SUPPLEMENTARY INFORMATION

Compounds targeting disulfide bond forming enzyme DsbB of multiple Gram-negative bacteria

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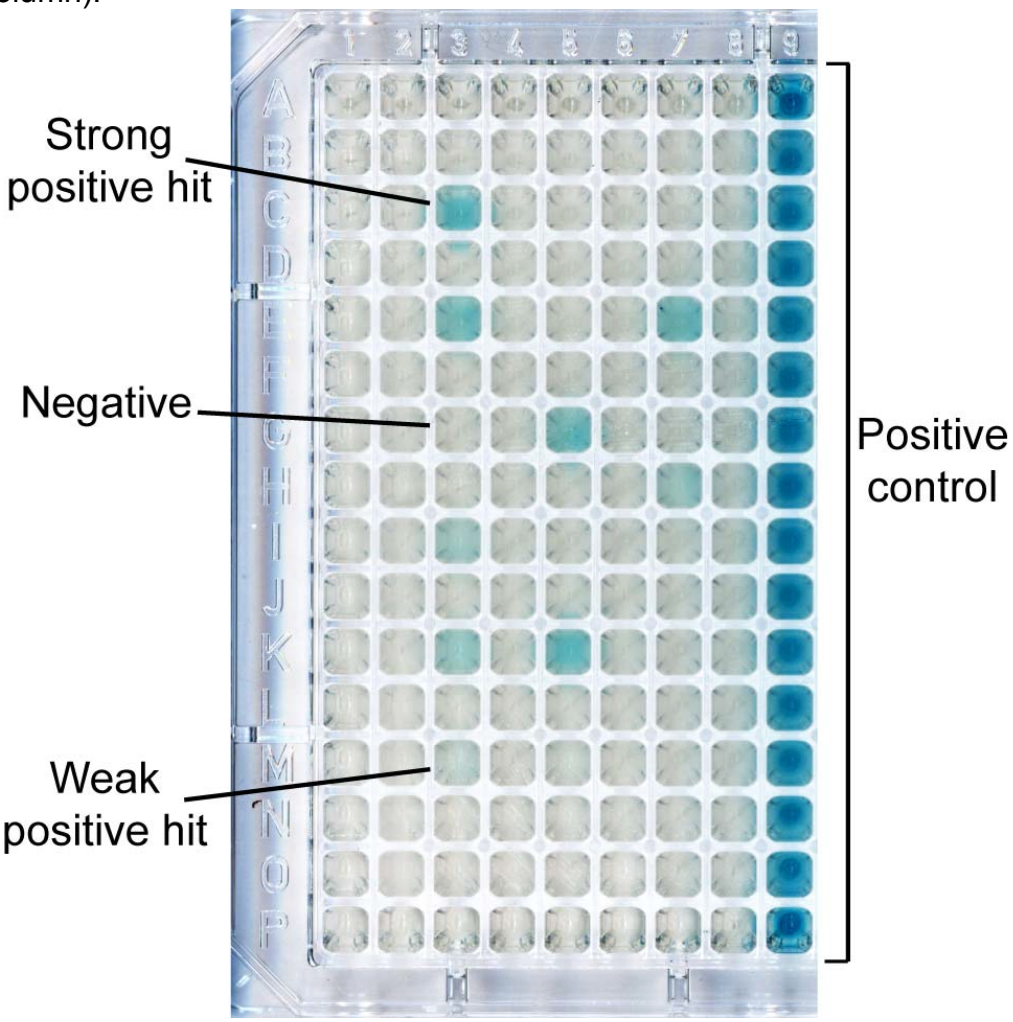
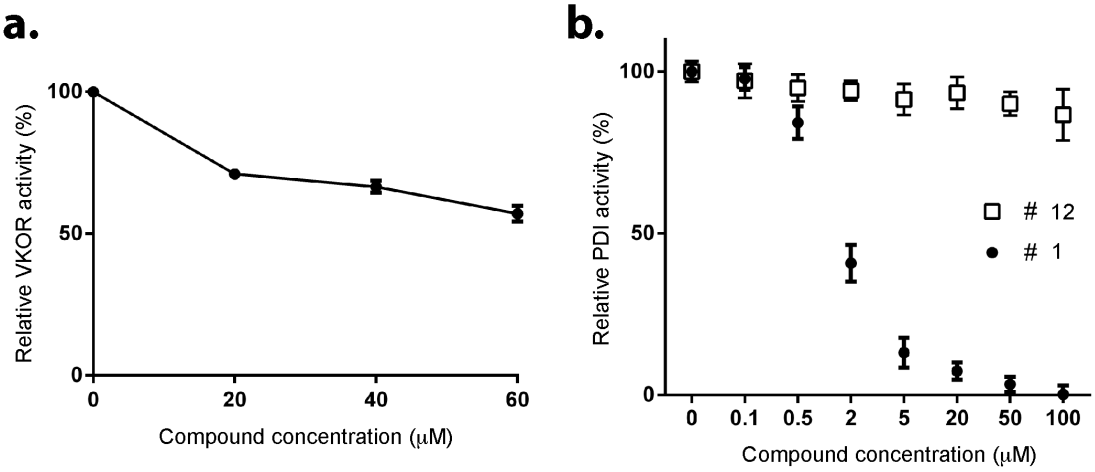
SUPPLEMENTARY RESULTS

Supplementary Figures

Supplementary Figure 1. Screening-plate example. A 384-multiwell plate filled with agar growth media plus chemical compounds and inoculated with E. coli strain expressing β-Gal^{dbs}. A strong positive hit shows up as as a dark blue well; a weak positive hit shows up as light blue and a negative hit as white. The positive control strain is an *E. coli* dsbB mutant expressing β-Gal^{dbs} (last blue-well column).

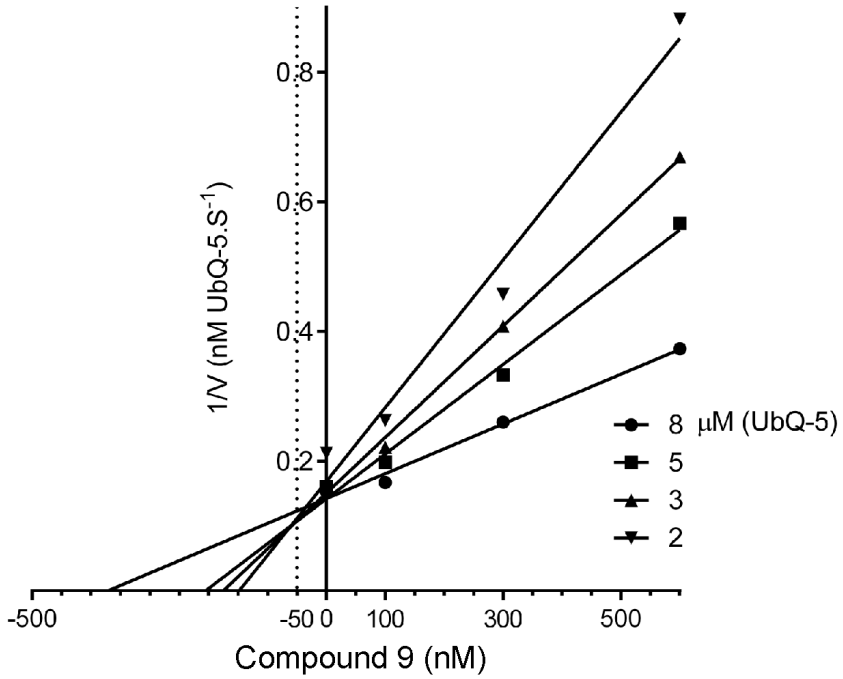


Supplementary Figure 2. Inhibition of mouse VKOR and human PDI by compound 1. (a) Effect of compound 1 on the activity of VKOR preparations from mouse liver microsomes by quantification of the reduction of vitamin K epoxide to vitamin K. Values represent the average of two independent experiments ± SD. (b) The effect of indicated concentrations of compounds 1 (circles) and 12 (squares) on the activity of PDI was measured by the insulin reduction assay. Values represent the average of three independent experiments ± SD.

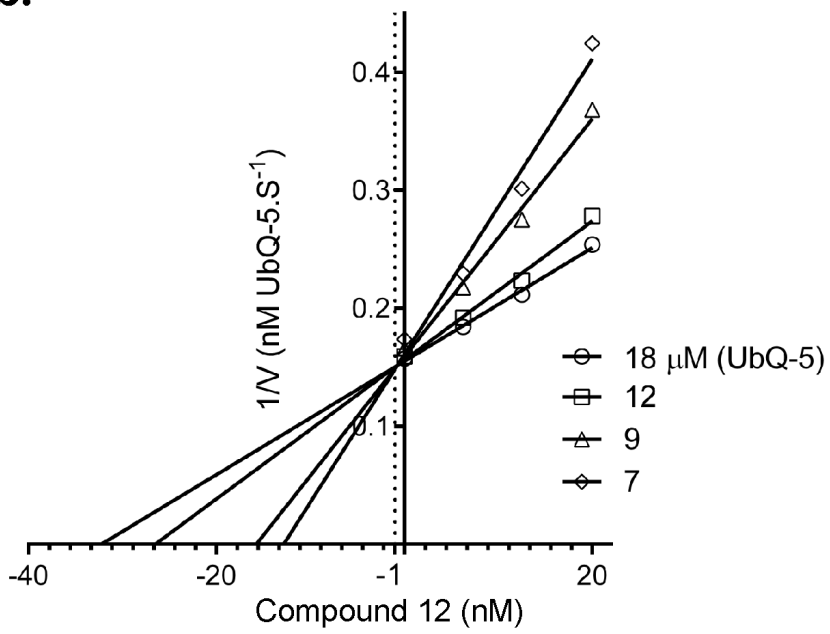


Supplementary Figure 3. Dixon plots of DsbB activity (a)with compound 9, values represent the average of three independent experiments and (b)with compound 12, values represent the average of two independent experiments. See description of details in Online Methods.

a.

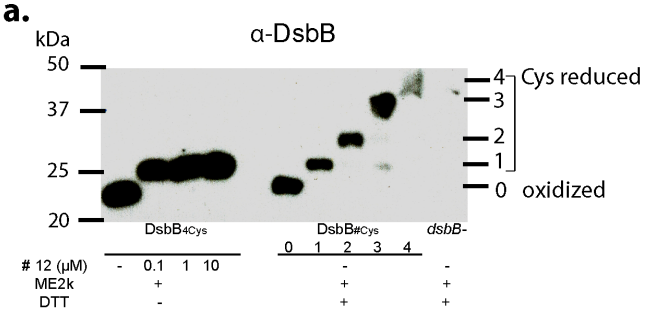


b.



Supplementary Figure 4. Mechanism of inhibition by compound 12. (a) *In vivo* accumulation of reduced DsbB when incubating cells with compound 12. Cells were grown aerobically with different concentrations of drug and precipitated proteins were treated with Maleimide-PEG2k (ME2k, 2kDa). Samples were run on reducing SDS-PAGE and immunoblotted against anti-DsbB. Dithiothreitol (DTT) was used for reducing disulfide bonds prior to alkylation. "oxidized" refers to the position of the oxidized protein which is the same as that of the protein with all four cysteines (Cys) mutated. "Reduced" refers to bands where the positions of the protein with the four or indicated number of reduced cysteines are detected due to alkylation which adds to the molecular weight. Gel shown is a representative immunoblot of two independent experiments. (b) Visible absorbance spectra of DsbB and DsbB-$DsbA_{C33A}$ dimer. The pink color of the DsbB-ubiquinone charge-transfer complex diminishes when compound 12 is added, indicating disruption of the interaction between Cys44 of DsbB and the cofactor ubiquinone. DsbB or DsbB-$DsbA_{C33A}$ complex (each at 100 μM) were mixed with compound 12 (or with DMSO) at 1:2 molar ratio in 50 mM Tris buffer pH 8.0 containing 300 mM NaCl and 0.05% DDM. Samples were incubated on ice for about 4 minutes before the spectra were recorded using 1 cm quarts cuvettes. (c) Summary of deconvoluted masses obtained from ESI-MS analysis of proteins treated with compound 12 (last column). (d) MS/MS fragmentation of DsbB peptide C*IYERVAL. Sequencing ions of the modified (44-51)-peptide gave information consistent with modification of Cys44 by compound 12. The calculated monoisotopic mass of modified b5 ion (residues 44-48, CIYER) is 917.293 Da and the observed mass is 917.295 Da. The calculated mass of the unmodified peptide is 664.300 Da. Thus the mass difference is 252.995 Da which is in agreement with the loss of a chloride ion from 12 upon binding to Cys44, 287.962 (mass of compound 12) - 34.969 (mass of chloride ion) =252.993 Da.

a.



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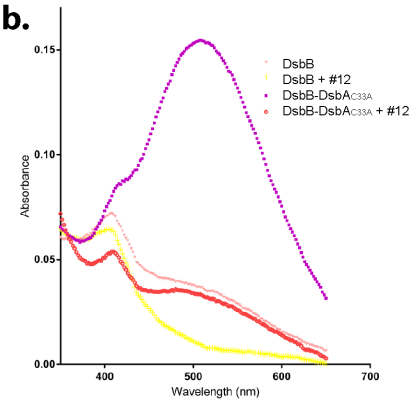
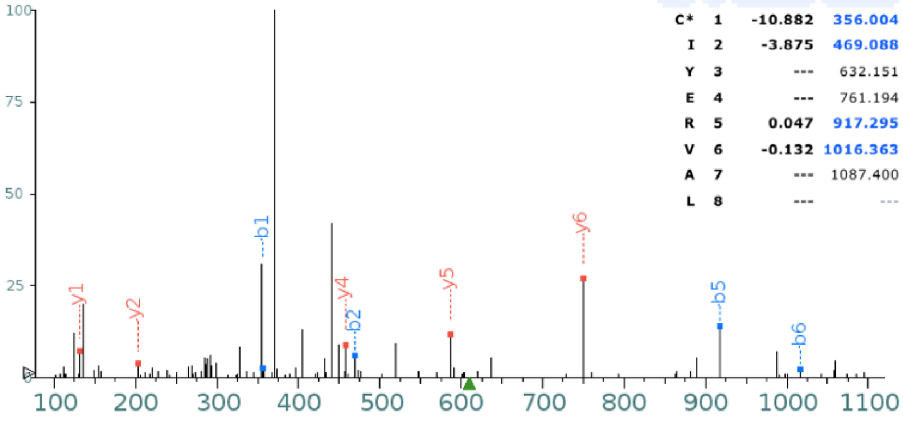


c.

Protein(s)	Theoretical calculated mass (Da)[a]	DMSO CONTROL Observed mass (Da)	COMPOUND 12 (Mw 289.54) Observed mass (Da)	Mass difference (Da)
DsbB	21168.17 (4 cys, disulfide bonded)	21200.6 (+2 ox [b])	No change	-
DsbA$_{C33A}$	22111.04	21984.7 (initiation methionine cleavage)	No change	-
DsbB-DsbA$_{C33A}$ Dimer	43279.21	43187.1 (initiation methionine cleavage)	43440.7	253.6
DsbA reduced	22143.16	22012.6 (initiation methionine cleavage)	No change	-

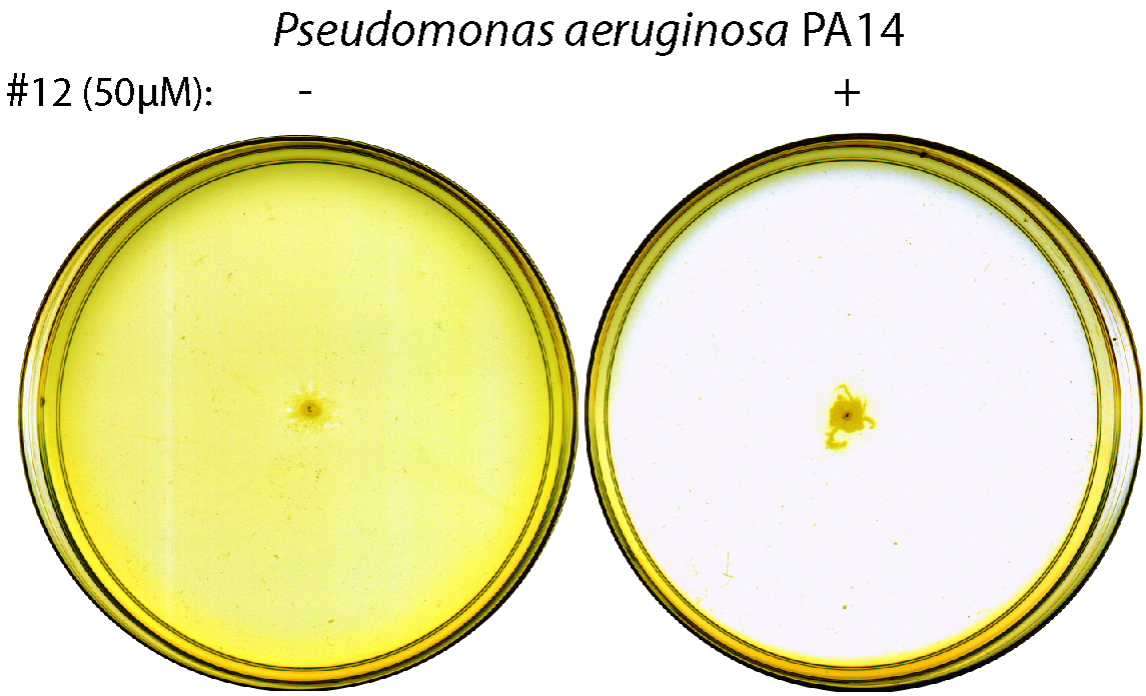
a Masses are +/- 2 Da.
b Ox stands for addition of 16 Da oxidation.

d.



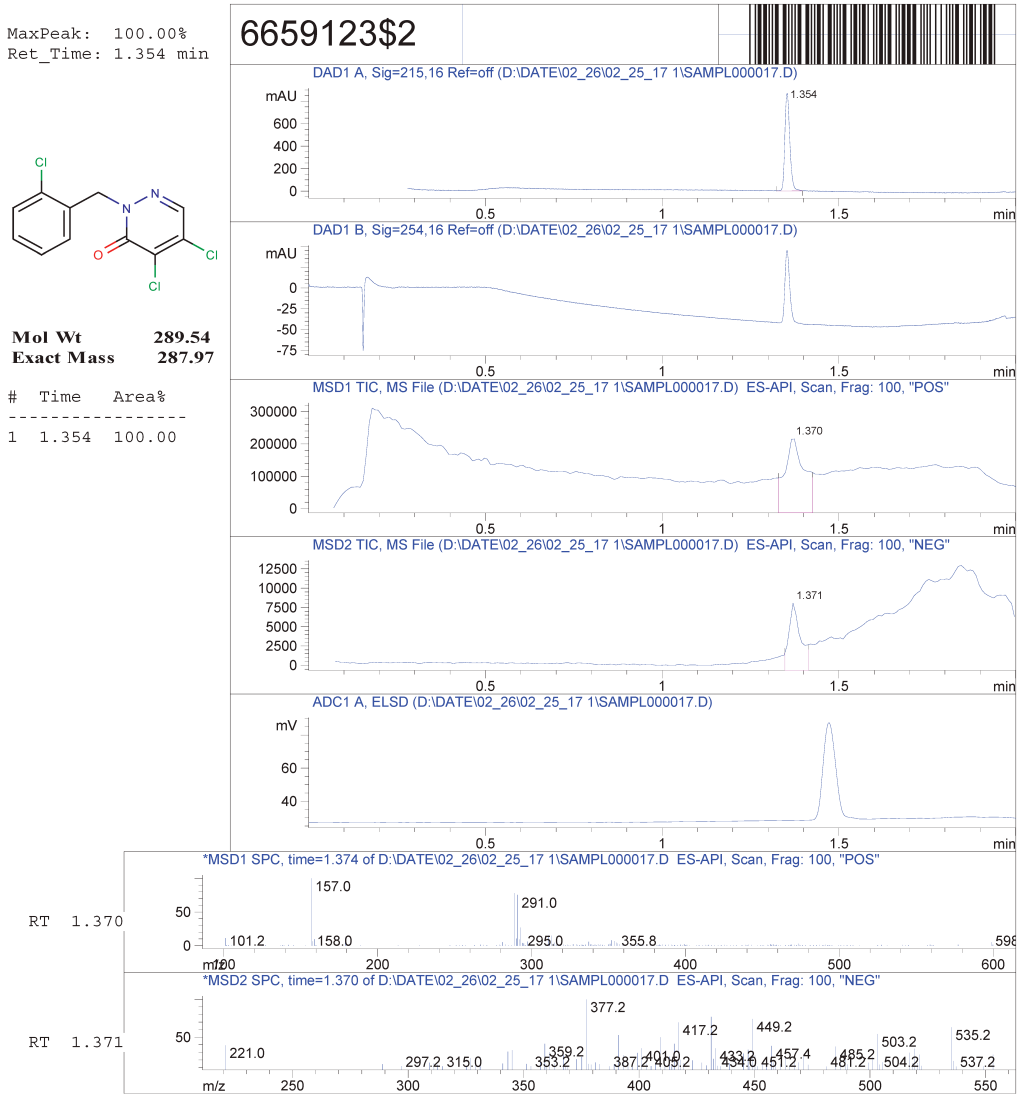
Seq	#	b: Δ Error	b	y	y: Δ Error	+1
C*	1	-10.882	356.004	---	---	8
I	2	-3.875	469.088	863.499	---	7
Y	3	---	632.151	750.414	2.228	6
E	4	---	761.194	587.351	0.932	5
R	5	0.047	917.295	458.309	0.707	4
V	6	-0.132	1016.363	302.207	---	3
A	7	---	1087.400	203.139	0.844	2
L	8	---	---	132.102	1.215	1

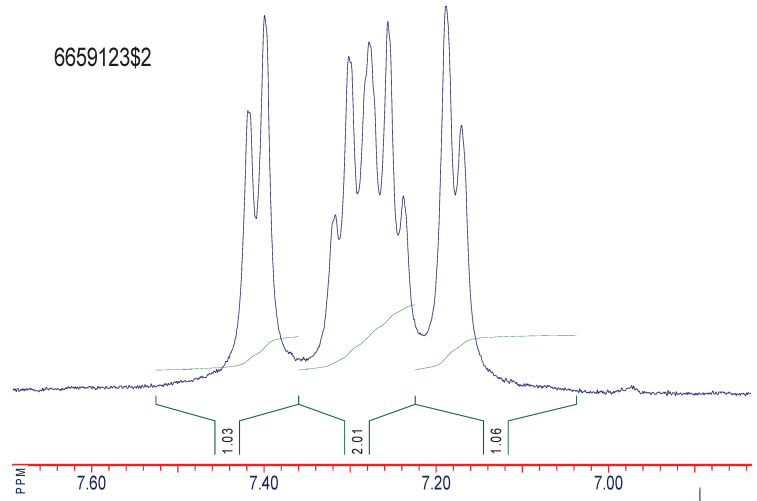
Supplementary Figure 5. Inhibition of *Pseudomonas aeruginosa* twitching motility by compound 12. Motility of *P. aeruginosa* on a hard and thin-layer of M63 minimal media was tested in the absence (left) or presence (right) of compound 12. The picture shows 10 cm-petri plates and is representative of three independent experiments.

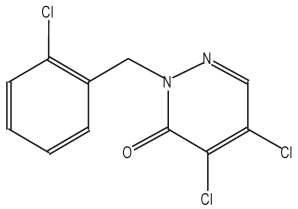


Supplementary analysis of compound 12 purity.

Purity of compound 12: 95%, assessed by HPLC-MS (Vendor and ICCB) and NMR (Vendor).







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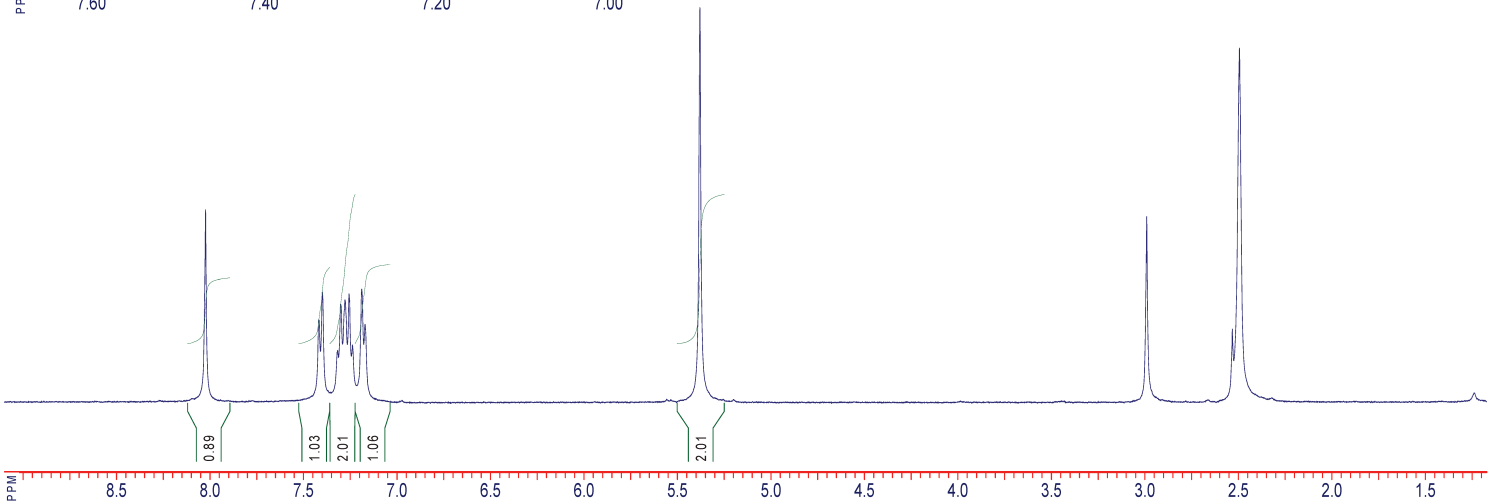

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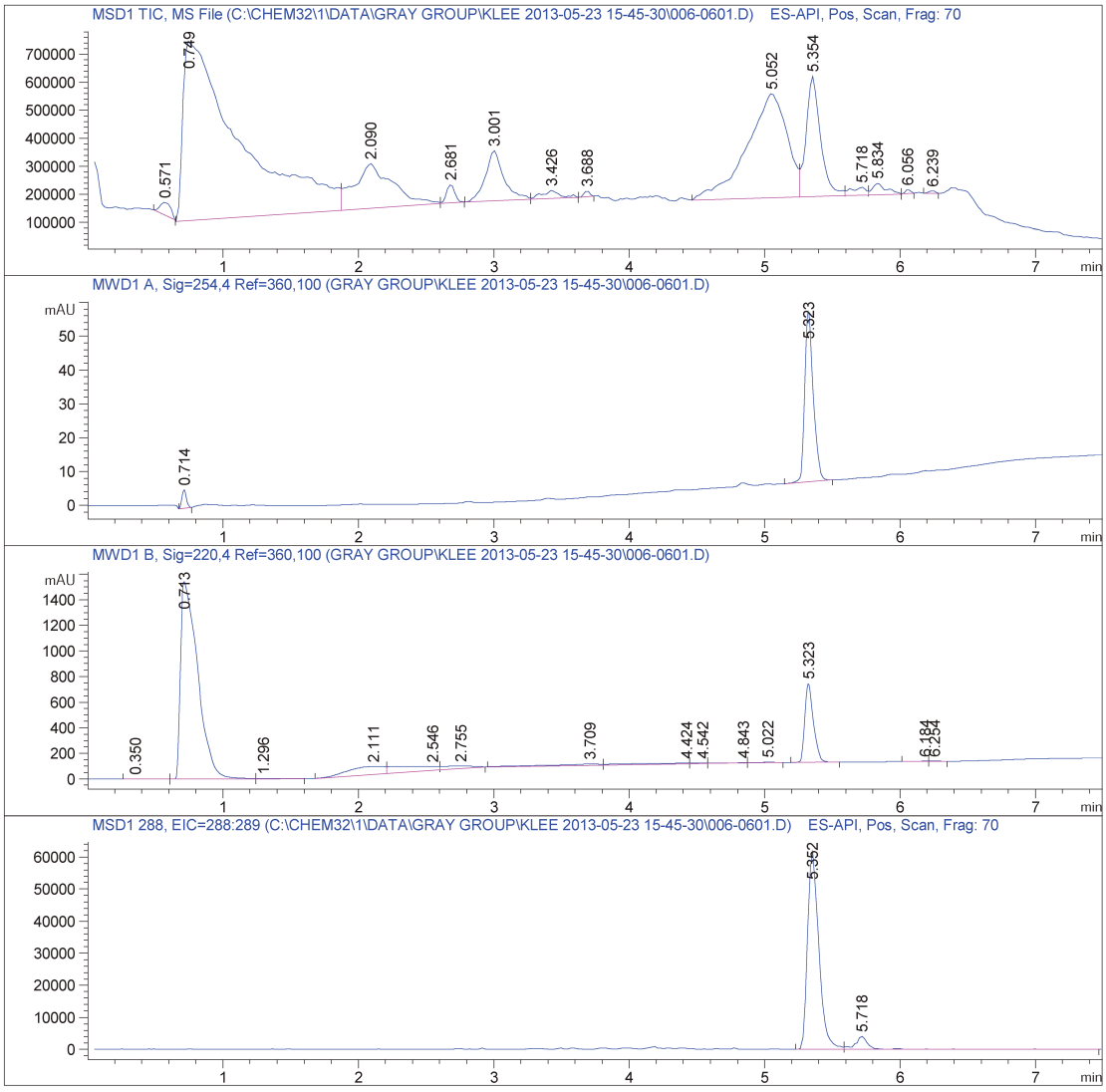

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Spectra averaged over upper half of peaks.
Noise Cutoff: 1000 counts.
Reportable Ion Abundance: > 10%.

Retention Time (MS)	MS Area	Mol. Weight or Ion
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		157.00 I
		101.00 I
0.749	18509582	157.00 I
		137.05 I
		101.00 I
2.090	3092101	179.00 I
		157.00 I
		137.00 I
		101.00 I
2.681	272354	325.20 I
		324.20 I
		179.05 I
		157.00 I
		137.00 I
		101.05 I
3.001	1674163	178.95 I
		157.00 I
		137.00 I
		101.00 I
3.426	262883	179.00 I
		157.00 I
		137.00 I
		101.00 I
3.688	59491	386.95 I
		179.00 I
		157.00 I
		137.00 I
		101.00 I
5.052	7634087	157.00 I
		137.00 I
		130.20 I
		101.00 I
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		416.95 I
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		314.95 I
		312.90 I

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		288.90 I
		157.00 I
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		101.05 I
5.718	222423	159.10 I
		157.00 I
		137.00 I
		101.00 I
5.834	282075	385.20 I
		364.20 I
		363.20 I
		159.05 I
		157.05 I
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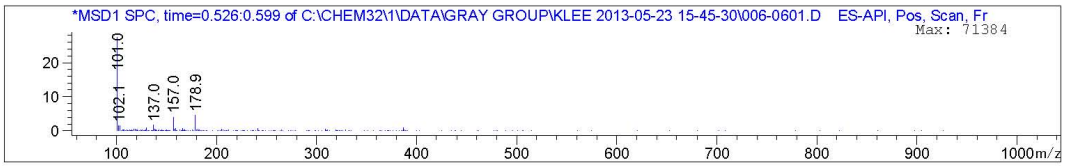
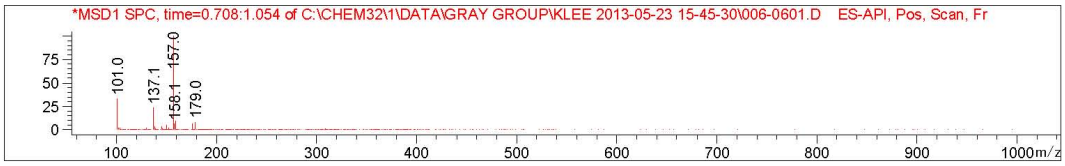
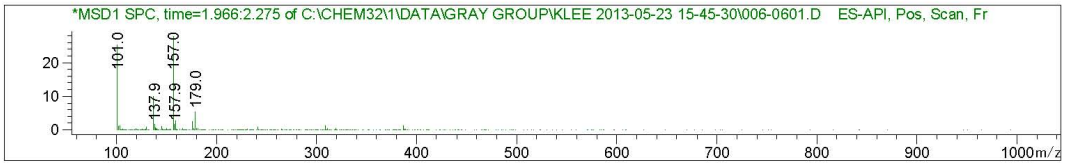
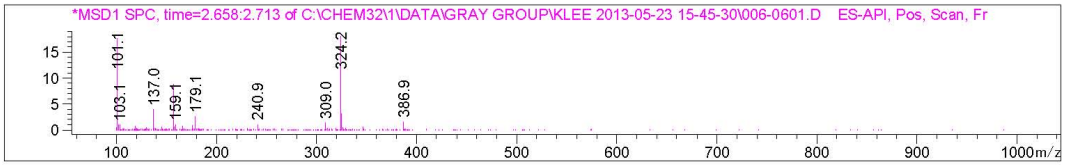
		101.10 I









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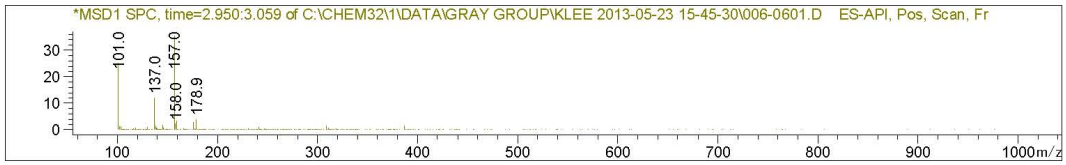

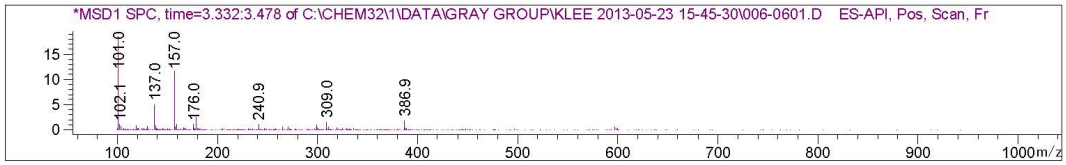

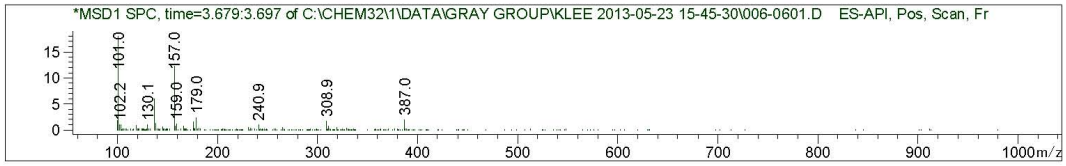

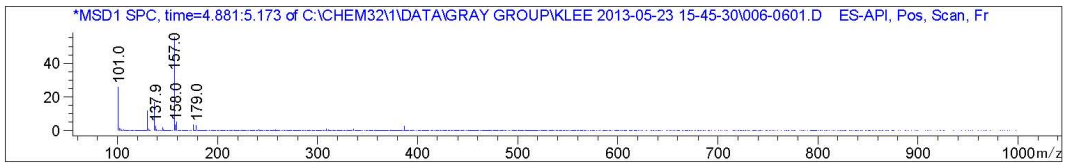

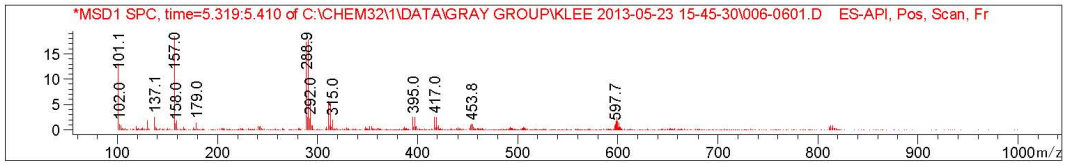

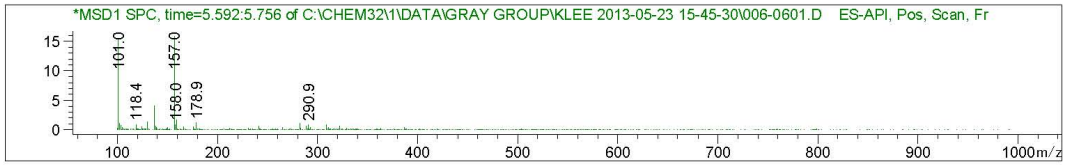

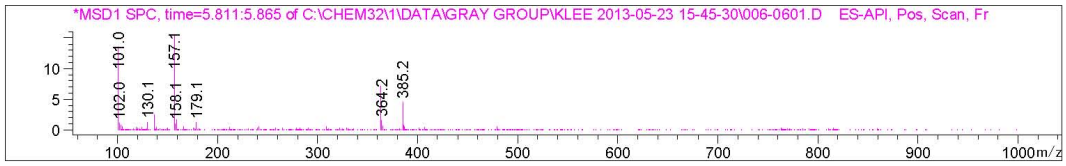

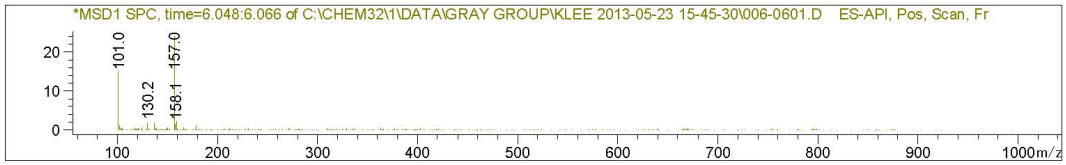

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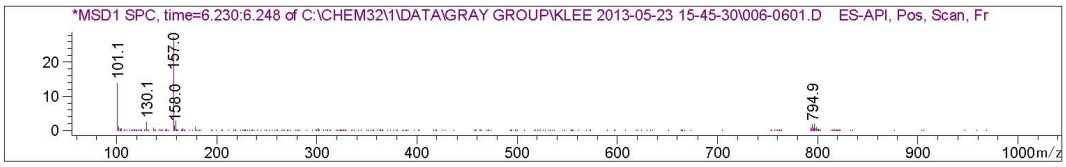
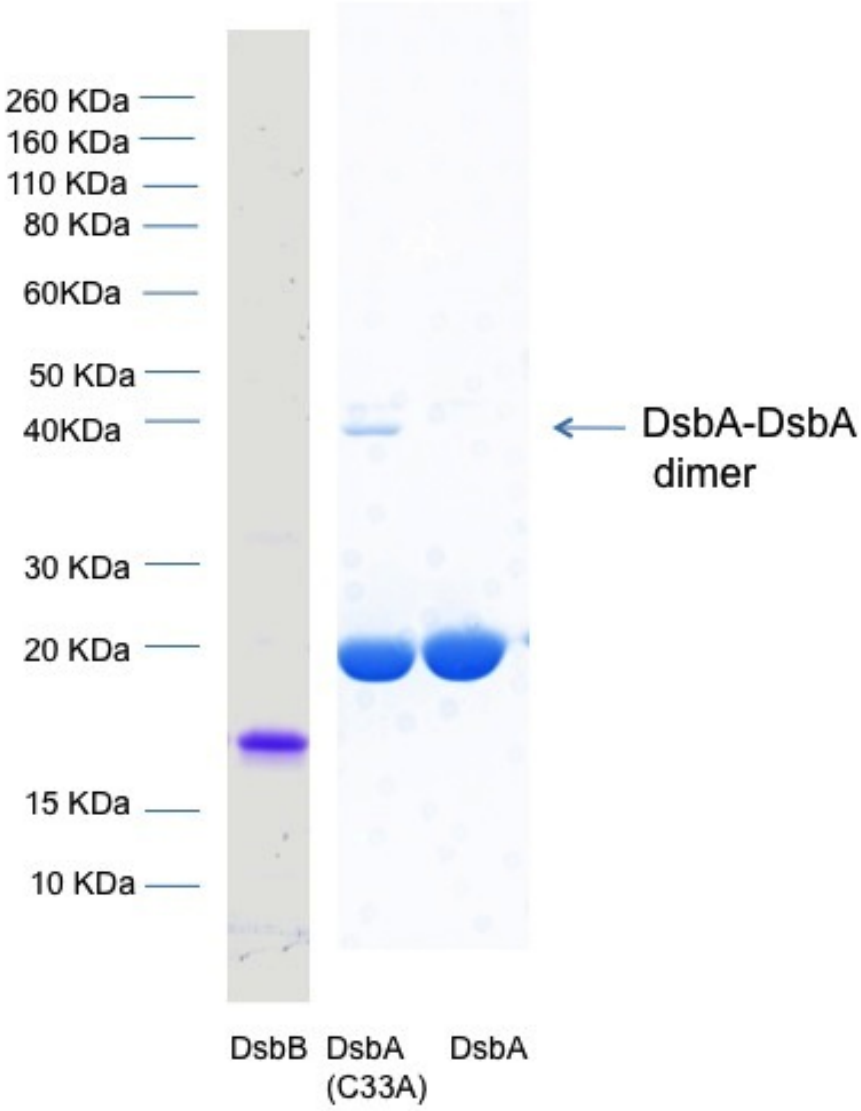


*** End of Report ***

Supplementary analysis of proteins purity.
The purity of the enzymes used in this study is over 95% for the three of them and was assessed by SDS-PAGE (12% acrylamide gels and Coomassie Briliant Blue staining). A representative image is shown below. The sequences of all of them are also indicated.



DsbB sequence (2 non-catalytic cysteines mutated)
MLRFLNQASQGRGAWLLMAFTALALELTALWFQHVMLLKPCVLCIYERVALFG
VLGAALIGAIAPKTPLRYVAMVIWLYSAFRGVQLTYEHTMLQLYPSPFATCDFMV
RFPEWLPLDKWVPQVFVASGDCAERQWDFLGLEMPQWLLGIFIAYLIVAVLVVI
SQPFKAKKRDLFGRRSHHHHHH
DsbA sequence
MGHHHHHHAQYEDGKQYTTLEKPVAGAPQVLEFFSFFCPHCYQFEEVLHISDN
VKKKLPEGVKMTKYHVNFMGGDLGKDLTQAWAVAMALGVEDKVTVPLFEGVQ

KTQTIRSASDIRDVFINAGIKGEEYDAAWNSFVVKSLVAQQEKAAADVQLRGVP
AMFVNGKYQLNPQGMDTSNMDVFVQQYADTVKYLSEKK

$DsbA_{C33A}$ sequence

MGHHHHHHAQYEDGKQYTTLEKPVAGAPQVLEFFSFFCPHAYQFEEVLHISDN
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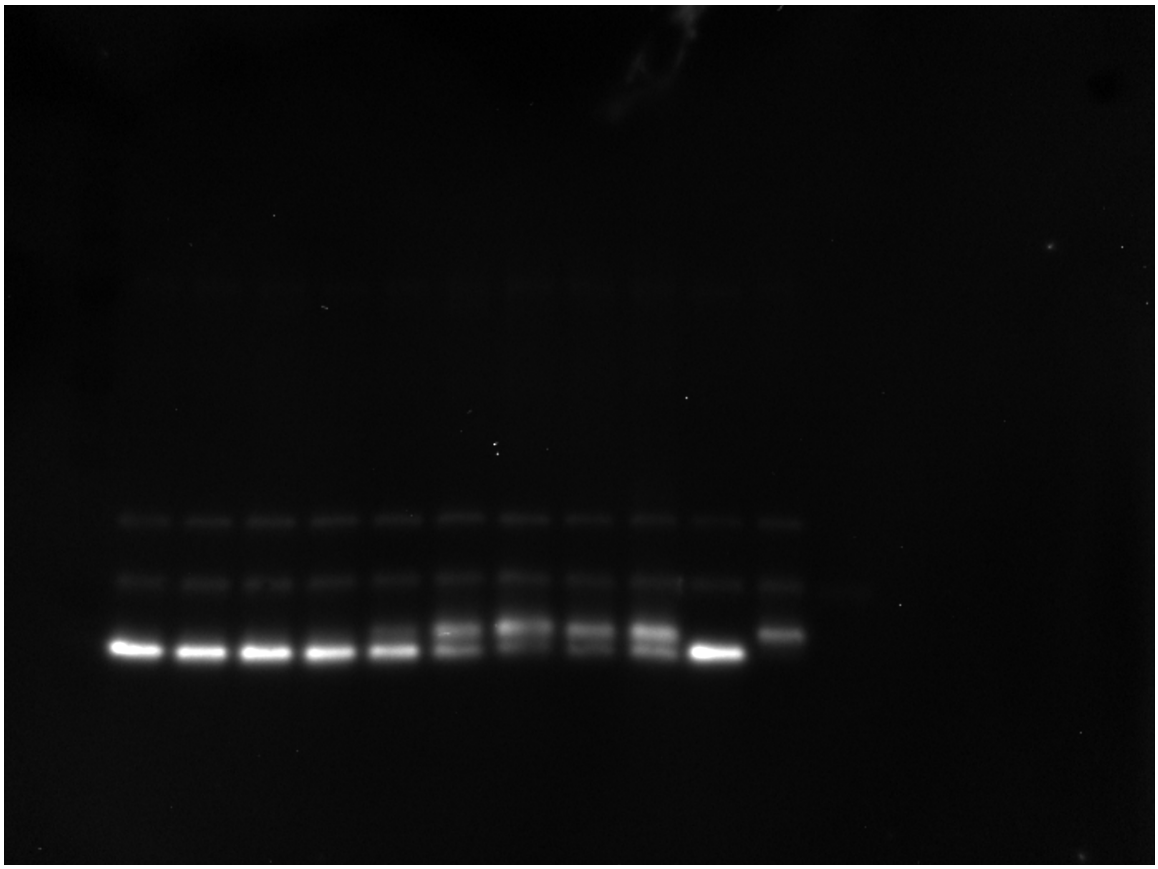
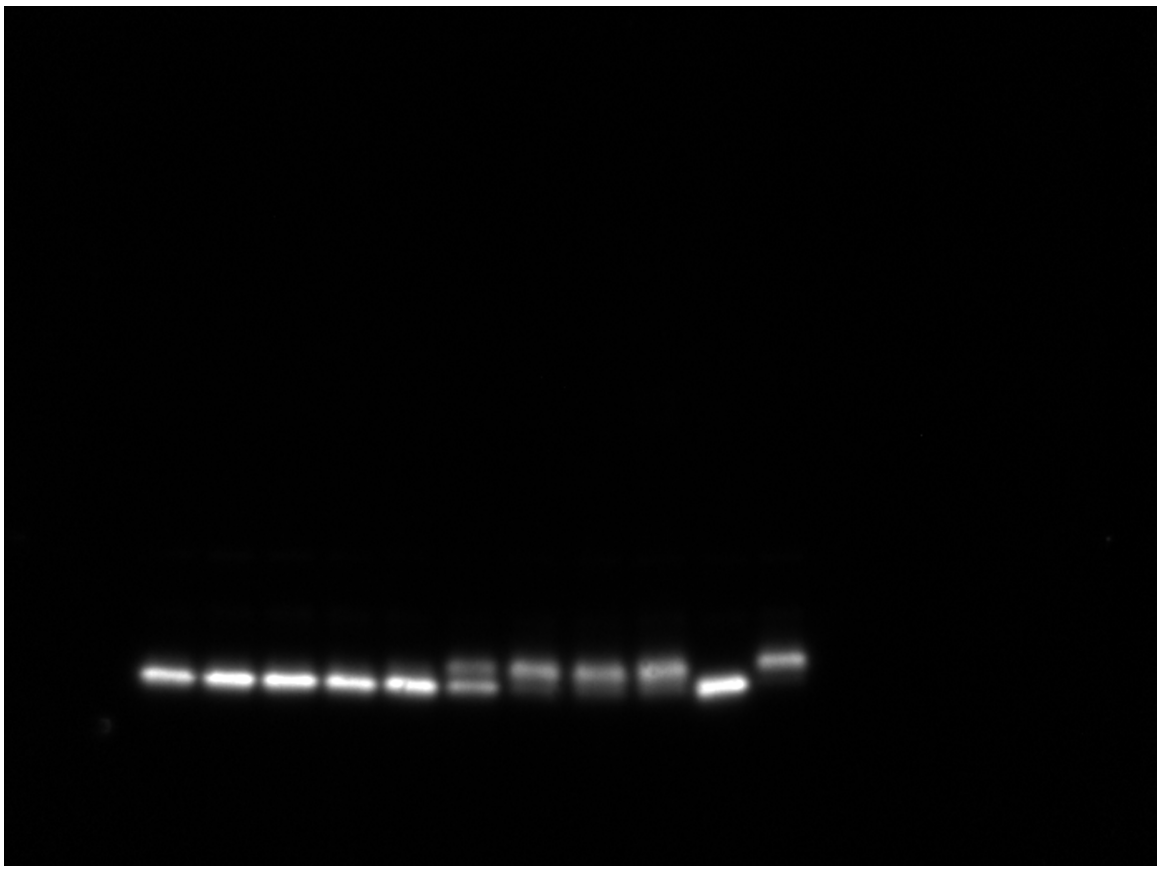
KTQTIRSASDIRDVFINAGIKGEEYDAAWNSFVVKSLVAQQEKAAADVQLRGVP
AMFVNGKYQLNPQGMDTSNMDVFVQQYADTVKYLSEKK

Original and complete pictures of western blots (taken with ChemiDoc of Image Lab 5.2, BIORAD) used to make Figure 2b.

TOP: cells treated with compound 9

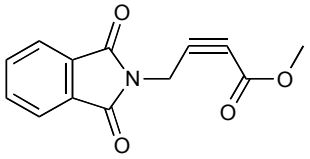
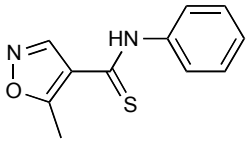
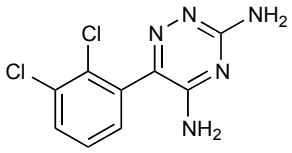
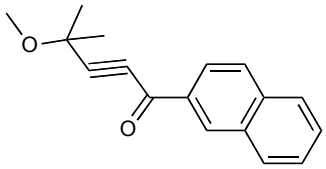
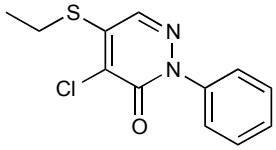
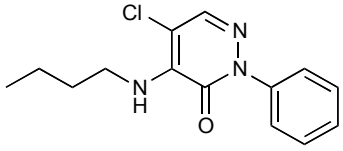
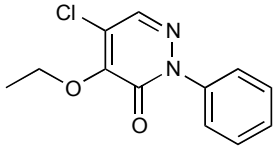


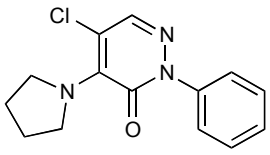
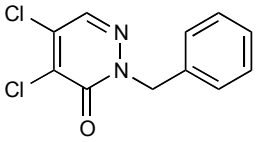
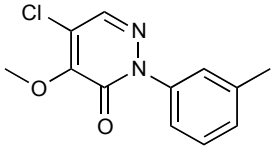
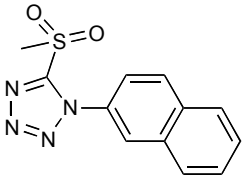
BOTTOM: cells treated with compound 12



Supplementary Tables

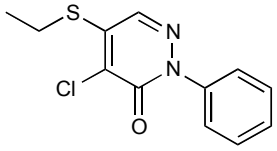
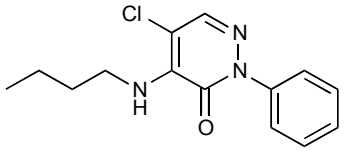
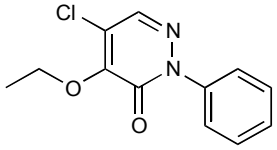
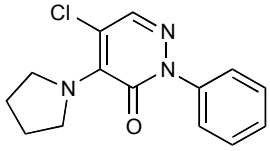
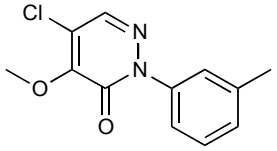
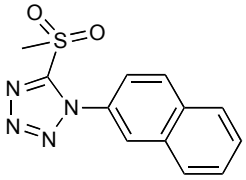
Supplementary Table 1. *M. tuberculosis* growth inhibition by compounds found as *Mtb*VKOR and *Ec*DsbB candidate inhibitors in the HTS.

ID No.	Structure	*Mycobacterium tuberculosis* growth (MIC in μM) in three different media*		
*Mtb*VKOR candidate inhibitors		7H9	7H9+OADC	Sauton's
1		128	510	1000
2		1100	>1100	>1100
3		>980	>980	>980
4		62	120	120
*Ec*DsbB candidate inhibitors				
5		470	>940	940
6		900	>900	900
7		500	1000	1000

8		450	910	910
9		250	490	490
10		>1000	>1000	>1000
11		57	110	110

* Minimal inhibitory concentrations are reported from two independent experiments with three replicas each. The MIC was exactly the same in the two independent experiments, except for compound 1 in Sauton's media that was 2 fold different between the two experiments so the highest MIC was reported.

Supplementary Table 2. IC50s of candidate inhibitors determined on purified *Ec*DsbB.

ID No.	Structure	*In vitro* IC50 (μM)*
*Ec*DsbB inhibitors		
5		9.12 to 15.99
6		6.69 to 10.29
7		5.62 to 7.911
8		7.16 to 9.71
10		4.87 to 6.12
11		5.01 to 7.68

*Results are the 95% confidence intervals of three independent experiments, with exception of compound 11 obtained from two independent experiments.

Supplementary Table 3. Strains used in this work.

Strain	Genotype	Reference
Escherichia coli strains		
HK295	MC1000 Δ*ara*714 *leu*$^{+}$	45
HK320	HK295 ΔdsbB	45
HK314	HK295 λ*att*::*malF-lacZ*102 (Km^{r})	H. Kadokura
HK325	HK295 ΔdsbB λ*att*::*malF-lacZ*102 (Km^{r})	H. Kadokura
MER672	HK295 λ*att*::*malF-lacZ*102 (Km^{r}) pTrc99a (Amp^{r}) *recA::*Cm	This study
DHB7657	HK295 ΔdsbB λ*att*::*malF-lacZ*102 (Km^{r}) pTrc99a*Mtb*VKOR (Amp^{r}) *recA::*Cm^{r}	This study
DHB7658	HK295 ΔdsbB λ::*malF-lacZ*102 (Km^{r}) pTrc99a (Amp^{r}) *recA::*Cm^{r}	This study
DHB7935	HK295 ΔdsbB λ*att*::*malF-lacZ*102 (Km^{r}) ϕ80::pDSW206dsbB (Amp^{r})	This study
DHB7936	HK295 ΔdsbB λ*att*::*malF-lacZ*102 (Km^{r}) ϕ80::pDSW206 (Amp^{r})	This study
CL315	HK295 λ*att*::*malF-lacZ*102 (Km^{r}) ΔdsbB::Trc204promoter-Pa*dsbB* (Amp^{r})	This study
CL320	HK295 λ*att*::*malF-lacZ*102 (Km^{r}) ΔdsbB::Trc204promoter-Kp*dsbB* (Amp^{r})	This study
CL377	HK295 ΔdsbB λ*att*::*malF-lacZ*102 (Km^{r}) pDSW204Pa*dsbH* (Amp^{r})	This study
CL378	HK295 ΔdsbB λ*att*::*malF-lacZ*102 (Km^{r}) pDSW204Ab*dsbB* (Amp^{r})	This study
CL369	HK295 ΔdsbB λ*att*::*malF-lacZ*102 (Km^{r}) pDSW204St*dsbB* (Amp^{r})	This study
CL368	HK295 ΔdsbB λ*att*::*malF-lacZ*102 (Km^{r}) pDSW204St*dsbI* (Amp^{r})	This study
CL373	HK295 ΔdsbB λ*att*::*malF-lacZ*102 (Km^{r}) pDSW204Vc*dsbB* (Amp^{r})	This study
CL370	HK295 ΔdsbB λ*att*::*malF-lacZ*102 (Km^{r}) pDSW204Ft*dsbB* (Amp^{r})	This study
CL371	HK295 ΔdsbB λ*att*::*malF-lacZ*102 (Km^{r}) pDSW204Hi*dsbB* (Amp^{r})	This study
CL379	HK295 ΔdsbB λ*att*::*malF-lacZ*102 (Km^{r}) pDSW204	This study
CL396	HK320 pCL68 (Sp^{r})	This study
CL397	HK320 pCL75 (Sp^{r})	This study

CL398	HK320 pCL73 (Sp^r)	This study
CL399	HK320 pCL74 (Sp^r)	This study
Pseudomonas aeruginosa strain		
PA14	*Pseudomonas aeruginosa* UCBPP-PA14	S. Lory Lab collection
Mycobacterium smegmatis strains		
RD263	*M. smegmatis* D*vkor* transformed with pRD43 (pTetG-*E. coli dsbB*)	12
RD265	*M. smegmatis* D*vkor* transformed with pRD42 (pTetG-*Mtb vkor*)	12
Plasmids		
pTrc99a	Expression vector, Amp^r	46
pDSW206	Promoter down mutation in -10 and -35 of pTrc99A	42
pDSW204	Promoter down mutation in -35 of pTrc99A	42
pHK517	pAM238 with *dsbB*-his_6-c-myc ($DsbB_{C9A,C49V}$), Sp^r	45
pKD46	Encodes lambda Red genes under arabinose promoter, Amp^r	47
pNG102	*malF-lacZ*102 fusion, Kan^r	14
pRD33	pTrc99a-his*Mtb*VKOR	12
pDHB7933	pDSW206-*dsbB*-his_6-c-myc	This study
pCL58	pKD46 Ampicillin cassette replaced by Chloramphenicol cassette, Cm^r	This study
pCL25	pDSW204Pa*dsbB* (*Pseudomonas aeruginosa* PA14 *dsbB* gene, PA14_07000)	This study
pCL26	pDSW204Pa*dsbH* (*Pseudomonas aeruginosa* PA14 *dsbH* gene, PA14_69400)	This study
pCL24	pDSW204Kp*dsbB* (*Klebsiella pneumoniae* W63917 *dsbB* gene)	This study
pCL27	pDSW204Ab*dsbB* (*Acinetobacter baumannii* A118 *dsbB* gene)	This study
pCL62	pDSW204St*dsbB* (*Salmonella typhimurium* LT2 *dsbB* gene, STM1807)	This study
pCL61	pDSW204St*dsbI* (*Salmonella typhimurium* LT2 *dsbI* gene, STM3194)	This study
pCL66	pDSW204Vc*dsbB* (*Vibrio cholerae* N16961 *dsbB* gene, VC1902)	This study
pCL63	pDSW204Ft*dsbB* (*Francisella tularensis* 1670 *dsbB* gene, FTL1670)	This study

pCL64	pDSW204Hi*dsbB* (*Haemophilus influenzae* RdKW20 *dsbB* gene, HI0428)	This study
pCL68	pHK517 *dsbB*-C41S	This study
pCL75	pHK517 *dsbB*-C41S,C44S	This study
pCL73	pHK517 *dsbB*-C41S,C44S, C104S	This study
pCL74	pHK517 *dsbB*-C41S,C44S,C104S,C130S	This study

Supplementary Table 4. Primers used in this work.

Name	Gene	Sequence	Restriction sites
Cl16	KpdsbB-1	gcgttcatgatgttgcaatatttaaaccagtgctca	BspHI
Cl17	KpdsbB-2	cggagctcttaacgaccaaacagatcgcgtt	SacI
Cl3	PadsbB-1	tcgaagctttcaggcggtgcggcggcc	HindIII
Cl10	PadsbB-2	gctgtcatgagcagcgctctcctcaa	BspHI
CL5	PadsbH-1	cagaagctttcaggcacgtcggaggaac	HindIII
Cl9	PadsbH-2	ctccatggtgcccctggccagcccc	NcoI
Cl19	AbdsbB-1	ctccatggtgcgattaagttaccgtttggt	NcoI
Cl20	AbdsbB-2	cggagctcttacttttttagccgtcttaa	SacI
Cl92	StdsbB-1	gactccatgggccattatttcatttcccgctagtggcg	NcoI
Cl93	StdsbB-2	cgtcggatccgatgtatttaatatacacctttaatcactggc	BamHI
Cl90	StdsbI-1	gactccatgggctcaacggcaagtaccttatctatacca	NcoI
Cl91	StdsbI-2	cgtcggatcctcgttcagtttcaaagaacgacgaata	BamHI
Cl94	VcdsbB-1	gactccatgggcatttcaattgaaactgaaactaatcca	NcoI
Cl95	VcdsbB-2	cgtcggatcctaaacagcagaaacaacaaaagtaa	BamHI
Cl100	FtdsbB-1	gactccatgggcaaactcagaaacacgctaaagcagc	NcoI
Cl101	FtdsbB-2	cgtcggatccagtttcttttgcttgagtttatttttttgtttaa	BamHI
Cl96	HidsbB-1	gactccatgggcctggctattgaatttattttaccag	NcoI
Cl97	HidsbB-2	cgtcggatcctagcaaaatcagttaccgttgaata	BamHI
Cl65	Ins ΔdsbB-1	attccggggatccgtcgacctgcagttcgaagttcctattctctatctaaagtatatatgagtaaacttggt	-
Cl66	Ins ΔdsbB-2	ttagtgtaggctggagctgcttcgaagttcctatactttctaccgggagctgcatgtgtcagaggttttc	-

Supplementary Table 5. Small molecule screening data

Category	Parameter	Description
Assay	Type of assay	Organism cell-based assay.
	Target	*Escherichia coli* DsbB enzyme (UniProtKB ID: P0A6M2). *Mycobacterium tuberculosis* VKOR enzyme.
	Primary measurement	Detection of β-galactosidase activity using X-Gal.
	Key reagents	384-well tissue culture-treated plates (BD Falcon #353961). M63 medium containing 0.2% glucose and 0.9% agar, supplemented with kanamycin (40 µg/mL), ampicillin (50 µg/mL), IPTG (1 mM), and X-Gal (120 µg/mL).Breathable sealing film (Axygen BF-400).
	Assay protocol	See Agar Screening in Online Methods.
	Additional comments	The medium was maintained at 57 ºC by a water bath throughout the pouring process. The Wellmate tubing was pre-warmed by washing with sterile hot water immediately prior to loading the agar medium. The plates were incubated in humidified boxes (i.e, plastic boxes with small containers of water and paper towels) and were not stacked.
Library	Library size	Complete ICCB-library: 663,363 small-molecule compounds. Screened ICCB-library: 50,374 compounds. Screened NIAID-library: 1,113 compounds.
	Library composition	The ICCB-library used in the screening contained mostly commercial libraries and small libraries of known bioactive and natural product extracts. See Online Methods.
	Source	Institute for Chemistry and Chemical Biology (ICCB) Longwood. Harvard Medical School. National Institute of Allergy and Infectious Diseases (NIAID).
	Additional comments	http://iccb.med.harvard.edu/libraries/compound-libraries/
Screen	Format	384-well plate format.

	Concentration(s) tested	Concentration of library: 5 mg/mL (≈10-20 mM) Amount of compound used: 0.5 μg (0.1 μL) Estimated final concentration of compound 8.3 ng/μL (≈16.7 μM) Final concentration of DMSO: 0.16%
	Plate controls	Positive control: *Escherichia coli* DsbB mutant. Negative control: DMSO.
	Reagent/ compound dispensing system	EPSON compound transfer robot.
	Detection instrument and software	The readout of the assay is a blue-color well; the detection is done by eye.
	Assay validation/QC	NA
	Correction factors	NA
	Normalization	NA
	Additional comments	
Post-HTS analysis	Hit criteria	Hits were categorized as strong (dark blue), medium (medium blue), weak (light blue) and very weak (very light blue).
	Hit rate	0.29% for VKOR inhibitors and 0.021% for DsbB inhibitors.
	Additional assay(s)	Compounds were re-tested in a cherry pick assay. See Online Methods.
	Confirmation of hit purity and structure	Compounds were purchased from ChemBridge (San Diego, CA); Asinex Ltd. (Moscow,Russia); Sequoia Research Products Ltd. (Pangbourne, UK); Key Organics Ltd. (Camelford, UK); AK Scientific (Union City, CA); Ambinter (Orleans, France); Ryan Scientific (Mt. Pleasant, SC); Enamine (Ukraine). All purchased compounds were analyzed by mass spectrometry to verify the molecular weights and to estimate purity (ICCB-Longwood). See compound resupply section in Online Methods.
	Additional comments	EcDsbB data.csv MtbVKOR data.csv