PNAS

Supplementary Information for

Ring-fused 2-pyridones effective against multidrug-resistant Gram-positive pathogens and synergistic with standard-of-care antibiotics

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Supporting Text
Figures S1 to S9
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SI References

Other supplementary materials for this manuscript include the following:

Dataset S1

Supporting Text

Cellular toxicity assays. HeLa 229 cells (CCL-2.1; ATCC) were maintained and passaged at 37°C (5% CO2) in RPMI-1640 medium (25 mM HEPES and L-Glutamine) (HyClone) supplemented with 10% fetal bovine serum (FBS) (Sigma). The Neutral Red (NR) cytotoxicity assay was used to study cellular toxicity of PS757. HeLa cells were seeded in 96-well plates and left to adhere overnight. Cells were then treated in triplicate with 10, 25, 50 and 100 μM of PS757 dissolved in 100% dimethyl sulfoxide (DMSO) (Sigma) and further diluted in complete media before being added to the test wells. After 48 hours, the cells were washed with Dulbecco's phosphate buffered saline (DPBS) ($CaCl_2$ and $MgCl_2$) and thereafter incubated with media containing neutral red dye (Sigma) for 3 hours. The cells were subsequently washed, the dye was extracted with NR-desorb (1% Glacial acetic acid solution, 50% EtOH, 49% H_2O), and absorbance was measured at 540 nm. Averages and standard deviations were calculated from three separate experiments performed in triplicate. The results are shown as a percentage of viable cells compared to the DMSO control sample. Clofazimine (Sigma), an antitubercular antibiotic, is included as a control.

Isolation of PS757-resistant mutants. BHI-agar medium was prepared, autoclaved, and cooled to 69°C in a hybridization oven. The cooled BHI-agar was added to a final volume of 30 mL into petri dishes containing PS757-IMD (the imidazole salt of PS757) at final concentrations of 10, 20, and 40 μM PS757-IMD. Overnight cultures of *E. faecalis* OG1RF (OD_{600} ~3.0) were serially diluted and plated on BHI-agar plates with and without PS757-IMD.

Genomic sequencing and mutation analysis for PS757-resistant mutants. Overnight cultures of OG1RF and PS757-resistant isolates HT061, HT062, and HT063 were grown at 37°C in BHI media. The cultures were harvested via centrifugation and the resulting cell pellets were stored at -80°C. Cells pellets were resuspended in 180 μL of lysis buffer (12.5 mM Tris-HCl pH 8.0, 2 mM EDTA, 1.2% Triton X100, and 20 mg/mL lysozyme) and incubated for 60 minutes at 37°C. DNA was extracted using the Qiagen DNeasy Blood & Tissue Kit (Cat. No. 69504) per the manufacturer's instructions. Genomic DNA was sent to MicrobesNG (Birmingham, UK) and sequenced using the standard workflow. Genomic DNA libraries were prepared using the Illumina Nextera XT Library Prep Kit following the manufacturer's protocol with the following modifications: input DNA was increased 2-fold and PCR elongation time was increased to 45 seconds. DNA quantification and library preparation were carried out on a Hamilton Microlab STAR automated liquid handling system. Libraries were sequenced using Illumina HiSeq sequencer using a 250bp paired-end protocol. Sequencing reads were deposited to the NCBI SRA database and can be accessed under BioProject ID PRJNA876221 and Biosample accessions SAMN40648114-117. The resulting paired end reads were compared to the *E. faecalis* OG1RF reference genome (CP002621) using breseq (v0.35.5) (1) with the dependencies bowtie2 (2.2.5) (2) and R (3.6.1) (3). The analysis was performed in Consensus/Mixed Base mode with default parameters.

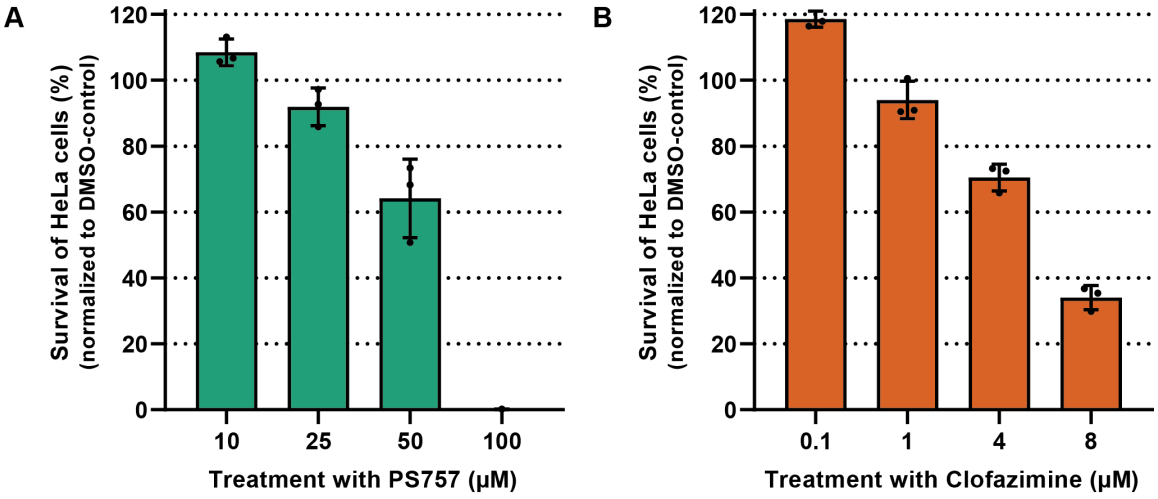


Fig. S1. HeLa cells show nearly complete survival with treatment of PS757 at concentrations up to 4x MIC of *E. faecalis* OG1RF. (A) Percent survival of HeLa cells treated with indicated concentrations of PS757 relative to the DMSO treated control condition. **(B)** Percent survival of HeLa cells treated with Clofazimine, an FDA approved antitubercular antibiotic, relative to the DMSO treated control condition. Black bars represent standard deviation of replicates.

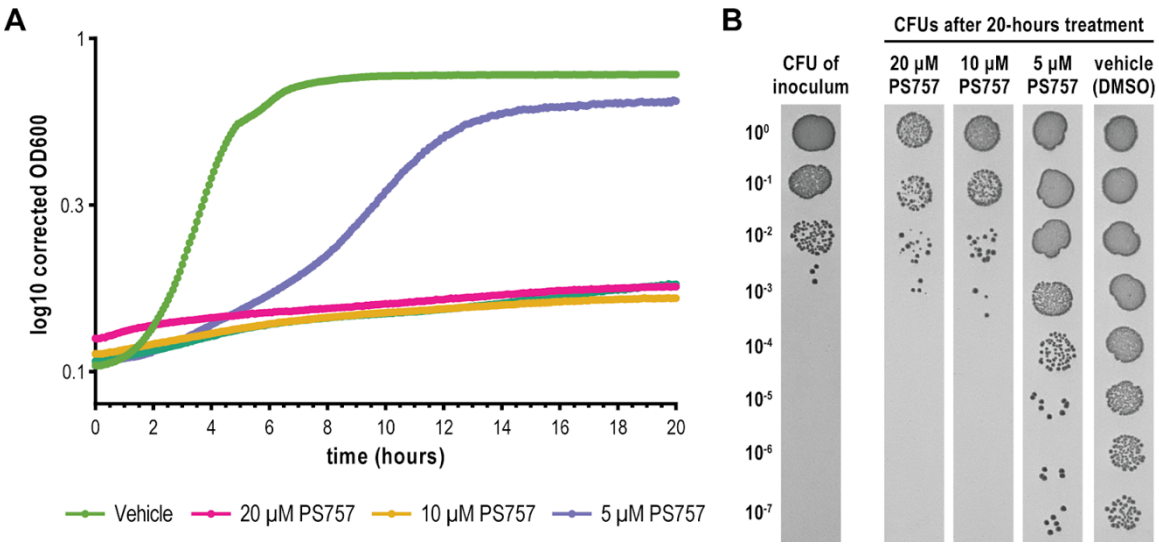


Fig. S2. PS757 treatment of exponential phase cultures does not result in significantly decreased CFUs over 20 hours. (A) Growth of exponential phase OG1RF culture in the presence of PS757. Exponential phase cultures of OG1RF were split and then treated with vehicle control (DMSO, green), 5 µM PS757 (purple), 10 µM PS757 (yellow), or 20 µM PS757 (pink) for 20 hours at 37°C. The OD_{600} was monitored once in every 1.5 minutes during the 20-hour incubation period. **(B)** Spot titer assays of OG1RF cells before (initial inoculum, t_0) and after 20 hours of treatment with 20 µM PS757, 10 µM PS757, 5 µM PS757, and vehicle control (DMSO).

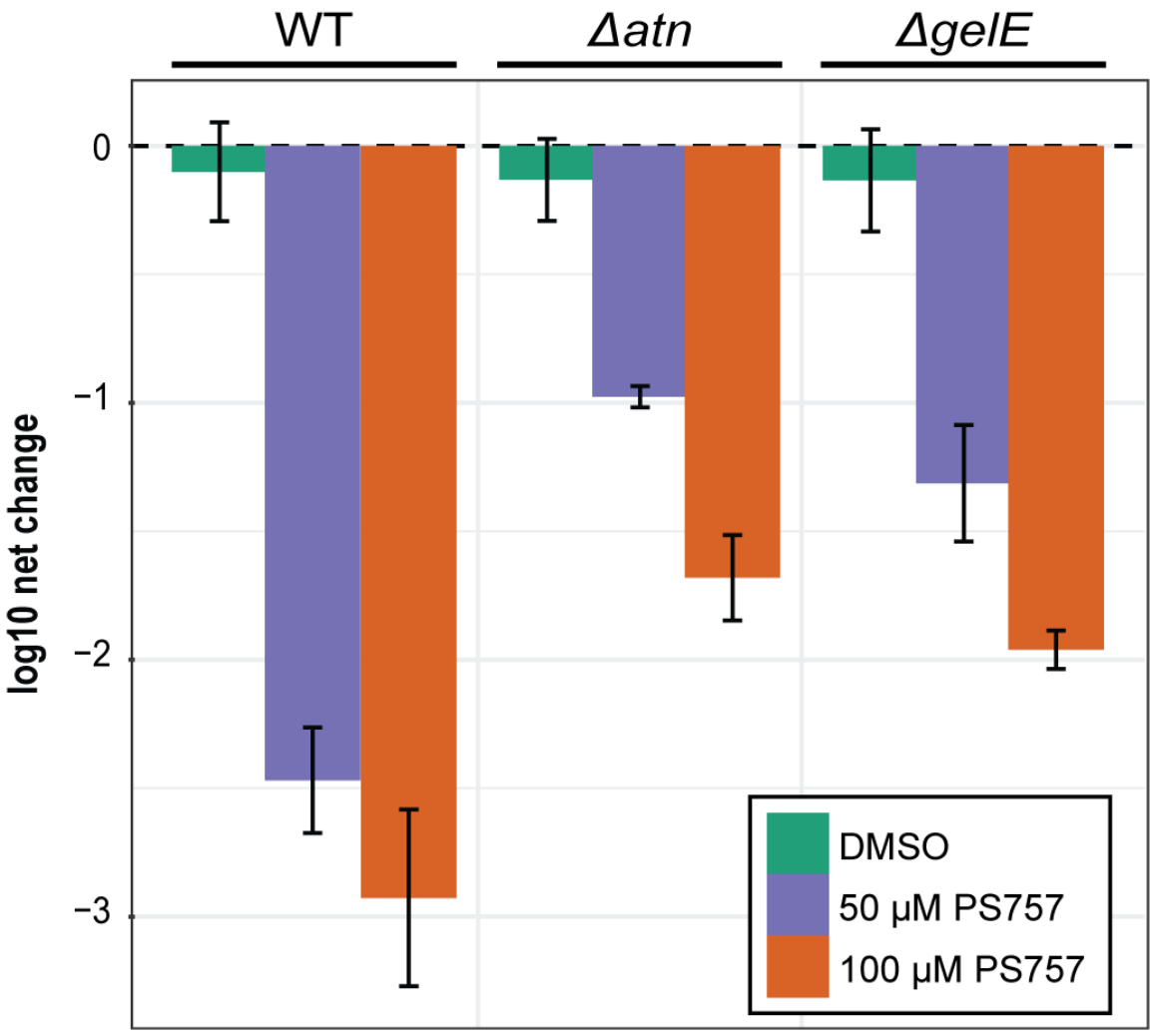
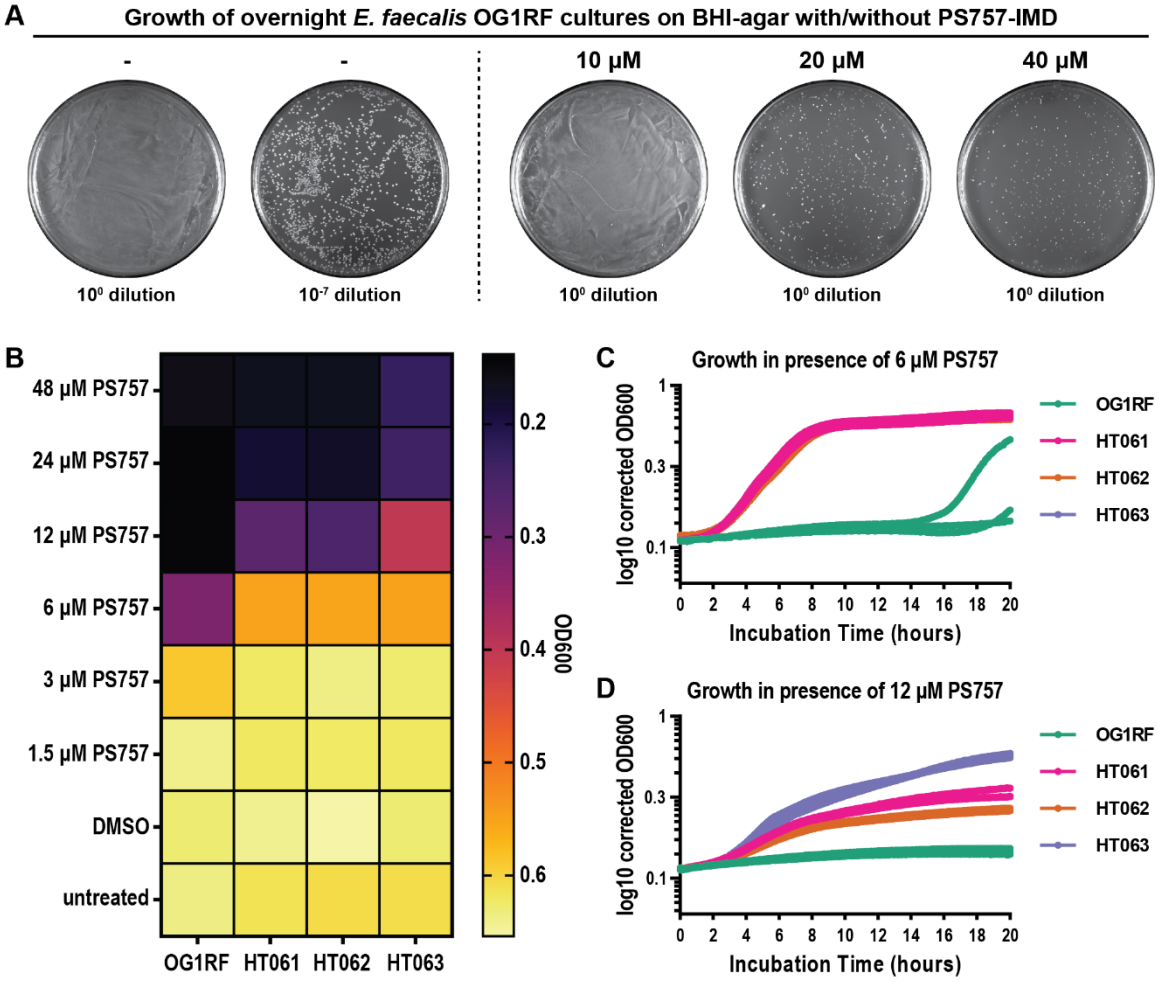


Fig. S3. PS757-dependent stationary phase cell death is mediated by the Atn autolysin and GelE protease. Quantification of CFUs from treated stationary phase cultures of mutant strains. Stationary phase (18 hour) cultures of the indicated strains were treated with the vehicle control DMSO (green), 50 µM PS757 (orange), or 100 µM PS757 (purple) for 20 hours. Net fold change (see materials and methods) is presented as the average change in final CFU/mL relative to the CFU/mL of the untreated initial inoculum. Black bars represent standard deviation of replicates.



E

				Mutation Frequencies:			
Position	**Gene(s)**	**Mutation**	**Annotation**	**OG1RF**	**HT061**	**HT062**	**HT063**
25660	*OG1RF_10021* →	G → T	R121L (CGT → CTT)	100%	100%	100%	100%
260670	*OG1RF_10252* →	(A)6 → (A)7	coding (129/852 nt)	100%	100%	100%	100%
1822399	*yihY* ←	G → A	P83L (CCA → CTA)	100%	100%	100%	100%
2108103	*est* ← / ← *secG*	(T)9 → (T)8	intergenic (-98/+168)	100%	100%	100%	100%
322641	*lmrB* ← / → *merR*	C → T	intergenic (-44/-57)	-	100%	-	100%
322660	*lmrB* ← / → *merR*	A → G	intergenic (-63/-38)	-	-	100%	-

Fig. S4. PS757-resistant isolates have mutations in the intergenic region of the gene encoding the LmrB efflux pump. (A) Isolation of PS757-resistant strains. Overnight stationary phase *E. faecalis* OG1RF cultures plated on BHI-agar plates containing the

indicated concentrations of PS757-imidazole salt (PS757-IMD), which improved the solubility of PS757 in the BHI-agar. **(B)** The growth of three strains (HT061-63) isolated on the 40 μM PS757-IMD BHI-agar plate was monitored by OD_{600} values as indicated after 20 hours in the presence of increasing concentrations of PS757 in liquid BHI media. **(C-D)** Exponential phase cultures of OG1RF and HT061-63 were back-diluted into fresh BHI media and growth was monitored by OD_{600} in the presence of 6 μM (1x MIC) or 12 μM (2x MIC) PS757. **(E)** Genomic mutations with 100% mutation frequency in PS757-resistant isolates HT061-63. Genomic DNA was purified from OG1RF and HT061-63 and sent for whole genome sequencing. The genome sequences of all strains were compared to the OG1RF reference sequence as described in the Supporting Text.

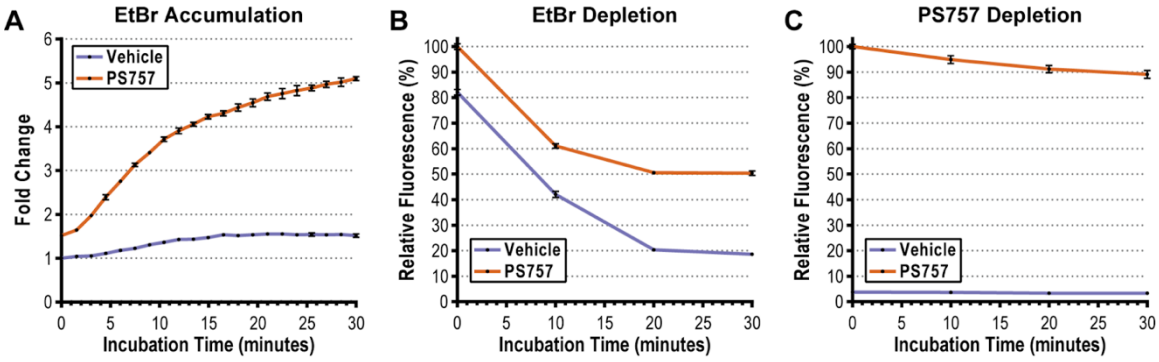


Fig. S5. Sublethal concentrations of PS757 increase influx and decrease efflux in V583. (A) EtBr accumulation in V583 cells treated with vehicle (DMSO, blue) or PS757 (orange). EtBr accumulation results are plotted as fold-change of EtBr fluorescence over a 30-minute time course. **(B)** Relative fluorescence of EtBr depletion of V583 cells pre-treated with EtBr and **i)** vehicle (DMSO, blue); or **ii)** PS757 (orange). **(C)** Relative fluorescence of PS757 depletion of V583 cells pre-treated with EtBr and **i)** vehicle (DMSO, blue); or **ii)** PS757 (orange). Cells were pre-treated with EtBr (1 µg/ml), DMSO (0.05%) and/or PS757 (5 µM). The fluorescence signals emitted from PS757 (λEx=400 nm and λEm=440 nm) and EtBr (λEx=544 nm and λEm=590 nm) were measured with SpectraMax iD3 microplate reader (Molecular Devices). All measurements were performed in four replicates and results were plotted using GraphPad Prism (version 9.3.1). Black bars represent standard deviation of replicates.

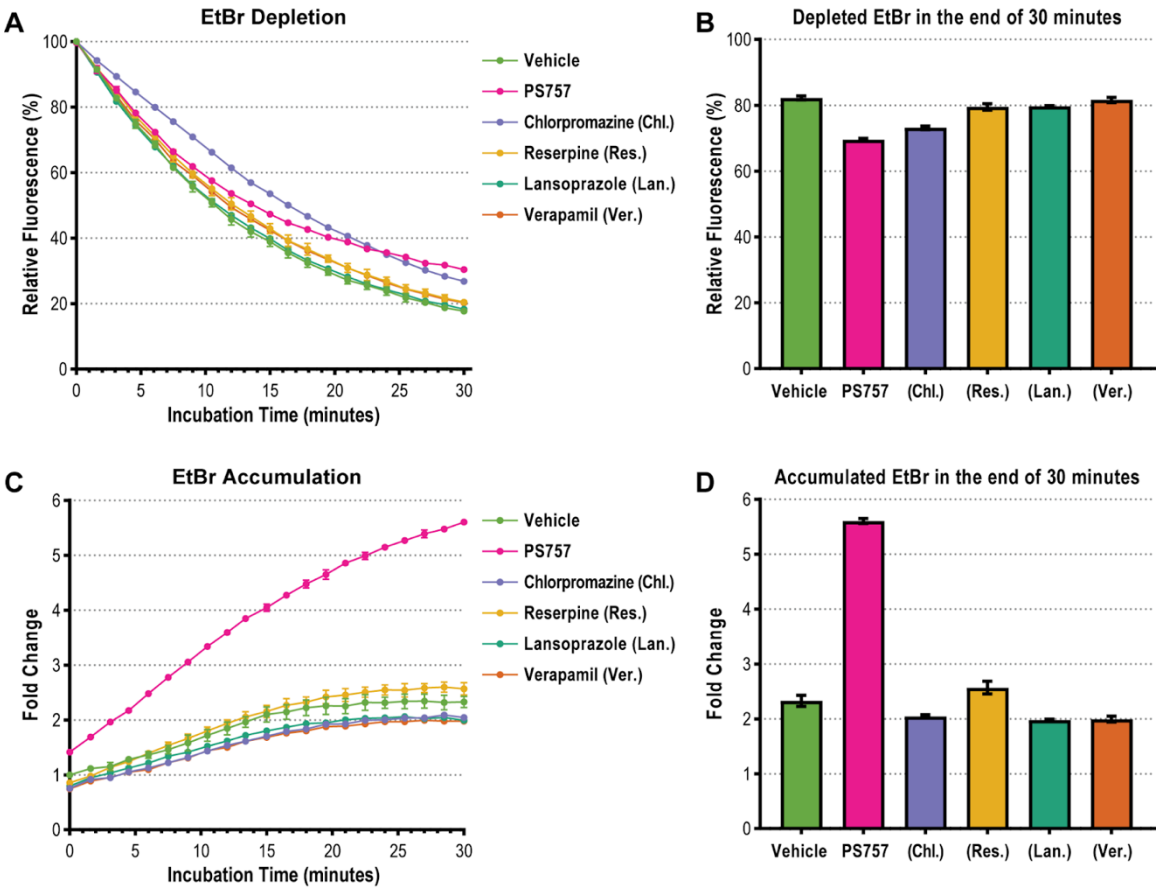


Fig. S6. EtBr Depletion and Accumulation Assay with OG1RF cells in presence of PS757 or other characterized efflux inhibitors. (A) Relative fluorescence of EtBr depletion of OG1RF cells treated with vehicle (DMSO, green), PS757 (pink), chlorpromazine-HCl (blue), reserpine (yellow), lansoprazole (cyan), or verapamil-HCl (orange) over a 30-minutes time course. EtBr depletion results are plotted as % relative EtBr fluorescence (respective to the initial fluorescence, 100% at t=0) over a 30-minute time course. **(B)** Quantification of % relative fluorescence after 30 minutes from **(A)** in OG1RF in the indicated conditions. **(C)** Fold-change of EtBr accumulation of OG1RF cells treated with vehicle (DMSO, green), PS757 (pink), chlorpromazine-HCl (blue), reserpine (yellow), lansoprazole (cyan), or verapamil-HCl (orange) over a 30-minutes time course. **(D)** Quantification of fold-change of EtBr fluorescence after 30 minutes from **(C)** in

OG1RF in the indicated conditions. Cells were pre-treated with EtBr (1 μg/ml), DMSO (0.05%), PS757 (5 μM) and/or other characterized efflux inhibitors (5 μM). The fluorescence signals emitted from EtBr (λEx=544 nm and λEm=590 nm) were measured with SpectraMax iD3 microplate reader (Molecular Devices). All measurements were performed in triplicates and results were plotted using GraphPad Prism (version 9.3.1). Black bars represent standard deviation of replicates.

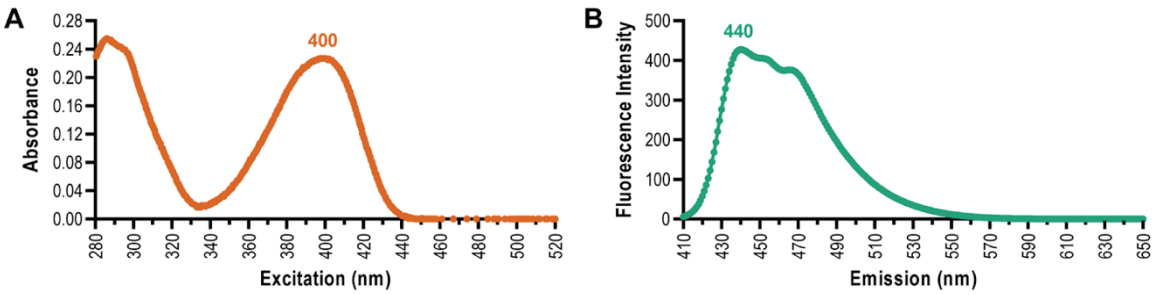


Fig. S7. Fluorescence properties of PS757. (A) The absorbance of PS757 (10 µM) dissolved in DMSO (vehicle). The absorption maxima (l_{max}) was determined as 400 nm from the maximum absorbance value against the wavelength. **(B)** The fluorescence emission of PS757 (10 µM, dissolved in DMSO) upon excitation at 400 nm. The emission maxima (l_{max}) was determined as 440 nm from the maximum emission value against the wavelength.

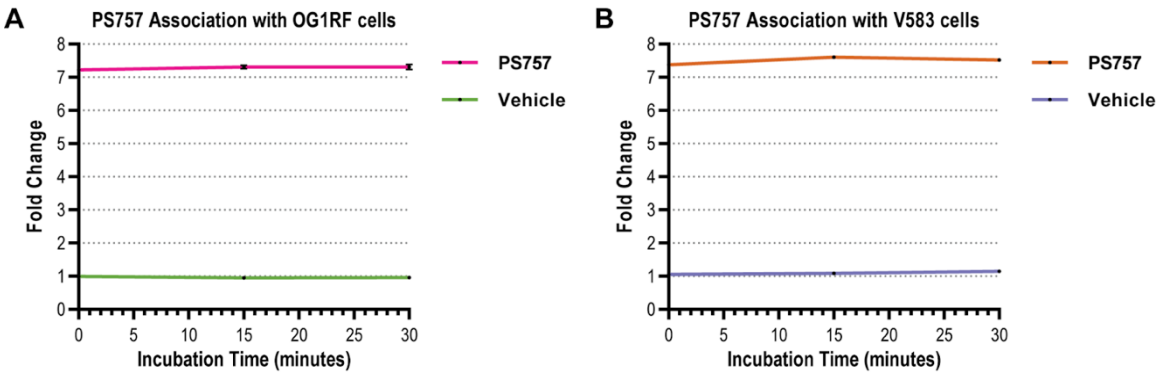


Fig. S8. PS757 associates rapidly with *E. faecalis* OG1RF and VRE V583 strains. (A) PS757 association with OG1RF cells treated with 0.01% DMSO (vehicle, green) and 1 μM PS757 (pink) over a 30-minute time course. **(B)** PS757 association with V583 cells treated with 0.01% DMSO (vehicle, purple) and 1 μM PS757 (orange) over a 30-minute time course. The fluorescence signals emitted from PS757 (λEx=400 nm and λEm=440 nm) were measured with SpectraMax iD3 microplate reader (Molecular Devices). All measurements were performed in triplicates and results were plotted using GraphPad Prism (version 9.3.1). Black bars represent standard deviation of replicates.

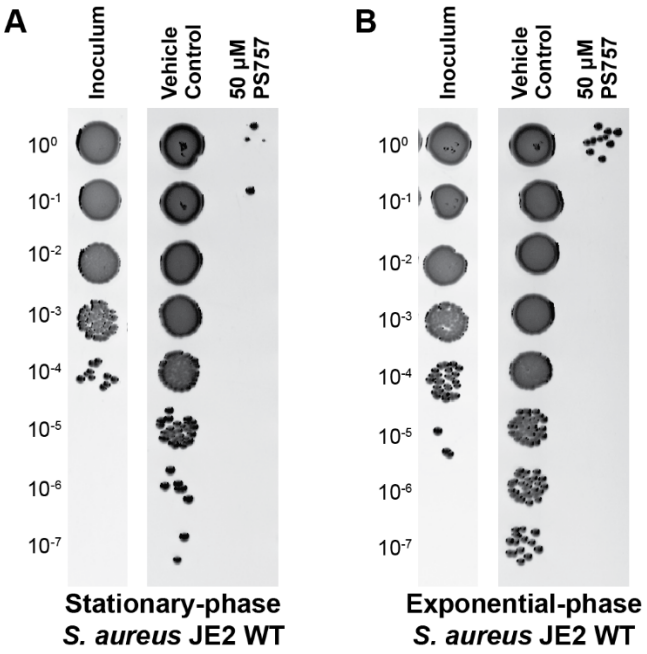
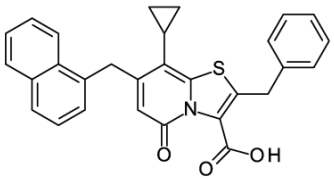
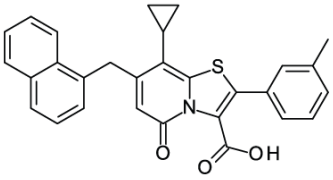
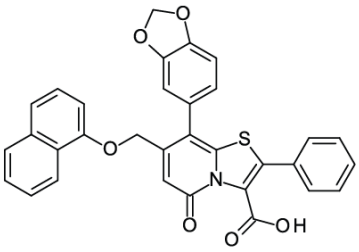
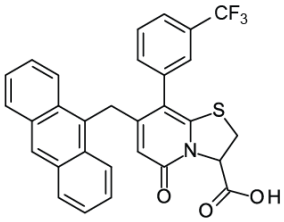
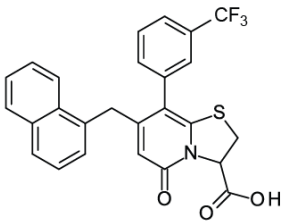


Fig. S9. PS757 has bactericidal effects against both stationary and exponential phase cultures of *S. aureus*. Spot plates of stationary **(A)** or exponential **(B)** phase cultures of *S. aureus* JE2 cells treated with either DMSO (vehicle control) or 50 µM PS757 for 20 hours. The initial inoculums and treated cultures were 10-fold serially diluted and plated on BHI-agar plates.

Table S1. Structure and MICs of first generation 2-pyridones

Structure	Name	MIC (μM)[1]
	EC240	25
	EC305	50
	EC260	400
	EC312	100
	FN075	200

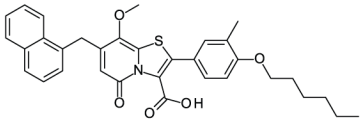
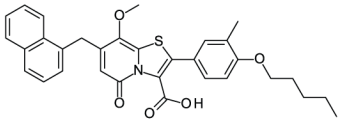
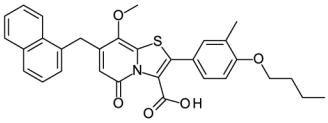
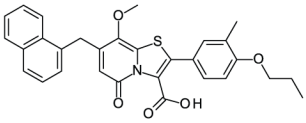
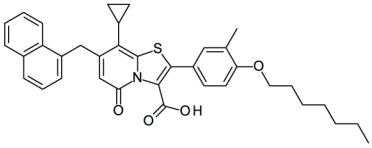
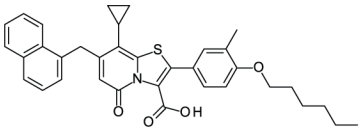
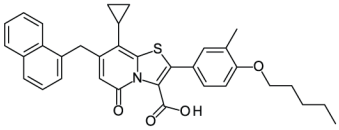
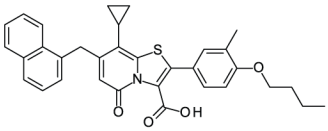
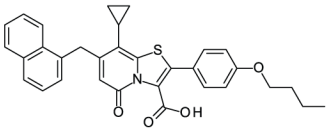
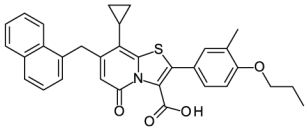
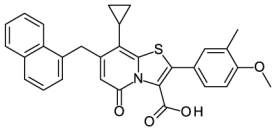
[1]MICs are determined as described in the Materials and Methods.

Table S2. List of strains used in this study

Species	Strain	Description	Resistance	Reference
Enterococcus faecalis	OG1RF	Laboratory strain	Rif, Fusidic acid	(4)
Enterococcus faecalis	V583	Blood isolate	Vanc	(5)
Enterococcus faecalis	VRE-1320	Blood isolate	Vanc	(6)
Enterococcus faecalis	VRE-1321	Blood isolate	Vanc	(6)
Enterococcus faecalis	VRE-1322	Clinical isolate	Vanc	(6)
Enterococcus faecalis	VRE-1323	Blood isolate		(6)
Enterococcus faecalis	VRE-1324	Blood isolate	Vanc	(6)
Enterococcus faecalis	VRE-1325	Blood isolate	Vanc	(6)
Enterococcus faecalis	VRE-1326	Blood isolate	Vanc	(6)
Enterococcus faecalis	VRE-1327	Blood isolate	Vanc	(6)
Enterococcus faecalis	VRE-1328	Blood isolate	Vanc	(6)
Enterococcus faecalis	VRE-1329	Blood isolate	Vanc	(6)
Enterococcus faecalis	VRE-1332	Gut isolate	Vanc	(6)
Enterococcus faecalis	VRE-1333	Gut isolate	Vanc	(6)
Enterococcus faecium	VRE-1379	Gut isolate	Vanc	(6)
Enterococcus faecium	VRE-1381	Clinical isolate	Vanc	(6)
Enterococcus faecalis	VRE-1386	Clinical isolate	Vanc	(6)
Enterococcus faecalis	OG1RF *Δatn*	*Δatn*		(7)
Enterococcus faecalis	OG1RF *ΔgelE*	*ΔgelE*		(8)
Enterococcus faecalis	OG1RF *ΔsprE*	*ΔsprE37-861*		(8)
Enterococcus faecalis	OG1RF *ΔgelE/pgelE*	*ΔgelE/pgelE-HA*	Kan, Cam	(8)
Enterococcus faecalis	OG1RF *ΔsprE/psprE*	*ΔsprE/psprE-HA*	Kan, Cam	(8)
Enterococcus faecalis	VRE-1388	Clinical isolate	Vanc	(6)
Stapylococcus aureus	MRSA-1369	Urine isolate	Methicillin	(9)
Streptococcus pyogenes	HSC5	Laboratory strain		(10)
Streptococcus agalactiae	GCP1418	Serotype 3, hyperpigmented		(11)
Streptococcus agalactiae	GCP1419	Serotype 5, nonpigmented		(11)
Streptococcus pneumoniae	JAM158	Clinical isolate		*
Streptococcus pneumoniae	JAM159	Clinical isolate		*
Staphylococcus aureus	JE2	Clinical isolate		(12)
Bacillus subtilis	168	Laboratory strain		(13)
Enterococcus faecalis	OG1RF	HT061		This work
Enterococcus faecalis	OG1RF	HT062		This work
Enterococcus faecalis	OG1RF	HT063		This work

*Gift of Celeste Morley, Dept. Pediatrics, Washington University School of Medicine
Rif (Rifampin), Vanc (Vancomycin), Kan (Kanamycin), Cam (Chloramphenicol)

Table S3. Structure and MICs of second generation 2-pyridones

Structure	Name	MIC (μM)[1]
	PS757	6
	PS627	6
	PS631	12
	PS756	25
	PS579	>50
	PS624	6
	PS581	6
	PS625	6
	PS749	>50
	PS623	>50
	PS583	>50

[1]MICs are determined as described in the Materials and Methods.

Table S4. Checkerboard assay to determine standard-of-care antibiotic interactions with PS757

Strain	PS757 MIC (μM)	PS757 combo MIC (μM)	Antibiotic*	Antibiotic MIC (μg/mL)	Antibiotic combo MIC (μg/mL)	FIC PS757	FIC Antibiotic	Sum of FIC	Interaction
OG1RF	16	1	Gent	64	8	0.0625	0.125	0.187	synergistic
V583	4	2	Gent	16,384	1,024	0.5	0.0625	0.562	additive
VRE-1332	4	1	Gent	64	16	0.25	0.25	0.5	synergistic
VRE-1333	4	2	Gent	64	8	0.5	0.125	0.625	additive
VRE-1379	4	1	Gent	4,069	512	0.25	0.125	0.375	synergistic
VRE-1381	4	1	Gent	16,384	1,024	0.25	0.0625	0.312	synergistic
VRE-1386	1	1	Gent	2,048	256	1	0.125	1.125	additive
VRE-1388	8	4	Gent	4,096	256	0.5	0.0625	0.562	additive
OG1RF	4	4	Vanc	4	1	1	0.25	1.25	additive
V583	4	2	Vanc	32	4	0.5	0.125	0.625	additive
VRE-1332	4	2	Vanc	4	2	0.5	0.5	1	additive
VRE-1333	4	2	Vanc	4	2	0.5	0.5	1	additive
VRE-1379	8	4	Vanc	256	16	0.5	0.0625	0.56	additive
VRE-1381	4	2	Vanc	2,048	1,024	0.5	0.5	1	additive
VRE-1386	8	2	Vanc	512	128	0.25	0.25	0.5	synergistic
VRE-1388	8	4	Vanc	512	64	0.5	0.125	0.625	additive
OG1RF	8	4	Cipro	2	0.25	0.5	0.125	0.625	additive
V583	16	4	Cipro	0.5	0.125	0.25	0.25	0.5	synergistic
VRE-1332	4	2	Cipro	0.5	0.25	0.5	0.5	1	additive
VRE-1333	4	2	Cipro	1	0.25	0.5	0.25	0.75	additive
VRE-1379	4	4	Cipro	256	32	1	0.125	1.125	additive
VRE-1381	4	4	Cipro	256	32	1	0.125	1.125	additive
VRE-1386	2	1	Cipro	512	256	0.5	0.5	1	additive
VRE-1388	4	4	Cipro	256	32	1	0.125	1.125	additive

*Gent (Gentamicin), Vanc (vancomycin), Cipro (Ciprofloxacin)

Table S5. Activity of GmPcide PS757 against Gram-positive bacteria

Species	Strain	PS757 MIC (μM)
Staphylococcus aureus	MRSA 1369	3.12
Streptococcus agalactiae	GCP1418	3.12
Streptococcus agalactiae	GCP1419	6.25
Streptococcus pneumoniae	JAM158	0.39
Streptococcus pneumoniae	JAM159	0.39
Streptococcus pyogenes	HSC5	0.78
Bacillus subtilis	168	3.12
Escherichia coli	UTI89	>500

* Structure: PS757, see Fig. 1.
**MICs determined as described in the Materials and Methods

Dataset S1 (separate file). Chemical synthesis and analytical data for 2-pyridone compounds.

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