

Structure-guided functional studies of plasmid-encoded dihydrofolate reductases reveal a common mechanism of trimethoprim resistance in Gram-negative pathogens

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a

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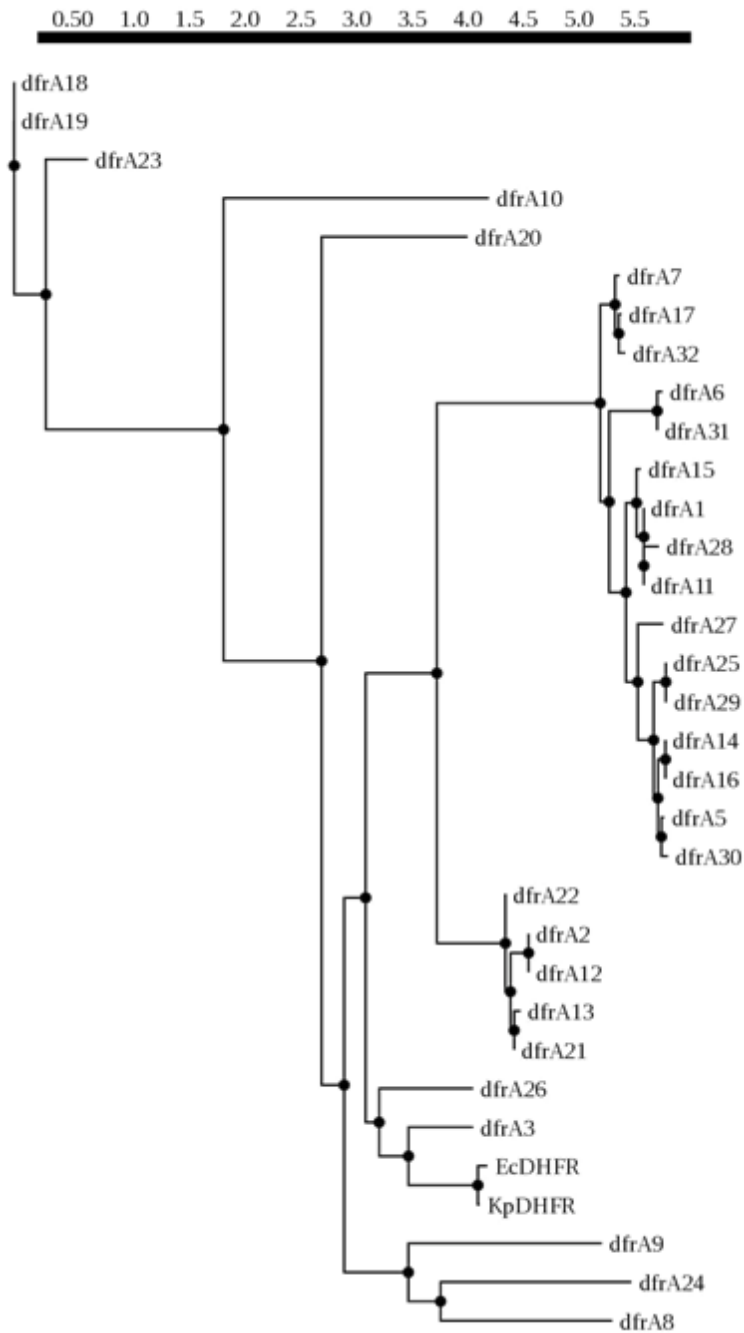
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dfrA21	FFPVLNA-AEFEVVS-----SETIQG---TITYTHSVYARRNG-----	165
dfrA26	FFPDVDL-SQYQETQ-----RQDFEPSGGNPYPFSFVVYQRT-----	183
dfrA3	HFPDYLS-LGWQELE-----RSTHPADDKNSYACEFVTLRQR-----	162
ScDHFR	HFPDYEP-DDWESVF-----SEPHDADAQNSHSYCFEILERR-----	159

dfrA18	-----	189
dfrA19	-----	189
dfrA23	-----	186
dfrA10	-----	187
dfrA24	-----	185
dfrA8	-----	169
dfrA6	-----	157
dfrA31	-----	157
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dfrA32	-----	157
dfrA28	V A S L A G	172
dfrA1	-----	157
dfrA11	-----	157
dfrA15	-----	157
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dfrA29	-----	152
dfrA14	-----	160
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dfrA5	-----	157
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dfrA9	-----	177
dfrA2	-----	165
dfrA12	-----	165
dfrA22	-----	165
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dfrA21	-----	165
dfrA26	-----	183
dfrA3	-----	162
ScDHFR	-----	159

b



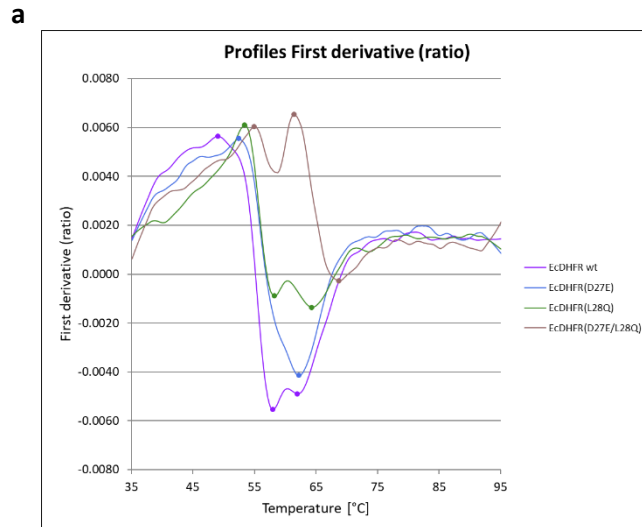
Supplementary Figure S1. Multiple sequence alignment of amino acid sequences by MUSCLE and a phylogenetic tree built off the standard Likelihood Ratio Test for all DfrA isoforms.

a A sequence alignment of all known DfrA enzymes and EcDHFR. Residues that interact with trimethoprim are boxed. **b** *K. pneumoniae* DHFR (KpDHFR) was used as a positive control in building the phylogenetic tree given the close relation to EcDHFR. Phylogenetic analysis was based on the sequence alignment.

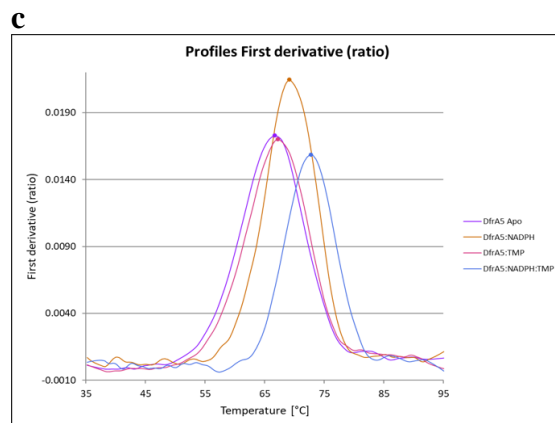
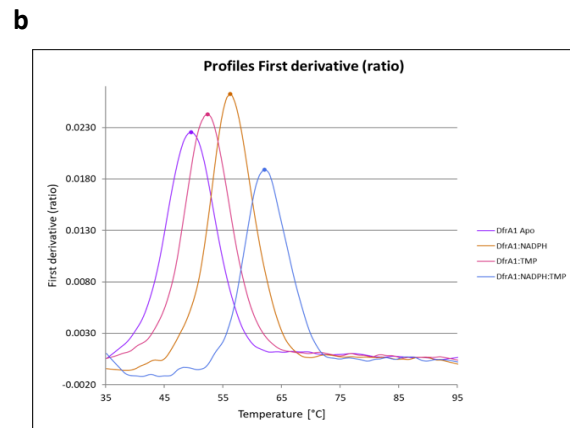
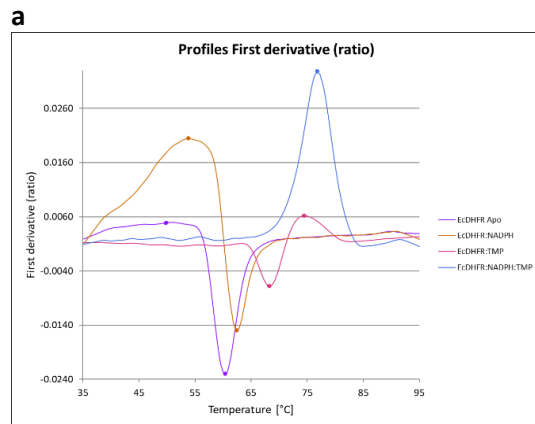
<i>E. coli</i> DHFR Mutant T _i (°C)			
	T _i 1	T _i 2	T _i 3
WT	50.6 ± 1.7	58.7 ± 0.5	62.3 ± 2.5
D27E	51.9 ± 1.7	62.1 ± 1.3	-
L28Q	52.0 ± 1.2	57.7 ± 0.8	63.9 ± 1.2
D27E/ L28Q	55.8 ± 0.8	62.0 ± 0.6	66.1 ± 2.1

Supplementary Table S1. Thermal unfolding data of EcDHFR wild type and its mutants.

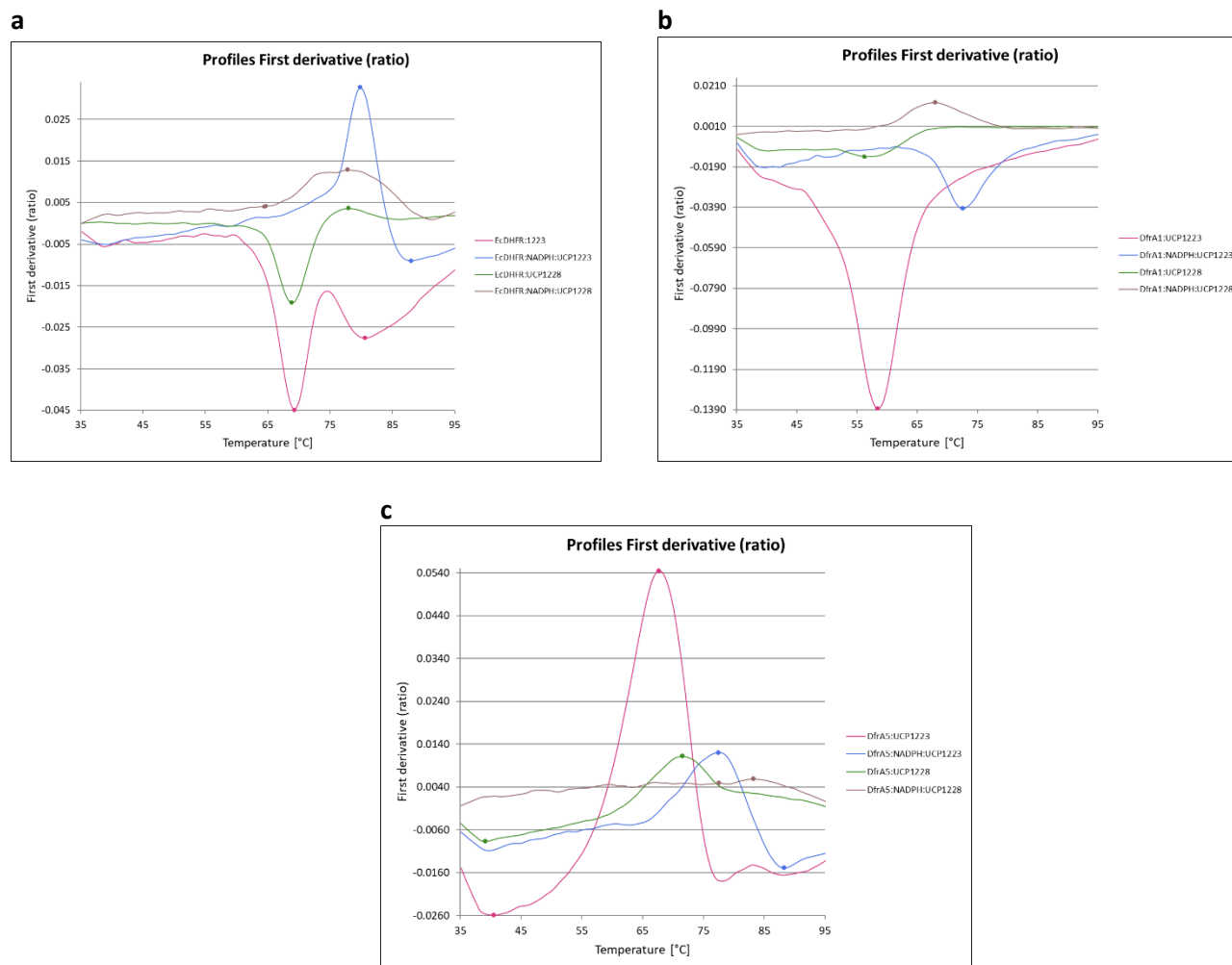
Data is shown as mean ± standard deviation for n = 2 independent replicates.



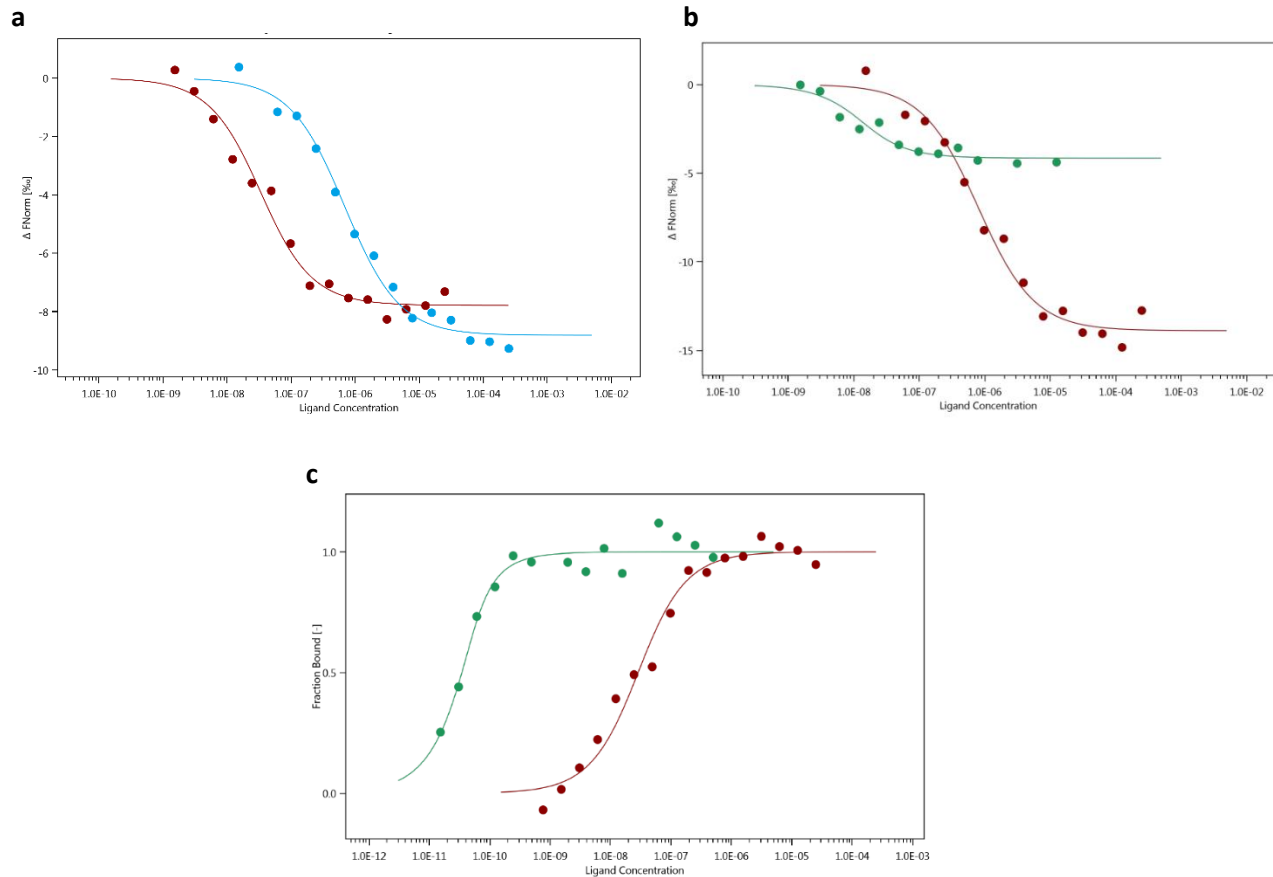
Supplementary Figure S2. Thermal stability of dihydrofolate reductases measured with label-free differential scanning fluorimetry. The first derivative of the fluorescence ratio ($\Delta F_{350nm}/\Delta F_{330nm}$) as a function of temperature are shown in the diagrams. Colored points represent T_i (inflection temperature at which an unfolding transition occurs). Maxima and minima indicate a transition point along the melting curve. Experiments were performed with two independent samples and one representative data for each sample is displayed. **a** Thermal unfolding profile of the wild-type *E. coli* DHFR (*EcDHFR*) (brown), *EcDHFR*(D27E) (blue) and *EcDHFR*(L28Q) (green) single mutants and *EcDHFR* (D27E/L28Q) (purple) double mutant.



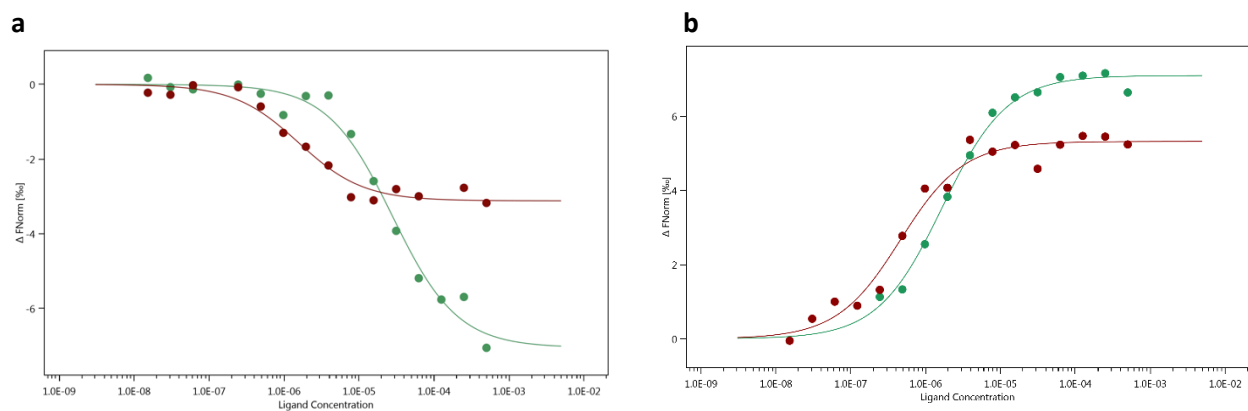
Supplementary Figure S3. Thermal stability profile of wild-type E. coli DHFR (EcDHFR), DfrA1 and DfrA5 upon binding to TMP in presence and absence of co-factor. Representative first derivative plots are shown for fluorescence ratio (350/330 nm) from which T_i values corresponding to the positive and negative peaks in the unfolding profile were determined (n=3). **a** Thermal shift first-derivative curves of EcDHFR Apo (purple), EcDHFR:NADPH (orange), EcDHFR:TMP (red), EcDHFR:TMP:NADPH (blue). **b** Thermal shift first-derivative curves of DfrA1 Apo (purple), DfrA1:NADPH (orange), DfrA1:TMP (red), DfrA1:TMP:NADPH (blue). **c** Thermal shift first-derivative curves of DfrA5 Apo (purple), DfrA5:NADPH (orange), DfrA5:TMP (red), DfrA5:TMP:NADPH (blue).



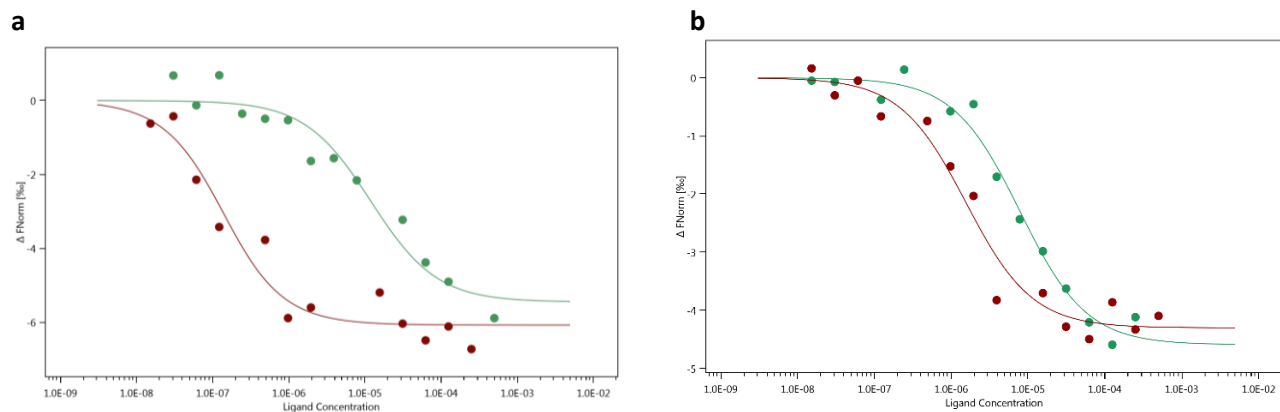
Supplementary Figure S4. Thermal stability profile of wild-type E. coli DHFR (EcDHFR), DfrA1 and DfrA5 upon binding to UCP1223 and UCP1228 in presence and absence of co-factor. Equivalent analysis for all three systems in the presence of PLAs as indicated (n=3). **a** Thermal shift first-derivative curves of EcDHFR:UCP1223 (red), EcDHFR:NADPH:UCP1223 (blue), EcDHFR:UCP1228 (green), EcDHFR:NADPH:UCP1228 (purple). **b** Thermal shift first-derivative curves of DfrA1:UCP1223 (red), DfrA1:NADPH:UCP1223 (blue), DfrA1:UCP1228 (green), DfrA1:NADPH:UCP1228 (purple). **c** Thermal shift first-derivative curves of DfrA5:UCP1223 (red), DfrA5:NADPH:UCP1223 (blue), DfrA5:UCP1228 (green), DfrA5:NADPH:UCP1228 (purple).



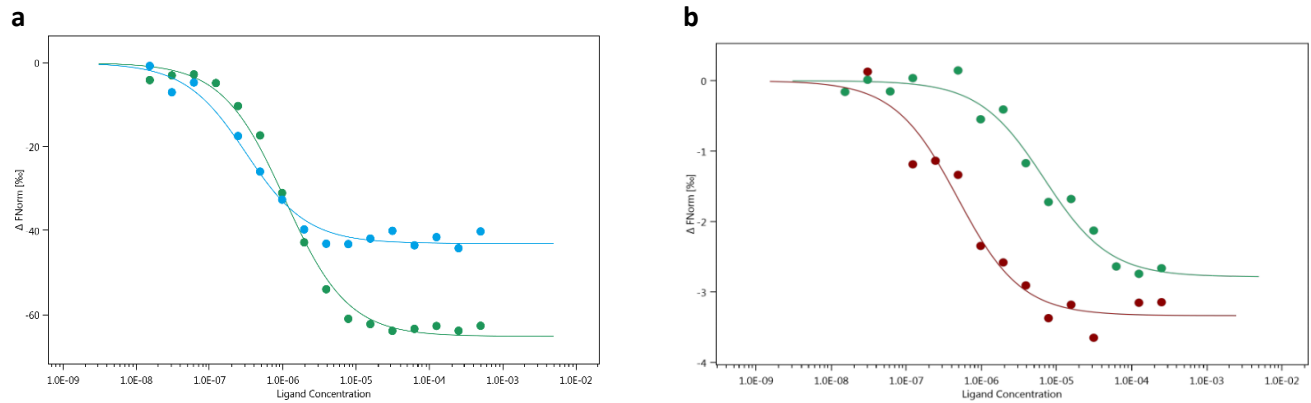
Supplementary Figure S5. Thermophoretic analysis of *EcDHFR* interactions with NADPH and TMP by MST. Normalized binding response (ΔF_{norm} of fraction bound) of the increasing concentrations of the non-fluorescent ligand to the constant concentration of the red-NTA labeled enzyme. The experiments were performed using medium MST power and between 40 to 80% LED power, at 23 °C. The MST traces were recorded with the following parameters: 5 s MST power of, 30 s MST power on and 5 s MST power of. Data collection was performed with MO.Control 2 (Nano-temper Technology GmbH). The reported measurements are the combination of the fast, local environment dependent responses of the fluorophore to the temperature jump and the slower diffusive thermophoretic fluorescence changes for the enzyme, pre and post incubation step. Equilibrium binding curves are depicted as thick lines and dots are representing up to 16 concentration sampling schemes, expressed as individual data points. The Data were fitted to a single-site binding model accounting for ligand depletion. The K_d values obtained from the best fit are reported in Table 4. **a** Titration of 50 nM red-NTA labeled *EcDHFR* apo form into a serial dilution of NADPH (blue) and TMP (red) ranging from 250 μM to 0.015 μM and 25 μM to 0.000763 μM respectively. Fit of the changes in the thermophoresis signal yielded a K_d of 661 nM \pm 98 nM for NADPH and 24.5 nM \pm 8.2 nM for TMP. **b** Binding of NADPH to red-NTA labeled *EcDHFR* with and without 1 μM TMP preincubation step. Shift in thermophoretic movement after titration of NADPH from 250 μM to 0.015 μM against 50 nM *EcDHFR* apo form resulted in K_d of 771 \pm 145 nM (red). Titration of NADPH from 12.5 μM to 0.00153 μM against 10 nM of the enzyme preincubated with TMP caused a less pronounced response in MST signal, yielding K_d of 8.1 \pm 4.0 nM (green). **c** Binding of TMP to red-NTA labeled *EcDHFR* in presence and absence of co-factor. Titration of TMP from 25 μM to 0.0007 μM against 50 nM apo enzyme yielded K_d of 23.57 nM \pm 5.15 nM (red), while addition of TMP from 0.5 μM to 0.000015 μM to 10 nM labeled, pre-formed binary complex with NADPH gave K_d of 0.011nM \pm 0.0075 nM (green). A high margin of error for the ternary binding events is due to a limitation of the instrument to accurately measure protein-ligand interactions in a low picomolar range.



Supplementary Figure S6. Thermophoretic analysis of DfrA1 interactions with NADPH and TMP by MST. Equivalent analysis for DfrA1 as indicated. **a** Binding of NADPH to red-NTA labeled DfrA1 apo form and to preformed DfrA1:TMP complex. Titration of NADPH from 500 μM to 0.0244 μM against 50 nM DfrA1 apo form resulted in K_d of $1.65 \pm 0.23 \mu\text{M}$ (green). Binding of NADPH from 500 μM to 0.015 μM to 50 nM DfrA1 preincubated with 100 μM TMP yielded K_d of $0.43 \pm 0.09 \mu\text{M}$ (red). **b** Binding of TMP to red-NTA labeled DfrA1 as a binary (DfrA1:TMP) and a ternary (DfrA1: NADPH:TMP) complex. Titration of TMP from 500 μM to 0.015 μM with 50 nM DfrA1 apo form gave K_d of $27.79 \mu\text{M} \pm 4.87 \mu\text{M}$ (green), while addition of TMP from 500 μM to 0.015 μM against the same enzyme preincubated with 100 μM NADPH gave K_d of $1.53 \mu\text{M} \pm 0.36 \mu\text{M}$ (red).



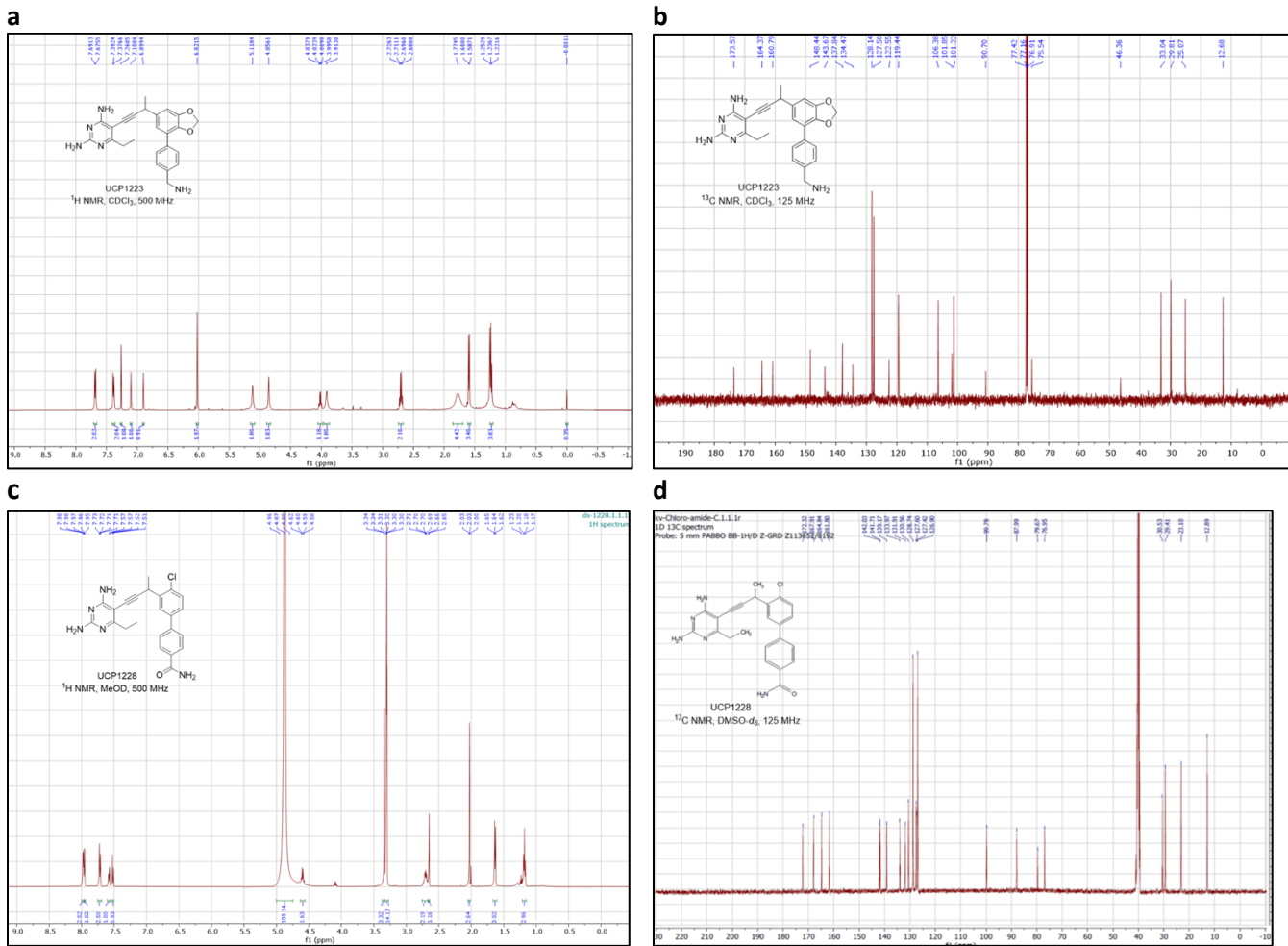
Supplementary Figure S7. Thermophoretic analysis of DfrA5 interactions with NADPH and TMP. Equivalent analysis for DfrA5 as indicated. **a** Binding of NADPH to red-NTA labeled DfrA5 apo form and to preformed DfrA5:TMP complex. Titration of NADPH from 500 μM to 0.015 μM against 50 nM labeled DfrA5 apo form yielded K_d of $7.47 \pm 1.66 \mu\text{M}$ (green), while titration of NADPH against 50 nM labeled DfrA5 preincubated with 50 μM TMP resulted in K_d equal $1.54 \mu\text{M} \pm 0.38 \mu\text{M}$ (red). **b** Binding of TMP to red-NTA labeled DfrA5 apo form and to preformed DfrA5:NADPH complex. Titration of TMP from 500 μM to 0.015 μM against 50 nM labeled DfrA5 apo form yielded K_d of $11.93 \mu\text{M} \pm 4.45 \mu\text{M}$ (green), while titration of TMP from 250 μM to 0.015 μM against 50 nM preformed complex of DfrA5 with 100 μM NADPH derived a K_d value of $0.113 \mu\text{M} \pm 0.056 \mu\text{M}$ (red).



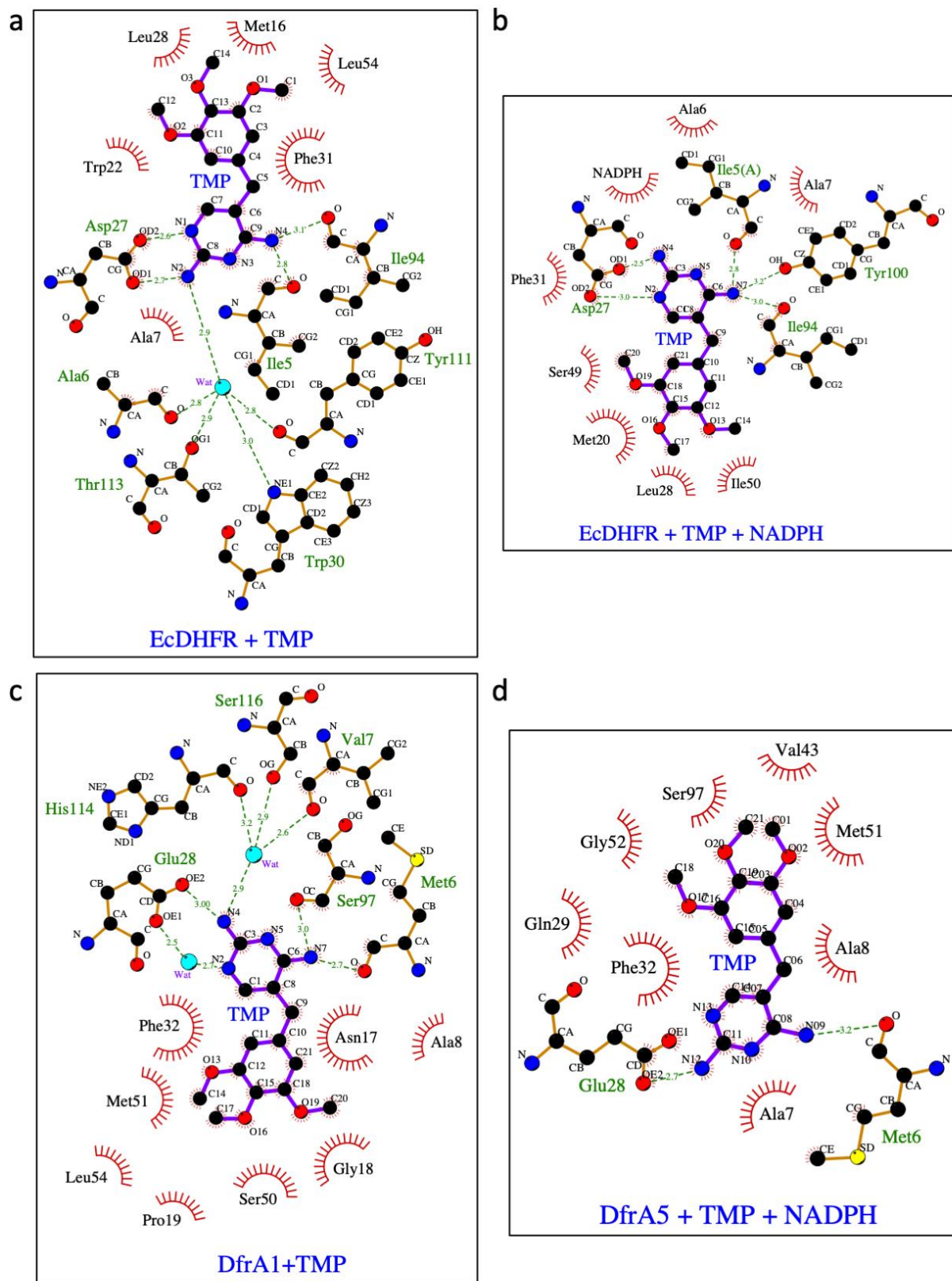
Supplementary Figure S8. MST binding response of NADPH to preformed complexes of DfrA1 and DfrA5 with 100 μM UCP1228 respectively. By fitting the change in the thermophoretic movement upon titration of NADPH to a constant amount of the labeled enzyme alone and in the presence of UCP1228, a different binding constant of K_d was determined. **a** Binding of NADPH to red-NTA labeled DfrA1 in presence and absence of UCP1228. Titration of NADPH from 500 μM to 0.015 μM against 50 nM labeled DfrA1 apo form yielded K_d of $1.04 \pm 0.11 \mu\text{M}$ (green), while titration of co-factor into 50 nM labeled protein preincubated with 100 μM UCP1228 complex resulted in K_d equal to $0.29 \mu\text{M} \pm 0.05 \mu\text{M}$ (blue). **b** Binding of NADPH to red-NTA labeled DfrA5 apo form and to preformed DfrA5:UCP1228 complex. Titration of NADPH from 250 μM to 0.015 μM against 50 nM labeled DfrA5 apo form yielded K_d of $6.84 \mu\text{M} \pm 1.60 \mu\text{M}$ (green), while titration of NADPH from 250 μM to 0.031 μM against 50 nM labeled DfrA5 preincubated with 100 μM UCP1228 derived a K_d value of $0.45 \mu\text{M} \pm 0.15 \mu\text{M}$ (red).

Protein	Inhibitor	Co-factor observed	Resolution (Å)	PDB ID
<i>E. coli</i> DHFR	Trimethoprim	-	2.4	7NAE
<i>E. coli</i> DHFR	Trimethoprim	+	3.0	7MYM
<i>E. coli</i> DHFR	UCP1223	-	2.1	7REB
<i>E. coli</i> DHFR	UCP1228	-	1.9	7MQP
DfrA1	Trimethoprim	-	2.2	7MYL
DfrA1	UCP1223	+	1.4	7RGJ
DfrA1	UCP1228	+	1.8	7REG
DfrA5	Trimethoprim	+	2.6	7R6G
DfrA5	UCP1223	+	2.2	7RGK
DfrA5	UCP1228	+	1.9	7RGO

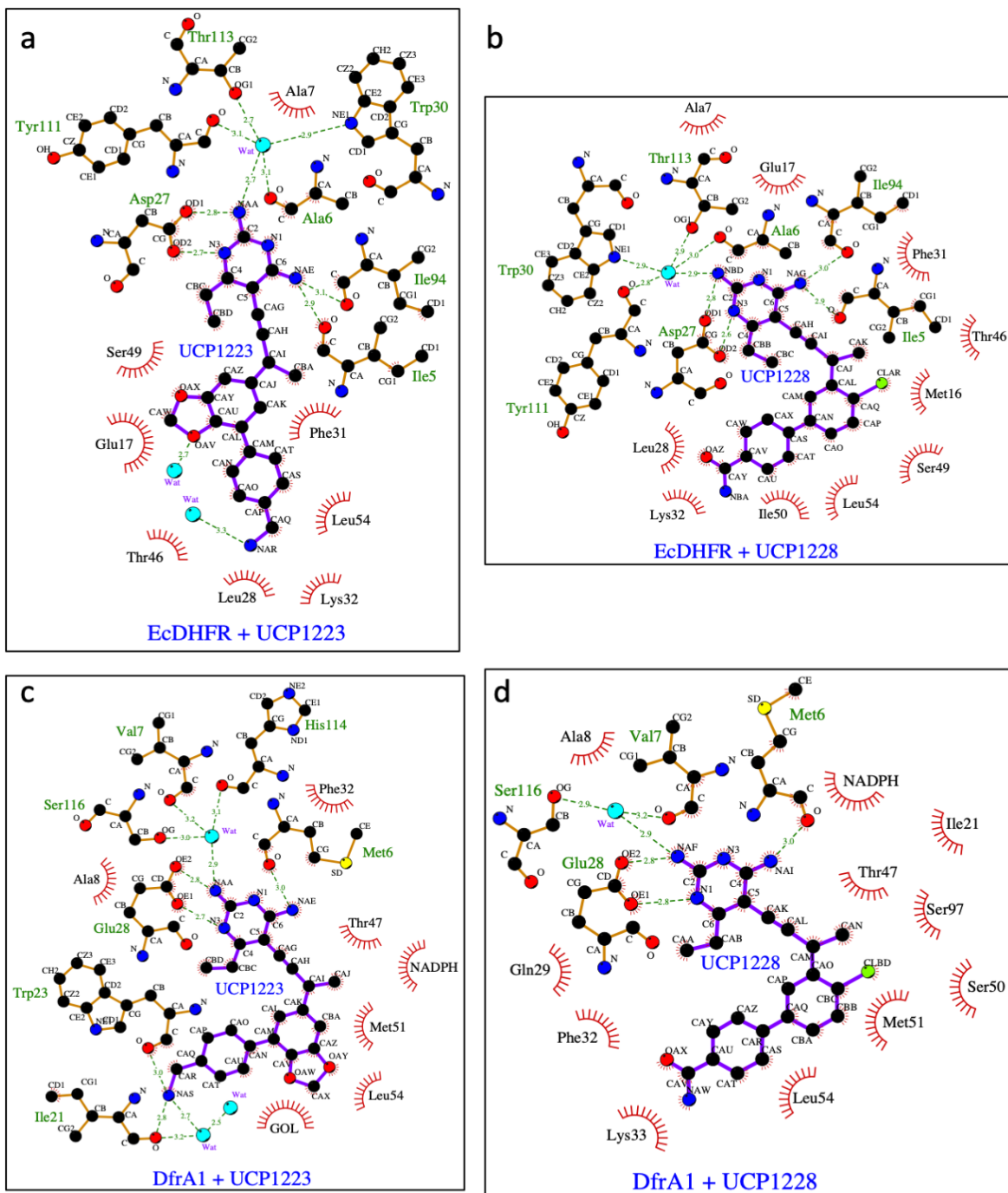
Supplementary Table S2. Summary of crystal structures described in this study



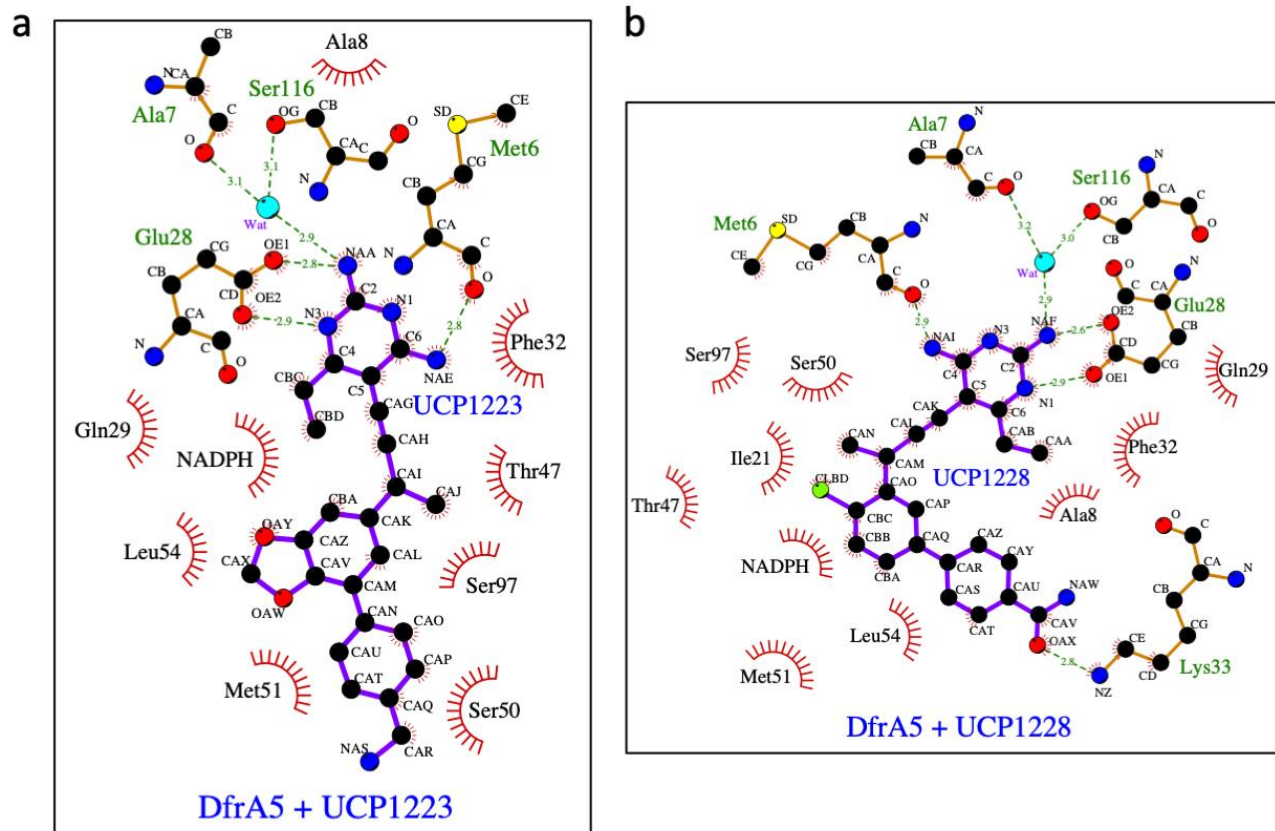
Supplementary Figure S9. Characterization of UCP1223 and UCP1228 by ¹H and ¹³C nuclear magnetic resonance (NMR). a ¹H and b ¹³C NMR spectra of UCP1223 in CDCl₃. c ¹H and d ¹³C NMR spectra of UCP1228 in MeOD and DMSO-d₆ respectively.



Supplementary Figure S10. Ligand-protein interaction diagrams for TMP-protein interactions from the crystal structures presented in this study. The complexes are: **a** EcdHFR:TMP (PDB entry 7NAE), **b** EcdHFR:TMP:NADPH (7MYM), **c** DfrA1:TMP (7MYL), **d** DfrA5:TMP:NADPH (7R6G). Residues involved in van der Waals contacts are depicted as red spoked arcs. Hydrogen bonds are shown as green dotted lines. Only conserved water molecules are included and are shown in cyan.



Supplementary Figure S11. Ligand-protein interaction diagrams for UCP1223 and UCP1228-protein interactions from the crystal structures presented in this study. The complexes are: **a** EcdHFR:UCP1223 (PDB entry 7REB), **b** EcdHFR:UCP1228:NADPH (7MQP), **c** DfrA1:UCP1223:NADPH (7RGJ), **d** DfrA1:UCP1228:NADPH (7REG). Residues involved in van der Waals contacts are depicted as red spoked arcs. Hydrogen bonds are shown as green dotted lines. Only conserved water molecules are included and are shown in cyan.



Supplementary Figure S12. Ligand-protein interaction diagrams for UCP1223 and UCP1228-protein interactions from the crystal structures of DfrA5 presented in this study. The complexes are: **a** DfrA5:UCP1223:NADPH (PDB entry 7RGK) **b** DfrA5:UCP1228:NADPH (7RGO). Residues involved in van der Waals contacts are depicted as red spoked arcs. Hydrogen bonds are shown as green dotted lines. Only conserved water molecules are included and are shown in cyan.

a

Compound ID	MIC ($\mu\text{g}/\text{mL}$) ^a			MIC ($\mu\text{g}/\text{mL}$) ^a		
	BW25113 w/o SMX	JW0451 w/o SMX	Fold difference	BW25113 w/o SMX	BW25113 w/ SMX	Fold difference
TMP	0.312	0.04	8	0.312	0.078	4
UCP1223	20	0.625	32	20	0.625	32
UCP1228	10	1.25	8	10	0.625	16

b

Compound ID	MIC ($\mu\text{g}/\text{mL}$) ^a			MIC ($\mu\text{g}/\text{mL}$) ^a		
	<i>EcDHFR</i> ^b	<i>DfrA1</i> ^c	<i>DfrA5</i> ^d	<i>EcDHFR</i> ^b	<i>DfrA1</i> ^c	<i>DfrA5</i> ^d
	w/o SMX	w/o SMX	w/o SMX	w/ SMX	w/ SMX	w/ SMX
TMP	0.625	>20	>20	0.078	>20	>20
UCP1223	20	>20	>20	1.25	20	10
UCP1228	20	>20	>20	1.25	20	20

Supplementary Table S3. Comparative antimicrobial activities of TMP, UCP1223 and UCP1228 alone and paired with SMX (1:19) a against *E. coli* BW25113 ATTC strain and *E. coli* JW0451 *acrB* mutant strain, **b** *E. coli* BL21(DE3) strain overexpressing *EcDHFR* wt, *DfrA1* and *DfrA5* respectively. ^aThe Minimum Inhibitory Concentration (MIC) values represent 80% inhibition of bacterial growth after 18 to 20 hours of incubation at 37°C from duplicates. ^bBL21(DE3) strain transformed with pET41a-*EcDHFR* wt plasmid, ^cBL21(DE3) strain transformed with pET41a-*DfrA1* plasmid and ^dBL21(DE3) strain transformed with pET24a-*DfrA5* plasmid.