</b> and analysis of the RmlA- <b>12b</b> complex.
Page S18	Analysis of the RmlA- <b>8j</b> complex.

Page S19	The structure of <b>8k</b> bound in the allosteric site of RmlA.
Page S20	Attenuation of <i>P. aeruginosa</i> infection in mice on RmlA-knockout.
Page S21	MIC values for selected RmlA inhibitors against <i>M. tuberculosis</i> .
Page S22	Determination of substrate $K_m$ values.
Page S23-24	Analysis of the binding of substrates to RmlA by SPR, $IC_{50}$ 's for biological ligands.
Page S25	SPR data showing binding of <b>8a</b> to RmlA.
Page S25	Lineweaver-Burk analysis of dTTP inhibition of RmlA.
Page S26-29	Unbiased original Fo-Fc maps for each compound
Pages S30-35	Detailed methods section
Pages S36-52	Protocols for the synthesis of RmlA inhibitors.
Pages S53-62	NMR spectra of inhibitors in Table 1.
Pages S63-64	References



**Figure S1. IC<sub>50</sub> and Hill Coefficient determination for 1 and 2.** Concentration response curves for the high throughput screening hits (inhibitors **1** and **2**) were generated using the BIOMOL Green assay and data analysed using a four-parameter logistic dose-response equation (as described in *Methods* section). **A** Representative IC<sub>50</sub> determination for compound **1**. Analysis of data from two experiments (each with  $n \geq 2$ ) returns a  $\text{pIC}_{50}$  of  $6.65 \pm 0.06$  and Hill slope of  $3.55 \pm 0.25$  (mean  $\pm$  SD;  $N=2$ ). **B** Representative IC<sub>50</sub> determination for compound **2**. Analysis of data from two experiments (each with  $n \geq 2$ ) returns a  $\text{pIC}_{50}$  of  $5.91 \pm 0.09$  and Hill slope of  $1.75 \pm 0.42$  (mean  $\pm$  SD;  $N=2$ ). Inhibitors **1** and **2** were purchased from Enamine and Early Discovery Chemistry Ltd respectively.



**D**

	RmlA [ $\mu\text{M}$ ]	Ligand [ $\mu\text{M}$ ]	N	$\Delta\text{H}$ [ $\text{kcal/mol}^{-1}$ ]	$-\text{T}\Delta\text{S}$ [ $\text{kcal/mol}^{-1}$ ]	$K_d$ [nM]
<b>8a</b>	25	450	1.74	-8.4	-2.0	$40 \pm 4$
<b>8a corr.</b>	25	259	1.00	-14.5	3.8	$28 \pm 3$
<b>1</b>	25	450	1.68	-7.4	-2.5	$119 \pm 14$
<b>1 corr.</b>	25	268	1.00	-12.4	1.2	$74 \pm 6$
<b>2</b>	25	900	3.28	-4.0	-4.3	$1326 \pm 70$
<b>2 corr.1</b>	25	540	1.98	-6.8	-1.8	$855 \pm 50$
<b>2 corr.2</b>	25	275	1.00	-13.1	4.1	$395 \pm 21$

**Figure S2: Isothermal Calorimetry Analysis of the binding of RmlA to 1, 2 and 8a.** ITC data obtained for the injection of ligands (A) **8a**, (B) **1** and (C) **2** into solutions of RmlA. The top panels show raw data representing the response to injections, the bottom panels show integrated heats of injections ( $\square$ ) and the best fit ( $-$ ) to a one-site binding model. **D** Thermodynamic parameters derived from ITC experiments

All ligand concentrations were adjusted to result in fits reflecting 1:1 stoichiometry. This was required due to the impossibility to ascertain ligand concentrations and it is justified by structural data supporting 1:1 complexes.

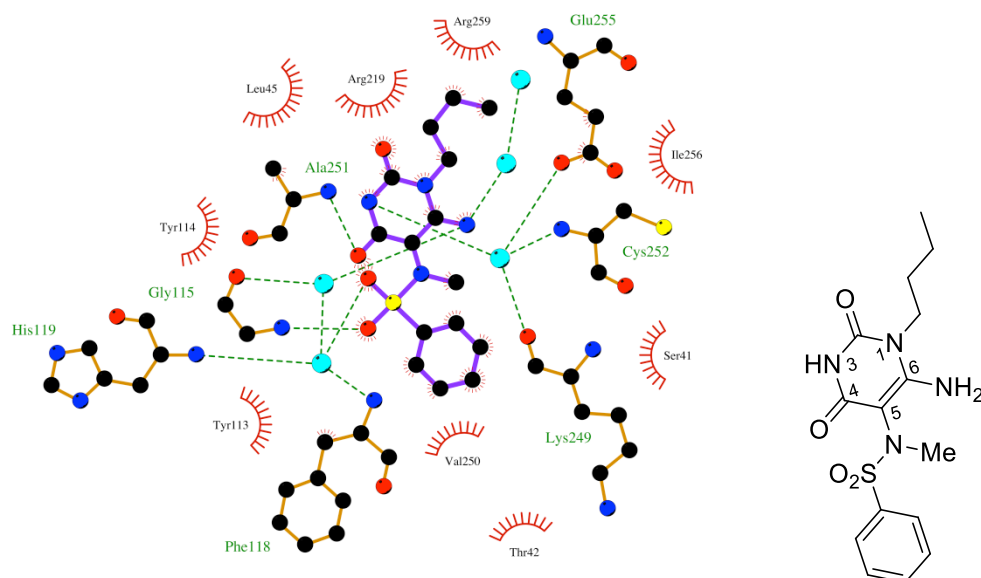
The different thermodynamic signature for **2**, i.e. higher entropic penalty, higher  $\Delta\text{H}$  but significantly lower affinity compared to **1**, is in agreement with structural data. **2** lacks the phenyl moiety of **1** (or **8a**) that is buried in the binding site, contributing favourably to  $\Delta\text{S}$  and unfavourably to  $\Delta\text{H}$ .

**Table S1:** Data collection and model refinement statistics for the complexes of RmlA with inhibitors **1** and **2**.

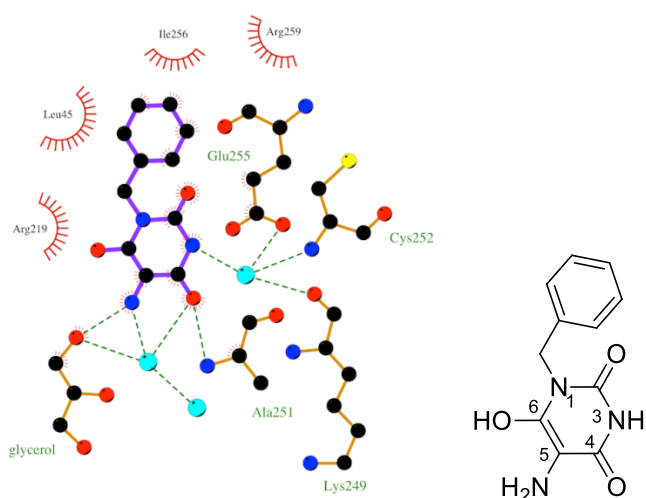
	RmlA-1 complex	RmlA-2 complex
Space group	C2	C2
X-ray source	In-house	Diamond (i04-1)
X-ray wavelength (Å)	1.542	0.917
Resolution (Å)	30.8-2.2 (2.26-2.2)	45.3-1.96 (2.01-1.96)
Unit cell dimensions		
<i>a</i> (Å)	64.0	64.1
<i>b</i> (Å)	153.8	154.1
<i>c</i> (Å)	134.7	134.7
$\beta$ (°)	92.3	92.5
No. Reflections	237477	637,510
Unique reflections	70491	88442
Redundancy	3.8 (3.2)	7.2 (7.1)
Completeness (%)	96.3 (79.1)	94.4 (91.6)
$R_{\text{merge}}$	0.064 (0.361)	0.05 (0.68)
$I/\sigma I$	17.1 (3.5)	18.5 (2.7)
 <i>B. Data refinement</i>		
No. reflections	62911	88442
No. Atoms		
Protein	9140	9276
Inhibitor	96	68
Water	547	456
Average <i>B</i> -factor (Å <sup>2</sup> )		
Protein	40.8	51.6
Inhibitor	34.5	60.2
Inhibitor <i>B</i> range	31 - 39	50 - 71
Water	46.1	54.7
<i>R</i> -factor	0.175	0.199
$R_{\text{free}}$	0.208	0.245
r.m.s.d from ideal		
Bond lengths (Å)	0.008	0.008
Bond angles (°)	1.228	1.206
% favoured	98.5	98.5
Ramachandran		
wwPDB code	4ARW	4AS6

Numbers in parentheses correspond to data in the highest resolution bin.

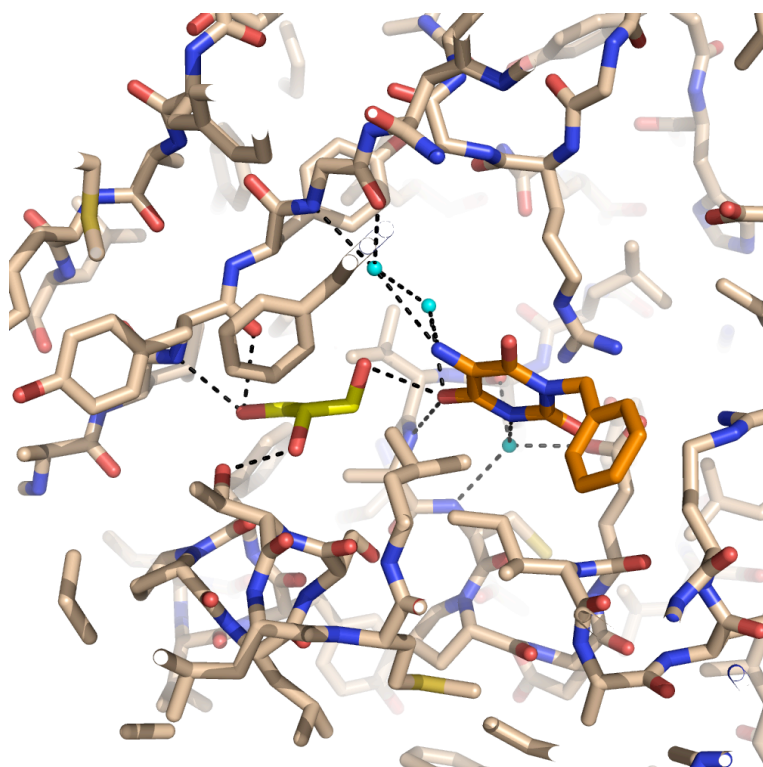
Inhibitor *B*-range are lowest and highest average *B* for one ligand, out of the four copies in the crystal



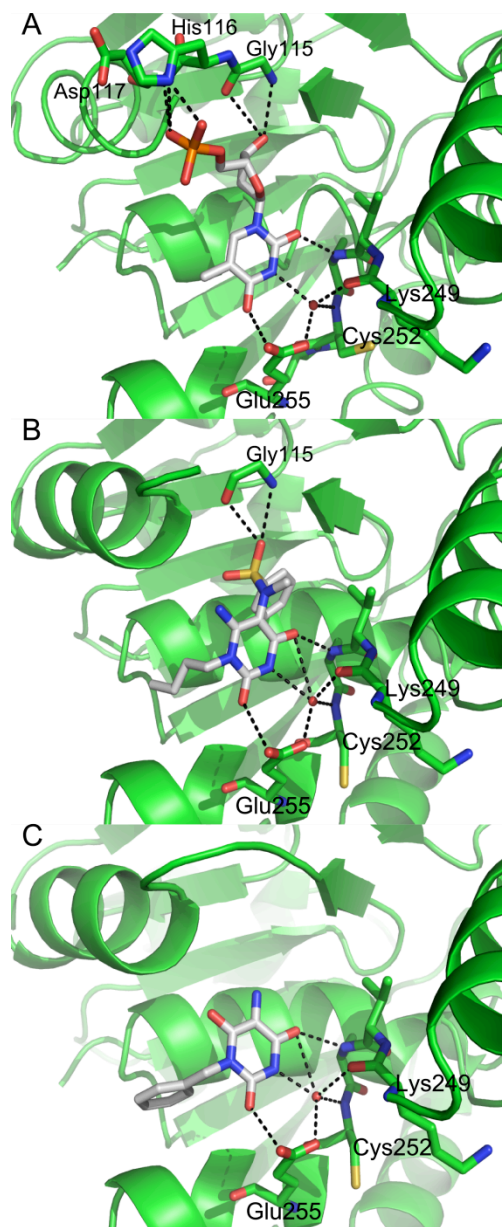
**Figure S3.** Schematic representation of the interactions between **1** and RmlA in the allosteric site. Hydrogen bonds are shown as green dotted lines, and residues involved in hydrophobic interactions are in red. Water molecules are shown as cyan circles, carbon atoms are black, oxygens are red, nitrogens are blue, and sulfurs yellow. Figure prepared with Ligplot <sup>1</sup>.



**Figure S4.** Schematic representation of the interactions between **2** and RmlA in the allosteric site. Hydrogen bonds are shown as green dotted lines, and residues involved in hydrophobic interactions are in red. Water molecules are shown as cyan circles, carbon atoms are black, oxygens are red, nitrogens are blue, and sulfurs yellow.

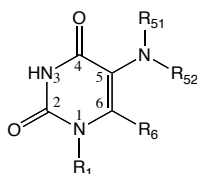


**Figure S5.** Crystal complex structures of inhibitor **2** (orange) and glycerol (yellow) bound in the secondary site of RmlA from *P. aeruginosa*. Hydrogen bonding interactions within 3.5 Å are shown as black dotted lines. The glycerol, originally added as crystal cryoprotectant during data collection, is seen to bind in the pocket occupied by the benzenesulfonamide moiety of inhibitor **1** (not shown, see Figure 2A).



**Figure S6. Interactions of 1 and 2 with RmlA.** Interactions between (A) deoxy-thymidine monophosphate (dTMP) and RmlA are compared with (B) **1** and (C) **2**. Protein is shown in cartoon form, except interacting residues and ligands which are shown as sticks. All ligands form a water-mediated hydrogen bond with residues Lys249, Cys252 and Glu255, deemed essential for affinity. Image generated using Pymol <sup>2</sup>.

**Table S2.** Inhibition data against RmlA for commercially available compounds identified using a 2D similarity search based on **1**.



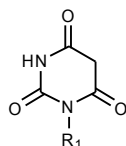
Compound	R <sub>51</sub>	R <sub>52</sub>	R <sub>6</sub>	R <sub>1</sub>	% inhibition		IC <sub>50</sub> (μM) <sup>b</sup>	PDB
					at 10 μM <sup>a</sup>	at 60 μM <sup>a</sup>		
<b>1</b>	<sup>n</sup> Bu	Me	SO <sub>2</sub> Ph	NH <sub>2</sub>	100	100	0.32 ± 0.1	4ARW
<b>S1</b>	H	<i>o</i> -OMe Benzyl	NH <sub>2</sub>	<sup>n</sup> Bu	35.7 ± 1.2	40.6 ± 5.1	n.d.	n.d.
<b>S2</b>	H	C(O)NHPh	NH <sub>2</sub>	<i>p</i> -OMePh	18.9 ± 0.7	77.6 ± 5.5	n.d.	n.d.
<b>S3</b>	H	2,2,6,6-TMP	NH <sub>2</sub>	Me	9.2 ± 0.4	17.2 ± 4.2	n.d.	n.d.
<b>S4</b>	H	C(O)furan	NH <sub>2</sub>	Me	17.1 ± 0.7	23.8 ± 4.1	n.d.	n.d.
<b>S5</b>	H	SO <sub>2</sub> - <i>p</i> -Me Ph	Me	H	6.1 ± 1.7	16.4 ± 1.9	n.d.	n.d.

TMP = tetramethylpiperidine; n.d. not determined

<sup>a</sup> SE, standard error (n=2)

<sup>b</sup> SD, standard deviation (n=3)

**Table S3.** Inhibition data against RmlA for commercially available compounds identified using a 2D similarity search based on **2**.



Compound	R <sup>1</sup>	% inhibition at		PDB
		10 μM <sup>a</sup>	60 μM <sup>a</sup>	
<b>6</b>	CH <sub>2</sub> CH <sub>2</sub> Ph	8.4 ± 4.7	23.4 ± 2.3	4B4B
<b>7</b>	CH <sub>2</sub> -CH <sub>2</sub> - <i>p</i> -F Ph	22.1 ± 2.5	62.9 ± 2.3	n.d.
<b>S6</b>	CH <sub>2</sub> CH <sub>2</sub> -cyclohex-1-ene	13.3 ± 2.5	26.7 ± 1.2	n.d.
<b>S7</b>	3,5-(OMe) <sub>2</sub> Benzyl	25.0 ± 4.1	42.3 ± 2.9	n.d.
<b>S8</b>	<i>p</i> -F Benzyl	16.8 ± 2.4	34.8 ± 3.2	n.d.
<b>S8</b>	<i>p</i> -OMe Benzyl	32.2 ± 5.1	64.7 ± 2.9	n.d.
<b>S10</b>	cyclohexyl	29.8 ± 5.1	49.3 ± 2.0	n.d.

n.d. not determined

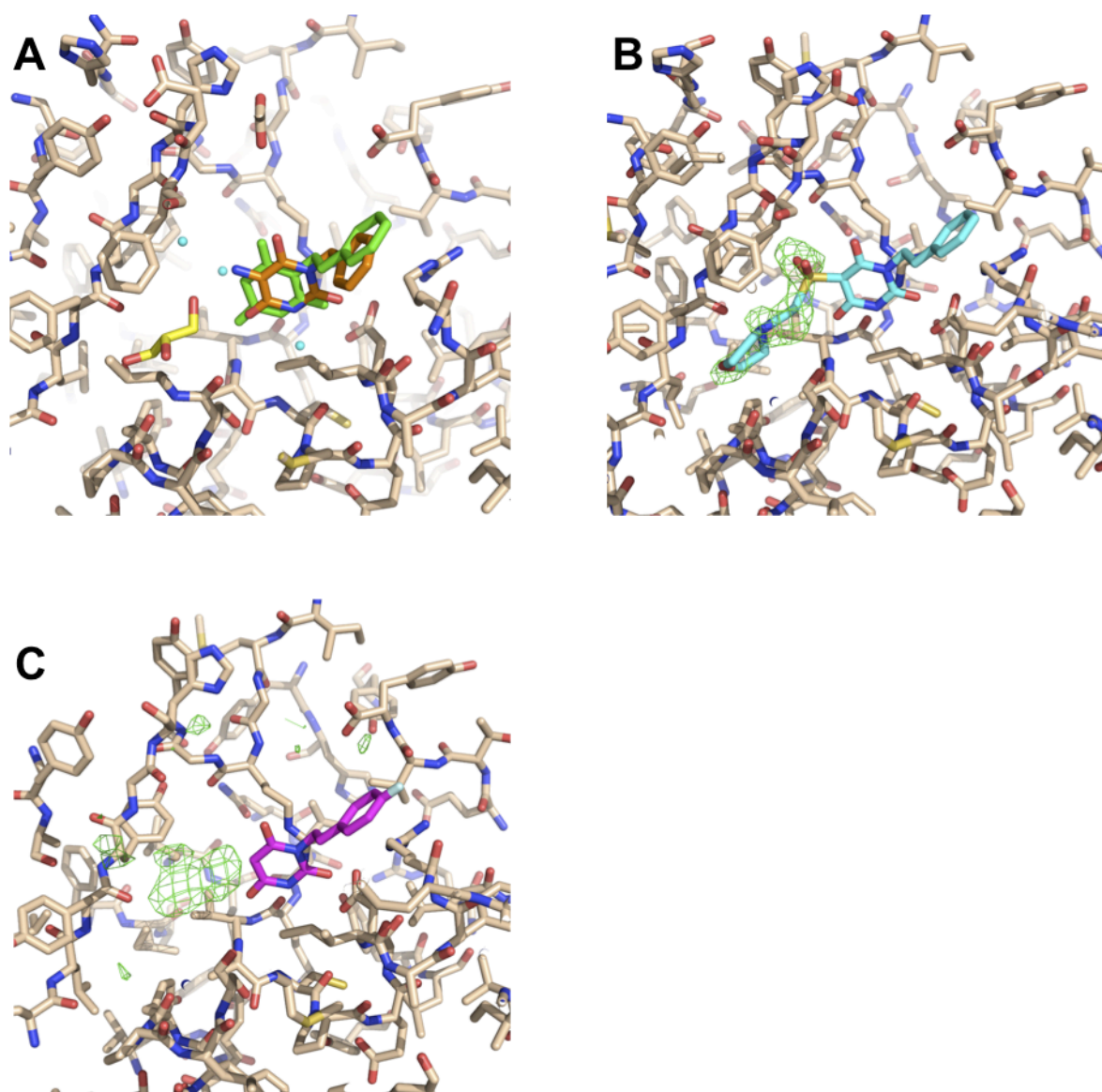
<sup>a</sup> SE, standard error (n=2)

**Table S4:** Data collection and model refinement statistics for the complexes of RmlA with inhibitors **3**, **4** and **6**. The five membered ring of **4** is disordered over two positions.

	RmlA-3 complex	RmlA-4 complex	RmlA-6 complex
Space group	C2	C2	C2
X-ray source	In-house	Diamond I03	Diamond I03
X-ray wavelength (Å)	1.542	0.9763	0.9763
Resolution (Å)	34.8-2.5 (2.57-2.5)	67.2-1.7 (1.74-1.7)	59.1-2.1 (2.15-2.1)
Unit cell dimensions			
<i>a</i> (Å)	64.2	64.2	64.1
<i>b</i> (Å)	153.4	154.8	154.2
<i>c</i> (Å)	134.7	134.5	134.9
$\beta$ (°)	92.5	92.2	92.8
No. Observations	138335	456998	545015
Unique reflections	43393	139268	72939
Redundancy	3.2 (3.2)	3.3 (3.4)	7.5 (7.9)
Completeness (%)	97.5 (97.1)	96.4 (99.1)	95.8 (99.9)
$R_{\text{merge}}$	0.1 (0.54)	0.05 (0.76)	0.082 (0.53)
$I/\sigma I$	10.2 (2.5)	13.7 (2.1)	13.7 (3.5)
<b>B. Data refinement</b>			
No. reflections	43492	131398	72228
No. Atoms			
Protein	9165	9341	9229
Inhibitor	92	76	68
Water	218	840	243
Average <i>B</i> -factor (Å <sup>2</sup> )			
Protein	46.2	32.7	64.8
Inhibitor	37.2	34.5	61.4
Inhibitor <i>B</i> range	31 - 43	31-38	55 - 72
Water	39.8	43.1	63.9
<i>R</i> -factor	22.3	21.8	22.6
$R_{\text{free}}$	28.3	25.3	26.6
r.m.s.d from ideal			
Bond lengths (Å)	0.014	0.007	0.012
Bond angles (°)	1.718	1.317	1.442
% favoured	97.8	98.7	98.3
Ramachandran			
wwPDB code	4B42	4B2X	4B4B

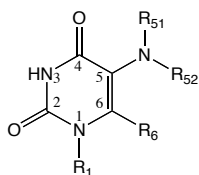
Numbers in parentheses correspond to data in the highest resolution bin.

Inhibitor *B*-range are lowest and highest average *B* for one ligand, out of the four copies in the crystal



**Figure S7.** **A** Overlay of crystal complex structures of N1-substituted analogue **6** with the RmlA-2 complex. **2** is shown in orange and **6** in green. **B** On determination of the crystal structure of RmlA-6, additional density was observed in the allosteric site. The model of RmlA-6 shows the compound core eclipsing the position of **2** (panel **A**), and the additional observed electron density indicates that the compound may have been derivatized in the crystal reacting with the MES buffer to form a diketone which has been modelled. The green Fo-Fc density in **B** is contoured at  $0.1 \text{ e}/\text{\AA}^3$  ( $5.2 \sigma$ ). **C** A complex of RmlA-7 also showed additional density in the allosteric site, but in this case we could not unambiguously place the core structure of the compound in the model so could not rely on the structural data. The green Fo-Fc electron density in **C** is contoured at  $0.2 \text{ e}/\text{\AA}^3$  ( $3.9 \sigma$ ). For both **6** and **7** we suspect the additional density may be due to a reaction of the 1,3-diketone moiety with MES buffer. Both compounds identity was verified by mass spectrometry prior to crystallization.

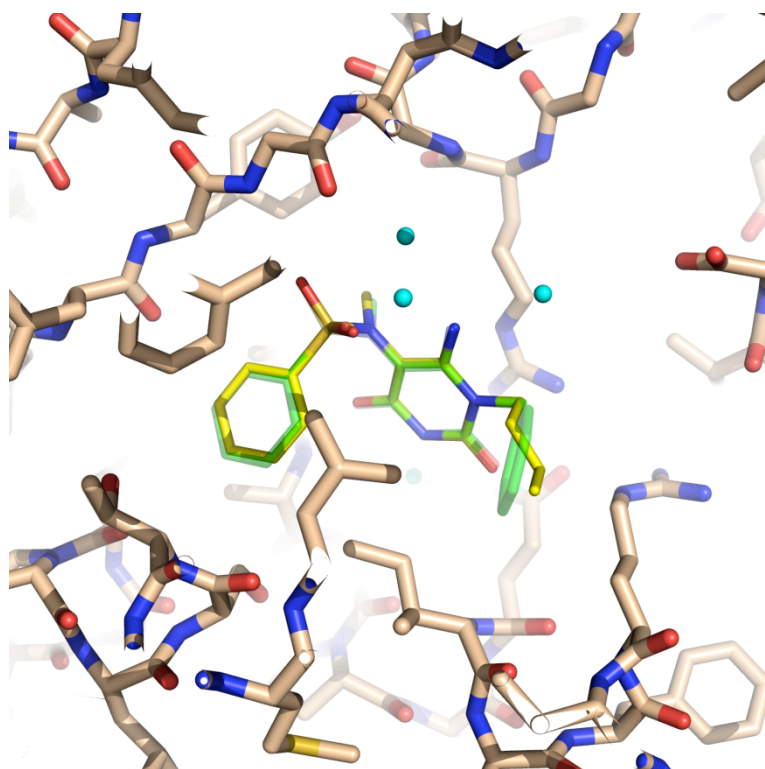
**Table S5.** Inhibition data against RmlA



Compound	R <sub>1</sub>	R <sub>51</sub>	R <sub>52</sub>	R <sub>6</sub>	%	%	IC <sub>50</sub> (μM) <sup>b</sup>
					inhibition at 10 μM <sup>a</sup>	inhibition at 60 μM <sup>a</sup>	
<b>1</b>	<i>n</i> Bu	Me	SO <sub>2</sub> Ph	NH <sub>2</sub>	100	100	0.32 ± 0.1
<b>2</b>	Benzyl	H	H	OH	100	85.7 ± 2.1	4.91 ± 0.1
<b>8d</b>	Benzyl	<i>n</i> Bu	SO <sub>2</sub> Ph	NH <sub>2</sub>	1.4 ± 2.1	0.2 ± 0.1	> 60
<b>8e</b>	Benzyl	<i>i</i> Bu	SO <sub>2</sub> Ph	NH <sub>2</sub>	1.2 ± 2.3	0.4 ± 1.1	> 60
<b>12c</b>	Benzyl	<i>n</i> Pr	H	NH <sub>2</sub>	100	38.2 ± 0.9	> 10
<b>12d</b>	Benzyl	<i>n</i> Bu	H	NH <sub>2</sub>	2.3 ± 1.2	0.5 ± 1.2	> 60
<b>12e</b>	Benzyl	<i>i</i> Bu	H	NH <sub>2</sub>	0.3 ± 1.2	0.4 ± 1.2	> 60
<b>14</b>	Benzyl	H	H	NH <sub>2</sub>	9.6 ± 2.1	8.9 ± 4.3	>60
<b>15b</b>	Benzyl	H	COPh	NH <sub>2</sub>	0	11.3 ± 0.3	> 60
<b>8g</b>	Benzyl	Me	SO <sub>2</sub> Me	NH <sub>2</sub>	97.7 ± 2.3	88.7 ± 4.3	3.25±0.15
<b>8h</b>	Benzyl	Me	SO <sub>2</sub> Et	NH <sub>2</sub>	93.9 ± 3.3	89.3 ± 0.8	3.72±0.15
<b>8i</b>	Benzyl	Me	SO <sub>2</sub> - <i>n</i> -Pr	NH <sub>2</sub>	100	100	0.452±0.015
<b>8l</b>	Benzyl	Me	SO <sub>2</sub> -4-BrPh	NH <sub>2</sub>	97.2 ± 3.7	83.5 ± 3.7	4.34 ± 1.01
<b>8m</b>	Benzyl	Me	SO <sub>2</sub> -4-MePh	NH <sub>2</sub>	62.6 ± 6.8	0	> 10
<b>8n</b>	Benzyl	Me	SO <sub>2</sub> -4-OMePh	NH <sub>2</sub>	96.2 ± 1.1	52.5 ± 1.8	9.57 ± 0.85
<b>8o</b>	Benzyl	Me	SO <sub>2</sub> -3-F Ph	NH <sub>2</sub>	100	100	0.425±0.004
<b>8q</b>	Benzyl	Me	SO <sub>2</sub> -3-OMePh	NH <sub>2</sub>	100	76.5 ± 2.4	3.71 ± 0.61
<b>8r</b>	Benzyl	Me	SO <sub>2</sub> -2-MePh	NH <sub>2</sub>	100	90.3 ± 0.8	5.49 ± 0.31
<b>8s</b>	Benzyl	Me	SO <sub>2</sub> -3,5-Me <sub>2</sub> Ph	NH <sub>2</sub>	95.6 ± 1.7	27.4 ± 5.6	> 10
<b>8u</b>	Benzyl	Me	SO <sub>2</sub> -1- naphthalene	NH <sub>2</sub>	40.2 ± 1.2	13.6 ± 8.0	> 60
<b>8v</b>	Benzyl	Me	SO <sub>2</sub> -2- naphthalene	NH <sub>2</sub>	20.8 ± 4.6	0	> 60
<b>8w</b>	Benzyl	Me	SO <sub>2</sub> -quinoline	NH <sub>2</sub>	57.2 ± 4.8	0	> 60
<b>S11</b>	Benzyl	Me	SO <sub>2</sub> -CH <sub>2</sub> Ph	NH <sub>2</sub>	85 ± 5.2	25 ± 1.2	> 10

<sup>a</sup> SE, standard error (n=2)

<sup>b</sup> SD, standard deviation (n=3)



**Figure S8.** Superposition of the structures of the RmlA-1 with RmlA-**8a** complexes highlighting the fact that no change in the interactions of the inhibitor core with the enzyme occurred on moving from inhibitor **1** (yellow) to inhibitor **8a** (green). The almost identical positioning of the C5-N-methyl-phenylsulfonamide substituent in **1** and **8a** is also shown, whilst the *n*-butyl of **1** is eclipsed by the phenyl of **8a**.

**Table S6:** Data collection and model refinement statistics for the complexes of RmlA with inhibitors **8a**, **8f**, **8j**, **8k** and **8p**.

	RmlA- <b>8a</b> complex	RmlA- <b>8f</b> complex	RmlA- <b>8j</b> complex	RmlA- <b>8k</b> complex	RmlA- <b>8p</b> complex
Space group	<i>C2</i>	<i>P21</i>	<i>C2</i>	<i>C2</i>	<i>C2</i>
X-ray source	In-house	In-house	In-house	In-house	In-house
X-ray wavelength (Å)	1.542	1.542	1.542	1.542	1.542
Resolution (Å)	37.14-2.25 (2.31-2.25)	44.5-2.3 (2.36-2.30)	29.11-2.49 (2.55-2.49)	33.66-2.34 (2.4-2.34)	31.44-2.05 (2.11-2.05)
Unit cell dimensions					
<i>a</i> (Å)	64.2	74.0	64.1	64.2	64.1
<i>b</i> (Å)	154.6	137.0	153.1	154.0	154.4
<i>c</i> (Å)	134.7	75.2	134.8	134.7	134.5
$\beta$ (°)	92.2	103.3	92.6	92.4	92.4
No. Observations	147712	233020	121293	184866	243698
Unique reflections	56889	62887	44901	53967	79243
Redundancy	2.6 (2.1)	3.7 (3.7)	2.7 (2.3)	3.4 (2.3)	3.1 (2.5)
Completeness (%)	88.7 (75.1)	97.2 (90.1)	98.9 (94.7)	98.0 (84.7)	97.7 (79.0)
$R_{\text{merge}}$	0.087 (0.23)	0.08 (0.58)	0.062 (0.48)	0.082 (0.54)	0.073 (0.482)
$I/\sigma I$	8.8 (4.7)	16.7 (2.9)	13.5 (2.4)	12.1 (2.1)	12.9 (2.3)
<b>B. Data refinement</b>					
No. reflections	55248	59742	44364	53397	79243
No. Atoms					
Protein	9122	9164	9146	9226	9181
Ligands	108	104	100	112	112
Water	373	396	160	261	273
Average <i>B</i> -factor (Å <sup>2</sup> )					
Protein	38.1	45.1	53.1	44.8	41.9
Inhibitor	28.7	48.7	42.6	39.4	46.8
Inhibitor <i>B</i> range	23 - 32	47 - 52	34 - 54	34 - 43	42 - 54
Water	37.9	48.6	46.7	42.9	41.7
<i>R</i> -factor	23.8	20.9	20.0	18.3	21.2
$R_{\text{free}}$	31.8	25.9	27.4	23.7	24.7
r.m.s.d from ideal					
Bond lengths (Å)	0.012	0.009	0.011	0.009	0.010
Bond angles (°)	1.580	1.339	1.617	1.346	1.345
% favoured	97.4	98.5	96.0	97.9	98.7
Ramachandran					
wwPDB code	4ASJ	4ASY	4B4G	4B4M	4B5B

Numbers in parentheses correspond to data in the highest resolution bin.

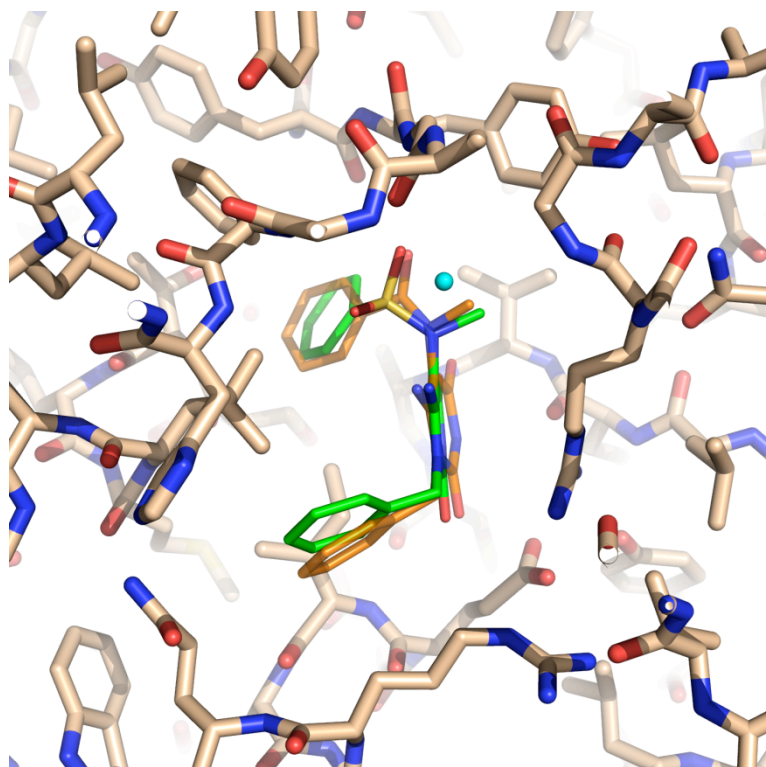
Inhibitor *B*-range are lowest and highest average *B* for one ligand, out of the four copies in the crystal

**Table S7:** Data collection and model refinement statistics for the complexes of RmlA with inhibitors **15a** and **12b**.

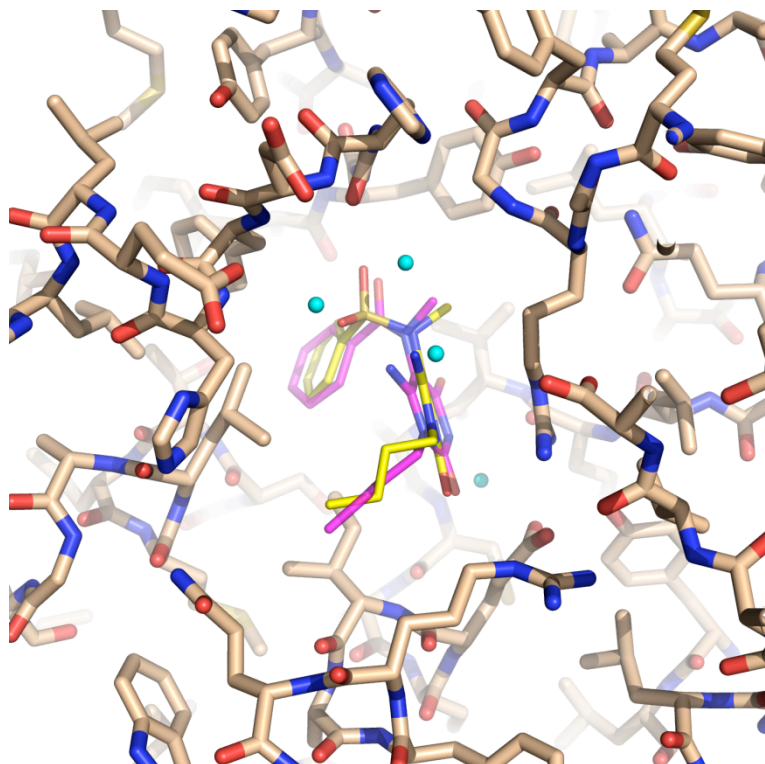
	RmlA- <b>15a</b> complex	RmlA- <b>12b</b> complex
Space group	<i>C</i> 2	<i>C</i> 2
X-ray source	In-house	Diamond i03
X-ray wavelength (Å)	1.542	0.9763
Resolution (Å)	37.23-1.95 (2.00-1.95)	45.13-1.75 (1.80-1.75)
Unit cell dimensions		
<i>a</i> (Å)	64.3	64.2
<i>b</i> (Å)	155.0	154.6
<i>c</i> (Å)	134.5	134.0
$\beta$ (°)	92.0	92.1
No. Observations	206783	390465
Unique reflections	81490	118780
Redundancy	2.5 (2.3)	3.3 (3.2)
Completeness (%)	85.5 (69.0)	91.1 (98.8)
$R_{\text{merge}}$	0.04 (0.47)	0.04 (0.68)
$I/\sigma I$	14.7 (2.0)	14.9 (1.7)
<b>B. Data refinement</b>		
No. reflections	80615	103188
No. Atoms		
Protein	9224	9312
Inhibitor	104	76
Water	726	802
Average <i>B</i> -factor (Å <sup>2</sup> )		
Protein	35.6	40.2
Inhibitor	30.7	40.4
Inhibitor <i>B</i> range	27 - 34	37 - 44
Water	42.9	49.3
<i>R</i> -factor	23.3	21.8
$R_{\text{free}}$	27.7	26.2
r.m.s.d from ideal		
Bond lengths (Å)	0.008	0.008
Bond angles (°)	1.244	1.476
% favoured	98.5	98.4
Ramachandran		
wwPDB code	4ASP	4B3U

Numbers in parentheses correspond to data in the highest resolution bin.

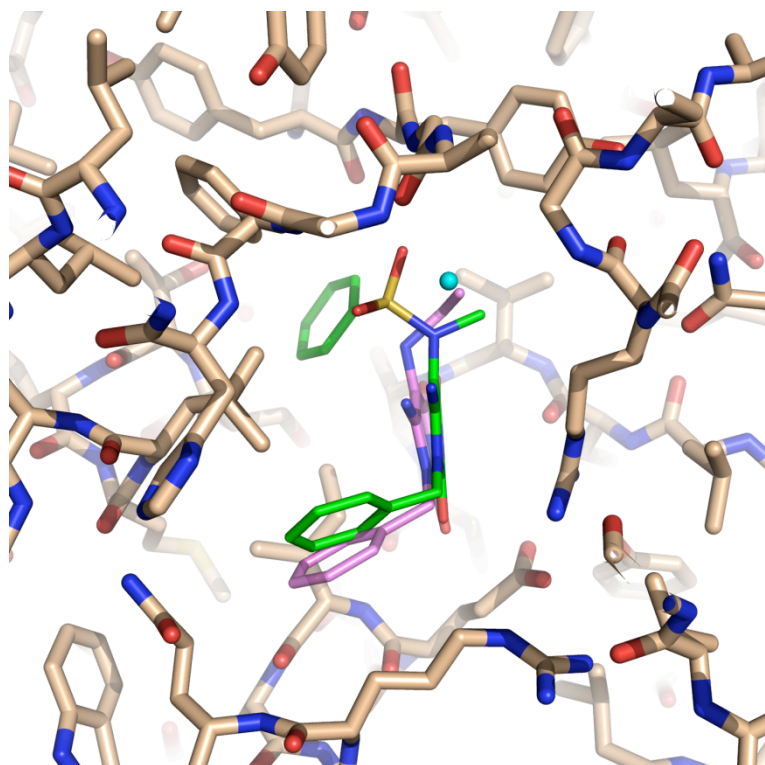
Inhibitor *B*-range are lowest and highest average *B* for one ligand, out of the four copies in the crystal



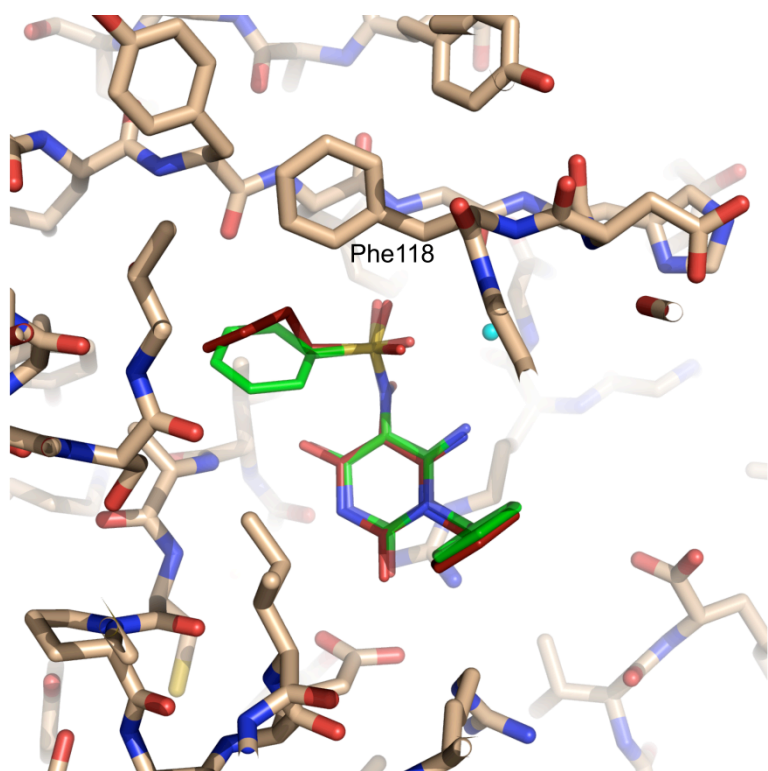
**Figure S9.** An overlay of the structures of the RmlA-**8a** (in green) and RmlA-**8f** (in orange) complexes showed that the contacts made between the enzyme and the pyrimidinedione rings of **8a** and **8f** were preserved. However, replacement of the sulfonamide in **8a** by the amide in **8a** results in movement of the N-methyl and the N1-benzyl groups away from their optimal alignment in the allosteric site. As the amide of **8f** prefers to adopt a planar *trans*-conformation, **8f** must pay an entropic penalty to achieve a binding conformation similar to **8a**. A combination of this factor coupled with the difference in the hydrogen bonding networks and the enforced repositioning of the N-methyl group (see Figure 3 in manuscript) may explain the lowered potency for **8f** against RmlA compared to **8a**.



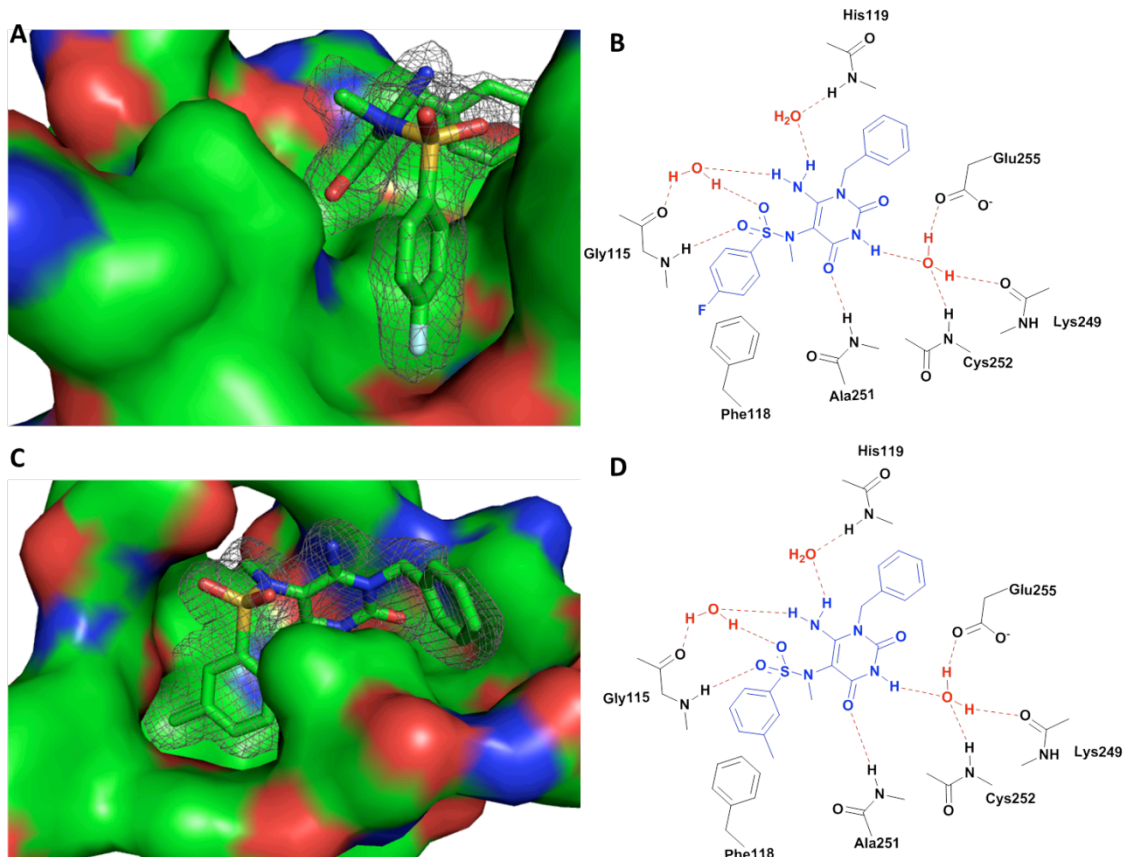
**Figure S10.** Comparison of complex structures of RmlA-1 (yellow) and RmlA-3 (magenta). Fewer hydrogen bonding interactions and a rotation of the N5-methyl in **3** were seen when the structures of the complexes were compared.



**Figure S11.** Analysis of the RmlA-12b complex showed that the N5-ethyl no longer occupied the previously identified hydrophobic pocket but instead was positioned in a nearby cleft. **8a** is shown in green and **12b** in violet.

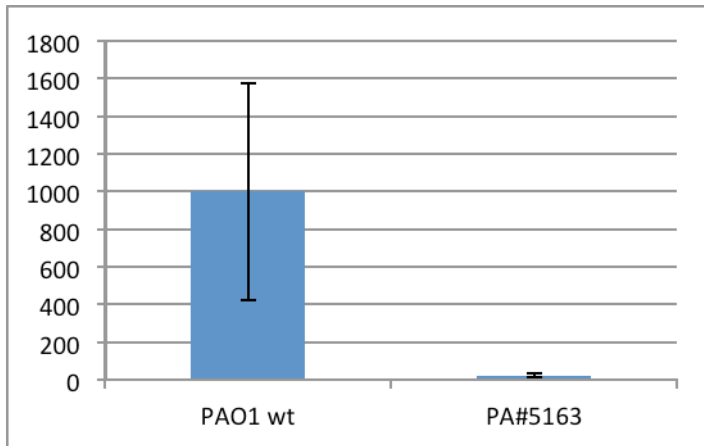


**Figure S12.** Analysis of the RmlA-**8j** complex showed that hydrophobic interactions between the alkyl side chain and Phe118 were present. The *n*-butyl chain extends towards the same pocket in which the phenyl substituent was previously bound in **8a** having hydrophobic interactions with the side chain of Phe118. The major difference is the loss of the face-edge  $\pi$ -stack interaction of the phenyl substituent in **8a** with Phe118. However, the sulfonamide alkyl chain is still able to make hydrophobic interactions with this residue. It is predicted that analogues **8g-i** (Table S7) bind in the same manner as **8j** and in doing so retain the key hydrogen bonding interactions of the core structure. However, **8g-i** were less active than **8j** due to the loss of the face-edge  $\pi$ -stack interaction with Phe118. The longer chain analogues **8i** and **8j**, having stronger hydrophobic interactions with Phe118, are more active than the shorter chain analogues **8g** and **8h** since the Me and Et groups would not extend deeply enough into this pocket to interact with Phe118.



**Figure S13.** **A** The structure of analog **8k** bound in the allosteric site of RmlA. 2Fo-Fc electron density contoured at  $1\sigma$  is shown around inhibitor. **B.** Schematic representation of the key interactions between **8k** and the enzyme allosteric pocket. Hydrogen bonds are shown as red dotted lines. **C** The structure of analogue **8p** bound in the allosteric pocket of RmlA. 2Fo-Fc electron density contoured at  $1\sigma$  is shown around inhibitor. **D.** Schematic representation of the key interactions between **8p** and the enzyme allosteric pocket. Green is indicative of hydrophobic regions, red shows polar oxygen-rich areas, and blue shows polar nitrogen-rich areas.

The RmlA-**8k** complex shows the same binding conformation as analogue **8a**. The hydrogen bonding interactions between the core of **8k** and the protein were retained. As shown in Figure S13A the pocket into which the 4-position of the phenyl binds is relatively shallow. This suggests that incorporation of a larger substituent in this position would not be accommodated, and would lead to twisting of the phenyl group to allow the compound to bind resulting in a loss of the face-edge  $\pi$ -stack interaction between the phenyl and the side chain of Phe118. This should result in a loss in activity of the analogs. This is observed for analogues **8l-m**. The structure of **8p** bound in the allosteric site of RmlA leads to a comparable rationalisation. As shown in Figures S13C and S13D the key interactions of the core are retained and the 3-Me substituent is accommodated in the phenyl-binding pocket. The face-edge  $\pi$ -stack interaction is also retained. However it is evident that a substituent larger than Me would not be accommodated without movement of the phenyl ring within this pocket. The predicted loss of the face-edge  $\pi$ -stack interaction between the phenyl ring and the side chain of Phe118 would be expected to lead to a loss in potency of the compound, and this observation may explain the difference in the potency of the 3-F containing **8o** which retains potency against RmlA compared to analog **8q**, containing the larger 3-OMe group which is 10 fold less active.



**Figure S14.** Quantification of colonies grown (Y-axis colony forming units) in the lungs of mice intratracheally infected with agarose beads loaded with the *Pseudomonas* mutant lacking RmlA (PA#5163) in comparison to PAO1 wildtype (wt).

#### **Intratracheal lung infection of NMRI mice with *Pseudomonas aeruginosa* and the RmlA mutant**

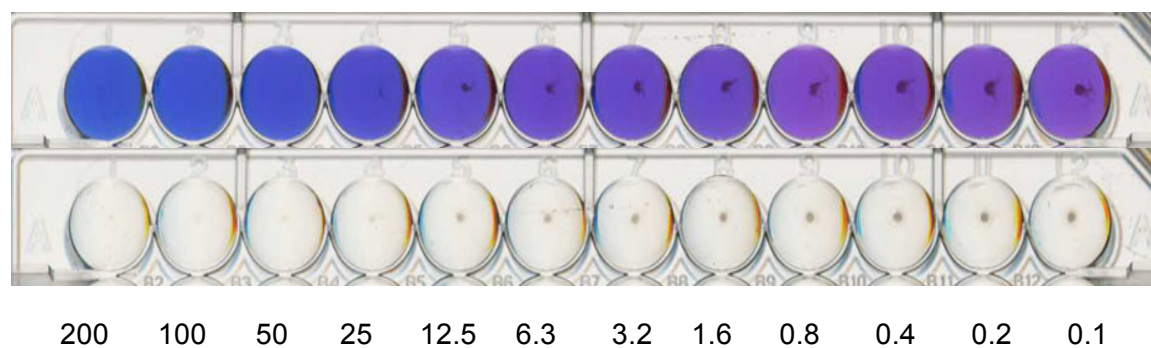
NMRI mice were intratracheally infected with bacterial loaded agarose beads. Mice were separated in groups per ten animals each. The single groups were infected with bacterial beads loaded with the mutant RmlA and PAO1 wildtype as control. 72 hours post infection mice were sacrificed by CO<sub>2</sub> inhalation and final bleeding. Lungs were isolated and their homogenates were plated in different dilutions for quantification of the bacterial load.

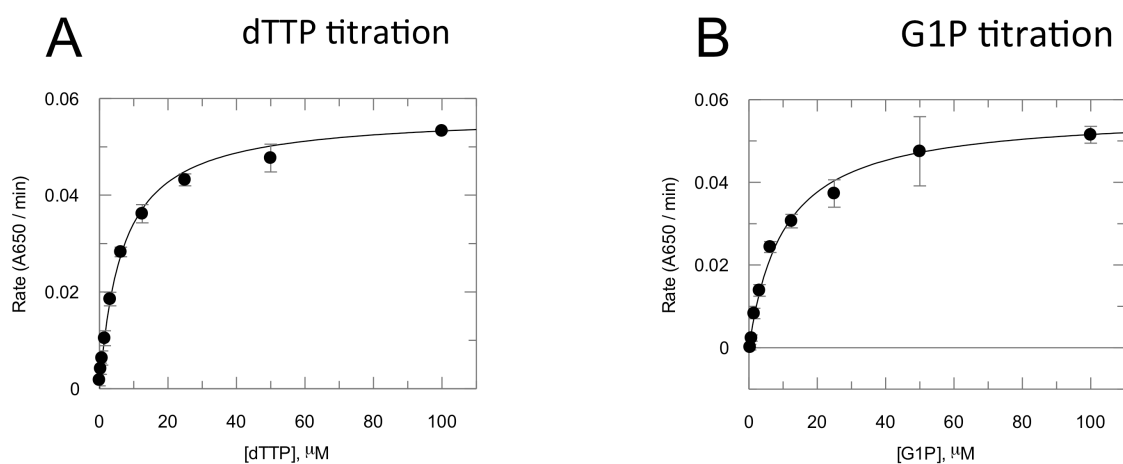
The RmlA lacking mutant (PA#5163) showed significant reduced numbers of colonies compared to the Wildtype. Figure 1 shows total amounts of bacterial colonies grown in the lung after intratracheal installation in NMRI outbred mice.

**Table S8.** MIC values for selected RmlA inhibitors against *Mycobacterium tuberculosis*.

Compound	MIC <sub>100</sub> ( $\mu\text{g mL}^{-1}$ )
Isoniazid	0.078
<b>1</b>	>200
<b>2</b>	>200
<b>8a</b>	100
<b>8f</b>	25
<b>15a</b>	>200
<b>8b</b>	200
<b>8c</b>	n.d.
<b>12a</b>	n.d.
<b>12b</b>	100
<b>8j</b>	200
<b>8k</b>	200
<b>8p</b>	50
<b>8t</b>	200

The MIC plates for compound **8f**, top panel has resazurin added, the bottom does not. The concentration of compound in  $\mu\text{g mL}^{-1}$  is shown below each well.





**Figure S15. Substrate  $K_m$  Determinations.** Substrate Michaelis constants were determined in the presence of a fixed, saturating concentration of the second substrate using a BIOMOL Green assay. Data was analysed using the Michaelis Menten equation and the Hill equation (equations 1 and 2 in *Methods* section). **A** Representative  $K_m$  determination for dTTP. Analysis of data from three experiments (each with  $n=3$ ) returns a  $K_m$  of  $7.8 \mu\text{M}$  (95% CI  $4.2 - 14.4 \mu\text{M}$ ) and a hill coefficient of  $1.05 \pm 0.12$  (mean  $\pm$  SD;  $N=3$ ). **B** Representative  $K_m$  determination for G1P. Analysis of data from three experiments (each with  $n=3$ ) returns a  $K_m$  of  $9.5 \mu\text{M}$  (95% CI  $6.8 - 13.4 \mu\text{M}$ ) and a hill coefficient of  $1.19 \pm 0.20$  (mean  $\pm$  SD;  $N=3$ ).

**Table S9.** Analysis of the binding of RmlA to its substrates in the absence or presence of other substrates and inhibitors by Surface Plasmon Resonance.

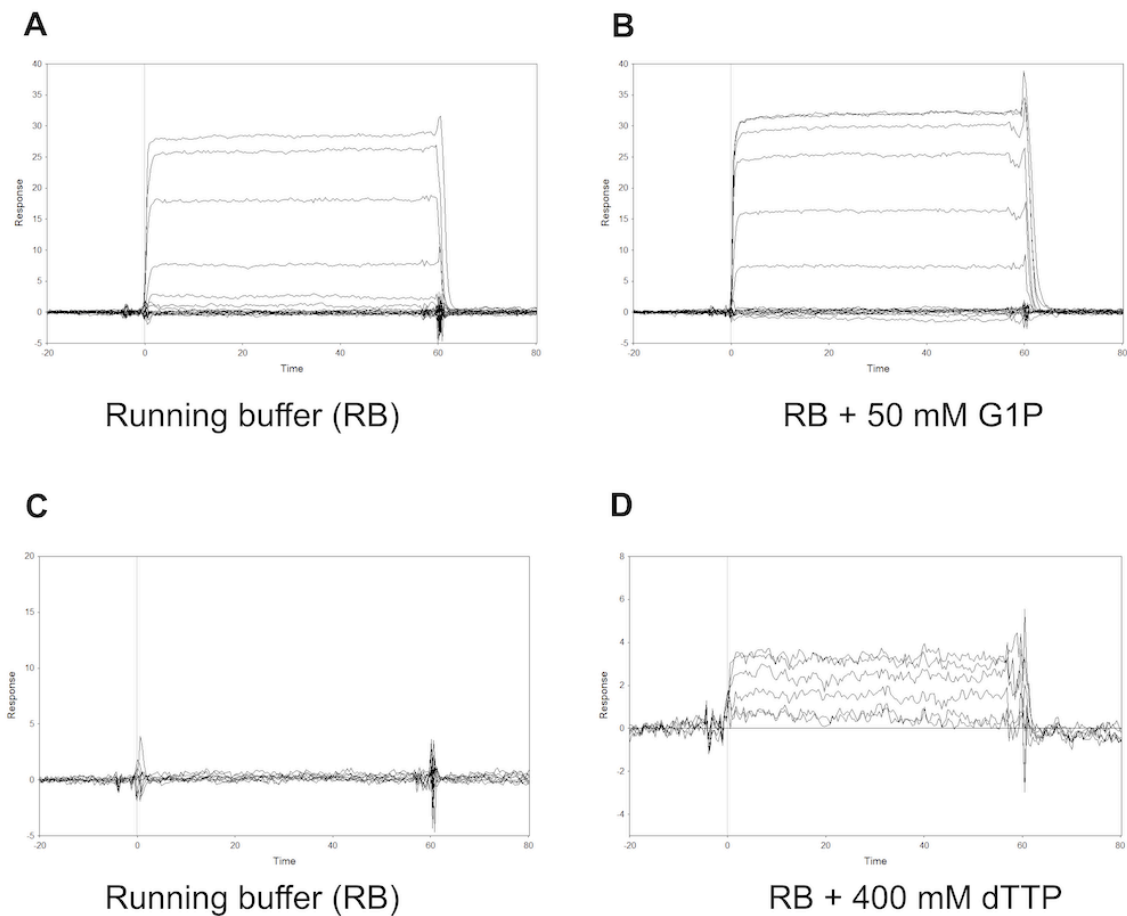
<b>Titration</b>	<b>Running buffer additions</b>	<b>K<sub>D</sub> (95% CI)</b>
dTTP	Nothing	39.9 μM (35.9 - 44.3 μM)
G1P	Nothing	No binding
dTTP	G1P (50 μM)	6.02 μM (5.03 - 7.20 μM)
G1P	dTTP (400 μM)	6.70 μM (5.55 - 8.10 μM)
G1P	1. <b>8a</b> (1 μM) + dTTP (400 μM)	No binding
dTTP	2. <b>8a</b> (1 μM)	92.0 μM (81.6 - 103.7 μM)
dTTP	3. <b>8a</b> (1 μM) + G1P (50 μM)	101 μM (79 - 130 μM)
G1P	4. <b>9a</b> (1 μM)	No binding

**Table S10.** IC<sub>50</sub> for natural ligands. Note these values likely reflect inhibition by binding at both the active and second (allosteric) site.

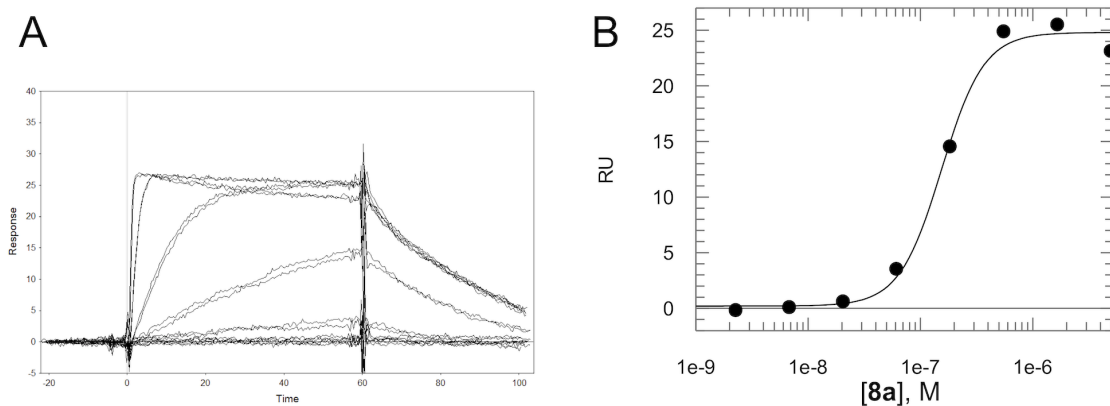
<b>Compound</b>	<b>% inhibition at 60 μM<sup>a</sup></b>	<b>IC<sub>50</sub> (μM)<sup>b</sup></b>
<b>dTMP</b>	22.3 ± 1.9	>90
<b>dTTP</b>	21.2 ± 3.3	>90
<b>dDTP-D-glucose</b>	49.1 ± 2.3	63.3 ± 5.9
<b>dTDP-L-rhamnose</b>	50.8 ± 4.2	60.3 ± 7.5

<sup>a</sup> SE, standard error (n=2)

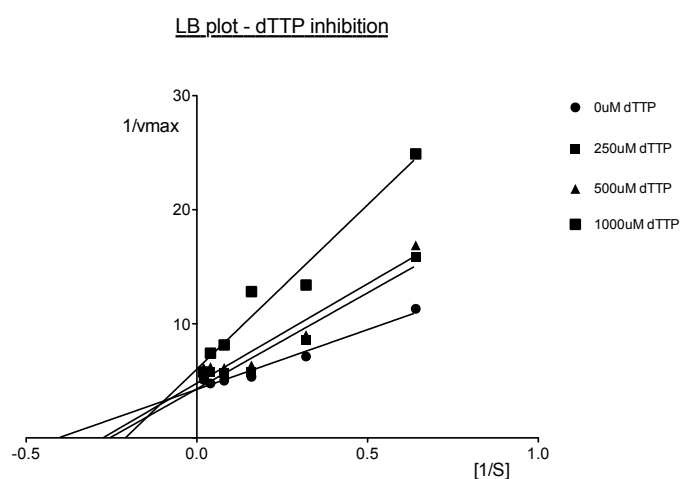
<sup>b</sup> SD, standard deviation (n=3)



**Figure S16. SPR data for substrate binding to RmlA.** Panels **A** and **B** show dTTP titrations in the absence (**A**) and presence (**B**) of 50  $\mu\text{M}$  G1P. These data reveal dTTP binding affinities of 39.9  $\mu\text{M}$  (95% CI 35.9 – 44.3  $\mu\text{M}$ ) (N=3) in the absence of G1P and 6.02  $\mu\text{M}$  (95% CI 5.03 – 7.2  $\mu\text{M}$ ) (N=3) in the presence of G1P. Panels **C** and **D** show G1P titrations in the absence (**C**) and presence (**D**) of 400  $\mu\text{M}$  dTTP. Binding of G1P ( $K_D$  6.70  $\mu\text{M}$  (95% CI 5.55 – 8.10  $\mu\text{M}$ ) (N=3)) is only observed when dTTP is present illustrating the sequential ordered bi-bi mechanism of catalysis employed by RmlA.

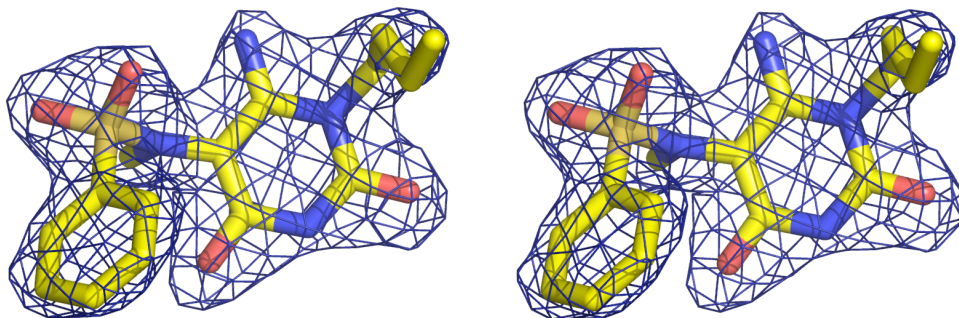


**Figure S17. SPR data showing binding of 8a to RmlA.** **A** Representative SPR sensorgram shows binding of 8a to RmlA (concentrations range tested, 2.3 nM – 5  $\mu$ M. n=2). **B** Plotting the SPR response at equilibrium against inhibitor concentration returns a dose response curve with a  $K_D$  of 163 nM (95% CI 146 – 182 nM) and a Hill slope of  $2.67 \pm 0.51$  (mean  $\pm$  SD, N=2).

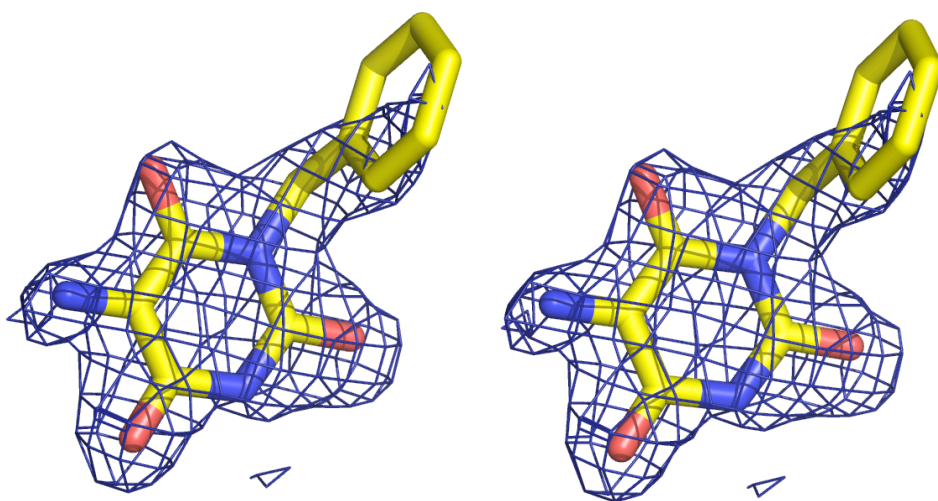


**Figure S18.** Lineweaver-Burk showing high concentrations of dTTP inhibit G1P binding to RmlA, suggesting a feedback mechanism.

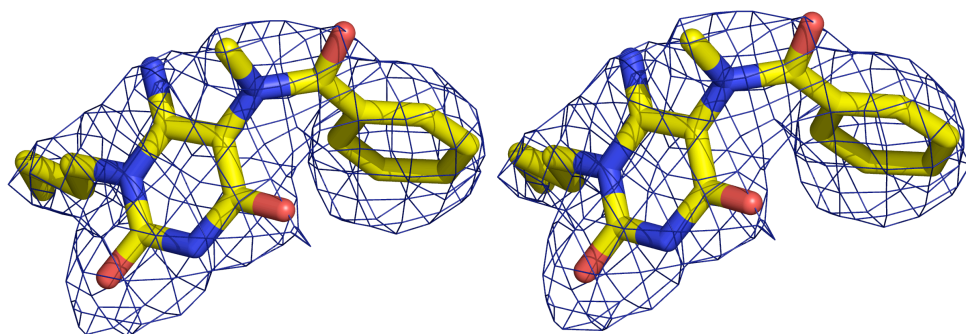
**Figure S19** Stereo Fo-Fc difference electron density maps calculated for each structure reported here. The phases of the map are calculated from the initial molecular replacement solution which had been refined for one cycle. The model used to phase these maps therefore has never included any ligand, water molecule or buffer. Underneath each image is given the PDB code, the compound code, the contour level and the subunit from which the molecule is shown. The carve radius in Pymol was 1.9 Å.



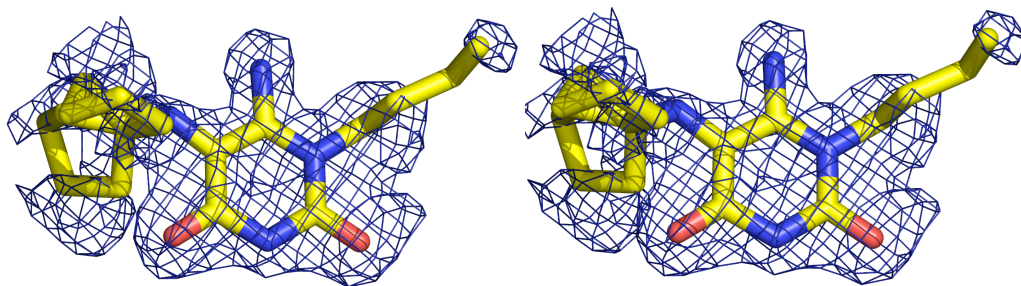
Compound 1, PDB 4arw, 3 $\sigma$ , subunit C



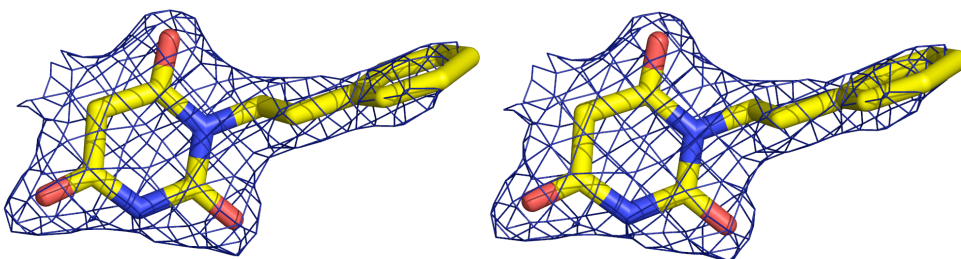
Compound 2, PDB 4as6, 3 $\sigma$ , subunit B, (further refinement improves density for phenylalanine ring).



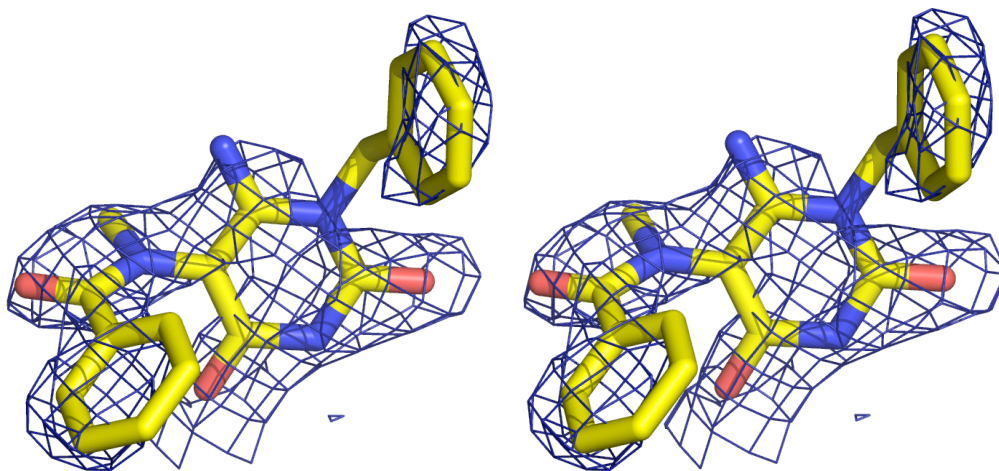
Compound 3, PDB 4b42, 3 $\sigma$ , subunit B



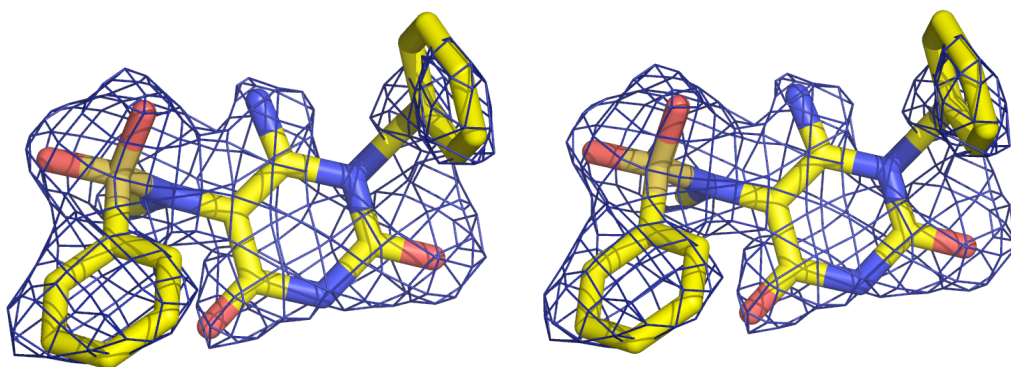
Compound **4**, PDB 4b2x,  $2\sigma$ , subunit A. The five membered ring was fitted as disordered over two positions.



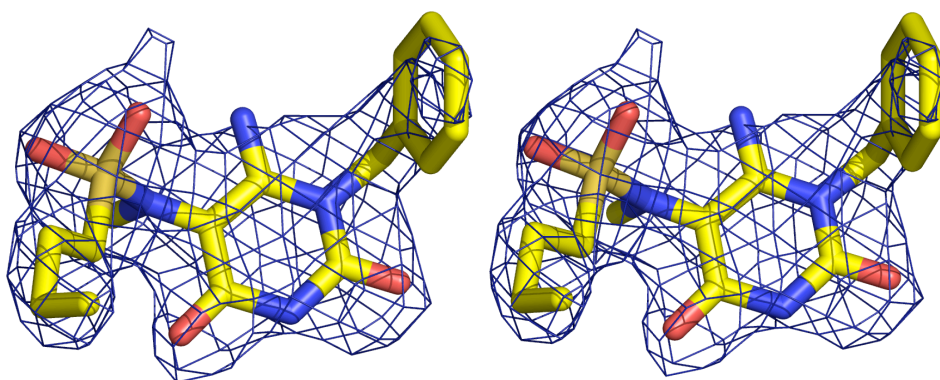
Compound **6**, PDB 4b4b,  $3\sigma$ , subunit A. The final  $2F_o-F_c$  and  $F_o-F_c$  electron density (Figure S7) suggest an adduct between the buffer (MES) and the compound (at the 1,3 diketone).



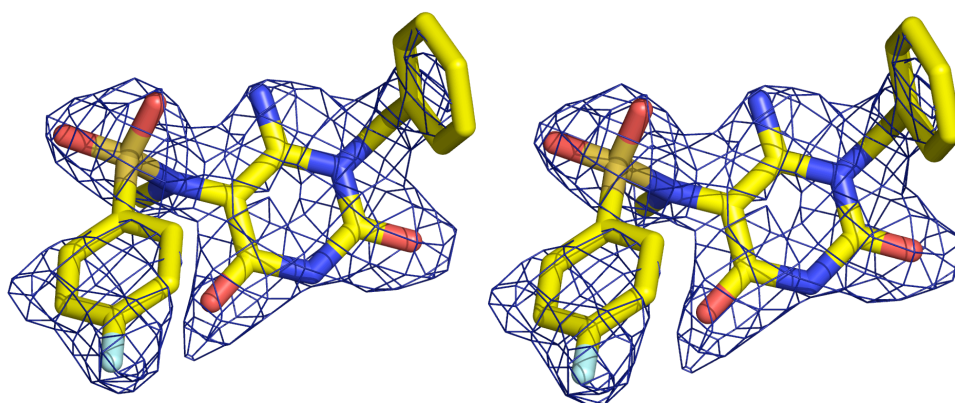
Compound **8f**, PDB 4asy,  $1.75\sigma$ , subunit B.



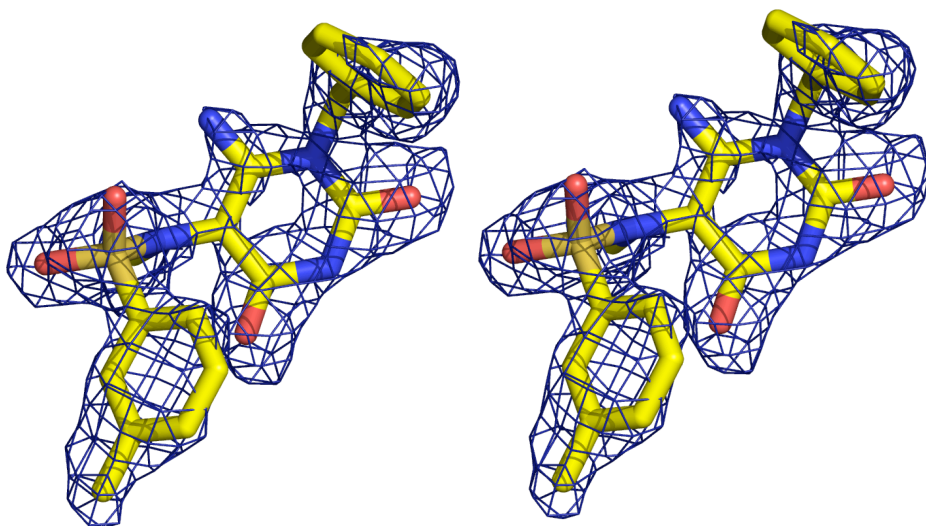
Compound **8a**, PDB 4asj,  $2.5\sigma$ , subunit C.



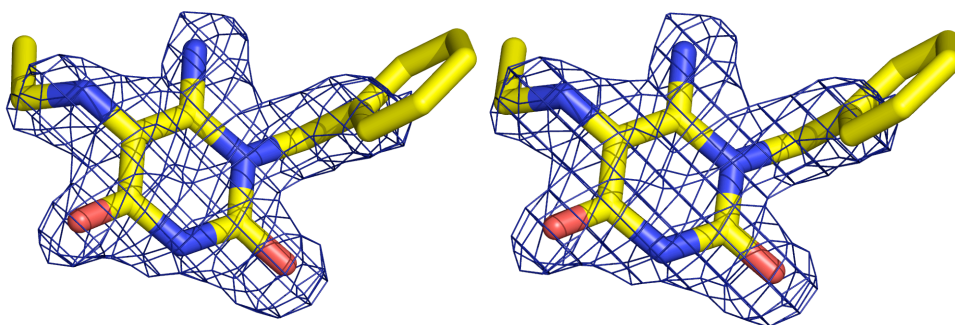
Compound **8j**, PDB 4b4g,  $2.75\sigma$ , subunit B.



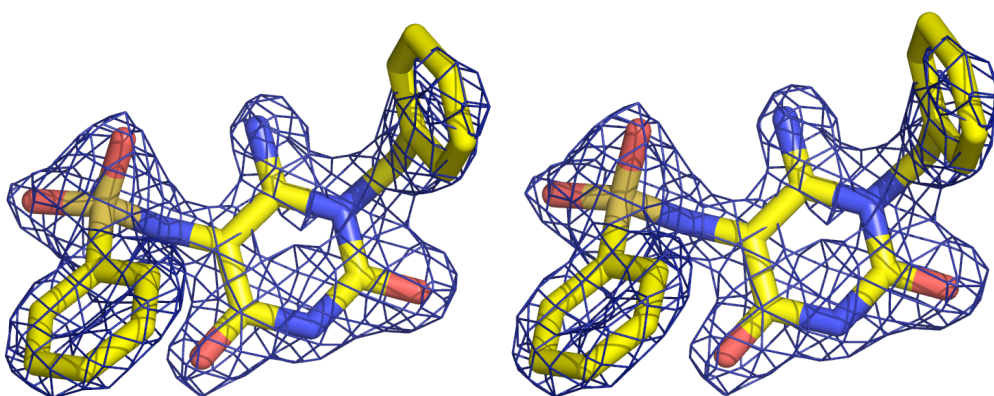
Compound **8k**, PDB 4b4m,  $2.75\sigma$ , subunit C.



Compound **8p**, PDB 4b5b,  $2.5\sigma$ , subunit C.



Compound **12b**, PDB 4b3u,  $2.75\sigma$ , subunit A.



Compound **15a**, PDB 4asp,  $3\sigma$ , subunit C.

## Methods

### Cloning, expression and purification of RmlA

The gene corresponding to *P. aeruginosa* RmlA was cloned as described previously (Blankenfeldt et al, 2000). Cells from a 1 L culture were resuspended in 30 ml buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, DNaseI and an EDTA-free protease cocktail inhibitor tablet (Roche)), then lysed at 16000 psi using a French Press. The resulting lysate was centrifuged at 20000g for 40 minutes to remove particulate matter and the supernatant filtered through a 0.45  $\mu$ m syringe filter. The supernatant was then applied, using a syringe, to a 5mL HiTrap FF column (GE Healthcare) pre-loaded with 0.1 M  $\text{Ni}^{2+}\text{SO}_4^{2-}$  and pre-equilibrated with buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl). The column was washed firstly with 20 column volumes of buffer containing 10 mM imidazole and followed by 30 column volumes of buffer containing 30mM imidazole to remove non-specifically bound, histidine-rich proteins. RmlA protein was eluted with 15 column volumes of buffer containing 500 mM imidazole. Purity was assessed by SDS-PAGE. The purified RmlA was dialysed for 2 hrs against 2 L of 50mM Tris-HCl, pH 7.5, 10 mM EDTA, and then overnight against 2 L of 50 mM Tris-HCl, pH 7.5. Dialysed, purified protein was then concentrated to 10 mg/mL for crystallization.

### Cloning, expression and purification of biotinylated RmlA

The biotinylated RmlA required for SPR experiments was cloned, expressed and purified as follows. The gene was ordered (codon optimized for *E. coli*) from GenScript with an XbaI AAATAATTTTGTTTAACTTTAAGAAGGAGATATACCATGGGCAGCAGCCATCATCATCATCACAG CAGCGGCGAAAACCTGTATTTTCAGGGCGGCCTGAACGATATTTTTGAAGCGCAGAAAATTGAATG GCATGAATCGGCCGCCGGCGGCAGCCAT leader sequence (containing a BAP peptide sequence and linker) and a BamHI site following the stop codon. The gene was cloned into a pET15b TEV (Tobacco Etch Virus) resulting in a His<sub>6</sub>-TEV-BAP-RmlA plasmid. The plasmid was transformed into BL21 DE3 Gold cells that were grown at 37 °C until OD<sub>600</sub> = 0.6. Cultures were induced with 250  $\mu$ M IPTG for 16 hours at 20°C and harvested by centrifugation. The cell pellet was resuspended in 25mM Tris-HCl, pH 8.5, 500 mM NaCl, 20 mM imidazole (Buffer A) containing DNAase, lysozyme and protease inhibitors, lysed, centrifuged at 40000 g for 20 mins and filtered (0.45  $\mu$ m). The supernatant was applied to a 5mL HiTrap HP column (GE Healthcare) pre-loaded with 100 mM  $\text{NiCl}_2$  and pre-equilibrated with buffer A. After washing with 50 mL buffer A, the target enzyme was eluted using an imidazole gradient. The His<sub>6</sub>-tag was cleaved using TEV protease during dialysis against 25 mM Tris-HCl pH 7.5, 150 mM NaCl, and removed using the HiTrap column. Purified RmlA was concentrated and dialysed into 10 mM Tris-HCl pH 7.4, 200 mM KCl. RmlA was biotinylated by adding ATP (200 mM),  $\text{MgCl}_2$  (50 mM), Biotin (100 mM) and BirA enzyme (130 $\mu$ M) and incubating at 37 °C for 1.5 hrs. Gel purification using a Superdex75 26/60 column equilibrated in 25 mM Tris-HCl pH7.5, 150 mM NaCl gave purified biotinylated RmlA, confirmed by mass spectrometry.

## **RmlA Hit Identification**

The enzymatic activity of RmlA was determined by monitoring release of pyrophosphate using BIOMOL Green reagent (BIOMOL International UK) coupled with a pyrophosphatase enzyme, which gives an absorbance read-out at 650 nm. The RmlA high throughput screen was performed using a Dundee Drug Discovery Unit in-house diverse compound collection of 15,667 molecules. All library compounds were solubilised in 100% DMSO to a concentration of 10 mM. Single point inhibition assays were carried out at room temperature in clear, flat-bottomed, polystyrene, 384-well plates (Matrix). Each assay was performed in a 50  $\mu$ L reaction volume containing containing 50 mM Tris, pH 7.4, 5 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 0.05% NP-40, 0.1 mM EDTA, 0.1 mM EGTA, 1.5 nM recombinant RmlA, 0.8  $\mu$ g mL<sup>-1</sup> pyrophosphatase, 5  $\mu$ M dTTP, 5  $\mu$ M G-1P and 30  $\mu$ M test compound. Test compound was transferred to all assay plates using an Echo 550 acoustic dispenser (Labcyte) before 25  $\mu$ L of enzyme mix was added to the plate using a Thermo Scientific WellMate (Matrix). The reaction was initiated and stopped with the additions of 25  $\mu$ L substrate mix and 50  $\mu$ L BIOMOL Green, respectively, on a Thermo Scientific WellMate (Matrix). The assay was run for 30 min at room temperature and the BIOMOL Green signal was allowed to develop for 30 min before the absorbance of each well was read at 650 nm using an EnVision multilabel plate reader (PerkinElmer Life Sciences).

ActivityBase from IDBS was used for data processing and analysis. Compounds were designated hits if the percentage inhibition (PI) was >30% (this value represents 3 standard deviations from the mean data set PI).

## **RmlA Inhibitor Studies**

Hit compounds identified from the primary screen were either cherry picked from original library plates using a JANUS work station or re-purchased from Vitas, Enamine, or Cheshire Sciences. To generate IC<sub>50</sub> data for these putative RmlA inhibitors, 10-point inhibitor curves (consisting of half-log serial dilutions of compound in DMSO) were prepared in 384-well plates using a JANUS work station (PerkinElmer Life Sciences). Following preparation of the inhibitor curves, assays were carried out using the BIOMOL Green reagent as described above.

ActivityBase from IDBS was used for data processing and analysis. All IC<sub>50</sub> curve fitting was undertaken using ActivityBase XE from IDBS with curves fitted to the four-parameter logistic dose-response curve. All test compound curves had floating top and bottom, and prefit was used for all four parameters.

## **Isothermal Titration Calorimetry**

ITC experiments were carried out using a VP-ITC instrument (MicroCal) in PBS, pH 7.4 at 37 °C. Protein concentrations were determined using absorbance at 280 nm and predicted extinction coefficients

(ProtParam, ExPASy). Concentrations of ligands were based on dry weight. The ligands were prepared from stock solutions prepared in DMSO, resulting in a final DMSO concentration of 1.125% (v/v). Titrations were performed as follows: one injection of 2  $\mu\text{L}$  followed by injections of 7.5  $\mu\text{L}$  at an injection speed of 0.5  $\mu\text{L sec}^{-1}$ . The stirring speed was 300 rpm; the delay between the injections was 3 min. To take into account heats of dilution, blank titrations were performed by injecting ligand solution into phosphate buffer and the averaged heat of dilution was subtracted from the main experiment (negligible for **1** and **8a**). Raw data were processed using MicroCal Origin software. The baseline was adjusted and integrations were carried out manually. Integrated data were analyzed using the OneSite binding model. Ligand concentrations were adjusted to result in fits reflecting 1:1 stoichiometry.

## 2D-similarity screens

2D similarity searches were run on our in-house database of 6168759 compounds using the Dotmatics browser ([www.dotmatics.com/products\\_browser.jsp](http://www.dotmatics.com/products_browser.jsp)) for scaffolds 1, 2 & 3 (figure S6). The compounds obtained were tested experimentally in an activity assay (see below).

## Crystallization

Initial crystallization conditions were found by sparse matrix screening. Precipitant conditions were optimized to 4% PEG 6000, 100 mM MES pH 6.0, 50 mM  $\text{MgCl}_2$ , 100 mM NaBr and 1%  $\beta$ -mercaptoethanol. Crystals were grown using the sitting drop method with drops comprising 1  $\mu\text{L}$  protein (10 mg  $\text{mL}^{-1}$ ) mixed with 1  $\mu\text{L}$  precipitant. Crystals grow overnight to dimensions of 0.2 x 0.2 x 0.1 mm, and cryoprotected in precipitant substituted with 25% glycerol for 5 seconds, prior to be frozen in a stream of nitrogen gas at 100 K for data collection.

## Soaking/co-crystallization

Complexes of enzyme and inhibitor were prepared by soaking or co-crystallization. For soaking, apo-crystals were grown as described above. Once crystals were present in the drop, solid compound was added and left to incubate with the crystals overnight. The crystal was then cryo-protected and frozen in a stream of nitrogen gas at 100 K for data collection. For co-crystallization, drops were set up as before, but solid compound added to the drop prior to sealing and incubation. Crystals typically grew overnight.

## Data collection

Data were processed with iMOSFLM<sup>3</sup> or XIA2<sup>4</sup> incorporating XDS<sup>5</sup>. Processing statistics are shown in Table 1. Each structure was solved using MOLREP<sup>6</sup>, initially using a monomer from 1FZW<sup>7</sup>, (apo *P. aeruginosa* RmlA) and subsequent complexes using a subunit of the **1** complex stripped off inhibitor. Rigid body and restrained refinement was done with REFMAC5<sup>8</sup> and statistics shown in Tables S4-6. Model building was performed using COOT<sup>9</sup>, and ligands built using the PRODRG server<sup>10</sup>. Structural figures were prepared with PyMOL.<sup>2</sup> Ligands were added when  $F_o - F_c$  difference electron density was

clear. In all cases the central 'thymidine' ring mimic was often clear in the density, the quality of the electron density of the substituents varied, with some flexible parts poorly ordered. As noted in Figure S7 compound **6** and **7** had additional density suggesting an 1,3 diketo adduct. We were unable to chemically identify any such a compound and all ligands were assessed as pure and authentic prior to incubation with RmlA. Ligand atoms were included at full occupancy, with one exception compound **4** has a five membered ring on the amine, this appeared to disordered over two sites, consequently each site was assigned an occupancy of 0.5 (occupancy was not refined). % of most favoured Ramachandran angles calculated using MOLPROBITY ([www.molprobity.duke.edu](http://www.molprobity.duke.edu)).<sup>11, 12</sup>.

### Surface Plasmon Resonance

Binding of analytes (substrates and inhibitor) to RmlA was investigated using a Biacore™ 3000 instrument (GE Healthcare). Recombinant, biotinylated RmlA was diluted to a concentration of 40  $\mu\text{g mL}^{-1}$  in running buffer (50 mM Tris, pH7.4, 150 mM NaCl, 5 mM  $\text{MgCl}_2$ , 0.1 mM EDTA, 0.1 mM EGTA, 0.05% Tween-20, 1 mM DTT and 3% DMSO) and immobilised to a streptavidin sensor chip surface by means of the N-terminal biotin moiety. Following a 10 min injection of protein (flow rate 5  $\mu\text{l/min}$ ) RmlA was immobilised to a density of approximately 3,000 resonance units (RU) on flow cell 4, leaving flow cell 3 as a reference surface.

For standard binding assays RmlA analytes were diluted in running buffer to give the following concentration ranges: dTTP, 1.6 – 400  $\mu\text{M}$ ; G-1P, 0.4 – 100  $\mu\text{M}$  and inhibitor **8a**, 2-2500 nM. For competition studies, analytes were diluted to the desired concentration in running buffer containing either 400  $\mu\text{M}$  dTTP, 50  $\mu\text{M}$  G-1P, 1  $\mu\text{M}$  inhibitor **8a**, or a combination of these analytes. In all cases binding assays were carried out at 25°C, with a constant flow rate of 50  $\mu\text{l min}^{-1}$ . Each analyte sample was injected for 60 s, with a 120 s dissociation time.

Data processing and kinetic analysis was performed using Scrubber software (version 2.0c). Responses were corrected for signals from the reference surface before fitting to a 1:1 binding model.

### Kinetic Parameter Determinations

Enzyme assays were carried out at room temperature in a 50  $\mu\text{l}$  reaction volume containing 50 mM Tris, pH 7.4, 5 mM  $\text{MgCl}_2$ , 1 mM dithiothreitol, 0.05% NP-40, 0.1 mM EDTA, 0.1 mM EGTA, 1.5 nM recombinant RmlA, 0.8  $\mu\text{g ml}^{-1}$  pyrophosphatase and varying concentrations of substrates. Reactions were stopped at various time points by the addition of 50  $\mu\text{l}$  BIOMOL Green. The BIOMOL Green signal was allowed to develop for 30 min before the absorbance of each well was read at 650 nm using an EnVision multilabel plate reader (PerkinElmer Life Sciences). Michaelis constants for each substrate (dTTP and G-1P) were determined with data fitted to the following Michaelis–Menten equation using GraFit (version 6.0.12)<sup>13</sup>.

$$v = \frac{V_{\max} \cdot [S]}{K_m + [S]} \text{ (equation 1)}$$

Data sets for each substrate (dTTP and G-1P) were also fitted to the following Hill Equation using GraFit (version 6.0.12)<sup>13</sup> to ascertain the Hill coefficient indicating the level of cooperativity within the tetrameric protein.

$$v = \frac{V_{\max} [S]^n}{K^n + [S]^n} \text{ (equation 2)}$$

where  $n$  = Hill coefficient.

### ***Mycobacterium tuberculosis* MIC determination**

MIC values were determined against *M. tuberculosis* (H37Rv) by the microbroth dilution method <sup>14</sup>. A broth culture of *M. tuberculosis* was grown in Middlebrook 7H9 medium with 10 % OADC supplement with 0.05 % Tween-80 to an OD<sub>600</sub> of 0.4–0.6. The culture was diluted with the same 7H9 medium to an OD<sub>600</sub> of 0.004 (~2x10<sup>5</sup> CFU/ml), and 200 μL was added to a microtiter plate containing two-fold serial dilutions of the tested compounds (in 4 μl of DMSO) for a final volume of 204 μL. The plates were incubated at 37 °C for 10 days. Resazurin was added and the plates incubated an additional 24 hours. The MIC<sub>100</sub> was determined by visual inspection and defined as the concentration that inhibited 100 % of growth.

### **Construction of suicide vector for knockout of *rmIA***

For gene replacement of *rmIA* (PA5163) the *sacB*-based previously described strategy <sup>15, 16</sup> was used. In order to construct the suicide vector for deletion of *rmIA* (PA5163) regions of approximately 400 bp flanking the target gene were amplified using primers 88PA5163 (5'-GGAGGAGAATTCCGACGAACTCAAGGTCGTG-3') with *EcoRI* site at 5'-end and 111PA5163 (5'-GGAGGAAGATCTGTGGTCCCTGCTCGCTCAG-3') containing a *BglII* site for upstream fragment and primers 133PA5163 (5'-GGAGGAAGATCTGAAAGCGACCCGCCTGGC-3') containing *BglII* site and 155PA5163 (5'-GGAGGGAAGCTTCCAGAAGTCGGTGGTCTTG-3') with *HindIII* site for downstream fragment of *rmIA*. After digest with corresponding endonucleases up- and downstream fragments were inserted into multiple cloning site of vector pEX18Ap <sup>17</sup>. The Gentamicin resistance cassette of pPS858 <sup>17</sup> was excised using *BamHI* and introduced into *BglII* site between the two flanking fragments.

Constructs were confirmed by sequencing and then transformed into *E. coli* donor strain ST18 that was used for conjugational transfer of the plasmid into *P. aeruginosa* PAO1 as previously described <sup>16</sup>.

After counter-selection on LB-agar plates containing 5 % sucrose and 80 µg/mL gentamycin that should force loss of plasmid borne DNA by recombinational events obtained clones were first tested with respect to carbenicillin sensitivity by replica plating. In order to confirm the carbenicillin sensitive and sucrose resistant phenotype total DNA was isolated from the probable KO mutants and used in PCR together with primers specific for internal sequences of *sacB* and *bla* genes. Additionally the correct replacement of the target gene by the gentamycin cassette was examined also by PCR. Therefore primers located in approximately 200 base pairs distance from the ends of the cassette and directed outwards were used together with primers located about 500 base pairs up- and downstream, respectively, from target gene. For final confirmation obtained PCR products were sequenced.

After third conjugation and testing about 100 transconjugants one *rImA* KO mutant was obtained and confirmed. In comparison to wild type that forms turquoise colonies on M9 agar plates colonies of  $\Delta rImA$  mutant were smaller and showed a light lime green colour.

### **Agarose bead preparation for infection with PAO1 wt and PA mutants**

Agarose beads were prepared using a modified method previously described by Starke et al. 18. *Pseudomonas aeruginosa* and its mutant strains were cultured over night at 37°C in LB and LB+Gentamycin [80µg/ml], respectively. After centrifugation at 4000 rpm for 10 min the sedimented bacteria were resuspended in 1 ml sterile PBS (pH 7,4) and added to 5 ml 2% agarose pre-warmed to 50°C. The bacteria-agarose mixture was pipetted rapidly to 5 ml 50°C pre-warmed heavy white mineral oil. After intensive vortexing the mixture was cooled on ice for 5 minutes followed by centrifugation at 4000 rpm for 10 min. The resulting agarose beads were washed three times in sterile PBS (pH 7.4).

The load of bacteria in the agarose beads was quantified by plating 10-fold serial dilutions on Columbia blood agar plates. The inoculums for infection were prepared by diluting the bead suspension with PBS (pH7.4) to  $2 \times 10^8$  CFU/ml.

### **Lung infection of mice**

For the infection studies we used female NMRI outbred mice at the age of min. 6 weeks. The animals were anesthetized using a Ketamin-Xylazin mixture applied by i.p. injection. Mice were intratracheally infected with 50µl of the bead suspension at a concentration of  $2 \times 10^8$  CFU/ml resulting in  $1 \times 10^7$  CFU.

### **Quantification of bacteria in the lung**

72 hours after infection, the mice were sacrificed by CO<sub>2</sub> inhalation. Lungs were isolated and homogenized through microfilters. Mouse lung homogenates were diluted 1:1, 1:10 and 1:100 with sterile PBS (pH 7,4). The different dilutions were plated on Columbia blood agar plates in case of PAO1 wildtype, and on agar plates containing Gentamycin for *Pseudomonas* mutants. After overnight incubation at 37°C colony counts were determined for each individual dilution.

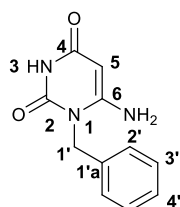
## Compound synthesis

### General Methods

All chemicals and solvents were purchased from Aldrich (UK), Alfa Aesar, Apollo Scientific or Fluorochem and used without further purification. All reactions were carried out under a positive pressure of nitrogen or argon in oven-dried glassware. Thin layer chromatography (TLC) analysis was performed using glass plates coated with silica gel (with fluorescent indicator UV<sub>254</sub>). Developed plates were air dried and analysed under a UV lamp (254/365 nm). Flash chromatography was performed using silica gel (40-63  $\mu\text{m}$ , Fluorochem).

Melting points were measured using Electrothermal 9100 capillary melting point apparatus and are uncorrected. Fourier Transform infra-red spectra (FT IR) were acquired on a Perkin Elmer paragon 1000 FT spectrometer. Absorption maxima are reported in wavenumbers ( $\text{cm}^{-1}$ ). Nuclear magnetic resonance (NMR) spectra were recorded at room temperature on a Bruker Avance 400 ( $^1\text{H}$ , 400;  $^{13}\text{C}$ , 101 MHz) and Bruker Avance 300 ( $^1\text{H}$ , 300;  $^{13}\text{C}$ , 75 MHz) instruments. Deuterated solvents were used and  $^1\text{H}$  NMR shifts were internally referenced to  $\text{CD}_2\text{HCl}_3$  (7.26 ppm) in  $d_1$ -chloroform and to  $\text{CD}_2\text{HSO}_2\text{CHD}_2$  (2.50 ppm) in  $d_6$ -dimethylsulphoxide. Chemical shifts are expressed as  $\delta$  in units of ppm.  $^{13}\text{C}$  NMR spectra were recorded under the same conditions and solvents using the PENDANT sequence mode. Data processing was carried out using the TOPSPIN 3.1 NMR program (Bruker UK Ltd). For  $^1\text{H}$  NMR, the multiplicity used for assignment is indicated by the following abbreviations: s = singlet, d = doublet, t = triplet, q = quadruplet, m = multiplet, br = broad. Signals of protons and carbons were assigned, as far as possible, by using the following two dimensional NMR spectroscopy techniques: [ $^1\text{H}$ ,  $^1\text{H}$ ] COSY, [ $^1\text{H}$ ,  $^{13}\text{C}$ ] HSQC (Heteronuclear Single Quantum Coherence) and long range [ $^1\text{H}$ ,  $^{13}\text{C}$ ] HMBC (Heteronuclear Multiple Bond Connectivity). Mass spectrometry (electrospray mode, ES) was carried out on a high performance orthogonal acceleration reflecting TOF mass spectrometer operating in positive and negative mode, coupled to a Waters 2975 HPLC.

### 6-Amino-1-benzylpyrimidine-2,4(1*H*,3*H*)-dione (**11**)<sup>19</sup>

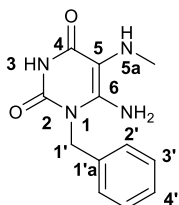


A mixture of benzylurea (**9**) (5.0 g, 33.3 mmol, 1.0 equiv.), 2-cyanoacetic acid (**10**) (2.6 g, 36.6 mmol, 1.1 equiv.) and acetic anhydride (20 mL) was heated at 80  $^{\circ}\text{C}$  for 2 hours. After cooling to room temperature,  $\text{Et}_2\text{O}$  (30 mL) was added and the resulting suspension was stirred for 1 hour. The solid intermediate was filtered, suspended in a mixture of water (30 mL) and ethanol (15 mL) and heated at 85  $^{\circ}\text{C}$ . NaOH (10% aq. soln., 10 mL) was slowly added and the resulting solution stirred for 1 hour. After cooling, the reaction mixture was concentrated and acidified by dropwise addition of conc. HCl. The resulting white

precipitate was collected by filtration and dried to afford the product as an off-white solid (3.7 g, 17.0 mmol, 51 %). Mp 268-270 °C (*lit.* 260-270 °C)<sup>20</sup>;  $\delta_{\text{H}}$  (400 MHz, DMSO-*d*<sub>6</sub>) 10.47 (1H, s, H3), 7.37-7.19 (5H, m, ArH), 6.78 (2H, s, C6-NH<sub>2</sub>), 5.03 (2H, s, H1'), 4.60 (1H, d, *J* = 1.8 Hz, H5); *m/z* (ES<sup>+</sup>) 218.09 [(M+H)<sup>+</sup>, 100%].

## Synthesis of 12a-12e

### 6-Amino-1-benzyl-5-(methylamino)pyrimidine-2,4(1H,3H)-dione (12a)<sup>21</sup>

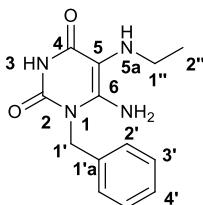


To a suspension of **11** (3.0 g, 13.8 mmol, 1 equiv.) in methanol (15 mL) was added NaHCO<sub>3</sub> (1.2 g, 13.8 mmol, 1 equiv.) and the mixture cooled to 4 °C. Bromine (2.2 g, 0.7 mL, 13.8 mmol, 1 equiv.) was added dropwise and the solution stirred at room temperature for 30 minutes. The resulting solution was cooled to 4 °C to afford precipitation of bromo intermediate. The light yellow solid was filtered and suspended in a 30 % aqueous solution of methylamine (6.0 mL). The suspension was heated to 70 °C and stirred for 4 hours. After this time the resulting white solid precipitate was filtered and dried to afford the product as an off-white solid (2.3 g, 9.5 mmol, 69 %). Mp 218-219 °C (*lit.* 220-221 °C)<sup>22</sup>;  $\delta_{\text{H}}$  (400 MHz, DMSO-*d*<sub>6</sub>) 10.53 (1H, br. s., H3), 7.35-7.17 (5H, m, ArH), 6.43 (2H, s, C6-NH<sub>2</sub>), 5.05 (2H, s, H1'), 2.80 (1H, br. s., H5a), 2.34 (3H, s, CH<sub>3</sub>); *m/z* (ES<sup>+</sup>) 247.12 [(M+H)<sup>+</sup>, 100%].

### General Procedure A - Synthesis of amines 12b-e

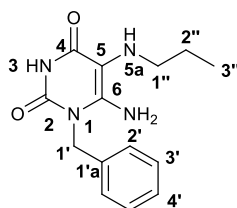
To a suspension of **11** (500 mg, 2.3 mmol, 1 equiv.) in methanol (2.5 mL) was added sodium hydrogen carbonate (193 mg, 2.3 mmol, 1 equiv.) and the mixture cooled to 4 °C. Bromine (370 mg, 120  $\mu$ L, 2.3 mmol, 1 equiv.) was added dropwise and the solution stirred at room temperature for 30 minutes. The resulting solution was cooled to 4 °C to afford precipitation of the corresponding bromide. The light yellow solid was filtered and suspended in a 30 % aqueous solution of the corresponding amine (1.0 mL). The suspension was heated to 70 °C and stirred for 4-6 hours. After this time the resulting white solid precipitates were filtered and dried to afford products **12a-2**.

### 6-Amino-1-benzyl-5-(ethylamino)pyrimidine-2,4(1H,3H)-dione (12b)



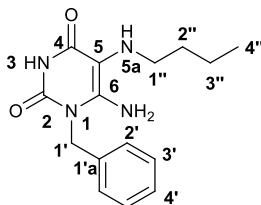
**12b** was obtained by reaction with ethylamine (1 mL) using general procedure A as a white solid (182 mg, 0.67 mmol, 30 %). Mp 212-214 °C;  $\nu_{\max}$   $\text{cm}^{-1}$  (KBr) 3385 (N-H), 2965 (aromatic C-H), 1808, 1705 (C=O), 1632 (C=O), 1489, 1402, 1268, 1173, 1101 and 738;  $\delta_{\text{H}}$  (400 MHz, DMSO- $d_6$ ) 10.48 (1H, app. br. s., H3), 7.35-7.16 (5H, m, ArH), 6.40 (2H, s, C6-NH<sub>2</sub>), 5.05 (2H, s, H1'), 2.77 (1H, br. s., H5a), 2.65 (2H, q,  $J = 6.9$  Hz, H1''), 0.98 (3H, t,  $J = 6.9$  Hz, H2'');  $\delta_{\text{C}}$  (100 MHz, DMSO- $d_6$ ) 160.9 (C4), 152.4 (C2), 150.1 (C6), 136.7 (C1'a), 128.4 (C2'), 127.0 (C4'), 126.2 (C3'), 97.1 (C5), 44.2 (C1'), 42.2 (C1''), 15.0 (C2'');  $m/z$  (ES<sup>+</sup>) 261.13 [(M+H)<sup>+</sup>, 100%]; HRMS (ES<sup>+</sup>) [Found: (M+H)<sup>+</sup>, 261.1346, C<sub>13</sub>H<sub>17</sub>N<sub>4</sub>O<sub>2</sub> requires 261.1349].

#### 6-Amino-1-benzyl-5-(propylamino)pyrimidine-2,4(1H,3H)-dione (12c)



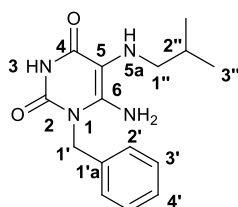
**12c** was obtained by reaction with propylamine (1 mL) using general procedure A as an off-white solid (175 mg, 0.64 mmol, 28 %). Mp 202-203 °C (*lit.* 204-206 °C)<sup>23</sup>;  $\delta_{\text{H}}$  (400 MHz, DMSO- $d_6$ ) 10.51 (1H, app. br. s., H3), 7.35-7.16 (5H, m, ArH), 6.36 (2H, s, C6-NH<sub>2</sub>), 5.06 (2H, s, H1'), 2.79 (1H, br. s., H5a), 2.57 (2H, t,  $J = 7.3$  Hz, H1''), 1.40 (2H, app. sextet,  $J = 7.3$  Hz, H2''), 0.85 (3H, t,  $J = 7.3$  Hz, H3'');  $m/z$  (ES<sup>+</sup>) 275.15 [(M+H)<sup>+</sup>, 100%]; HRMS (ES<sup>+</sup>) [Found: (M+H)<sup>+</sup>, 275.1503, C<sub>14</sub>H<sub>19</sub>N<sub>4</sub>O<sub>2</sub> requires 275.1503].

#### 6-Amino-1-benzyl-5-(butylamino)pyrimidine-2,4(1H,3H)-dione (12d)



**12d** was obtained by reaction with <sup>n</sup>butylamine (1 mL) using general procedure A to afford the product as a white solid (153 mg, 0.53 mmol, 11 %). Mp 189-191 °C;  $\nu_{\max}$   $\text{cm}^{-1}$  (KBr) 3403 (N-H), 2963 (aromatic C-H), 1810, 1701 (C=O), 1621 (C=O), 1479, 1409, 1270, 1171, 1102 and 736;  $\delta_{\text{H}}$  (400 MHz, DMSO- $d_6$ ) 10.60 (1H, br. s., H3), 7.34-7.16 (5H, m, ArH), 6.36 (2H, s, C6-NH<sub>2</sub>), 5.06 (2H, s, H1'), 2.77 (1H, br. s., H5a), 2.60 (2H, t,  $J = 7.3$  Hz, H1''), 1.42-1.23 (4H, m, H2'', H3''), 0.84 (3H, t,  $J = 7.2$  Hz, H4'');  $\delta_{\text{C}}$  (100 MHz, DMSO- $d_6$ ) 161.3 (C4), 152.6 (C2), 150.5 (C6), 137.1 (C1'a), 128.8 (C2'), 127.4 (C4'), 126.6 (C3'), 97.9 (C5), 48.3 (C1''), 44.6 (C1'), 32.2 (C2''), 20.4 (C3''), 14.4 (C4'');  $m/z$  (ES<sup>+</sup>) 311.17 [(M+Na)<sup>+</sup>, 100%]; HRMS (EI<sup>+</sup>) [Found: M, 288.1583, C<sub>15</sub>H<sub>20</sub>N<sub>4</sub>O<sub>2</sub> requires 288.1586].

#### 6-Amino-1-benzyl-5-(isobutylamino)pyrimidine-2,4(1H,3H)-dione (12e)



**12e** was obtained by reaction with *i*-butylamine (1 mL) using general procedure A to afford the product as a white solid (142 mg, 0.49 mmol, 8 %). Mp 206-207 °C;  $\nu_{\max}$   $\text{cm}^{-1}$  (KBr) 3401 (N-H), 2921 (aromatic C-H), 1807, 1697 (C=O), 1619 (C=O), 1408, 1260, 1158, 1092 and 758;  $\delta_{\text{H}}$  (400 MHz, DMSO- $d_6$ ) 9.50 (1H, br. s., H3), 7.35-7.17 (5H, m, ArH), 6.30 (2H, s, C6-NH<sub>2</sub>), 5.05 (2H, s, H1'), 2.76 (1H, br. s., H5a), 2.45-2.40 (2H, m, H1''), 1.64 (1H, app. septet,  $J = 6.7$  Hz, H2''), 0.87 (6H, d,  $J = 6.7$  Hz, H3'');  $\delta_{\text{C}}$  (100 MHz, DMSO- $d_6$ ) 160.9 (C4), 151.8 (C2), 150.1 (C6), 136.7 (C1'a), 128.4 (C2'), 127.0 (C4'), 126.3 (C3'), 97.9 (C5), 56.4 (C1''), 48.8 (C1'), 28.3 (C2''), 20.7 (C3'');  $m/z$  (ES<sup>+</sup>) 289.23 [(M+H)<sup>+</sup>, 100%]; HRMS (EI<sup>+</sup>) [Found: M, 288.1586, C<sub>15</sub>H<sub>20</sub>N<sub>4</sub>O<sub>2</sub> requires 288.1586].

### Synthesis of 8a-8f

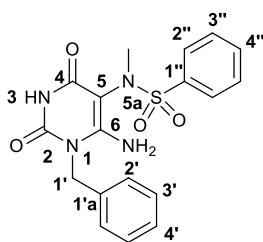
#### General procedure B - Synthesis of analogs 8a, 8f, 15a and

To a stirred solution of amine **12a** or **14** (1 equiv.) in dry DCM (7 mL per mmol) was added pyridine (5 equiv.) followed by the acyl or sulfonyl chloride (1.5 equiv.). The resulting yellow solution was stirred at rt for 16 hours. The solvent was removed *in vacuo* and water (7 mL per mmol) added to the resulting residue followed by addition of 1M HCl until the pH was determined to be acidic. The resulting solid precipitate was filtered and recrystallised from ethanol.

#### General Procedure C - Preparation of Sulfonamides 8b-e

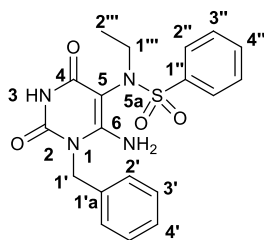
To a stirred solution of amine **12b-e** (0.47 mmol, 1.0 equiv.) in dry dichloromethane (4 mL) was added pyridine (187 mg, 190  $\mu\text{L}$ , 2.36 mmol, 5.0 equiv.) followed by benzene sulfonyl chloride (125 mg, 90  $\mu\text{L}$ , 0.71 mmol, 1.5 equiv.). The resulting yellow solution was stirred at rt for 16 hours. The solvent was removed *in vacuo* and water added to the residue. 1M HCl was added until the pH was determined to be acidic. The water was decanted and the residues recrystallised from ethanol.

#### *N*-(6-Amino-1-benzyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)-*N*-methylbenzene sulfonamide (8a)



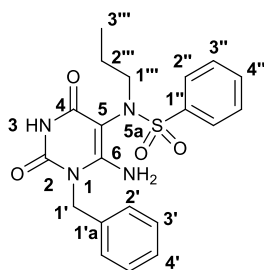
**8a** was obtained following reaction of amine **12a** (80 mg, 0.33 mmol) and benzene sulfonyl chloride (86.5 mg, 62.5  $\mu\text{L}$ , 0.49 mmol) using general procedure B. The product was obtained as a light yellow crystalline solid (83 mg, 0.21 mmol, 65 %). Mp 206-208  $^{\circ}\text{C}$ ;  $\nu_{\text{max}}$   $\text{cm}^{-1}$  (KBr) 3402 (N-H), 3067 (aromatic C-H), 2801, 1705 (C=O), 1622 (C=O), 1567 (N-H), 1501, 1450 (C-N), 1331, 1156 (S=O), 1052 (S=O) and 734 (S-N);  $\delta_{\text{H}}$  (400 MHz,  $\text{DMSO-}d_6$ ) 10.61 (1H, s, H3), 7.76-7.73 (2H, m, H2''), 7.63-7.56 (3H, m, H3'', H4''), 7.38-7.18 (5H, m, H2', H3', H4'), 7.00 (2H, s, C6-NH<sub>2</sub>), 5.17-5.02 (2H, m, AB system, H1'), 3.04 (3H, s, N5a-CH<sub>3</sub>);  $\delta_{\text{C}}$  (100 MHz,  $\text{DMSO-}d_6$ ) 158.7 (C4), 155.7 (C2), 149.9 (C6), 139.2 (C1''), 136.2 (C1'a), 132.4 (C2''), 128.5 (C4''), 128.4 (C2'), 127.7 (C3''), 127.1 (C4'), 126.1 (C3'), 91.0 (C5), 44.5 (C1'), 36.9 (NCH<sub>3</sub>);  $m/z$  (ES<sup>+</sup>) 409.17 [(M+Na)<sup>+</sup>, 100%]; HRMS (ES<sup>+</sup>) [Found: (M+Na)<sup>+</sup>, 409.0946, C<sub>18</sub>H<sub>18</sub>N<sub>4</sub>O<sub>4</sub>NaS requires 409.0946].

***N*-(6-Amino-1-benzyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)-*N*-ethylbenzene sulfonamide (8b)**



**8b** was obtained by reaction of amine **12b** using general procedure C as a yellow solid (101 mg, 0.25 mmol, 54 %). Mp 169-172  $^{\circ}\text{C}$ ;  $\nu_{\text{max}}$   $\text{cm}^{-1}$  (KBr) 3436 (N-H), 3333 (aromatic C-H), 2977, 1806, 1714 (C=O), 1630 (C=O), 1567 (N-H), 1500, 1451 (C-N), 1318, 1155 (S=O), 1053 (S=O) and 732 (S-N);  $\delta_{\text{H}}$  (400 MHz,  $\text{DMSO-}d_6$ ) 10.62 (1H, br. s., H3), 7.75-7.15 (10H, m, ArH), 6.96 (2H, s, C6-NH<sub>2</sub>), 5.20-5.04 (2H, m, AB system, H1'), 3.62-3.25 (2H, m, AB system, H1'''), 1.00 (3H, t,  $J = 7.0$  Hz, H2'');  $\delta_{\text{C}}$  (100 MHz,  $\text{DMSO-}d_6$ ) 159.1 (C4), 157.4 (C2), 150.4 (C6), 140.0 (C1''), 136.6 (C1'a), 132.7 (C2''), 128.9 (C4''), 128.8 (C2'), 127.9 (C3''), 127.4 (C4'), 126.2 (C3'), 87.9 (C5), 44.8 (C1'), 43.4 (C1'''), 13.5 (C2''');  $m/z$  (ES<sup>+</sup>) 401.13 [(M+H)<sup>+</sup>, 100%]; HRMS (ES<sup>+</sup>) [Found: (M+H)<sup>+</sup>, 401.1279, C<sub>19</sub>H<sub>21</sub>N<sub>4</sub>O<sub>2</sub>S requires 401.1278].

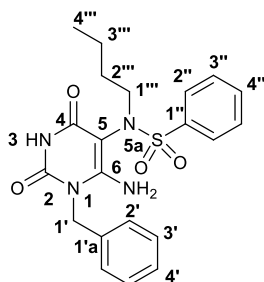
***N*-(6-Amino-1-benzyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)-*N*-propylbenzene sulfonamide (8c)**



**8c** was obtained by reaction of amine **12c** using general procedure C as a light yellow solid (106 mg, 0.26 mmol, 54 %). Mp 194-196  $^{\circ}\text{C}$ ;  $\nu_{\text{max}}$   $\text{cm}^{-1}$  (KBr) 3480 (N-H), 3367 (aromatic C-H), 2968, 1803, 1718 (C=O), 1617 (C=O), 1567 (N-H), 1498, 1452 (C-N), 1325, 1156 (S=O), 1052 (S=O) and 734 (S-N);  $\delta_{\text{H}}$  (400 MHz,  $\text{DMSO-}d_6$ ) 10.63 (1H, s, H3), 7.75-7.15 (10H, m, ArH), 6.91 (2H, s, C6-NH<sub>2</sub>), 5.20-5.06 (2H, m, AB system, H1'), 3.51-3.59 (1H, m, AB system, H1'''), 3.20-3.29 (1H, m, AB system, H1'''), 1.60-1.29 (2H, m, H2'''), 0.82 (3H, t,  $J = 7.4$  Hz, H3''');  $\delta_{\text{C}}$  (100 MHz,  $\text{DMSO-}d_6$ ) 159.0 (C4), 157.0 (C2), 150.3 (C6), 139.8 (C1''), 136.6 (C1'a), 132.8 (C2''), 128.9 (C4''), 128.8 (C2'), 128.0 (C3''), 127.5 (C4'), 126.3

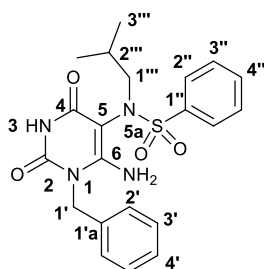
(C3'), 88.8 (C5), 50.6 (C1'''), 44.9 (C1'), 21.6 (C2'''), 11.7 (C3''');  $m/z$  (ES<sup>+</sup>) 415.14 [(M+H)<sup>+</sup>, 100%]; HRMS (ES<sup>+</sup>) [Found: (M+H)<sup>+</sup>, 415.1436, C<sub>20</sub>H<sub>23</sub>N<sub>4</sub>O<sub>2</sub>S requires 415.1435].

***N*-(6-Amino-1-benzyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)-*N*-butylbenzene sulfonamide (8d)**



**8d** was obtained by reaction of amine **12d** using general procedure C as a light yellow solid (121 mg, 0.28 mmol, 60 %). Mp 206-208 °C;  $\nu_{\max}$  cm<sup>-1</sup> (KBr) 3448 (N-H), 3355, 3064 (aromatic C-H), 2814, 1711 (C=O), 1623 (C=O), 1570 (N-H), 1493, 1454 (C-N), 1322, 1156 (S=O), 1056 (S=O) and 733 (S-N);  $\delta_{\text{H}}$  (400 MHz, DMSO-*d*<sub>6</sub>) 10.63 (1H, s, H3), 7.75-7.15 (10H, m, ArH), 6.91 (2H, s, C6-NH<sub>2</sub>), 5.21-5.05 (2H, m, AB system, H1'), 3.65-3.56 (1H, m, AB system, H1'''a), 3.32-3.23 (1H, m, AB system, H1'''b), 1.54-1.42 (1H, m, AB system, H2'''a), 1.39-1.15 (3H, m, H2'''b, H3''') 0.81 (3H, t,  $J$  = 7.1 Hz, H4''');  $\delta_{\text{C}}$  (100 MHz, DMSO-*d*<sub>6</sub>) 158.6 (C4), 156.7 (C2), 149.9 (C6), 139.5 (C1''), 136.2 (C1'a), 132.4 (C2''), 128.5 (C4''), 128.4 (C2'), 127.6 (C3''), 127.1 (C4'), 125.9 (C3'), 88.3 (C5), 48.1 (C1'''), 44.4 (C1'), 30.0 (C2'''), 19.5 (C3'''), 13.8 (C4''');  $m/z$  (ES<sup>+</sup>) 429.16 [(M+H)<sup>+</sup>, 100%]; HRMS (ES<sup>+</sup>) [Found: (M+H)<sup>+</sup>, 429.1594, C<sub>21</sub>H<sub>25</sub>N<sub>4</sub>O<sub>2</sub>S requires 429.1591].

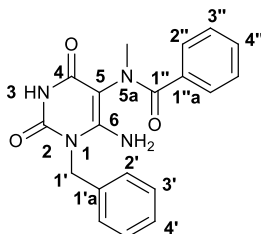
***N*-(6-Amino-1-benzyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)-*N*-isobutylbenzene sulfonamide (8e)**



**8e** was obtained by reaction of amine **12e** using general procedure C as a yellow solid (103 mg, 0.24 mmol, 51 %). Mp 184-186 °C;  $\nu_{\max}$  cm<sup>-1</sup> (KBr) 3448 (N-H), 3355, 3064 (aromatic C-H), 2814, 1710 (C=O), 1621 (C=O), 1572 (N-H), 1491, 1456 (C-N), 1328, 1153 (S=O), 1037 (S=O) and 738 (S-N);  $\delta_{\text{H}}$  (400 MHz, DMSO-*d*<sub>6</sub>) 10.64 (1H, s, H3), 7.77-7.14 (10H, m, ArH), 6.85 (2H, s, C6-NH<sub>2</sub>), 5.21-5.07 (2H, m, AB system, H1'), 3.43-3.34 (1H, m, AB system, H1'''a), 3.24-3.18 (1H, m, AB system, H1'''b), 1.69-1.55 (1H, m, C2'''), 0.92 (3H, d,  $J$  = 6.5 Hz, H3'''a), 0.79 (3H, d,  $J$  = 6.5 Hz, H3'''b);  $\delta_{\text{C}}$  (100 MHz, DMSO-*d*<sub>6</sub>) 158.3 (C4), 156.3 (C2), 149.8 (C6), 139.0 (C1''), 136.2 (C1'a), 132.5 (C2''), 128.6 (C4''), 128.4 (C2'), 127.1 (C4'), 127.0 (C3''), 125.9 (C3'), 89.4 (C5), 56.4.1 (C1'''), 44.6 (C1'), 27.3 (C2'''), 20.8 (C3'''), 20.5

(CH<sub>2</sub>CH(C<sub>2</sub>H<sub>5</sub>))<sub>2</sub>; *m/z* (ES<sup>+</sup>) 429.19 [(M+H)<sup>+</sup>, 100%]; HRMS (ES<sup>+</sup>) [Found: (M+H)<sup>+</sup>, 429.1593, C<sub>21</sub>H<sub>25</sub>N<sub>4</sub>O<sub>2</sub>S requires 429.1591].

### ***N*-(6-Amino-1-benzyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)-*N*-methylbenzamide (8f)**



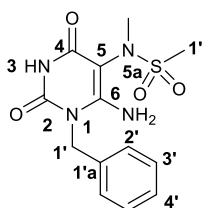
**8f** was obtained following reaction of amine **12a** (100 mg, 0.41 mmol) and benzoyl chloride (85 mg, 70.5  $\mu$ L, 0.61 mmol) using general procedure B. The product was obtained as a light yellow solid (105 mg, 0.30 mmol, 73 %). Mp 317-319 °C;  $\nu_{\max}$  cm<sup>-1</sup> (KBr) 3400 (N-H), 3030 (aromatic C-H), 2807, 1706 (C=O), 1647 (C=O), 1501, 1436 (C-N), 1333, 1097, 807 and 697;  $\delta_{\text{H}}$  (400 MHz, DMSO-*d*<sub>6</sub>) 10.62 (1H, s, H3), 7.42-7.20 (10H, m, ArH), 7.77 (2H, app. dd., *J* = 1.9 Hz, 6.0 Hz, C6-NH<sub>2</sub>), 5.14-4.84 (2H, m, AB system, H1'), 2.98 (3H, s, N5a-CH<sub>3</sub>);  $\delta_{\text{C}}$  (100 MHz, DMSO-*d*<sub>6</sub>) 173.3 (C1''), 159.7 (C4), 152.6 (C2), 150.2 (C6), 137.9 (C1''a), 136.5 (C1'a), 129.3 (C2''), 128.7 (C2'), 127.6 (C4''), 127.2 (C4'), 125.8 (C3'), 125.7 (C3''), 96.5 (C5), 44.4 (C1'), 34.7 (NCH<sub>3</sub>); *m/z* (ES<sup>+</sup>) 351.15 [(M+H)<sup>+</sup>, 100%]; HRMS (ES<sup>+</sup>) [Found: (M+H)<sup>+</sup>, 351.1455, C<sub>19</sub>H<sub>19</sub>N<sub>4</sub>O<sub>3</sub> requires 351.1452].

### **Synthesis of sulfonamide analogs 8g-8w**

#### **General Procedure D - Parallel Synthesis of Sulfonamides 8g-8w**

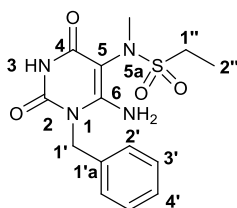
To a stirred solution of **12a** (100 mg, 0.41 mmol, 1.0 equiv.) in dry DCM (3 mL) was added pyridine (161 mg, 164  $\mu$ L, 2.03 mmol, 5.0 equiv.) followed by the corresponding sulfonyl chloride (0.61 mmol, 1.5 equiv.). The resulting solution was stirred at rt for 16 hours. The solvent was removed by blowing air through the tubes in the Greenhouse apparatus to afford residues. Water (3 mL) added to the residue and 1M HCl was added until the pH was determined to be acidic. The water was decanted and the residues recrystallised from ethanol to afford the products.

### ***N*-(6-Amino-1-benzyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)-*N*-methylmethane sulfonamide (8g)**



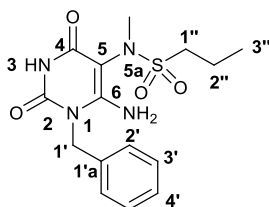
**8g** was obtained by reaction of amine **12a** with methanesulfonyl chloride (70 mg, 47  $\mu$ L, 0.61 mmol) using general procedure D to afford the product as a light yellow solid (105 mg, 0.32 mmol, 79 %). Mp 244-246  $^{\circ}$ C;  $\nu_{\max}$   $\text{cm}^{-1}$  (KBr) 3419 (N-H), 3300, 3034 (aromatic C-H), 2815, 1719 (C=O), 1620 (C=O), 1568 (N-H), 1507, 1449 (C-N), 1319, 1142 (S=O), 1056 (S=O), 774 (S-N) and 743 (S-N);  $\delta_{\text{H}}$  (400 MHz, DMSO- $d_6$ ) 10.93 (1H, s, H3), 7.44-7.23 (5H, m, ArH), 7.09 (2H, s, C6-NH<sub>2</sub>), 5.20-5.12 (2H, m, AB system, H1'), 3.08 (3H, s, H1''), 3.08 (3H, s, N5a-CH<sub>3</sub>);  $\delta_{\text{C}}$  (100 MHz, DMSO- $d_6$ ) 159.6 (C4), 155.7 (C2), 149.9 (C6), 136.1 (C1'a), 128.4 (C2'), 127.0 (C4'), 126.4 (C3'), 91.8 (C5), 44.4 (C1'), 38.5 (NCH<sub>3</sub>), 36.5 (C1'');  $m/z$  (ES<sup>+</sup>) 346.95 [(M+Na)<sup>+</sup>, 100%]; HRMS (ES<sup>+</sup>) [Found: (M+Na)<sup>+</sup>, 347.0784, C<sub>13</sub>H<sub>16</sub>N<sub>4</sub>O<sub>4</sub>NaS requires 347.0790].

***N*-(6-Amino-1-benzyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)-*N*-methylethane sulfonamide (8h)**



**8h** was obtained by reaction of amine **12a** with ethanesulfonyl chloride (78 mg, 58  $\mu$ L, 0.61 mmol) using general procedure D to afford the product as a light yellow solid (102 mg, 0.30 mmol, 74 %). Mp 241-243  $^{\circ}$ C;  $\nu_{\max}$   $\text{cm}^{-1}$  (KBr) 3418 (N-H), 3306, 3036 (aromatic C-H), 1717 (C=O), 1622 (C=O), 1566 (N-H), 1506, 1451 (C-N), 1317, 1139 (S=O), 1052 (S=O), 776 (S-N) and 736 (S-N);  $\delta_{\text{H}}$  (400 MHz, DMSO- $d_6$ ) 10.85 (1H, s, H3), 7.36-7.16 (5H, m, ArH), 7.02 (2H, s, C6-NH<sub>2</sub>), 5.13-5.03 (2H, m, AB system, H1'), 3.27-3.05 (2H, m, H1''), 3.02 (3H, s, N5a-CH<sub>3</sub>), 1.26 (3H, t,  $J$  = 7.4 Hz, H2'');  $\delta_{\text{C}}$  (100 MHz, DMSO- $d_6$ ) 159.7 (C4), 155.7 (C2), 149.9 (C6), 136.2 (C1'a), 128.4 (C2'), 127.1 (C4'), 126.2 (C3'), 91.4 (C5), 47.2 (C1''), 44.4 (C1'), 36.8 (NCH<sub>3</sub>), 7.8 (C2'');  $m/z$  (ES<sup>+</sup>) 360.98 [(M+Na)<sup>+</sup>, 100%]; HRMS (ES<sup>+</sup>) [Found: (M+Na)<sup>+</sup>, 361.0940, C<sub>14</sub>H<sub>18</sub>N<sub>4</sub>O<sub>4</sub>NaS requires 361.0946].

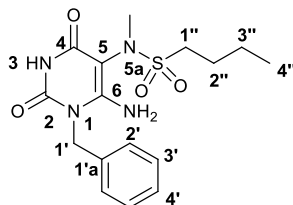
***N*-(6-Amino-1-benzyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)-*N*-methylpropane-1-sulfonamide (8i)**



**8i** was obtained by reaction of amine **12a** with 1-propanesulfonyl chloride (87 mg, 69  $\mu$ L, 0.61 mmol) using general procedure D to afford the product as a yellow solid (91 mg, 0.26 mmol, 64 %). Mp 239-241  $^{\circ}$ C;  $\nu_{\max}$   $\text{cm}^{-1}$  (KBr) 3425 (N-H), 3242, 3037 (aromatic C-H), 2812, 1721 (C=O), 1636 (C=O), 1562 (N-H), 1505, 1450 (C-N), 1389, 1137 (S=O), 1054 (S=O), 782 (S-N) and 731 (S-N);  $\delta_{\text{H}}$  (400 MHz, DMSO- $d_6$ ) 10.84 (1H, s, H3), 7.35-7.16 (5H, m, ArH), 7.02 (2H, s, C6-NH<sub>2</sub>), 5.13-5.04 (2H, m, AB system, H1'), 3.22-3.05 (2H, m, H1''), 3.02 (3H, s, N5a-CH<sub>3</sub>), 1.86-1.65 (2H, m, H2''), 0.96 (3H, t,  $J$  = 7.5 Hz, H3'');  $\delta_{\text{C}}$

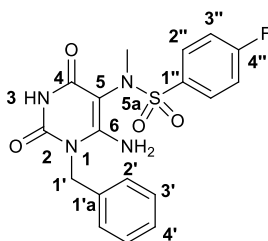
(100 MHz, DMSO-*d*<sub>6</sub>) 159.7 (C4), 155.7 (C2), 149.9 (C6), 136.2 (C1'a), 128.4 (C2'), 127.1 (C4'), 126.2 (C3'), 91.5 (C5), 52.9 (C1''), 44.5 (C1'), 36.7 (NCH<sub>3</sub>), 16.7 (C2''), 13.0 (C3''); *m/z* (ES<sup>+</sup>) 374.99 [(M+Na)<sup>+</sup>, 100%]; HRMS (ES<sup>+</sup>) [Found: (M+Na)<sup>+</sup>, 375.1102, C<sub>15</sub>H<sub>20</sub>N<sub>4</sub>O<sub>4</sub>NaS requires 375.1103].

***N*-(6-Amino-1-benzyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)-*N*-methylbutane-1-sulfonamide (8j)**



**8j** was obtained by reaction of amine **12a** with 1-butanefulfonyl chloride (96 mg, 80  $\mu$ L, 0.61 mmol) using general procedure C to afford the product as a bright yellow solid (92 mg, 0.26 mmol, 61 %). Mp 221-223 °C;  $\nu_{\max}$  cm<sup>-1</sup> (KBr) 3435 (N-H), 3249, 3036 (aromatic C-H), 1717 (C=O), 1621 (C=O), 1564 (N-H), 1501, 1448 (C-N), 1314, 1136 (S=O), 1054 (S=O), 789 (S-N) and 723 (S-N);  $\delta_{\text{H}}$  (400 MHz, DMSO-*d*<sub>6</sub>) 10.92 (1H, s, H3), 7.43-7.24 (5H, m, ArH), 7.09 (2H, s, C6-NH<sub>2</sub>), 5.20-5.11 (2H, m, AB system, H1'), 3.34-3.15 (2H, m, H1''), 3.09 (3H, s, N5a-CH<sub>3</sub>), 1.90-1.65 (2H, m, H2''), 1.44 (2H, app. sextet, *J* = 7.3 Hz, H3''), 0.95 (3H, t, *J* = 7.3 Hz, H4'');  $\delta_{\text{C}}$  (100 MHz, DMSO-*d*<sub>6</sub>) 159.7 (C4), 155.7 (C2), 149.9 (C6), 136.2 (C1'a), 128.4 (C2'), 127.1 (C4'), 126.2 (C3'), 91.5 (C5), 50.9 (C1''), 44.5 (C1'), 36.7 (NCH<sub>3</sub>), 24.9 (C2''), 21.2 (C3''), 13.5 (C4''); *m/z* (ES<sup>+</sup>) 367.14 [(M+H)<sup>+</sup>, 100%]; HRMS (ES<sup>+</sup>) [Found: (M+H)<sup>+</sup>, 367.1439, C<sub>16</sub>H<sub>23</sub>N<sub>4</sub>O<sub>4</sub>S requires 367.1435].

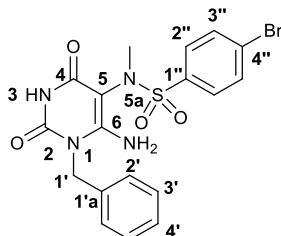
***N*-(6-Amino-1-benzyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)-4-fluoro-*N*-methylbenzene sulfonamide (8k)**



**8k** was obtained by reaction of amine **12a** with 4-fluorobenzene sulfonyl chloride (119 mg, 0.61 mmol) using general procedure D to afford the product as a light yellow solid (135 mg, 0.33 mmol, 80 %). Mp 221-223 °C;  $\nu_{\max}$  cm<sup>-1</sup> (KBr) 3459 (N-H), 3336, 3032 (aromatic C-H), 2808, 1715 (C=O), 1621 (C=O), 1567 (N-H), 1498, 1454 (C-N), 1339, 1154 (S=O), 1056 (S=O), 834 and 740 (S-N);  $\delta_{\text{H}}$  (400 MHz, DMSO-*d*<sub>6</sub>) 10.64 (1H, s, H3), 7.84-7.78 (2H, m, H2''), 7.39-7.18 (7H, m, ArH), 7.07 (2H, s, C6-NH<sub>2</sub>), 5.15-5.06 (2H, m, AB system, H1'), 3.06 (3H, s, N5a-CH<sub>3</sub>);  $\delta_{\text{C}}$  (100 MHz, DMSO-*d*<sub>6</sub>) 164.7 (d, *J* = 252.3 Hz, C4''), 159.1 (C4), 156.1 (C2), 150.3 (C6), 136.6 (C1'a), 135.9 (d, *J* = 2.8 Hz, C1''), 131.1 (d, *J* = 9.6 Hz, C2''), 128.8 (C2'), 127.5 (C4'), 126.5 (C3'), 116.0 (d, *J* = 23.1 Hz, C3''), 91.2 (C5), 44.8 (C1'), 37.4 (NCH<sub>3</sub>);  $\delta_{\text{F}}$

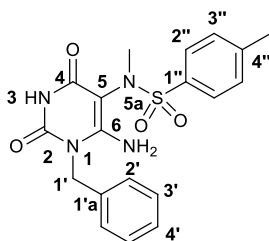
(376 MHz, DMSO- $d_6$ ) -107.93 (1F, m);  $\delta_{F\{H\}}$  (376 MHz, DMSO- $d_6$ ) -107.93 (1F, s);  $m/z$  (ES<sup>+</sup>) 426.93 [(M+Na)<sup>+</sup>, 100%]; HRMS (ES<sup>+</sup>) [Found: (M+Na)<sup>+</sup>, 427.0851, C<sub>18</sub>H<sub>17</sub>N<sub>4</sub>O<sub>4</sub>NaFS requires 427.0852].

***N*-(6-Amino-1-benzyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)-4-bromo-*N*-methylbenzene sulfonamide (8l)**



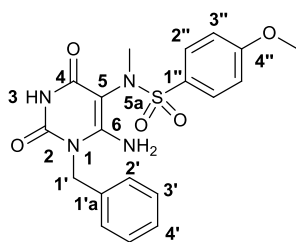
**8l** was obtained by reaction of amine **12a** with 4-bromobenzene sulfonyl chloride (156 mg, 0.61 mmol) using general procedure D to afford the product as a bright yellow solid (103 mg, 0.22 mmol, 54 %). Mp 223-224 °C;  $\nu_{\max}$  cm<sup>-1</sup> (KBr) 3439 (N-H), 3334, 3095 (aromatic C-H), 2809, 1714 (C=O), 1620 (C=O), 1569 (N-H), 1499, 1452 (C-N), 1335, 1155 (S=O), 1066 (S=O), 818 and 747 (S-N);  $\delta_H$  (400 MHz, DMSO- $d_6$ ) 10.65 (1H, s, H3), 7.71 (2H, d,  $J$  = 8.8 Hz, H2''), 7.65 (2H, d,  $J$  = 8.8 Hz, H3''), 7.39-7.17 (5H, m, ArH), 7.09 (2H, s, C6-NH<sub>2</sub>), 5.13-5.04 (2H, m, AB System, H1'), 3.05 (3H, s, N5a-CH<sub>3</sub>);  $\delta_C$  (100 MHz, DMSO- $d_6$ ) 158.7 (C4), 155.7 (C2), 149.9 (C6), 138.5 (C1''), 136.2 (C1'a), 131.6 (C3''), 129.7 (C2''), 128.4 (C2'), 127.1 (C4'), 126.3 (C4''), 126.1 (C3'), 90.6 (C5), 44.4 (C1'), 37.0 (NCH<sub>3</sub>);  $m/z$  (ES<sup>+</sup>) 465.02 [(M+H)<sup>+</sup>, 100%]; HRMS (ES<sup>+</sup>) [Found: (M+H)<sup>+</sup>, 465.0226, C<sub>18</sub>H<sub>18</sub>N<sub>4</sub>O<sub>4</sub><sup>79</sup>BrS requires 465.0227].

***N*-(6-Amino-1-benzyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)-*N*,4-dimethylbenzene sulfonamide (8m)**



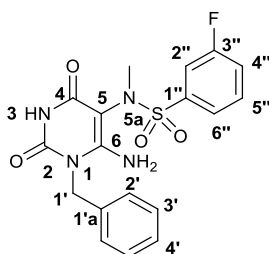
**8m** was obtained by reaction of amine **12a** with *p*-toluenesulfonyl chloride (116 mg, 0.61 mmol) using general procedure C to afford the product as a yellow solid (94 mg, 0.24 mmol, 59 %). Mp 226-227 °C;  $\nu_{\max}$  cm<sup>-1</sup> (KBr) 3441 (N-H), 3380, 3012 (aromatic C-H), 2810, 1712 (C=O), 1623 (C=O), 1565 (N-H), 1499, 1451 (C-N), 1330, 1155 (S=O), 1051 (S=O), 897, 813 and 738 (S-N);  $\delta_H$  (400 MHz, DMSO- $d_6$ ) 10.62 (1H, s, H3), 7.63 (2H, d,  $J$  = 8.2 Hz, H2''), 7.37-7.18 (7H, m, ArH), 6.97 (2H, s, C6-NH<sub>2</sub>), 5.13-5.05 (2H, m, AB system, H1'), 3.02 (3H, s, N5a-CH<sub>3</sub>), 2.35 (3H, s, C4''-CH<sub>3</sub>);  $\delta_C$  (100 MHz, DMSO- $d_6$ ) 158.7 (C4), 155.7 (C2), 149.9 (C6), 142.5 (C4''), 136.4 (C1''), 136.2 (C1'a), 129.0 (C3''), 128.4 (C2'), 127.7 (C2''), 127.1 (C4'), 126.1 (C3'), 91.1 (C5), 44.5 (C1'), 36.8 (NCH<sub>3</sub>), 30.0 (C4''-CH<sub>3</sub>);  $m/z$  (ES<sup>+</sup>) 422.97 [(M+Na)<sup>+</sup>, 100%]; HRMS (ES<sup>+</sup>) [Found: (M+Na)<sup>+</sup>, 423.1111, C<sub>19</sub>H<sub>20</sub>N<sub>4</sub>O<sub>4</sub>NaS requires 423.1103].

***N*-(6-Amino-1-benzyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)-4-methoxy-*N*-methyl benzenesulfonamide (8n)**



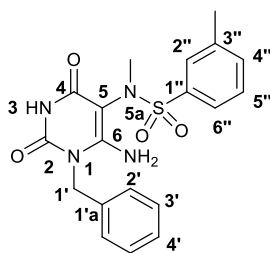
**8n** was obtained by reaction of amine **12a** with 4-methoxybenzene sulfonyl chloride (126 mg, 0.61 mmol) using general procedure D to afford the product as a light yellow solid (126 mg, 0.30 mmol, 74 %). Mp 225-226 °C;  $\nu_{\max}$   $\text{cm}^{-1}$  (KBr) 3447 (N-H), 3354, 3016 (aromatic C-H), 2818, 1713 (C=O), 1632 (C=O), 1566 (N-H), 1502, 1459 (C-N), 1310, 1146 (S=O), 1047 (S=O), 817 and 744 (S-N);  $\delta_{\text{H}}$  (400 MHz, DMSO- $d_6$ ) 10.62 (1H, s, H3), 7.67 (2H, d,  $J$  = 8.8 Hz, H2''), 7.38-7.16 (5H, m, ArH), 7.01 (2H, d,  $J$  = 8.8 Hz, H3''), 6.96 (2H, s, C6-NH<sub>2</sub>), 5.13-5.04 (2H, m, AB system, H1'), 3.81 (3H, s, OCH<sub>3</sub>), 3.02 (3H, s, N5a-CH<sub>3</sub>);  $\delta_{\text{C}}$  (100 MHz, DMSO- $d_6$ ) 162.3 (C4''), 158.7 (C4), 155.7 (C2), 149.9 (C6), 136.3 (C1'a), 130.9 (C1''), 129.8 (C2''), 128.4 (C2'), 127.0 (C4'), 126.5 (C3''), 126.1 (C3'), 91.2 (C5), 55.5 (OCH<sub>3</sub>), 44.5 (C1'), 36.8 (NCH<sub>3</sub>);  $m/z$  (ES<sup>+</sup>) 438.99 [(M+Na)<sup>+</sup>, 100%]; HRMS (ES<sup>+</sup>) [Found: (M+Na)<sup>+</sup>, 439.1055, C<sub>19</sub>H<sub>20</sub>N<sub>4</sub>O<sub>5</sub>NaS requires 439.1052].

***N*-(6-Amino-1-benzyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)-3-fluoro-*N*-methyl benzenesulfonamide (8o)**



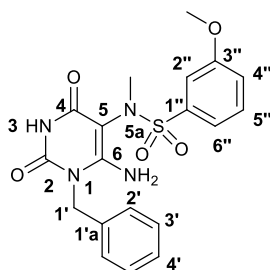
**8o** was obtained by reaction of amine **12a** with 3-fluorobenzene sulfonyl chloride (119 mg, 81  $\mu\text{L}$ , 0.61 mmol) using general procedure D to afford the product as a bright yellow solid (104 mg, 0.26 mmol, 63 %). Mp 217-219 °C;  $\nu_{\max}$   $\text{cm}^{-1}$  (KBr) 3445 (N-H), 3354, 3015 (aromatic C-H), 2805, 1714 (C=O), 1630 (C=O), 1563 (N-H), 1500, 1459 (C-N), 1314, 1146 (S=O), 1046 (S=O), 815 and 743 (S-N);  $\delta_{\text{H}}$  (400 MHz, DMSO- $d_6$ ) 10.64 (1H, s, H3), 7.70-7.19 (7H, m, ArH), 7.09 (2H, s, C6-NH<sub>2</sub>), 7.01 (2H, d,  $J$  = 8.9 Hz, ArH), 5.13-5.04 (2H, m, AB system, H1'), 3.03 (3H, s, N5a-CH<sub>3</sub>);  $\delta_{\text{C}}$  (100 MHz, DMSO- $d_6$ ) 161.5 (d,  $J$  = 246.2 Hz, C3''), 158.8 (C4), 155.7 (C2), 149.9 (C6), 136.2 (d,  $J$  = 6.1 Hz, C1''), 130.8 (C1'a), 129.8 (d,  $J$  = 2.9 Hz, C6''), 128.5 (C2'), 127.1 (C4'), 126.1 (C3'), 119.5 (d,  $J$  = 7.9 Hz, C5''), 114.7 (d,  $J$  = 21.3 Hz, C4''), 113.7 (d,  $J$  = 24.6 Hz, C2''), 91.1 (C5), 44.6 (C1'), 36.9 (NCH<sub>3</sub>);  $\delta_{\text{F}}$  (376 MHz, DMSO- $d_6$ ) -112.32 (1F, m);  $\delta_{\text{F}\{\text{H}\}}$  (376 MHz, DMSO- $d_6$ ) -112.32 (1F, s);  $m/z$  (ES<sup>+</sup>) 427.10 [(M+H)<sup>+</sup>, 100%]; HRMS (ES<sup>+</sup>) [Found: (M+H)<sup>+</sup>, 427.0849, C<sub>18</sub>H<sub>17</sub>N<sub>4</sub>O<sub>4</sub>NaSF requires 427.0852].

***N*-(6-Amino-1-benzyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)-*N*,3-dimethylbenzene sulfonamide (8p)**



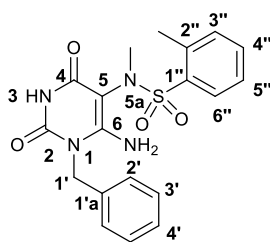
**8p** was obtained by reaction of amine **12a** with *m*-toluenesulfonyl chloride (116 mg, 89  $\mu$ L, 0.61 mmol) using general procedure D to afford the product as a light yellow solid (73 mg, 0.18 mmol, 44 %). Mp 221-222  $^{\circ}$ C;  $\nu_{\max}$   $\text{cm}^{-1}$  (KBr) 3471 (N-H), 3380, 3058 (aromatic C-H), 2811, 1712 (C=O), 1624 (C=O), 1568 (N-H), 1499, 1453 (C-N), 1328, 1151 (S=O), 1054 (S=O), 898, 800 and 741 (S-N);  $\delta_{\text{H}}$  (400 MHz, DMSO- $d_6$ ) 10.64 (1H, s, H3), 7.57-7.52 (2H, m, H2'', H6''), 7.39-7.17 (7H, m, ArH), 6.97 (2H, s, C6-NH<sub>2</sub>), 5.14-5.04 (2H, m, AB system, H1') 3.03 (3H, s, N5a-CH<sub>3</sub>), 2.35 (3H, s, CH<sub>3</sub>);  $\delta_{\text{C}}$  (100 MHz, DMSO- $d_6$ ) 158.8 (C4), 155.6 (C2), 149.9 (C6), 139.1(C1''), 137.9 (C3''), 136.2 (C1'a), 133.0 (C4''), 128.4 (C2'), 128.4 (C5''), 127.9 (C2''), 127.1 (C4'), 126.1 (C3'), 125.0 (C6''), 91.1 (C5), 44.5 (C1'), 36.8 (NCH<sub>3</sub>), 20.8 (CH<sub>3</sub>);  $m/z$  (ES<sup>+</sup>) 422.98 [(M+Na)<sup>+</sup>, 100%]; HRMS (ES<sup>+</sup>) [Found: (M+Na)<sup>+</sup>, 423.1095, C<sub>19</sub>H<sub>20</sub>N<sub>4</sub>O<sub>4</sub>NaS requires 423.1103].

***N*-(6-Amino-1-benzyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)-3-methoxy-*N*-methyl benzenesulfonamide (8q)**



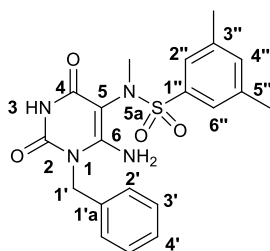
**8q** was obtained by reaction of amine **12a** with 3-methoxybenzene sulfonyl chloride (126 mg, 86  $\mu$ L, 0.61 mmol) using general procedure D to afford the product as a bright yellow solid (75 mg, 0.18 mmol, 44 %). Mp 209-211  $^{\circ}$ C;  $\nu_{\max}$   $\text{cm}^{-1}$  (KBr) 3474 (N-H), 3382, 3098 (aromatic C-H), 2807, 1713 (C=O), 1623 (C=O), 1562 (N-H), 1430 (C-N), 1324, 1251, 1151 (S=O), 1034 (S=O), 899, 865, 744 (S-N) and 706;  $\delta_{\text{H}}$  (400 MHz, DMSO- $d_6$ ) 10.67 (1H, s, H3), 7.45-7.13 (9H, m, ArH), 6.00 (2H, s, C6-NH<sub>2</sub>), 5.14-5.05 (2H, m, AB system, H1'), 3.78 (3H, s, OCH<sub>3</sub>), 3.02 (3H, s, N5a-CH<sub>3</sub>);  $\delta_{\text{C}}$  (100 MHz, DMSO- $d_6$ ) 159.0 (C3''), 158.8 (C4), 155.6 (C2), 149.9 (C6), 140.2(C1''), 136.2 (C1'a), 129.7.0 (C5''), 128.5 (C2'), 127.1 (C4'), 126.1 (C3'), 120.0 (C6''), 118.5 (C4''), 112.6 (C2''), 91.1 (C5), 55.4 (OCH<sub>3</sub>), 44.5 (C1'), 36.8 (NCH<sub>3</sub>);  $m/z$  (ES<sup>+</sup>) 438.99 [(M+Na)<sup>+</sup>, 100%]; HRMS (ES<sup>+</sup>) [Found: (M+Na)<sup>+</sup>, 439.1048, C<sub>19</sub>H<sub>20</sub>N<sub>4</sub>O<sub>5</sub>NaS requires 439.1052].

***N*-(6-Amino-1-benzyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)-*N*,2-dimethylbenzene sulfonamide (8r)**



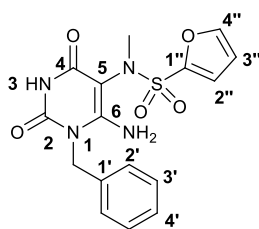
**8r** was obtained by reaction of amine **12a** with *o*-toluenesulfonyl chloride (116 mg, 88  $\mu$ L, 0.61 mmol) using general procedure D to afford the product as a light yellow solid (91 mg, 0.23 mmol, 55 %). Mp 212-213  $^{\circ}$ C;  $\nu_{\max}$   $\text{cm}^{-1}$  (KBr) 3477 (N-H), 3383, 3100 (aromatic C-H), 2805, 1714 (C=O), 1634 (C=O), 1565 (N-H), 1499, 1453 (C-N), 1326, 1156 (S=O), 1062 (S=O), 893, 806 and 746 (S-N);  $\delta_{\text{H}}$  (400 MHz, DMSO- $d_6$ ) 10.60 (1H, s, H3), 7.79 (1H, dd,  $J$  = 1.6 Hz, 6.9 Hz, H6''), 7.46-7.27 (6H, m, ArH), 7.18-7.12 (2H, m, ArH), 7.03 (2H, s, C6-NH<sub>2</sub>), 5.09 (2H, br. s, H1'), 3.10 (3H, s, N5a-CH<sub>3</sub>), 2.55 (3H, s, CH<sub>3</sub>);  $\delta_{\text{C}}$  (100 MHz, DMSO- $d_6$ ) 158.9 (C4), 155.5 (C2), 149.9 (C6), 138.1 (C1''), 137.7 (C2''), 136.2 (C1'a), 132.3 (C4''), 129.9 (C3'', C6''), 128.4 (C2'), 127.1 (C4'), 126.0 (C3'), 125.6 (C5''), 90.3 (C5), 44.4 (C1'), 37.0 (NCH<sub>3</sub>), 21.0 (CH<sub>3</sub>);  $m/z$  (ES<sup>+</sup>) 422.96 [(M+Na)<sup>+</sup>, 100%]; HRMS (ES<sup>+</sup>) [Found: (M+Na)<sup>+</sup>, 423.1113, C<sub>19</sub>H<sub>20</sub>N<sub>4</sub>O<sub>4</sub>NaS requires 423.1103].

***N*-(6-Amino-1-benzyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)-*N*,3,5-trimethylbenzenesulfonamide (8s)**



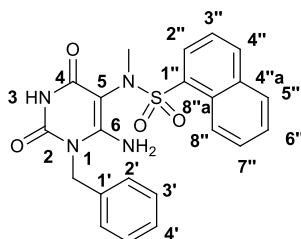
**8s** was obtained by reaction of amine **12a** with 3,5-dimethylbenzenesulfonyl chloride (125 mg, 0.61 mmol) using general procedure D to afford the product as a light yellow solid (113 mg, 0.27 mmol, 67 %). Mp 160-162  $^{\circ}$ C;  $\nu_{\max}$   $\text{cm}^{-1}$  (KBr) 3441 (N-H), 3365, 3019 (aromatic C-H), 1712 (C=O), 1622 (C=O), 1567 (N-H), 1499, 1453 (C-N), 1328, 1150 (S=O), 1051 (S=O) and 735 (S-N);  $\delta_{\text{H}}$  (400 MHz, DMSO- $d_6$ ) 10.66 (1H, s, H3), 7.38-7.18 (8H, m, ArH), 6.93 (2H, s, C6-NH<sub>2</sub>), 5.13-5.05 (2H, m, AB system, H1'), 3.02 (3H, s, N5a-CH<sub>3</sub>), 2.31 (6H, s, 2 x CH<sub>3</sub>);  $\delta_{\text{C}}$  (100 MHz, DMSO- $d_6$ ) 158.9 (C4), 155.6 (C2), 149.9 (C6), 139.0(C1''), 137.7 (C3'' or C5''), 137.5 (C3'' or C5''), 136.2 (C1'a), 133.8 (C4''), 128.4 (C2'), 127.1 (C4'), 126.1 (C3'), 125.3 (C2'', C6''), 91.3 (C5), 44.5 (C1'), 36.7 (NCH<sub>3</sub>), 20.7 (2 x CH<sub>3</sub>);  $m/z$  (ES<sup>+</sup>) 437.01 [(M+Na)<sup>+</sup>, 100%]; HRMS (ES<sup>+</sup>) [Found: (M+Na)<sup>+</sup>, 437.1258, C<sub>20</sub>H<sub>22</sub>N<sub>4</sub>O<sub>4</sub>NaS requires 437.1259].

***N*-(6-Amino-1-benzyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)-*N*-methylfuran-2-sulfonamide (8t)**



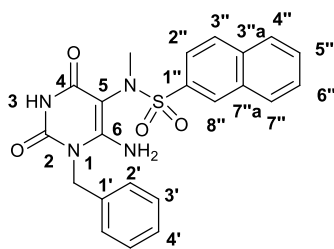
**8t** was obtained by reaction of amine **12a** with furan-2-sulfonyl chloride (102 mg, 0.61 mmol) using general procedure D to afford the product as a yellow solid (132 mg, 0.35 mmol, 85 %). Mp 215-217 °C;  $\nu_{\max}$   $\text{cm}^{-1}$  (KBr) 3448 (N-H), 3317, 3052 (aromatic C-H), 1710 (C=O), 1622 (C=O), 1571 (N-H), 1453 (C-N), 1331, 1154 (S=O), 1048 (S=O) and 773 (S-N);  $\delta_{\text{H}}$  (400 MHz, DMSO- $d_6$ ) 10.75 (1H, s, H3), 7.95 (1H, app. q.,  $J = 0.8$  Hz, H4''), 7.44-7.21 (5H, m, ArH), 7.17 (2H, s, C6-NH<sub>2</sub>), 7.07 (1H, dd,  $J = 0.8$  Hz, 2.7 Hz, H2''), 6.65 (1H, app. q.,  $J = 1.8$  Hz, H3''), 5.24-5.07 (2H, m, AB system, H1'), 3.16 (3H, s, N5a-CH<sub>3</sub>);  $\delta_{\text{C}}$  (100 MHz, DMSO- $d_6$ ) 159.1 (C4), 155.8 (C2), 150.4 (C6), 148.0 (C1''), 147.2 (C4''), 136.6 (C1'a), 128.8 (C2'), 127.5 (C4'), 126.6 (C3'), 116.5 (C2''), 111.6 (C3''), 91.0 (C5), 44.8 (C1'), 37.6 (NCH<sub>3</sub>);  $m/z$  (ES<sup>+</sup>) 399.10 [(M+Na)<sup>+</sup>, 100%]; HRMS (ES<sup>+</sup>) [Found: (M+Na)<sup>+</sup>, 399.0727, C<sub>16</sub>H<sub>16</sub>N<sub>4</sub>O<sub>5</sub>NaS requires 399.0739].

***N*-(6-Amino-1-benzyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)-*N*-methylnaphthalene-1-sulfonamide (8u)**



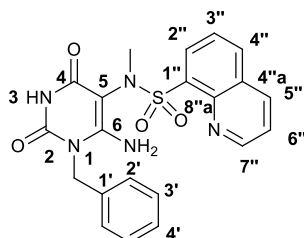
**8u** was obtained by reaction of amine **12a** with 1-naphthalene sulfonyl chloride (138 mg, 0.61 mmol) using general procedure D to afford the product as a yellow solid (152 mg, 0.35 mmol, 85 %). Mp 180-181 °C;  $\nu_{\max}$   $\text{cm}^{-1}$  (KBr) 3464 (N-H), 3344, 3033 (aromatic C-H), 2808, 1712 (C=O), 1623 (C=O), 1559 (N-H), 1499, 1453 (C-N), 1330, 1158 (S=O), 1057 (S=O), 804, 770 and 741 (S-N);  $\delta_{\text{H}}$  (400 MHz, DMSO- $d_6$ ) 10.37 (1H, s, H3), 8.64 (1H, d,  $J = 8.4$  Hz, H8''), 8.14 (2H, dd,  $J = 1.9$  Hz, 7.4 Hz, H4'', H5''), 7.98 (1H, d,  $J = 7.5$  Hz, H2''), 7.62-7.21 (8H, m, ArH), 7.06 (2H, s, C6-NH<sub>2</sub>), 5.11-5.06 (2H, m, AB system, H1'), 3.12 (3H, s, N5a-CH<sub>3</sub>);  $\delta_{\text{C}}$  (100 MHz, DMSO- $d_6$ ) 159.1 (C4), 155.8 (C2), 150.3 (C6), 136.7 (C1''), 135.7 (C1'a), 134.3 (C8''a), 134.2 (C8''), 130.0 (C5''), 129.6 (C4''a), 128.9 (C4''), 128.8 (C2'), 127.5 (CH), 127.5 (C4'), 126.7 (CH), 126.5 (C3'), 125.5 (CH), 124.8 (C2''), 91.0 (C5), 44.8 (C1'), 37.6 (NCH<sub>3</sub>);  $m/z$  (ES<sup>+</sup>) 458.96 [(M+Na)<sup>+</sup>, 100%]; HRMS (ES<sup>+</sup>) [Found: (M+Na)<sup>+</sup>, 459.1107, C<sub>22</sub>H<sub>20</sub>N<sub>4</sub>O<sub>4</sub>NaS requires 459.1103].

***N*-(6-Amino-1-benzyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)-*N*-methylnaphthalene-2-sulfonamide (8v)**



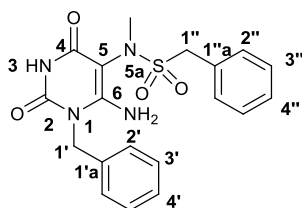
**8v** was obtained by reaction of amine **12a** with 2-naphthalene sulfonyl chloride (138 mg, 0.61 mmol) using general procedure D to afford the product as a bright yellow solid (128 mg, 0.29 mmol, 72 %). Mp 220-221 °C;  $\nu_{\max}$   $\text{cm}^{-1}$  (KBr) 3452 (N-H), 3361, 3020 (aromatic C-H), 2809, 1717 (C=O), 1628 (C=O), 1562 (N-H), 1457 (C-N), 1307, 1149 (S=O), 1070 (S=O), 816, 763 and 744 (S-N);  $\delta_{\text{H}}$  (400 MHz, DMSO- $d_6$ ) 10.59 (1H, s, H3), 8.40 (1H, d,  $J = 1.2$  Hz, H8''), 8.14 (1H, dd,  $J = 1.2$  Hz, 7.7 Hz, H3''), 8.04-7.99 (3H, m, H2'', H4'', H7''), 7.77-7.60 (2H, m, H5'', H6''), 7.40-7.20 (5H, m, H2', H3', H4'), 7.07 (2H, s, C6-NH<sub>2</sub>), 5.17-5.06 (2H, m, AB system, H1'), 3.13 (3H, s, N5a-CH<sub>3</sub>);  $\delta_{\text{C}}$  (100 MHz, DMSO- $d_6$ ) 159.1 (C4), 156.0 (C2), 150.3 (C6), 136.9 (C1''), 136.6 (C1'a), 134.7 (C3''a), 132.1 (C7''a), 129.7 (C3''), 128.8 (C4''), 128.8 (C5'' or C6''), 128.7 (C2'), 128.0 (C5'' or C6''), 127.5 (C2''), 127.4 (C4'), 126.5 (C3'), 124.2 (C8''), 91.4 (C5), 44.9 (C1'), 37.4 (NCH<sub>3</sub>);  $m/z$  (ES<sup>+</sup>) 437.13 [(M+H)<sup>+</sup>, 100%]; HRMS (ES<sup>+</sup>) [Found: (M+H)<sup>+</sup>, 437.1278, C<sub>22</sub>H<sub>21</sub>N<sub>4</sub>O<sub>4</sub>S requires 437.1278].

***N*-(6-Amino-1-benzyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)-*N*-methylquinoline-8-sulfonamide (**8w**)**



**8w** was obtained by reaction of amine **12a** with 8-quinoline sulfonyl chloride (139 mg, 0.61 mmol) using general procedure D to afford the product as a yellow solid (93 mg, 0.21 mmol, 52 %). Mp 191-193 °C;  $\nu_{\max}$   $\text{cm}^{-1}$  (KBr) 3451 (N-H), 3390, 3061 (aromatic C-H), 2812, 1718 (C=O), 1635 (C=O), 1568 (N-H), 1497, 1452 (C-N), 1323, 1160 (S=O), 1054 (S=O), 838, 790 and 740 (S-N);  $\delta_{\text{H}}$  (400 MHz, DMSO- $d_6$ ) 10.65 (1H, s, H3), 9.01 (1H, dd,  $J = 1.8$  Hz, 2.5 Hz, H7''), 8.59 (1H, dd,  $J = 1.8$  Hz, 6.6 Hz, H5''), 8.47 (1H, dd,  $J = 1.5$  Hz, 6.0 Hz, H2''), 8.34 (1H, dd,  $J = 1.3$  Hz, 6.8 Hz, H4''), 8.11 (2H, s, C6-NH<sub>2</sub>), 7.80-7.71 (2H, m, H3'', H6''), 7.40-7.17 (5H, m, H2', H3', H4'), 5.15-5.05 (2H, m, AB system, H1'), 2.92 (3H, s, NCH<sub>3</sub>);  $\delta_{\text{C}}$  (100 MHz, DMSO- $d_6$ ) 159.4 (C4), 155.7 (C2), 150.6 (C7''), 150.1 (C6), 142.9 (C8''a), 137.8 (C5''), 136.2 (C1'a), 134.8 (C1''), 134.7 (C3''), 134.5 (C2''), 128.9 (C4''a), 128.5 (C2'), 127.1 (C4'), 126.2 (C3'), 122.4 (C4'', C6''), 89.9 (C5), 44.6 (C1'), 36.4 (NCH<sub>3</sub>);  $m/z$  (ES<sup>+</sup>) 438.12 [(M+H)<sup>+</sup>, 100%]; HRMS (ES<sup>+</sup>) [Found: (M+H)<sup>+</sup>, 438.1231, C<sub>21</sub>H<sub>20</sub>N<sub>5</sub>O<sub>4</sub>S requires 438.1231].

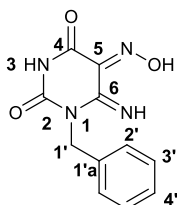
***N*-(6-Amino-1-benzyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)-*N*-methyl-1-phenylmethanesulfonamide (\*\*)**



\*\*) was obtained by reaction of amine **12a** with phenylmethane sulfonyl chloride (116 mg, 0.61 mmol) using general procedure D to afford the product as a light yellow solid (85 mg, 0.21 mmol, 52 %). Mp 235-237 °C;  $\nu_{\max}$   $\text{cm}^{-1}$  (KBr) 3440 (N-H), 3346, 3037 (aromatic C-H), 2818, 1712 (C=O), 1670 (C=O), 1619 (C=O), 1566 (N-H), 1454 (C-N), 1321, 1136 (S=O), 1043 (S=O), 777 (S-N) and 734 (S-N);  $\delta_{\text{H}}$  (400 MHz, DMSO- $d_6$ ) 10.95 (1H, s, H3), 7.48-7.20 (10H, m, ArH), 7.08 (2H, s, C6-NH<sub>2</sub>), 5.18-5.07 (2H, m, AB system, H1'), 4.62-4.46 (2H, m, AB system, H1''), 3.05 (N5a-CH<sub>3</sub>);  $\delta_{\text{C}}$  (100 MHz, DMSO- $d_6$ ) 159.9 (C4), 155.7 (C2), 150.0 (C6), 136.2 (C1'a), 131.0 (C2''), 129.9 (C1''a), 128.4 (C2'), 128.2 (C4''), 127.8 (C3''), 127.1 (C4'), 126.2 (C3'), 91.8 (C5), 56.9 (C1''), 44.5 (C1'), 37.0 (NCH<sub>3</sub>);  $m/z$  (ES<sup>+</sup>) 401.13 [(M+H)<sup>+</sup>, 100%]; HRMS (ES<sup>+</sup>) [Found: (M+H)<sup>+</sup>, 401.1280, C<sub>19</sub>H<sub>21</sub>N<sub>4</sub>O<sub>4</sub>S requires 401.1278].

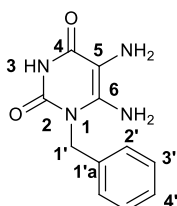
**Synthesis of sulfonamide analogs **14** and **15a,b****

**6-Amino-1-benzyl-5-(hydroxyamino)pyrimidine-2,4(1H,3H)-dione (**13**)<sup>24</sup>**



To a suspension of **11** (500 mg, 2.3 mmol, 1 equiv.) in water (5.8 mL) and acetic acid (5.8 mL) at 70 °C was added slowly a solution of sodium nitrite (317.5 mg, 4.6 mmol, 2 equiv.) in water (2 mL). The resulting solution was stirred at room temperature for 1 hour before being cooled to 4 °C. The suspension was filtered to afford the product as a bright purple solid (410 mg, 1.6 mmol, 72 %). Mp 242-244 °C (*lit.* 243-244 °C)<sup>20</sup>;  $\delta_{\text{H}}$  (400 MHz, DMSO- $d_6$ ) 13.33 (1H, br. s., C6=NH), 11.65 (1H, s, H3), 9.14 (1H, br. s., OH), 7.35-7.23 (5H, m, ArH), 5.07 (2H, s, H1');  $m/z$  (ES<sup>+</sup>) 247.08 [(M+H)<sup>+</sup>, 100%].

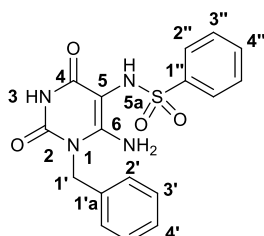
**5,6-Diamino-1-benzylpyrimidine-2,4(1H,3H)-dione (**14**)<sup>24</sup>**



To a stirred, bright purple solution of **13** (300 mg, 1.2 mmol, 1 equiv.) in aq. ammonia (4 mL) was added a solution of sodium hydrosulfite (424.5 mg, 2.4 mmol, 2 equiv.) dropwise. The resulting solution was

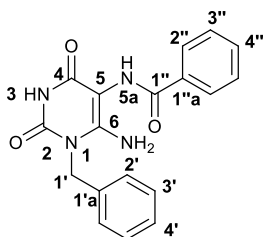
stirred at room temperature for 6 hours after which time the solution appeared colourless. The solution was cooled to 4 °C and the resulting precipitate filtered and dried to afford the product as a white solid (210 mg, 0.9 mmol, 75 %). Mp 248-251 °C (*lit.* 250-252 °C)<sup>25</sup>;  $\delta_{\text{H}}$  (400 MHz, DMSO-*d*<sub>6</sub>) 10.69 (1H, s, H3), 7.34-7.18 (5H, m, ArH), 6.09 (2H, s, C6-NH<sub>2</sub>), 5.06 (2H, s, H1'), 2.90 (2H, s, C5-NH<sub>2</sub>); *m/z* (ES<sup>+</sup>) 233.10 [(M+H)<sup>+</sup>, 100%].

***N*-(6-Amino-1-benzyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)benzenesulfonamide (15a)**

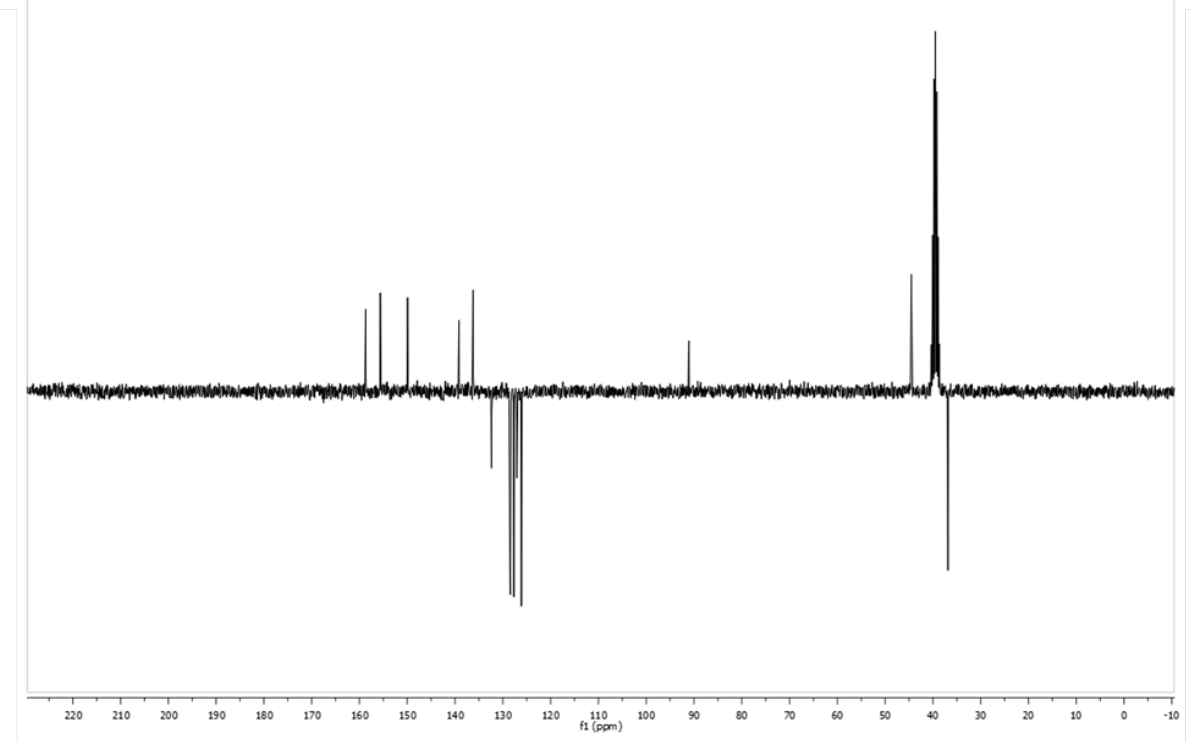
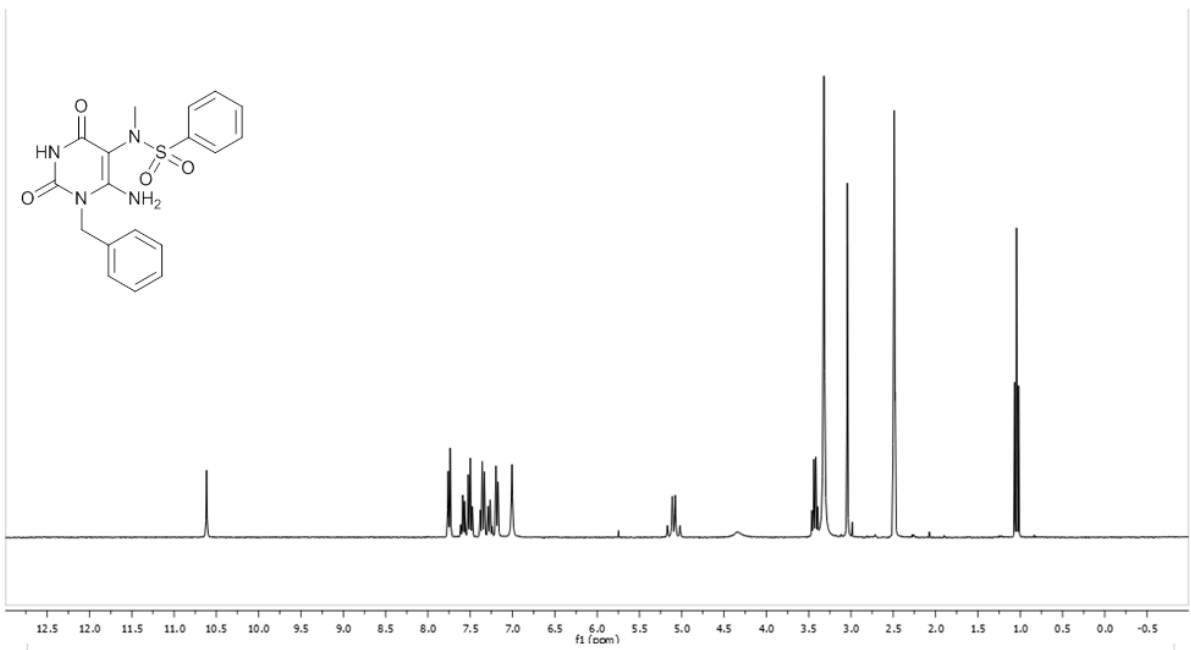


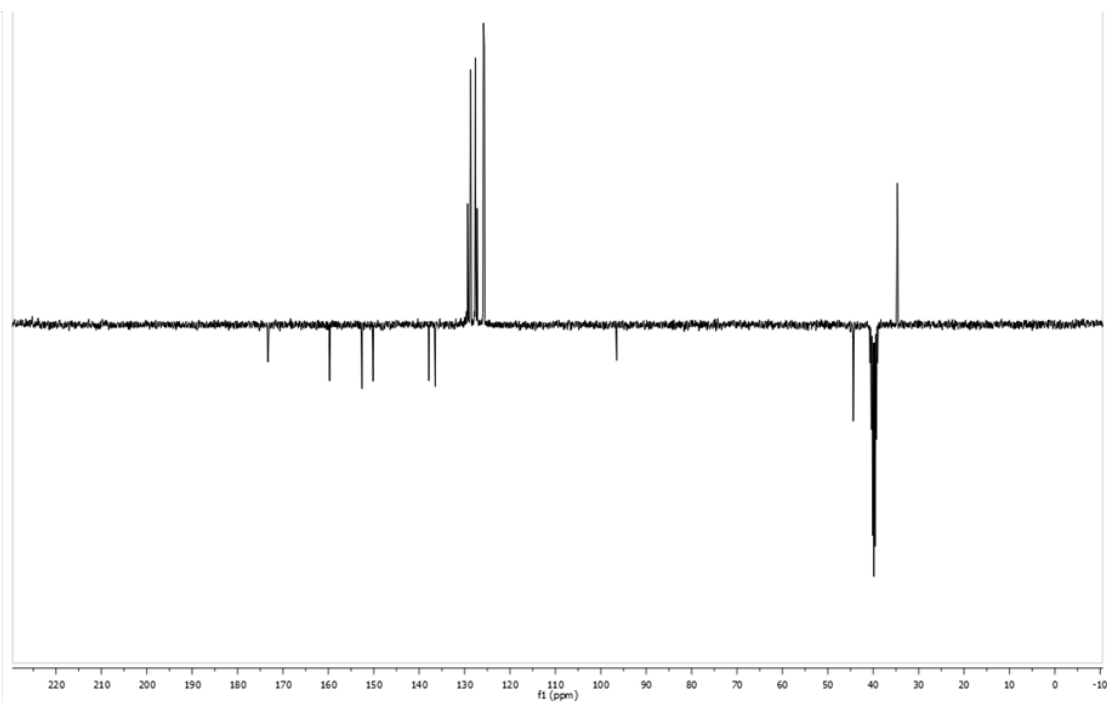
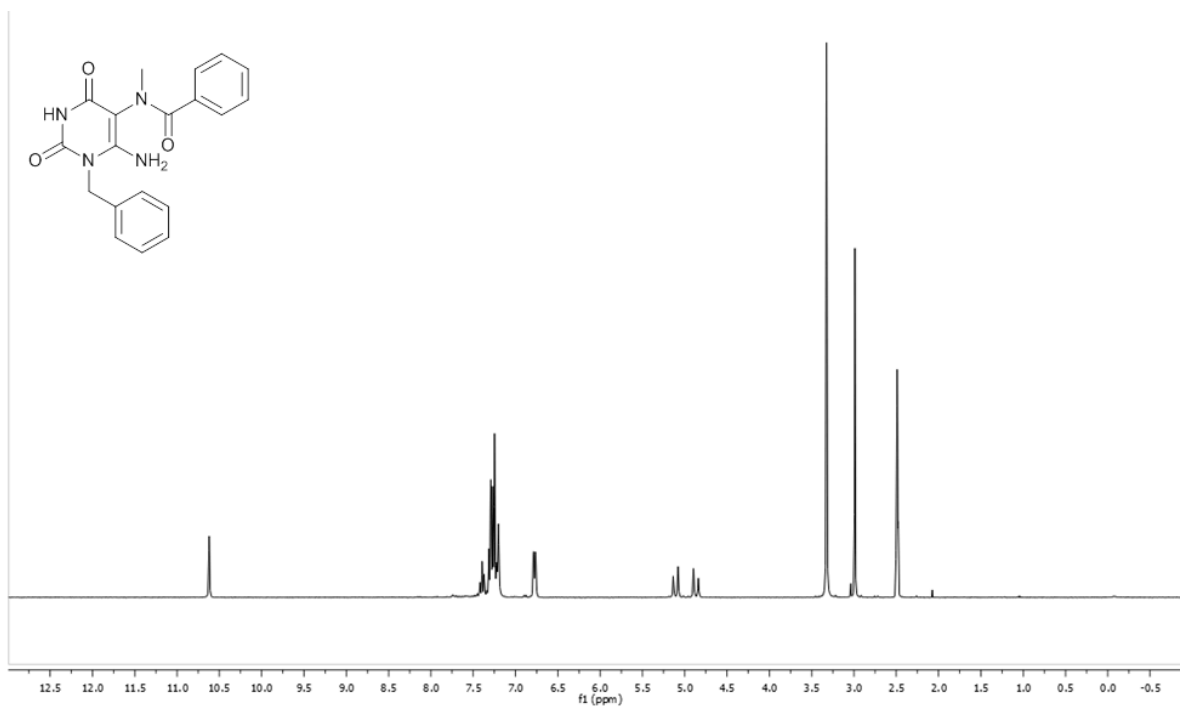
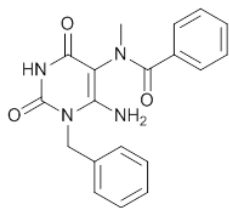
**15a** was obtained following reaction of amine **14** (100 mg, 0.43 mmol) and benzene sulfonyl chloride (115 mg, 82.5  $\mu$ L, 0.65 mmol) using general procedure B. The product was obtained as a cream solid (115 mg, 0.31 mmol, 71 %). Mp 249-250 °C;  $\nu_{\text{max}}$  cm<sup>-1</sup> (KBr) 3431 (N-H), 3063 (aromatic C-H), 2823, 1685 (C=O), 1621 (C=O), 1562 (N-H), 1451 (C-N), 1317, 1163 (S=O), 1093 (S=O) and 733 (S-N);  $\delta_{\text{H}}$  (400 MHz, DMSO-*d*<sub>6</sub>) 10.59 (1H, s, H3), 8.67 (1H, s, H5a), 7.77-7.74 (2H, m, H2''), 7.58-7.11 (8H, m, ArH), 6.75 (2H, s, C6-NH<sub>2</sub>), 5.08 (2H, s, H1');  $\delta_{\text{C}}$  (100 MHz, DMSO-*d*<sub>6</sub>) 159.2 (C4), 155.0 (C2), 149.9 (C6), 140.6 (C1''), 136.2 (C1'a), 132.2 (C2''), 128.4 (C2'), 128.3 (C4''), 127.3 (C3''), 127.1 (C4'), 126.1 (C3'), 86.2 (C5), 44.4 (C1'); *m/z* (ES<sup>+</sup>) 394.99 [(M+Na)<sup>+</sup>, 100%]; HRMS (ES<sup>+</sup>) [Found: (M+Na)<sup>+</sup>, 395.0793, C<sub>17</sub>H<sub>16</sub>N<sub>4</sub>O<sub>4</sub>NaS requires 395.0790].

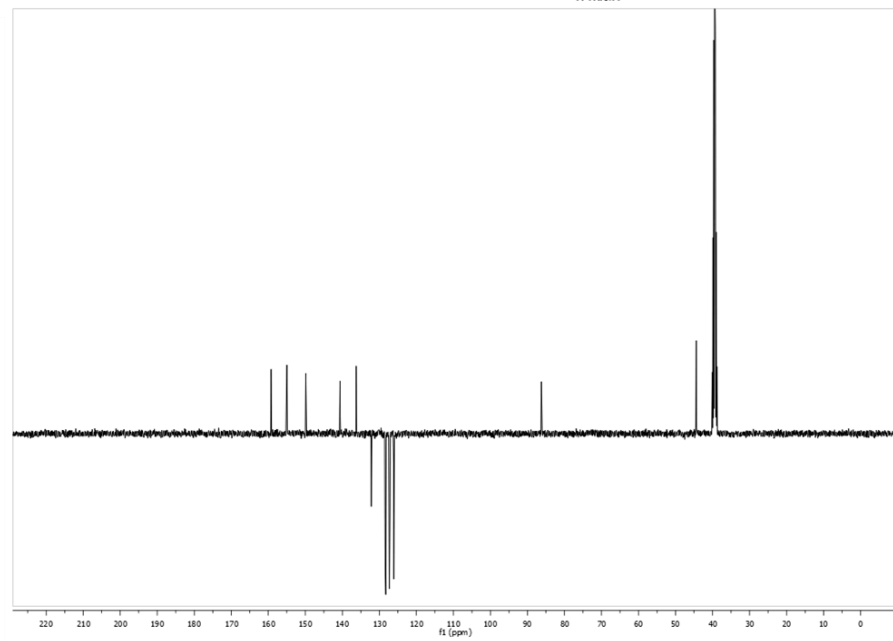
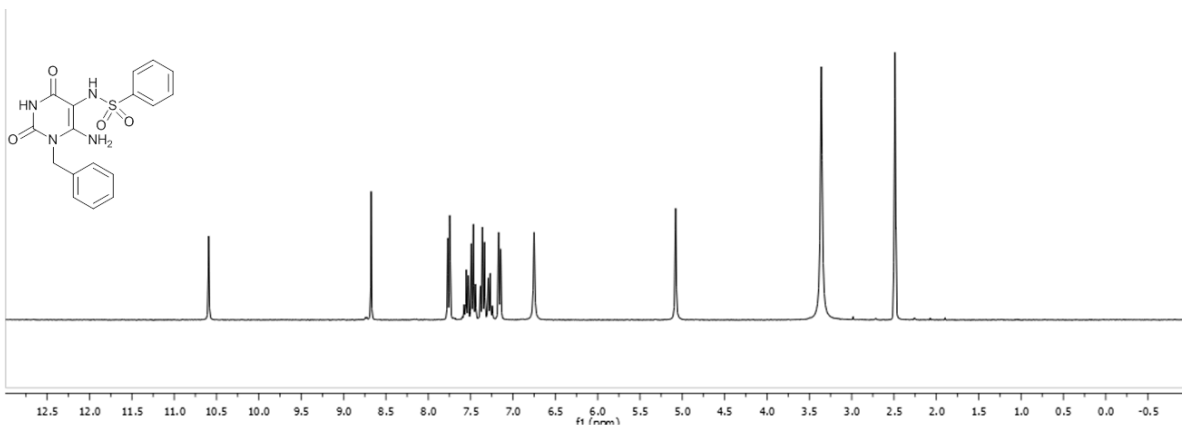
***N*-(6-Amino-1-benzyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)benzamide (15b)**

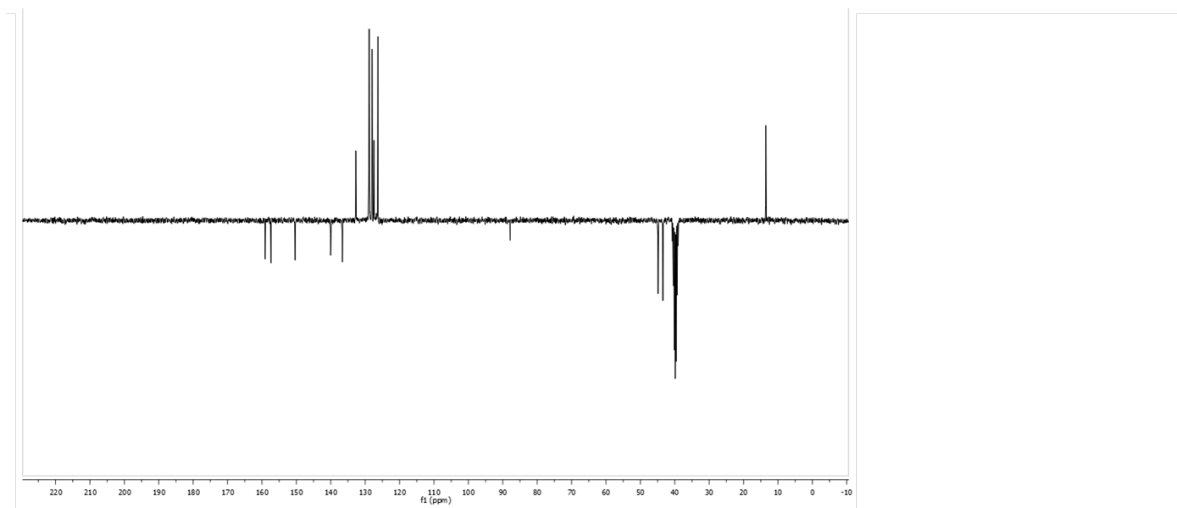
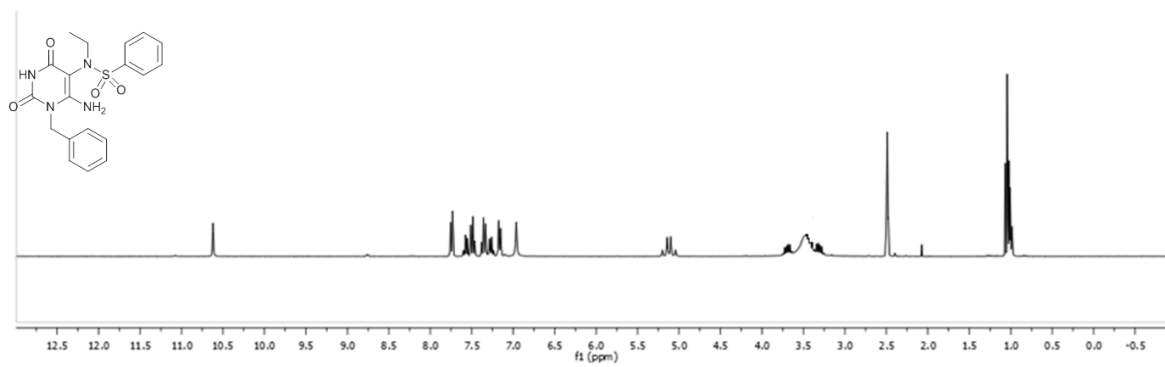


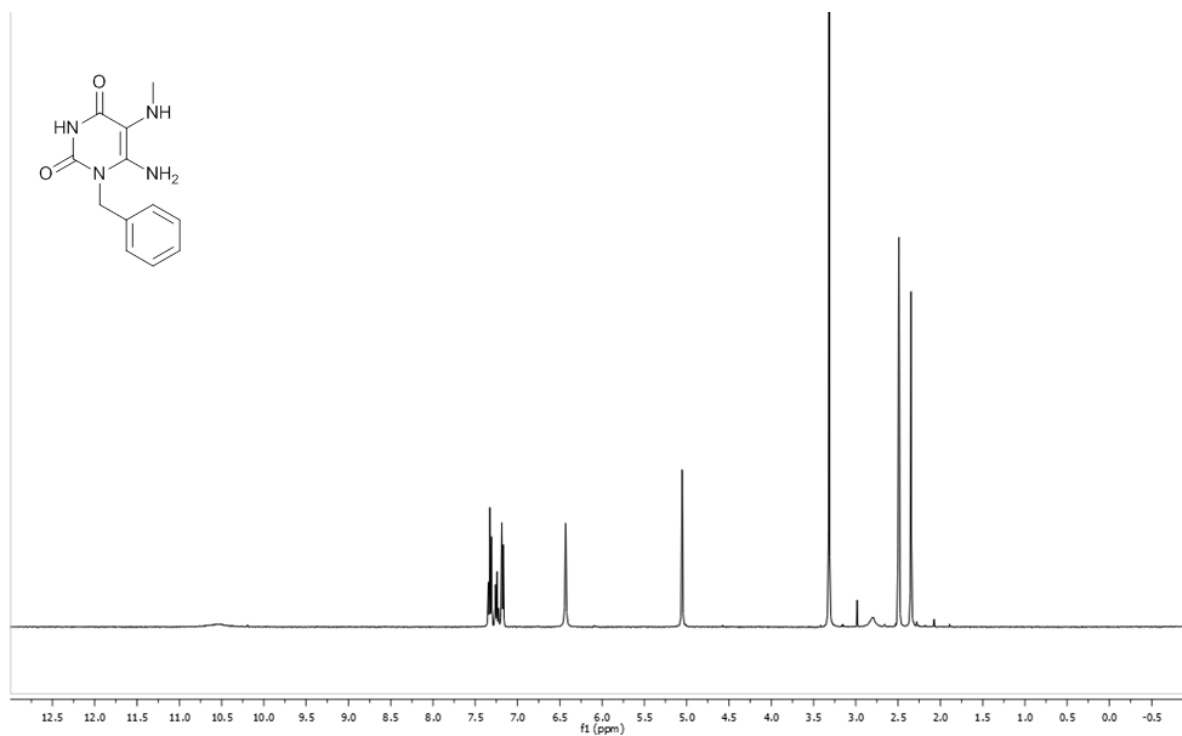
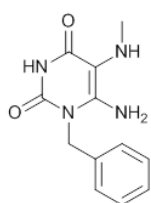
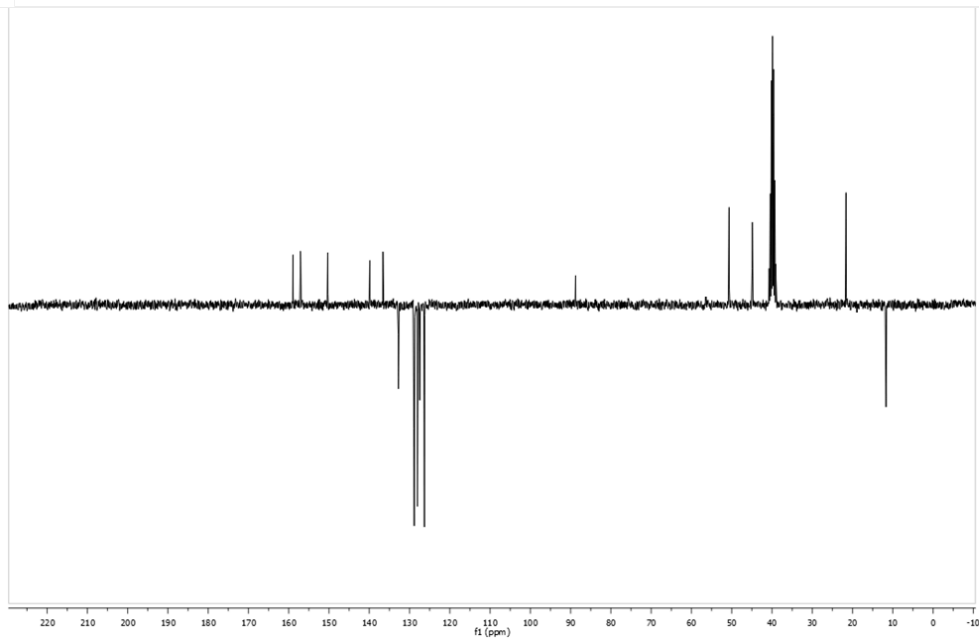
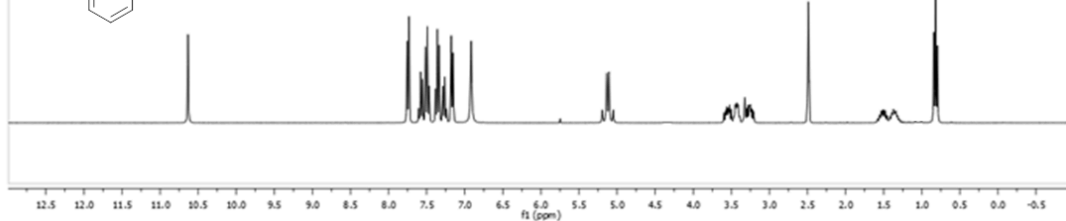
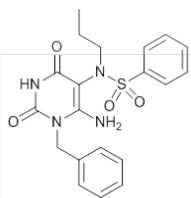
**15b** was obtained following reaction of amine **14** (100 mg, 0.43 mmol) and benzoyl chloride (91 mg, 75  $\mu$ L, 0.65 mmol) using general procedure A. The product was obtained as a light yellow solid (108 mg, 0.32 mmol, 75%). Mp 285-286 °C;  $\nu_{\text{max}}$  cm<sup>-1</sup> (KBr) 3405 (N-H), 3209, 3065 (aromatic C-H), 1704 (C=O), 1635 (C=O), 1578 (N-H), 1507, 1456 (C-N), 1390, 1291 and 731;  $\delta_{\text{H}}$  (400 MHz, DMSO-*d*<sub>6</sub>) 10.77 (1H, s, H3), 8.87 (1H, s, H5a), 7.99-7.94 (2H, m, H2''), 7.55-7.18 (8H, m, ArH), 6.69 (2H, br. s., C6-NH<sub>2</sub>), 5.13 (2H, s, H1');  $\delta_{\text{C}}$  (100 MHz, DMSO-*d*<sub>6</sub>) 166.3 (C1''), 159.9 (C4), 153.0 (C2), 150.4 (C6), 136.5 (C1''a), 134.4 (C1'), 131.1 (C2''), 128.4 (C2'), 127.9 (C3'', C4''), 127.1 (C4'), 126.3 (C3'), 87.7 (C5), 44.1 (C1'); *m/z* (ES<sup>+</sup>) 337.13 [(M+H)<sup>+</sup>, 100%]; HRMS (ES<sup>+</sup>) [Found: (M+H)<sup>+</sup>, 337.1299, C<sub>18</sub>H<sub>17</sub>N<sub>4</sub>O<sub>3</sub> requires 337.1295].

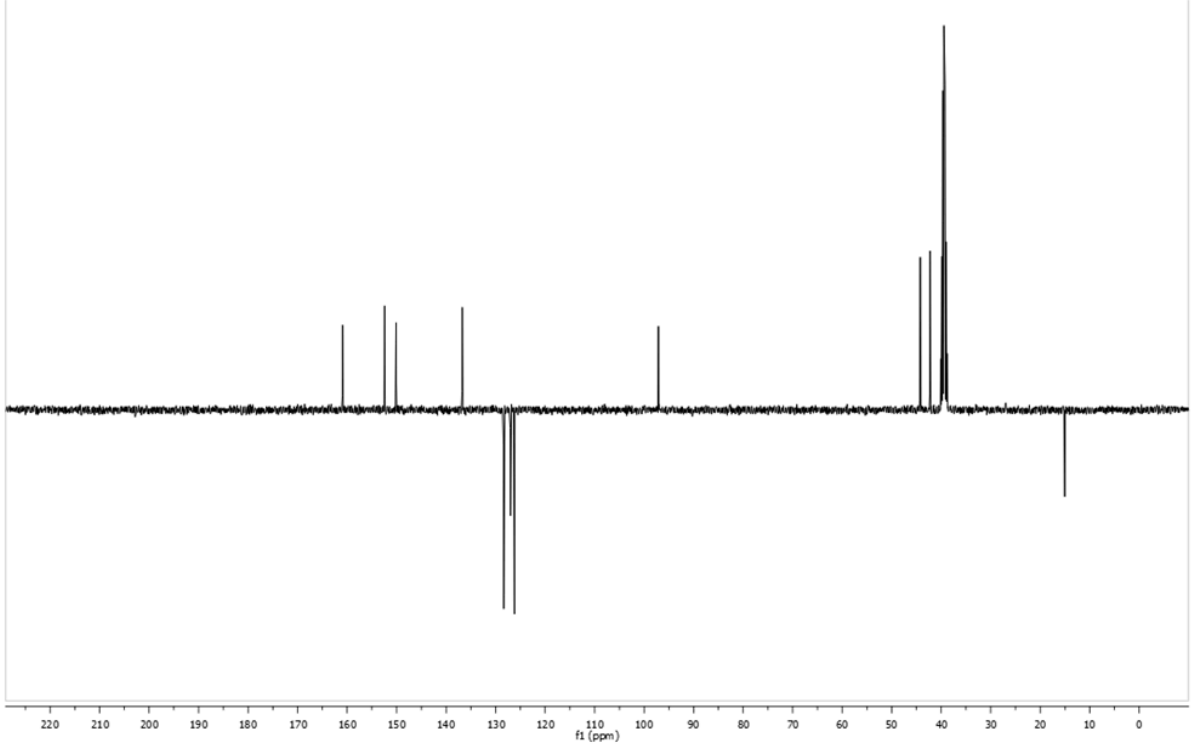
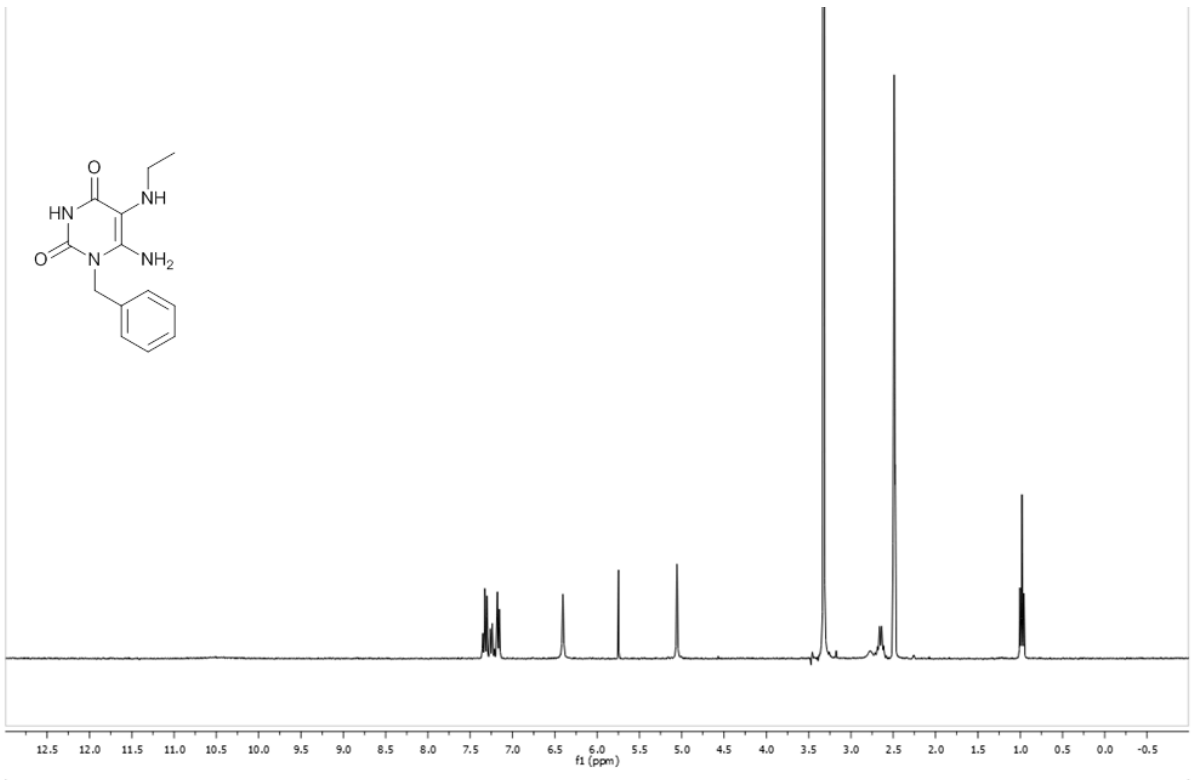


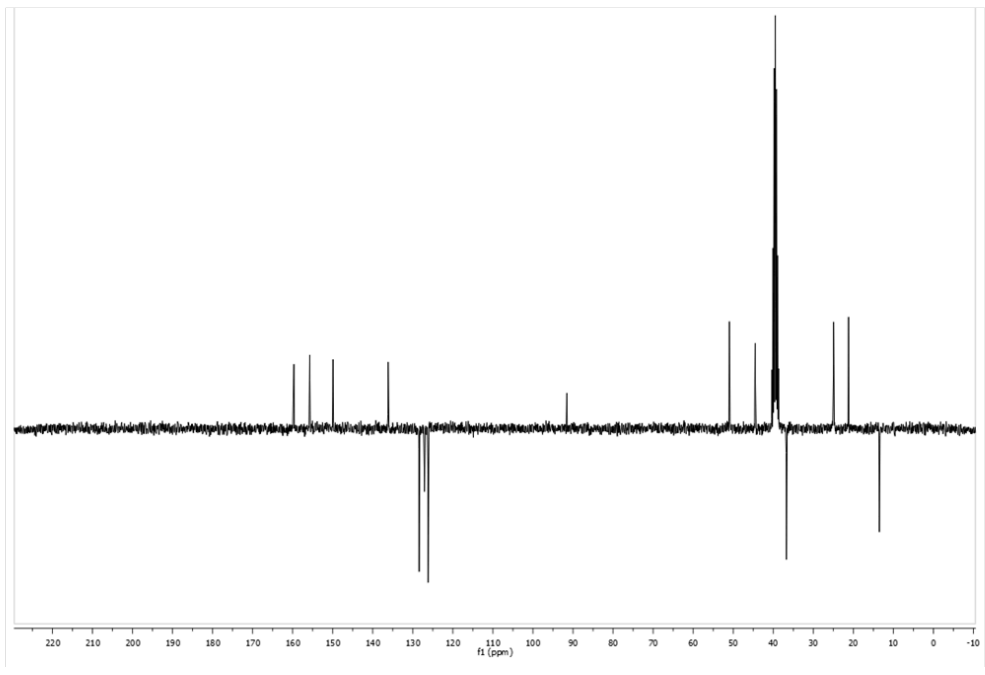
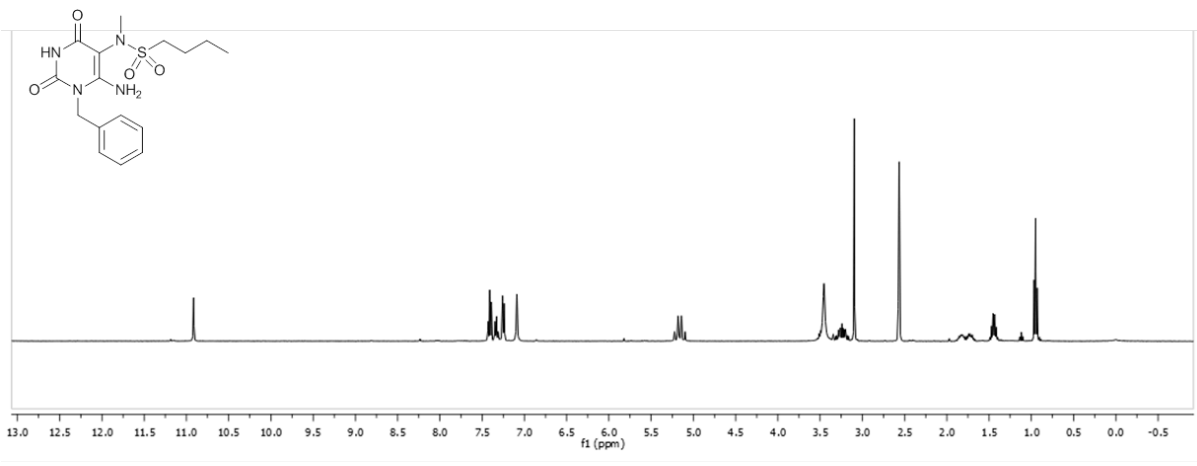


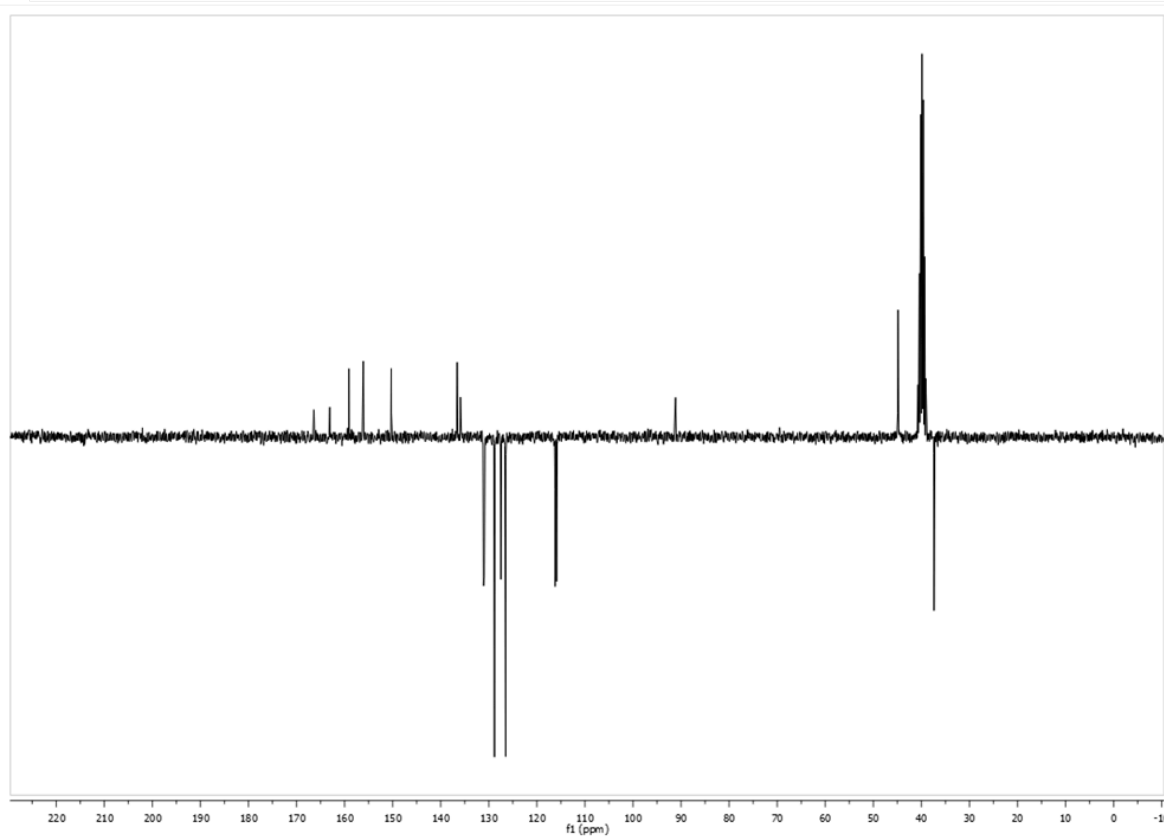
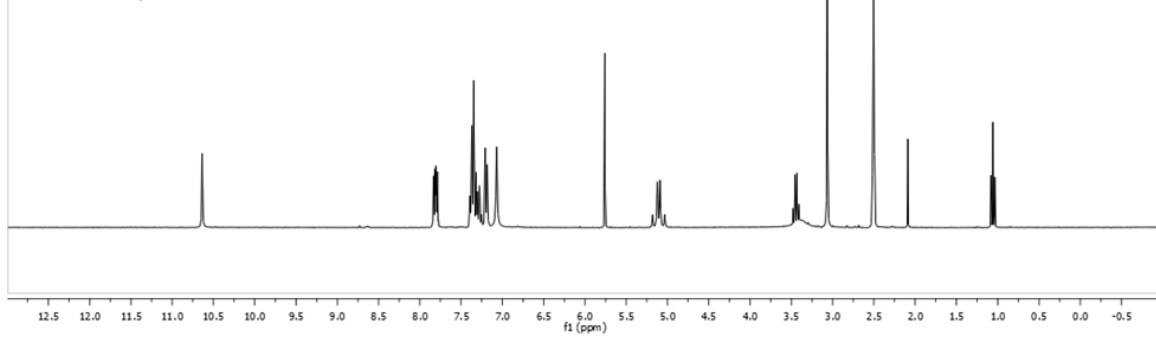
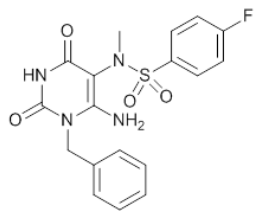


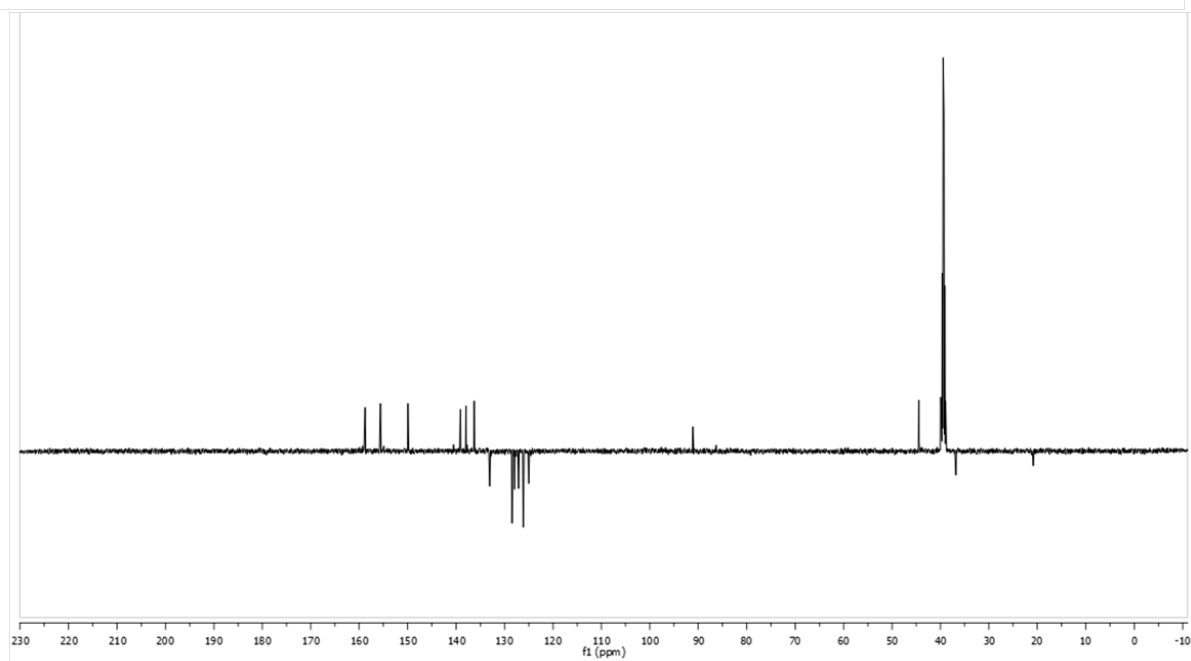
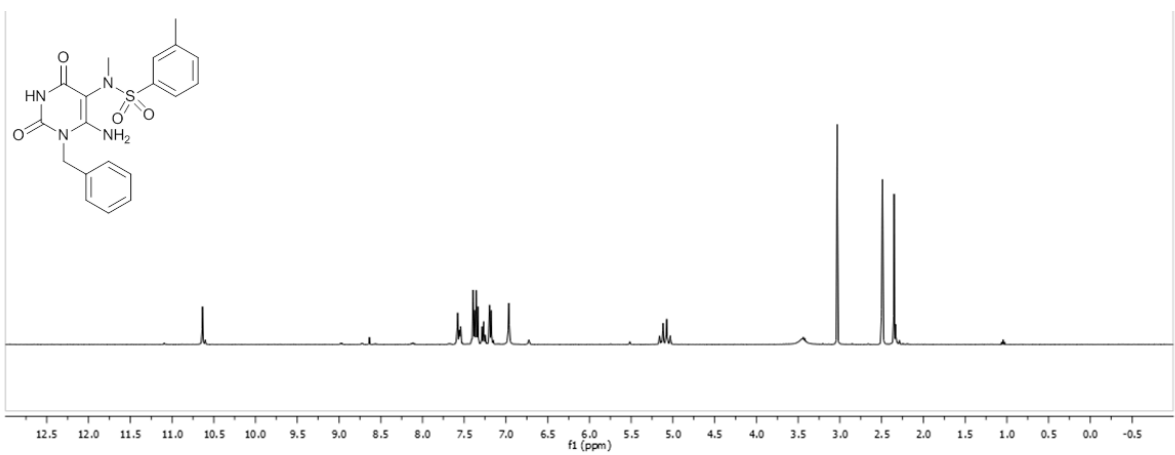


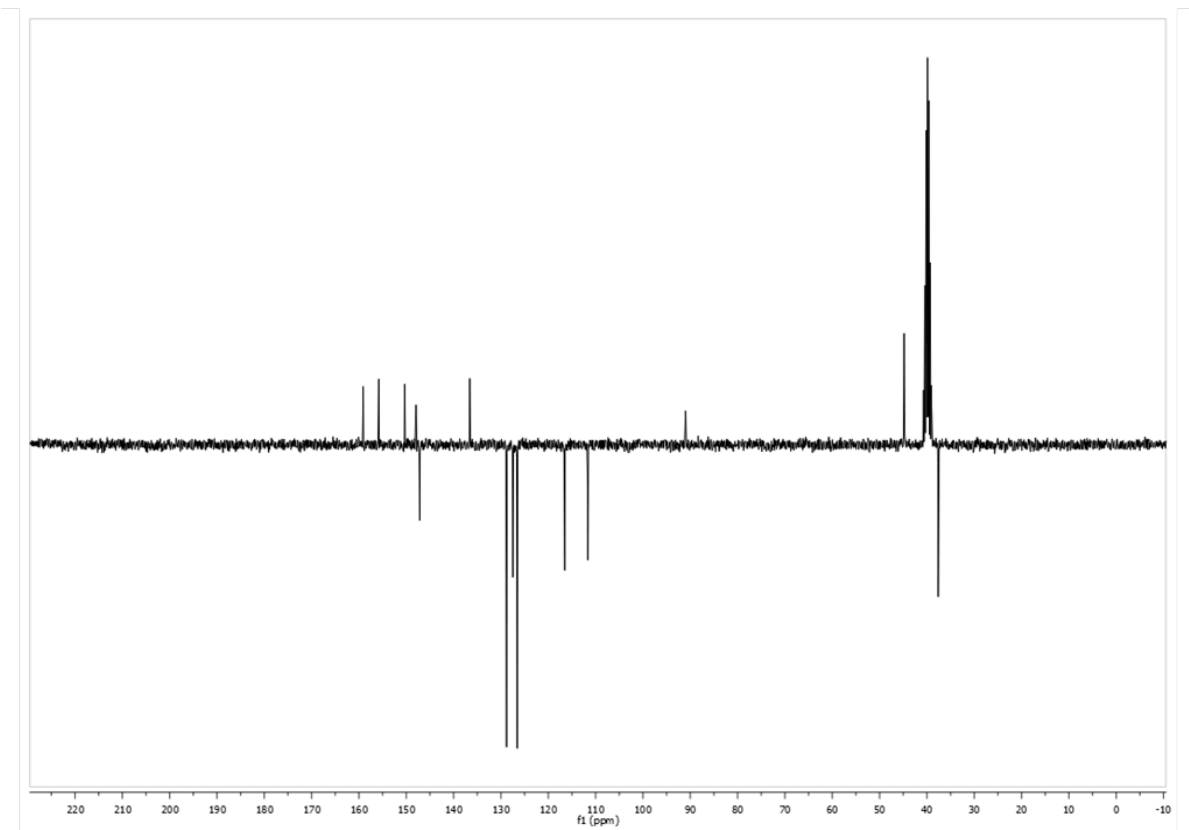
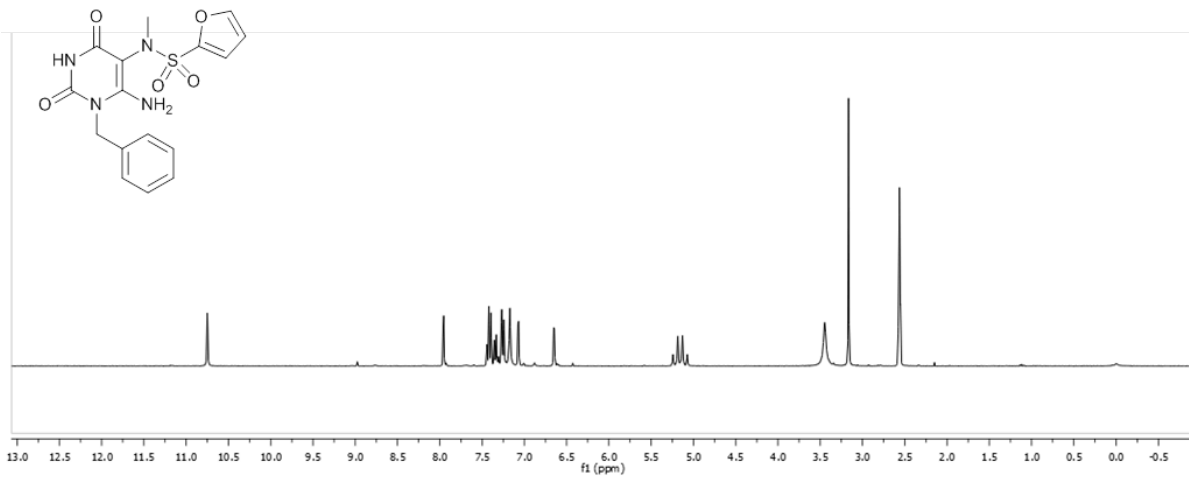












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