Discovery and characterization of the antimetabolite action of thioacetamide-linked 1,2,3-triazoles as disruptors of cysteine biosynthesis in Gram-negative bacteria

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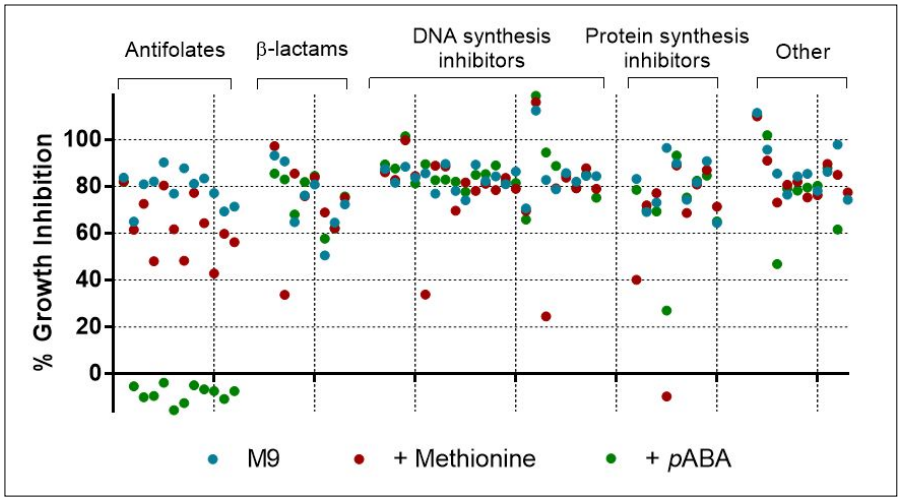
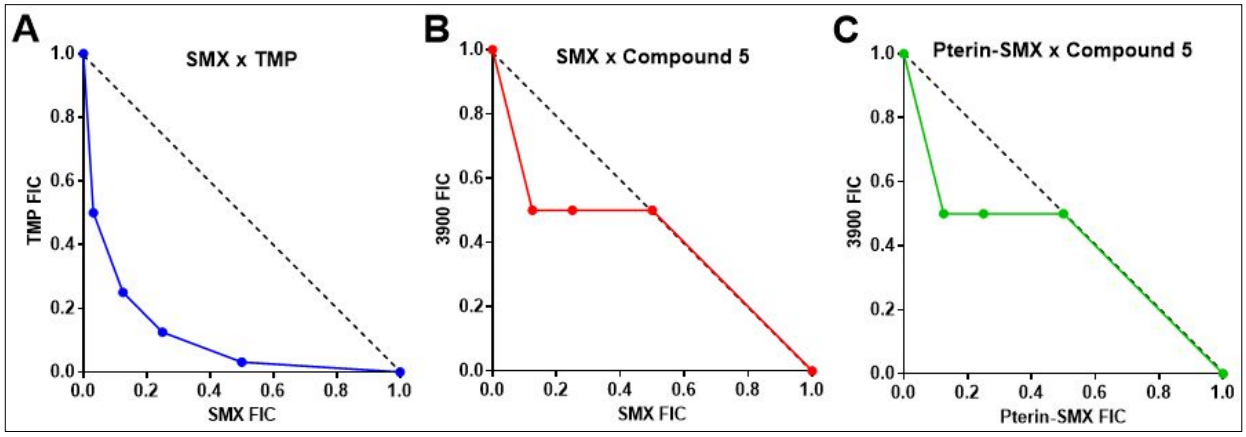
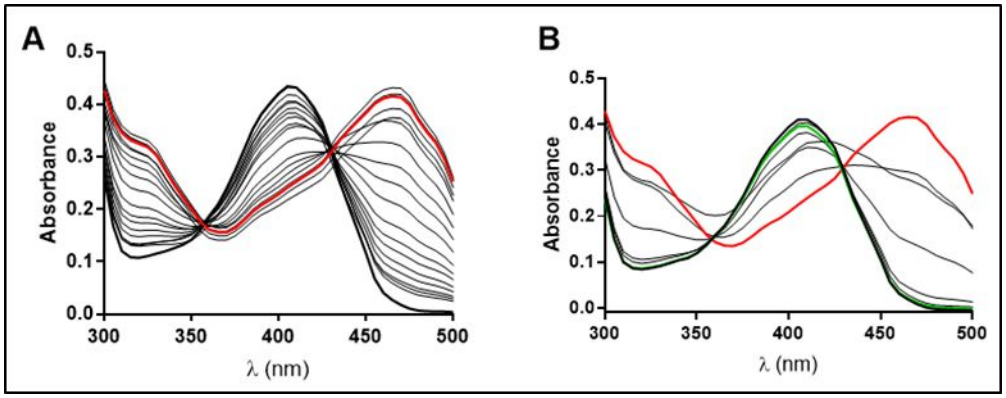


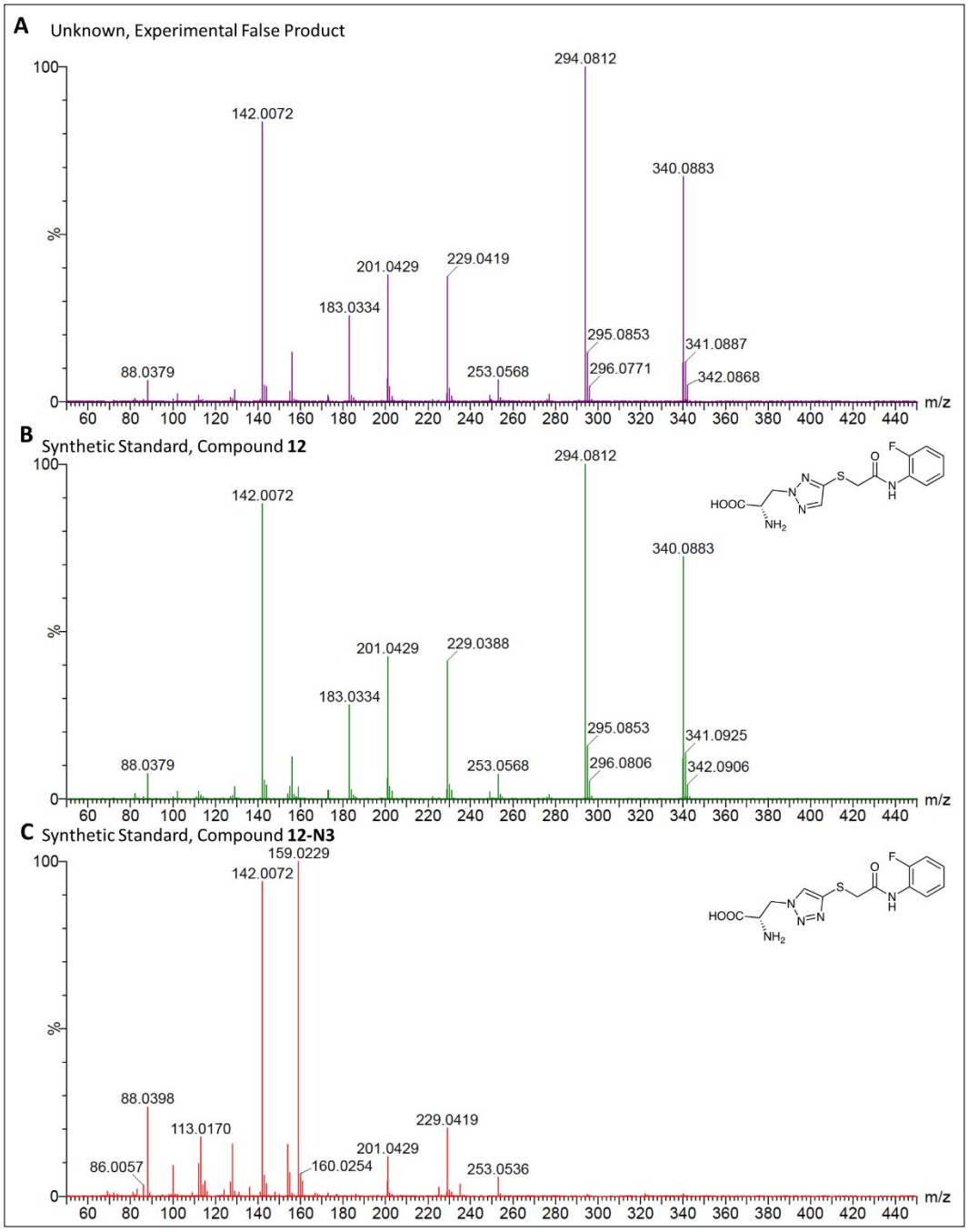
Supplementary Figure 1. Folate biosynthesis metabolite growth inhibition rescue of known antibiotics. Blue dots represent % growth inhibition in unsupplemented M9 media. Red dots represent supplementation with 20 μg/mL L-methionine and green dots represent supplementation with 5 μg/mL *p*ABA.



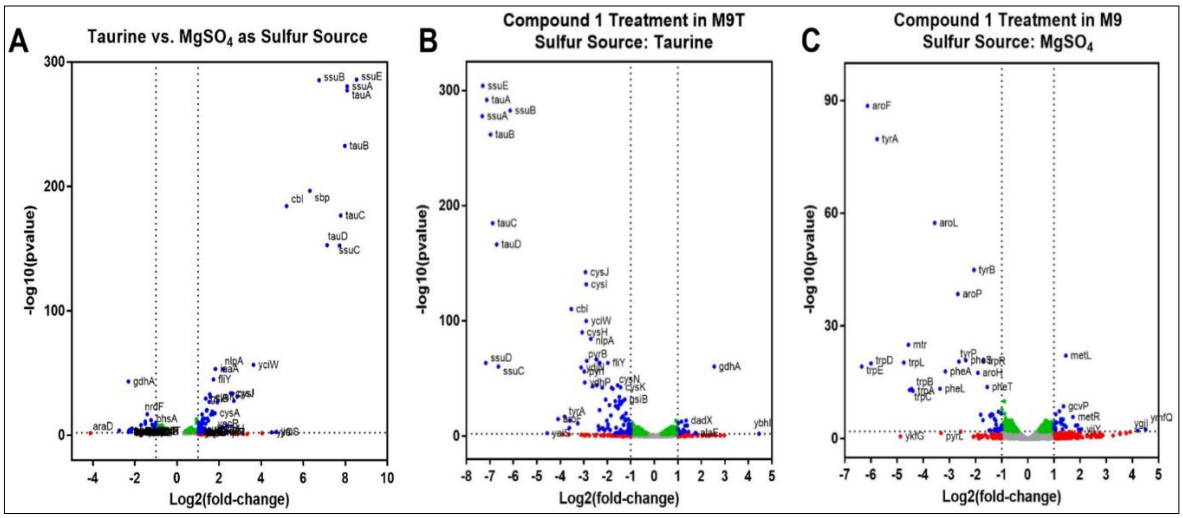
Supplementary Figure 2. TAT and antifolate synergy plots. Using checkerboard assays compound 5 potentiates the action of two DHPS inhibitors, sulfamethoxazole and pterin-SMX. SMX = sulfamethoxazole; TMP = trimethoprim; FIC = fractional inhibitory concentration.



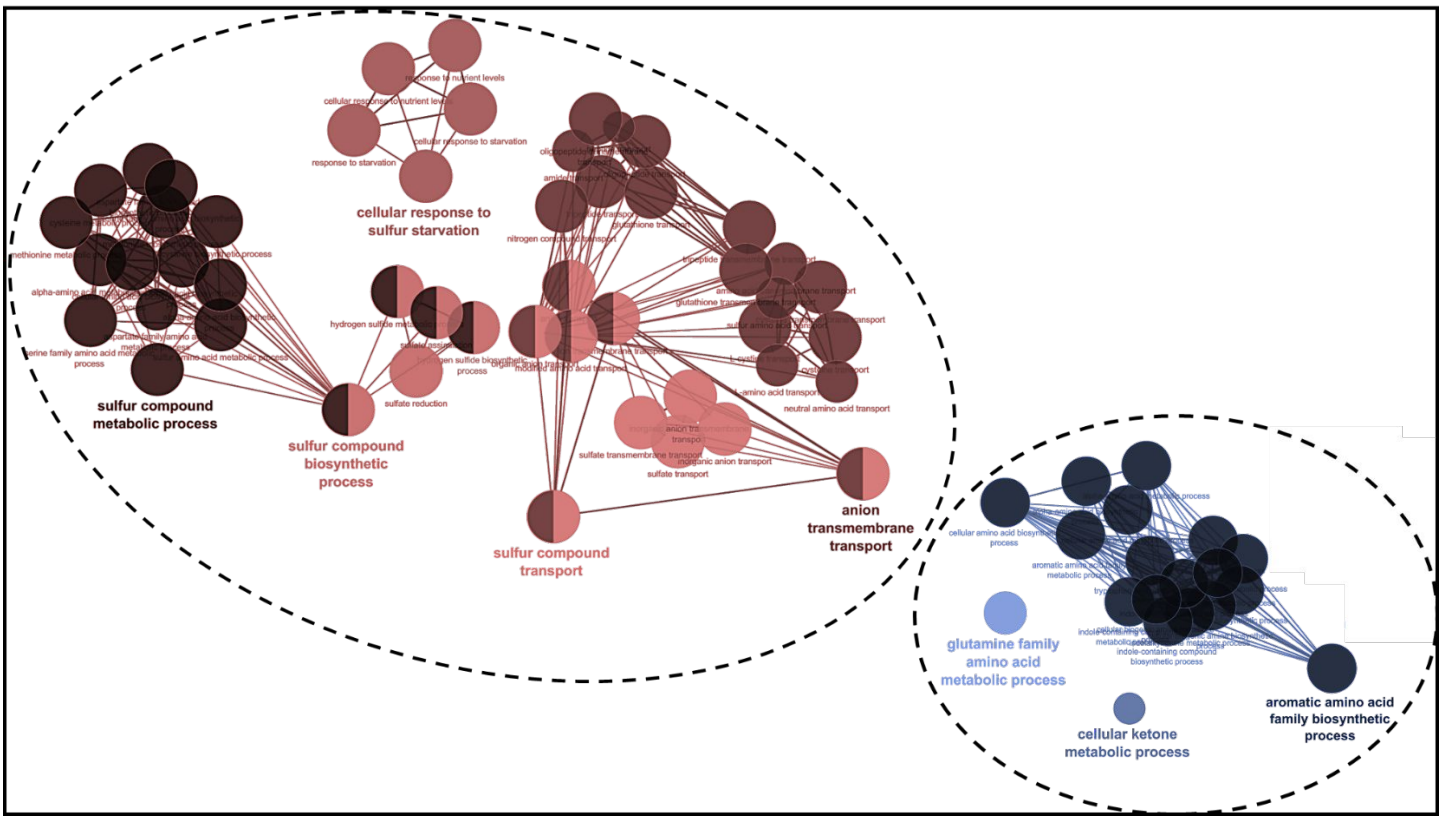
Supplementary Figure 3. Spectroscopic concentration-dependent effects of ligand binding to EcCysK. (**A**) OALS gradient. Apo-EcCysK is represented by a bold black line. Bold red line represents highest concentration of OALS at 1 mM, and other black lines represent intermediate concentrations of OALS. (**B**) Gradient of compound **1** on OALS-EcCysK complex. Apo-EcCysK is again represented by bold black line, OALS-EcCysK complex with no inhibitor is represented by a red line, a bold green line represents compound **1** and EcCysK without OALS, and all other black lines are intermediate concentrations of compound **1** on the OALS-EcCysK complex.



Supplementary Figure 4. LC-MS/MS determination of molecular structure of false product. **(A)** Spectra of compound **1** metabolite from *E. coli* lysate after cell treatment. **(B)** and **(C)** are spectra determined from synthetic, analytical standards of two potential isomeric forms of the CysK-mediated false product.



Supplementary Figure 5. Volcano plots of RNA-seq data from *E. coli* treated with compound 1. Dashed lines represent cutoffs for significant fold-change of expression at 2 and a P-value of 0.01. Genes with significantly altered transcription and P-values are in blue, genes with significant change in transcription but a P-value of > 0.01 are in red. Green dots represent genes with insignificant changes in transcription in terms of both fold-change and P-value. Grey dots represent genes with a P-value > 0.01 and no significant transcriptional change. Data is shown for (**A**) *E. coli* global transcriptional response in media environment, (**B**) treatment with compound **1** in M9T, and (**C**) treatment with compound **1** in M9.



Supplementary Figure 6. Pathway analysis of transcriptomic changes in M9T relative to M9. Only genes with at least a 2-fold change in expression and P-value ≤ 0.01 were chosen for the pathway analysis. Blue indicates downregulation and red indicates upregulation.

Supplementary Table 1. Validated hits from *E. coli* whole cell antimetabolite screen

Common Name/SJ#[a]	MIC[b] (μM)	Fold Shift MIC[c]		Screening Library	Mechanism of Action
		Methionine	*p*ABA		
5-fluorouracil	0.16	-	-	Bioactives	Thymidylate synthase inhibitor
Floxuridine	0.21	1.3	-	Bioactives	Thymidylate synthase inhibitor
Decitabine	0.32	1.6	0.83	Bioactives	DNA methyltransferase inhibitor
Azacytidine	1.6	0.71	0.71	Lead-Like	Cytidine analog, treats myelodysplastic syndrome
Zebularine	1.8	-	0.75	Bioactives	Cytidine analog, epigenetic therapy
Bleomycin	3.1	-	0.67	Bioactives	Induces DNA strand breaks
STATTIC	4.5	2.0	-	Bioactives	STAT3 inhibitor
Pifithrin	6.5	2.0	-	Bioactives	HSP70 inhibitor
Cladribine	8.9	1.7	-	Bioactives	Purine analog, inhibits adenosine deaminase
SJ000208968 (**1**)	12.6	4.0	-	Lead-Like	Unknown
Doxifluridine	14.3	-	0.88	Bioactives	Thymidylate synthase inhibitor
5-fluoro-1-(tetrahydro-2-furfuryl)uracil	18.1	1.3	-	Bioactives	Thymidylate synthase inhibitor
SJ000461885	21	4.8	-	Lead-Like	Unknown
SJ000209472 (**2**)	25	5.3	-	Lead-Like	Unknown
Cisplatin	42	2.4	-	Bioactives	Inhibits DNA replication, crosslinks purine bases
SJ000866485	44	-	-	Small Fragment	Unknown
SJ000208489 (**3**)	50	5.3	-	Lead-Like	Unknown
SJ000129420	50	3.3	1.7	Lead-Like	Unknown
Chloramphenicol	5.0	1.3	-	NA	Control antibiotic
Sulfamethoxazole	0.39	2.0	849	NA	Control antibiotic
Trimethoprim	0.69	-	-	NA	Control antibiotic

[a]Numbers designated in this study for the three selected hits **1**-**3** are noted in parenthesis.

[b]MIC values and MIC shifts are an average of three independent experiments.

[c]Fold shift MIC is the ratio of supplemented MIC over unsupplemented MIC. L-methionine was supplemented at a final concentration of 20 μg/mL. *p*ABA was supplemented at a final concentration of 5 μg/mL.

Supplementary Table 2. Susceptibility of *E. coli* K12 MG1655 to TAT analogs

Cpd. #	SMILE	*E. coli* K12 MG1655 MIC in M9[a] (μg/mL)
1	Fc1ccccc1NC(=O)CSc1c[nH]nn1	1.6
2	Fc1ccc(NC(=O)CSc2c[nH]nn2)cc1	3.1
3	O=C(CSc1c[nH]nn1)Nc1ccccc1C#N	3.1
4	FC1=CC=CC(NCCSC2=CN=NN2)=C1	1.6
5	O=C(CSC1=CN=NN1)NC1=NC=CS1	1.6
6	O=C(CSC1=CN=NN1)NC1=CC=CC=N1	1.6
7	CN1N=NC=C1SCC(=O)NC1=CC=CC=C1	>200
8	O=C(CSC1=NN=CN1)NC2=CC=CC=C2	12.5
9	FC1=C(NCCSC2=CN=NN2)C=CC=C1	12.5
10	O=C(COC1=NNC=N1)NC1=CC=CC=C1	>200
11	O=C(CNC1=NNC=N1)NC1=CC=CC=C1	>200
12	N[C@H](CN1N=NC=C1SCC(=O)NC1=CC=CC=C1F)C(O)=O	>200
CAM[b]		1.6
SMX		0.98
TMP		0.98

[a]Data represent an average of three independent experiments.
[b]CAM = Chloramphenicol; SMX = Sulfamethoxazole; TMP = Trimethoprim.

Supplementary Table 3A. Strains used in this study and growth inhibition by compound 1

Organism	Characteristic/Genotype	MIC Media	MIC of Cpd. 1 (μg/mL)
E. coli	K12 MG1655	M9	1.6
E. coli	BW25113	M9	1.6
E. coli	BW25113	M9AA-CMS[a]	3.1
E. coli ΔcysK	JW2407, Kan^R	M9AA-CMS	50
E. coli ΔcysM	JW2414, Kan^R	M9AA-CMS	1.6
E. coli pPJ131	BW25113, pPJ131 empty plasmid, CBN^R	M9AA-CMS	1.6
E. coli:*cysK*	BW25113, pPJ131:*cysK*, CBN^R	M9AA-CMS	1.6
E. coli ΔcysK pPJ131	JW2407, pPJ131 empty plasmid, KAN^R, CBN^R	ND	ND
E. coli ΔcysK:cysK	JW2407, pPJ131:*cysK*, Kan^R, CBN^R	M9AA-CMS	0.78
E. coli ΔcysK:cysK^{S70C}	JW2407, pPJ131:*cysK*^{S70C}, Kan^R, CBN^R	M9AA-CMS	>100
E. cloacae	ATCC 13047	M9AA-CMS	5.0
K. pneumoniae	ATCC 700603	M9AA-CMS	25
A. baumannii	ATCC 19606	M9AA-CMS	50
P. aeruginosa	PAO1	M9	200
S. aureus	ATCC 29213	SSM9PR	>200
E. faecalis	ATCC 33186	Mueller Hinton II	>200
M. tuberculosis	H37Rv	Sautons	>200

[a]M9AA-CMS is supplemented with all amino acids except for cysteine, serine, and methionine. Amino acids were supplemented at concentrations listed in Zlitni *et al*.[1] ND: Not determined.

Supplementary Table 3B. Strains used in this study and growth inhibition by compound12

Organism	Characteristic/Genotype	MIC Media	MIC of Cpd. 12 (μg/mL)
E. coli	BW25113	M9	>200
E. coli	BW25113	M9 +25uM PAβN	>200
E. coli	BW25113	M9 +0.1ug/mL colistin	>200
E. coli	GKCW102, BW25113, araC P_{araBAD} *fhuA⊿C/⊿4L*[2]	M9 +0.1% arabinose	>200

Supplementary Table 4. Cytotoxicity of lead TATs (IC_{50}, µM)

	Vero	HepG2
1	>200	>200
2	>200	>200
3	>200	>200
5	>200	>200
Nitrofurantoin	15.2 ±0.3	5.9 ±0.3
Verapamil	54.8 ±1.3	109.4 ±56.9
Thioridazine	6.1 ±0.9	5.6 ±1.7
Saponin	11.6 ±0.6	7.8 ±0.8

Vero and HepG2 IC_{50} values are an average of two technical replicates ±standard deviation.

Supplementary Table 5. Susceptibility of Gram-negative clinical isolates to lead TATs in M9AA-CMS.

	MIC (μg/μL)[a]						
Isolate	**AMK[b]**	**SMX**	**TMP**	**1**	**4**	**5**	**6**
E. coli							
BW25113	1.0	0.098	0.31	1.6	1.6	1.0	1.2
EcCI 1	6.3	>25	>25	1.6	1.3	1.6	1.3
EcCI 2	5.2	0.39	0.49	3.5	2.0	3.5	1.6
EcCI 3	3.1	0.78	>25	2.7	2.7	3.1	3.1
EcCI 4	3.1	>25	>25	1.3	1.0	1.6	1.3
EcCI 5	3.1	>25	>25	3.1	2.6	3.1	2.6
EcCI 6	9.4	>25	>25	1.6	1.6	1.6	1.6
K. pneumoniae							
ATCC 700603	0.78	>25	3.1	33.3	25	50	25
KpCI 1	1.6	0.78	0.68	15.6	12.5	25	12.5
KpCI 2	5.2	>25	>25	>100	>100	>100	>100
KpCI 3	1.6	>25	>25	12.5	12.5	25	12.5
KpCI 4	1.0	0.65	0.78	12.5	12.5	25	12.5
KpCI 5	0.78	>25	>25	20.8	16.7	25	16.7
KpCI 6	1.0	>25	>25	25	25	41.7	12.5

[a]MICs are an average of at least three independent experiments.

[b]AMK = amikacin; SMX = sulfamethoxazole; TMP = trimethoprim; EcCI = *E. coli* clinical isolate; KpCI = *K. pneumoniae* clinical isolate.

Supplementary Table 6. Location and MIC shifts of spontaneous *E. coli* MG1655 mutants

	CysK Mutation	1	2	3	SMX	TMP	CAM	KAN
1a	None	>64	>64	>64	1	2	1	2
1b	G71R	>64	>64	>64	1	1	0.5	1
2a	S70C	>64	>64	64	1	1	1	1
2b	S70C	>64	>64	64	1	1	1	2
2c	R305S	>64	>64	64	1	2	1	2
2d	P37H	>64	>64	64	1	1	0.5	1
2e	R44G	64	64	64	1	2	1	2
3a	R305S	>64	>64	64	1	1	1	1
3b	R305S	>64	>64	64	1	2	1	1
3c	R305L	>64	>64	64	1	1	0.5	1
3d	S70C	>64	>64	>64	1	1	1	1
3e	Y306F	>64	>64	64	1	1	1	2

[a]Each selected mutant named alphabetically relative to the compound (**1**-**3**) it was raised against.
[b]MIC shift of each mutant relative to WT MIC. MICs were determined at 24 hours of incubation at 37°C.
Notes: SMX: sulfamethoxazole, TMP: trimethoprim, CAM: chloramphenicol, KAN: kanamycin.

Supplementary Table 7. *E. coli* BW25113 susceptibilities and metabolite-induced MIC shifts

Cpd.	µg/mL MIC	*E. coli* BW25113 MIC Shift[a]							
		L-Cys[b]	L-Glutathione	L-Met	L-Ser	OALS	Na_2S	AA-CMS[c]	*p*ABA
1	0.78	>256	128	2	128	128	8	2	2
1,2,4-triazole	50	64	32	4	64	64	4	2	2
EcP10[d]	>200	NT	NT	NT	NT	NT	NT	NT	NT
SMX	0.10	0.5	2	8	2	2	2	2	>256
TMP	0.39	0.5	1	1	0.5	0.5	1	2	1
AMP	0.78	2	1.5	2	1	1	2	2	1

Notes: SMX = sulfamethoxazole; TMP = trimethoprim; AMP = ampicillin; NT = not tested.
[a]MIC shift is the ratio of averaged MICs from two experiments in supplemented M9 over unsupplemented M9. Dashes represent the absence of a shift of 2-fold or higher.
[b]L-Glutathione was supplemented at 10 µg/mL and $Na_2S•9H_2O$ was supplemented at 72 µg/mL. All other metabolites supplemented at concentrations previously specified.[1]
[c]AA-CMS is all twenty standard amino acids with the exception of cysteine, methionine, and serine. Amino acids are again supplemented at concentrations previously specified.[1]
[d]EcP10 is a peptide comprised of the last ten residues on the C-terminal tail of *E. coli* serine acetyltransferase (NHTFEYGDGI).

Supplementary Table 8. Susceptibility of the *E. coli cysK* and *cysM* knockout strains in M9AA-CMS.

Drug/Cpd.#	MIC (μg/mL)			ΔcysK fold decrease in MIC[a]
	WT	Δ*cysM*	Δ*cysK*	
Ampicillin	0.78	0.78	0.78	1
1	3.1	3.1	50	0.063
Sulfamethoxazole	0.098	0.098	0.024	4
Pterin-SMX	3.1	3.1	0.78	4
Trimethoprim	0.39	0.78	0.049	8
Tetracycline	0.78	1.6	0.39	2
Chloramphenicol	3.1	3.1	0.78	4

[a]Fold decrease in MIC represents the ratio of the Δ*cysK* MIC over the BW25113 MIC.

Supplementary Table 9. LC-MS/MS detection of compounds 1 and 12 (μM[a])

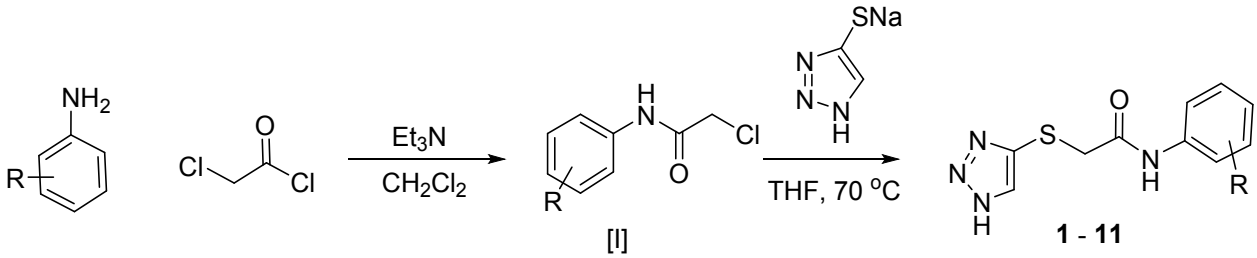
	In vivo[b]		*In vitro*[c]	
	1	**12**	**1**	**12**
Replicate 1	103.7	169.6	521.0	392.9
Replicate 2	132.5	198.5	499.7	431.2
Replicate 3	98.9	217.4	483.8	389.7
Average ±Std dev	**112 ±18**	**195 ±24**	**501 ±18**	**404 ±20**

[a]Experimental samples were quantified by standard curve of pure compound **1**, R^2 =0.9926 and the false product **12** R^2=0.9979 [b]*In vivo* experiments were treated with 0.5mM compound **1** [c]*In vitro* experiments were treated with 1mM compound **1**.

Supplementary Synthesis and characterization of compounds 1 - 12:

General experimental procedure: All solvents used for chromatography and liquid chromatography were purchased from Aldrich. Flash column chromatography silica cartridges were obtained from Biotage Inc. Reactions were monitored by thin-layer chromatography (TLC) on pre-coated Merch 60 F254 silica gel plates and visualized using UV light (254 nm). A Biotage FLASH column chromatography system was used to purify mixtures. 1H NMR spectra were recorded on a Varian INOVA-500 spectrometer or on a Bruker 400 MHz NMR spectrometer. Chemical shifts (δ) are reported in parts per million relative to the residual solvent peak or internal standard (tetramethylsilane), and coupling constants (*J*) are reported in hertz (Hz). Purity of the products was confirmed by UPLC/MS (the Waters Acquity).

Scheme 1: General method for the synthesis of compounds **1** - **11**



General procedure A: To a stirred solution of substituted amine (5.37 mmoL) in CH_2Cl_2 (7.0 mL) was added triethylamine (5.37 mmoL) and chloroacetyl chloride[3] (5.37 mmoL) at 0 °C. After stirring the reaction mixture for 1h, diluted with CH_2Cl_2 (20 mL), washed with $NaHCO_3$, organic layer was dried over Na_2SO_4, and concentrated under high vacuum to give crude chloroacetyl derivative [I]. To a crude chloroacetyl derivative (2.65 mmoL) in THF (6.0 mL) was added sodium 1H-1,2,3-triazole-5-thiolate (2.65 mmoL) and heated at 70 °C for 1h. The solids were filtered off and the filtrate was evaporated under high vacuum to give crude product. The crude product was purified over silica-gel column chromatography (Eluents: 20 – 50% EtOAc in hexane) to produce pure compound.

2-((1H-1,2,3-triazol-4-yl)thio)-N-(2-fluorophenyl)acetamide (1): Compound **1** (400 mg, 76%) was synthesized by following general procedure A, as a white solid. ^{1}H NMR (500 MHz, D_2O) δ 8.08 (s, 1H), 7.67 (td, *J* = 8.0, 1.9 Hz, 1H), 7.35 – 7.30 (m, 3H), 3.86 (s, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ 38.2, 115.0 (d, *J* = 19.8 Hz), 124.2 – 123.6 (m), 125.5 (d, *J* = 11.3 Hz), 125.7 (d, *J* = 7.6 Hz), 154.2 (d, *J* = 245.6 Hz), 168.5.

2-((1H-1,2,3-triazol-4-yl)thio)-N-(4-fluorophenyl)acetamide (2): Compound **2** (350 mg, 32%) was synthesized by following general procedure A, as a white solid. ^{1}H NMR (500 MHz, CD_3OD) δ 7.87 (s, 1H), 7.67 – 7.33 (m, 2H), 7.04 (tt, *J* = 8.7, 1.2 Hz, 2H), 3.71 (s, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ 38.6, 114.8, 115.0, 121.7, 121.8, 134.3, 134.3, 158.5, 160.4, 168.0.

2-((1H-1,2,3-triazol-4-yl)thio)-N-(2-cyanophenyl)acetamide (3): Compound **3** (160 mg, 38%) was synthesized by following general procedure A, as a white solid. ^{1}H NMR (500 MHz, CD_3OD) δ 7.93 (s, 1H), 7.70 (d, *J* = 8.2 Hz, 1H), 7.66 (dd, *J* = 7.8, 1.5 Hz, 1H), 7.60 (td, *J* = 8.0, 1.6 Hz, 1H), 7.35 – 7.23 (m, 1H), 3.83 (s, 2H). ^{13}C NMR (125 MHz, CD_3OD) δ 38.2, 106.9, 116.1, 124.7, 125.8, 132.9, 133.5, 139.5, 168.7, 171.7.

2-((1H-1,2,3-triazol-4-yl)thio)-N-phenylacetamide (4): Compound **4** (500 mg, 70%) was synthesized by following general procedure A, as a white solid. ^{1}H NMR (500 MHz, CD_3OD) δ 7.86 (s, 1H), 7.61 – 7.44 (m, 2H), 7.29 (tt, *J* = 8.5, 1.9 Hz, 2H), 7.21 – 7.01 (m, 1H), 3.71 (s, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ 38.7, 119.9, 119.9, 120.0, 124.1, 128.4, 131.2, 138.0, 138.1, 168.1.

2-((1H-1,2,3-triazol-4-yl)thio)-N-(thiazol-2-yl)acetamide (5): Compound **5** (350 mg, 55%) was synthesized by following general procedure A, as a white solid. ^{1}H NMR (500 MHz, D_2O) δ 8.01 (s, 1H), 7.46 (d, *J* = 3.6 Hz, 1H), 7.21 (d, *J* = 3.7 Hz, 1H), 3.82 (s, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ 38.0, 114.2, 135.0, 138.2, 158.2, 167.3.

2-((1H-1,2,3-triazol-4-yl)thio)-N-(pyridin-2-yl)acetamide (6): Compound **6** (405mg, 49%) was synthesized by following general procedure A, as a white solid. ^{1}H NMR (500 MHz, CD_3OD) δ 7.86 (s, 1H), 7.56 – 7.38 (m, 2H), 7.03 (td, *J* = 9.1, 2.1 Hz, 2H), 3.70 (s, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ 38.6, 114.8, 115.0, 121.8, 121.8, 134.3, 134.3, 158.5, 160.4, 168.0.

2-((1-methyl-1H-1,2,3-triazol-5-yl)thio)-N-phenylacetamide (7) and 2-((1-methyl-1H-1,2,3-triazol-4-yl)thio)-N-phenylacetamide (7-N1):

To a stirred solution of **4** (100 mg, 0.43 mmoL) in acetonitrile (3.0 mL) was added Cs_2CO_3 (139 mg, 0.43 mmoL) and iodomethane (0.029 mL, 0.43 mmoL) and stirred at room temperature for 2 h. Quenched with water (5.0 mL), extracted with EtOAc (2 X 10 mL), combined organic layer was dried over Na_2SO_4, and concentrated under high vacuum to give crude product. Chromatographic (eluents: 40% EtOAc in hexane) purification on silica-gel yielded compound **7** (30 mg, 26%) and **7-N1** (35 mg, 33%) as a white solid. Regio chemistry of methyl group is confirmed by HMBC correlation study.

7: ^{1}H NMR (500 MHz, CD_3OD) δ 7.64 (s, 1H), 7.50 (d, *J* = 8.0 Hz, 2H), 7.30 (t, *J* = 7.9 Hz, 2H), 7.10 (t, *J* = 7.4 Hz, 1H), 4.11 (s, 3H), 3.72 (s, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ 38.3, 40.8, 119.9, 124.1, 128.5, 135.9, 138.1, 140.1, 168.0.

7-N1: ^{1}H NMR (500 MHz, CD_3OD) δ 7.99 (s, 1H), 7.50 (d, *J* = 8.0 Hz, 2H), 7.29 (t, *J* = 7.8 Hz, 2H), 7.09 (t, *J* = 7.4 Hz, 1H), 4.05 (s, 3H), 3.66 (s, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ 35.9, 39.1, 119.8, 124.1, 128.3, 128.4, 138.1, 138.3, 168.1.

2-((1H-1,2,4-triazol-3-yl)thio)-N-phenylacetamide (8): Compound **8** (90 mg, 65%) was synthesized by following general procedure A using 1H-1,2,4-triazol-3-ol, as a white solid. ^{1}H NMR (500 MHz, CD_3OD) δ 8.36 (s, 1H), 7.52 (d, *J* = 8.1 Hz, 2H), 7.27 (t, *J* = 7.8 Hz, 2H), 7.07 (t, *J* = 7.5 Hz, 1H), 3.99 (s, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ 36.1, 119.8, 124.1, 128.5, 138.2, 145.8, 167.7.

N-(2-((1H-1,2,3-triazol-5-yl)thio)ethyl)-2-fluoroaniline (9):

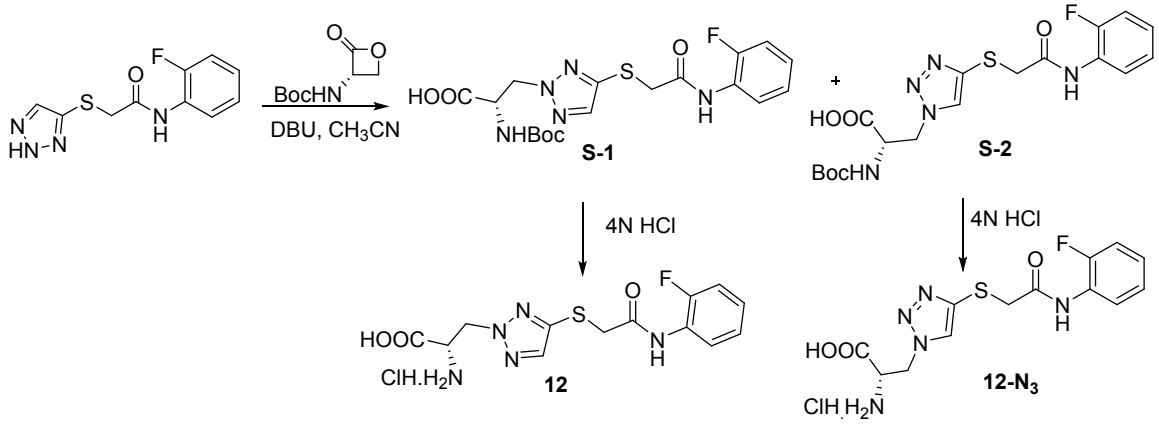
To a stirred solution of 2-chloro-N-(2-fluorophenyl)acetamide (50 mg, 0.27 mmoL) in THF (5 mL) was added $BH_3.SMe_3$ (0.25 mL, 2.67 mmoL) at 0 ^{o}C and the reaction mixture was stirred for 18 h at room temperature. Quenched with MeOH (2.0 mL) and the reaction mixture was evaporated to give crude residue which was dissolved in EtOAc, washed with 1N HCl solution, organic layer dried over Na_2SO_4, and concentrated to give amine. To a crude amine (60 mg) in THF (2.0 mL) was added sodium 1H-1,2,3-triazole-5-thiolate (63 mg, 1.5eq) and TBAI (5 mg) and the reaction mixture was heated at 70 ^{o}C for 30 min. Solids were filtered off and the solute was evaporated to give crude product which was purified on silica-gel column chromatography to yield **9** (25 mg, 39%) as a white solid. ^{1}H NMR (500 MHz, CD_3OD) δ 7.84 (s, 1H), 7.02 – 6.86 (m, 2H), 6.69 (td, *J* = 8.4, 1.5 Hz, 1H), 6.66 – 6.54 (m, 1H), 3.42 (t, *J* = 6.9 Hz, 2H), 3.11 (t, *J* = 6.9 Hz, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ 33.2, 42.6, 111.9, 111.9, 113.9, 114.0, 116.2, 116.3, 124.2, 124.3, 136.0, 136.1, 150.7, 152.6.

2-((1H-1,2,4-triazol-3-yl)oxy)-N-phenylacetamide (10): Compound **10** (21 mg, 16%) was synthesized by following general procedure A using 1H-1,2,4-triazol-3-ol, as a white solid. ^{1}H NMR (500 MHz, D_2O) δ 9.32 (s, 1H), 8.90 (d, *J* = 8.0 Hz, 2H), 8.82 (d, *J* = 7.7 Hz, 2H), 8.64 (t, *J* = 7.5 Hz, 1H), 6.05 (s, 2H); ^{13}C NMR (125 MHz, D_2O) δ 45.8, 122.4, 126.8, 130.5, 138.2, 140.7, 157.0, 167.7.

2-((1H-1,2,4-triazol-3-yl)amino)-N-phenylacetamide (11): Compound **11** (15 mg, 23%) was synthesized by following general procedure A using 1H-1,2,4-triazol-3-amine, as a white solid. ^{1}H

NMR (500 MHz, D_2O) δ 7.73 -7.72 (m, 2H), 7.70 (s, 1H), 7.43 (t, *J* = 7.6 Hz, 2H), 7.33 – 7.10 (m, 1H), 4.98 (s, 2H); ^{13}C NMR (125 MHz, D_2O) δ 122.9, 127.5, 131.7, 140.1, 151.3, 158.8, 168.3.

Scheme 2: Synthesis of **12**



(S)-2-((tert-butoxycarbonyl)amino)-3-(4-((2-((2-fluorophenyl)amino)-2-oxoethyl)thio)-2H-1,2,3-triazol-2-yl)propanoic acid (S-1) and (S)-2-((tert-butoxycarbonyl)amino)-3-(4-((2-((2-fluorophenyl)amino)-2-oxoethyl)thio)-1H-1,2,3-triazol-1-yl)propanoic acid (S-2): To a stirred solution of **1** (100 mg, 0.40 mmoL) L-serine-β-lactone[4,5] (223 mg, 1.19 mmoL) in CH_3CN (2.0 mL) was added DBU (0.059 mL, 0.40 mmoL) at room temperature for 1h. Quenched water and neutralized with 1N HCl, extracted with EtOAc (3 X 10 mL), dried over Na_2SO_4, and concentrated under high vacuum to give crude products. Purification on silica-gel column chromatography yielded pure products **S-1** (30 mg, 17%) and **S-2** (45 mg, 26%) as a colorless oil. Regio chemistry of isomers was confirmed from HMBC correlation experiment.

S-1: ^1H NMR (500 MHz, CD_3CN) δ 8.15 – 7.98 (m, 1H), 7.68 (s, 1H), 7.38 – 6.86 (m, 3H), 5.73 (dd, *J* = 14.1, 8.4 Hz, 1H), 5.02 – 4.62 (m, 3H), 3.79 (s, 2H), 1.38 (s, 9H); ^{13}C NMR (126 MHz, CD_3CN) δ 170.9, 167.8, 155.4 (d, *J* = 81.0 Hz), 141.6, 136.2, 126.0 (d, *J* = 7.7 Hz), 123.7, 115.8 (d, *J* = 19.4 Hz), 80.1, 56.0, 53.8, 38.7, 28.0.

S-2: ^1H NMR (500 MHz, CD_3OD) δ 8.00 (s, 1H), 7.96 – 7.84 (m, 1H), 7.26 – 7.03 (m, 3H), 4.88 (dd, *J* = 14.0, 4.4 Hz, 1H), 4.74 – 4.60 (m, 2H), 3.77 (s, 2H), 1.39 (s, 9H); ^{13}C NMR (125 MHz, CD_3OD) δ 172.76, 170.40, 157.62 (d, *J* = 109.8 Hz), 155.23, 140.44, 129.88, 127.81 (d, *J* = 7.7 Hz), 126.08 (dd, *J* = 26.8, 2.6 Hz), 117.19 (d, *J* = 19.7 Hz), 81.73, 55.61, 52.85, 40.76, 29.38.

(S)-2-amino-3-(4-((2-((2-fluorophenyl)amino)-2-oxoethyl)thio)-2H-1,2,3-triazol-2-yl)propanoic acid hydrochloride (12): A solution of **S-1** (20 mg, 0.08 mmoL) in 4N HCl in

dioxane (0.5 mL) was stirred for 1h at room temperature. Reaction mixture was evaporated and washed with EtOAc to give pure **12** (10 mg, 34%) as a white solid. ^{1}H NMR (500 MHz, D_2O) δ 7.86 (s, 1H), 7.61 – 7.43 (m, 1H), 7.43 – 7.27 (m, 1H), 7.28 – 7.19 (m, 2H), 5.03 – 4.93 (m, 2H), 4.38 (ddd, *J* = 6.9, 4.5, 2.2 Hz, 1H), 3.86 (s, 2H); ^{13}C NMR (126 MHz, D_2O) δ 170.6, 169.1, 155.3 (d, *J* = 246.3 Hz), 141.3, 137.7, 128.2 (d, *J* = 8.0 Hz), 126.0, 124.6 (d, *J* = 3.6 Hz), 116.0 (d, *J* = 19.6 Hz), 61.7, 53.4, 52.7, 37.6.

(S)-2-amino-3-(4-((2-((2-fluorophenyl)amino)-2-oxoethyl)thio)-1H-1,2,3-triazol-1-yl)propanoic acid hydrochloride (12-N_3): Compound **12**-N_3 (20 mg, 78%) was synthesized analogously as **12** from **S-2**, as a white solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.98 (s, 1H), 8.26 (s, 1H), 7.89 (d, *J* = 2.0 Hz, 1H), 7.49 – 6.99 (m, 3H), 4.87 (d, *J* = 5.2 Hz, 2H), 4.52 (t, *J* = 4.8 Hz, 1H), 3.86 (s, 2H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 168.6, 167.6, 153.9 (d, *J* = 245.2 Hz), 138.9, 128.2, 126.1 (dd, *J* = 51.5, 9.5 Hz), 124.8 (d, *J* = 3.5 Hz), 124.4, 116.0 (d, *J* = 19.2 Hz), 52.3, 49.4, 38.7.

Supplemental References

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