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Supporting Information for:

Development of 2nd generation aminomethyl spectinomycins that overcome native efflux in *Mycobacterium abscessus*

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Table S1. List of genetically engineered *M. abscessus* strains and primers used.

1A. *Mycobacterium abscessus* genetically engineered strains

Strain	Reference
M. abscessus ATCC19977	Type strain (parental)
Δ3508c (*whiB7*)	Selchow et al., 2022. (ref.[1])
Δ4532c (*eis2*)	Rominski et al. 2017. (ref.[2])
Δ2355c	This study
Δ1846	This study
Δ1409c (*tap*)	This study
Δ2780c (*tetV*)	This study
Δ3508c (*whiB7*) Δ2355c	This study
Δ3508c (*whiB7*) Δ1846	This study
Δ3508c (*whiB7*) Δ2780c (*tetV*)	This study
Δ4532 (*eis2*) Δ3508c (*whiB7*)	This study
Δ1409c (*tap*) Δ3508c (*whiB7*)	This study

1B. List of primers used for cloning of targeting vectors and lengths of amplification products

Name	Target	Primers	Amplification product (bp)
UP-MAB_2355c fwd	*MAB_2355c*	cacttcgcaatggccaagacCGTTCGGTCGGCAGGGTC	1530
UP-MAB_2355c rev		cggattccctGCCGATGGCATCGAGCTG	
DOWN-MAB_2355c fwd		tgccatcggcAGGGAATCCGTTGAACCC	1530
DOWN- MAB_2355c rev		ggcgtctgctaggacccgatGAAAGTCTGCCCCAGGATTG	
UP-MAB_1846 fwd	*MAB_1846*	cacttcgcaatggccaagacCGCCGCGGTGGGGGTGTG	1530
UP-MAB_1846 rev		gtctccagtcGAGTGCGCCGCAGGAGATGACAG	
DOWN-MAB_1846 fwd		cggcgcactcGACTGGAGACCCGATCCGG	1530
DOWN-MAB_1846 rev		ggcgtctgctaggacccgatACCGACCCTACCCGCTCG	
UP-MAB_1409c fwd	*MAB_1409c*	*AAGCTT*AGAAATGCCACCGCATAGTC (*HindIII*)	1538
UP-MAB_1409c rev		*GCGGCCGC*GCGACGACAGATGCGAAGAG (*NotI*)	
DOWN-MAB_1409c fwd		*GCGGCCGC*GTACTCGGATGCGTGTCTTG (*NotI*)	1556
DOWN-MAB_1409c rev		*ACATGT*GCAGAGTGACCTCGGTGAT(*PscI*)	
UP-MAB_2780c fwd	*MAB_2780c*	ggcgtctgctaggacccgatGATCCTGGTTCACGAGCG	1562
UP-MAB_2780c rev		actcacattaCAACTGTTCGGGGAAGTC	
DOWN-MAB_2780c fwd		cgaacagttgTAATGTGAGTGCCGATAAC	1630
DOWN-MAB_2780c rev		cacttcgcaatggccaagacCTGCTTACGGTGCACTAC	

1C. List of PCR amplification primers, restriction enzyme fragments for probe generation and digest for characterization of deletion mutant

Primer name	Target	Amplification primer/fragment	Amplification product (bp)	Digest*
Probe-MAB_2355c -DOWN-fwd	*MAB_2355c*	gagaattcggtgtaggagatg	409	*Eco47III*
Probe-MAB_2355c -DOWN-rev		gtgtgtatggagggtttg		
Probe-MAB_1846 -UP-fwd	*MAB_1846*	ccgccgcaatcacaat	778	*BfuI*
Probe-MAB_1846 -UP-rev		agcagacatggaaagaa		
Probe-MAB_2780c -UP-fwd	*MAB_2780c*	CGCCAATTTCGATGATGCG	378	*NsbI*
Probe-MAB_2780c -UP-rev		CCAATCCAGGGTACGAAAGC		
Probe – DOWN-MAB_1409c	*MAB_1409c*	pSE-Δ1409c vector cut with *ApaLI* / *StuI*	523	*PvuII*

1D. Primers used for subspecies identification and *erm*-typing

Primer name	Target	Primers	Amplification product (bp)
IMM-F	*rrs*	TTAACACATGCAAGTCGAACGGAA	550
IMM-R2		CAGTTA AGCCGTGAGATTTCACGAACA	
rpoB-2573f	*rpoB* (ref.[3])	GGCAAGGTCACCCCGAAGGG	764
rpoB-3337r		AGCGGCTGCTGGGTGATCATC	
erm41f2	*erm41* (ref.[4])	GACCGGGGCCTTCTTCGTGAT	673
erm41r2		GACTTCCCCGCACCGATTCC	
erm41f	*erm41*	TGGTATCCGCTCACTGATGA	451
erm41r:		GCGGTGGATGATGGAAAG	

Table S2. X-ray data collection and refinement statistics.

Crystals		**70S ribosome in complex with mRNA, A-site $tRNA^{Phe}$, P-site $tRNA_i^{Met}$, and SPC (PDB: 8UVR)**	**70S ribosome in complex with mRNA, A-site $tRNA^{Phe}$, P-site $tRNA_i^{Met}$, and 2694 (PDB: 8UVS)**
Diffraction data			
Space group		$P2_12_12_1$	$P2_12_12_1$
Unit cell dimensions, Å (a x b x c)		209.07 x 447.77 x 621.31	209.73 x 449.08 x 621.60
Wavelength, Å		0.9792	0.9792
Resolution range (outer shell), Å		311-2.60 (2.67-2.60)	311-2.75 (2.82-2.75)
I/σI (outer shell)		9.81 (1.13)	7.42 (0.87)
Resolution at which I/σI=1, Å		2.60	2.75
Resolution at which I/σI=2, Å		2.78	2.95
CC(1/2) at which I/σI=1, %		24.7	20.8
CC(1/2) at which I/σI=2, %		50.0	55.0
Completeness (outer shell), %		98.8 (98.5)	98.9 (99.1)
R_{merge} (outer shell)%		14.1 (128.3)	15.1 (158.8)
No. of crystals used		1	1
No. of reflections used:	Total	7,388,156	5,085,095
	Unique	1,741,371	1,483,558
Redundancy (outer shell)		4.24 (4.19)	3.43 (3.47)
Refinement			
Resolution range of used data, Å		255-2.60	122-2.75
No. of reflections used		1,741,205	1,483,279
R_{work}/R_{free}, %		21.9/26.5	21.3/26.1
No. of Non-Hydrogen Atoms			
RNA		201,803	201,803
Protein		90,996	90,996
Ions (Mg, K, Zn, Fe)		2,856	2,892
Waters		4,714	4,873
Ramachandran Plot			
Favored regions, %		91.53	90.93
Allowed regions, %		7.42	8.02
Outliers, %		1.06	1.06
Deviations from ideal values (RMSD)			
Bond, Å		0.004	0.004
Angle, degrees		0.843	0.834
Chirality		0.041	0.040
Planarity		0.005	0.005
Dihedral, degrees		16.881	16.834
Average B-factor (overall), $Å^2$		54.1	63.0

Table S3. Mutations within helix 34 confer resistance to eAmSPC.

			16S rRNA ref nucleotide position			
	Isolate	**SRA Accession**	**1064**	**1066**	**1191**	**1192**
	WT	SRX16983977	**G**	**C**	**A**	**C**
Low 2593 Trx (4X MIC)	L1	SRX16984018	T	C	A	C
	L2	SRX16984019	T	C	G	C
	L3	SRX16984020	G	C	A	A
	L4	SRX16983979	G	C	A	G
	L5	SRX16983980	G	C	A	T
	L6	SRX16983981	G	G	A	C
	L7	SRX16983982	G	A	A	C
	L8	SRX16983983	G	A	A	C
	L9	SRX16983984	G	A	A	C
	L10	SRX16983985	G	C	A	G
High 2593 Trx (10X MIC)	H1	SRX16983986	G	C	A	G
	H2	SRX16983987	G	A	A	C
	H3	SRX16983988	G	C	A	G
	H4	SRX16983990	G	A	A	C
	H5	SRX16983991	G	A	A	C
	H6	SRX16983992	G	C	A	G
	H7	SRX16983993	G	C	A	A

Whole genome sequencing results of spontaneously generated mutants detailing nucleotide changes in red within the Helix-34 loop of 16s rRNA observed that confer resistance to 2593 in *M. abscessus*. *E. coli* 16S rRNA numberings are shown. Corresponding numbering in *M. abscessus* are G1029 (G1064), C1031 (C1066), A1157 (A1191), and C1158 (C1192). Sequence read archive (SRA) accession numbers associated with the raw sequencing data provided. All 17 2593-resistant mutants registered an MIC >200 µg/mL to 2593. Abbreviations: H, High; L, Low; MIC, minimum inhibitory concentration; Trx, treatment; WT, *M. abscessus* Wild Type (ATCC 19977).

Table S4. Differential gene expression analysis summary.

DEGs vs DMSO [WhiB7 response genes in brackets]					
	SPC-1 µg/mL	SPC-10 µg/mL	2593-1 µg/mL	2694-1 µg/mL	2694-10 µg/mL
Up	844 [73]	975 [83]	987 [81]	729 [69]	1081 [83]
Down	679 [16]	873 [11]	888 [11]	627 [16]	1000 [14]
Total	1523 [89]	1848 [94]	1876 [92]	1356 [85]	2081 [97]
DEGs vs. SPC-1 µg/mL					
		2593-1 µg/mL	2694-1 µg/mL	2694-10 µg/mL	
Up		161 [37]	0 [0]	396 [53]	
Down		81 [2]	0 [0]	277 [9]	
Total		242 [39]	0 [0]	673 [62]	
DEGs vs. SPC-10 µg/mL					
		2593-1 µg/mL	2694-1 µg/mL	2694-10 µg/mL	
Up		3 [0]	72 [1]	55 [4]	
Down		0 [0]	205 [59]	6 [0]	
Total		3 [0]	277 [60]	61 [4]	

The number of differentially expressed genes, and direction of change, per treatment group compared to DMSO (top), SPC- µg/mL (middle), and SPC-10 µg/mL (bottom). Second number in brackets indicates the number of differentially expressed genes within the whiB7 regulon (whiB7 response genes identified in ref. (5)). Abbreviation: DEGs, differentially expressed genes; SPC, spectinomycin.

Table S5. eAmSPCs maintain activity against other NTMs and clinical isolates.

		Compound MIC (µg/mL)							
	Species	**SPC**	**1980**	**2593**	**2694**	**2845**	**2953**	**AMK**	**CLR**
Rapid Growing NTMs	*M. chelonae*	>200	6.3	6.3	25	12.5	>200	12.5	0.2
	M. peregrinum	>200	6.3	6.3	6.3	6.3	>200	0.2	0.1
	M. fortuitum	>200	12.5	12.5	6.3	12.5	>200	3.1	6.3
	M. smegmatis	200	6.3	3.1	6.3	6.3	>200	1.6	0.8
Slow Growing NTMs	*M. marinum*	>200	6.3	6.3	12.5	6.3	>200	1.6	ND
	M. ulcerans	25	0.8	<0.2	0.8	3.1	>200	ND	ND
	M. kansasii	>200	25	12.5	25	12.5	>200	ND	0.8
	M. avium	100	6.3	12.5	25	12.5	>200	3.1	1.6
Clinical Isolates	**CI1:** *M. chelonae*	>200	ND	1.6	6.3	6.3	>200	12.5	1.6
	CI2: *M. abs. subsp. massiliense*	>200	ND	6.3	6.3	3.1	>200	1.6	0.2
	CI3: *M. abs. subsp. abscessus*	>200	ND	3.1	12.5	6.3	>200	3.1	25
Resistant Isolates	eAmSPC^R	>200	>200	>200	>200	>200	>200	6.3	ND
	AMK^R	>200	3.1	3.1	12.5	3.1	>200	>200	ND

Whole cell antimicrobial activity of lead eAmSPC against a panel of NTM species, eAmSPC and AMK-resistant isolates, and clinical mycobacteria isolates. Strains tested: *M. chelonae* ATCC 35752, *M. peregrinum* ATCC 14467, *M. fortuitum* ATCC 6841, *M. smegmatis* ATCC 19420, *M. marinum* ATCC 927, *M. ulcerans* ATCC 19423, *M. kansasii* ATCC 12478, *M. avium* ATCC 25291, CI1: *M. chelonae* clinical isolate (CLR susceptible); CI2: *M. abscessus subsp. massiliense* (CLR susceptible); CI3: *M. abscessus subsp. abscessus* (CLR resistant; Erm(41) sequevar type 6 ref.(6)); eAmSPC^R: *M. abscessus* 19977 spontaneous 2593 mutant resistant to eAmSPC (L1 selected on 16 mg/L 2593 with rrs mutation: 1026G→T); AMK^R *M. abscessus* 19977 spontaneous mutant resistant to AMK. Abbreviations: AMK, amikacin; CI, clinical isolate; CLR, clarithromycin; MIC, minimum inhibitory concentration; ND, not determined; SPC, spectinomycin. MIC determined by microbroth dilution. Results of a single experiment are presented.

Table S6. eAmSPCs maintain activity against *M. abscessus* clinical isolates.

	Subspecies	Erm(41)	No.	AMK	CLR	SPC	1980	2592	2593	2694	2842	2845	2953
***Mycobacterium abscessus* complex clinical isolate MIC at Day 3 and (Day 5) [µg/mL]**	*abscessus*	28C	Z043	1 (2)	0.125 (0.25)	128 (512)	2-4 (8)	4 (8-16)	2-4 (8)	16 (32)	4 (8)	4 (8)	>256 (>256)
	abscessus	28T	Z042	8 (8)	Inducible (>64)	128 (512)	4 (8)	4 (8-16)	4 (8)	16 (32)	4 (8)	4 (8)	>256 (>256)
	bollettii	28T	Z09	1 (2)	Inducible (>64)	128 (512)	2 (4-8)	2-4 (8)	2 (4-8)	8-16 (32)	2-4 (8)	2-4 (8)	>256 (>256)
	bolletii	28T	Z39	4 (8)	Inducible (>64)	64-128 (256-512)	2 (4)	2-4 (4-8)	2 (4)	8-16 (16-32)	2 (8)	2 (8)	>256 (>256)
	massiliense	Deletion	Z44	2 (2)	0.25 (0.25)	128-256 (512)	2-4 (4)	4-8 (8)	2-4 (4)	8-16 (32)	4 (8)	4 (8)	>256 (>256)
	massiliense	Deletion	Z46	2 (4)	0.25 (0.25)	64-128 (512)	4-8 (16)	4-8 (16-32)	4-8 (16)	16 (32)	4-8 (16)	4-8 (16)	>256 (>256)

M. abscessus complex strains were isolated at the Institute of Medical Microbiology, University of Zurich and identified to the subspecies level by PCR amplification and subsequent sequencing of 16S rRNA (*rrs*), *rpoB* and *erm(41)*. Primers used for speciation shown in **Table S1**. The functionality of the *erm(41)*- encoded erythromycin resistance methyltransferase conferring macrolide resistance is indicated. Genotype 28C corresponds to non-inducible, 28T corresponds to inducible and deletion to a non-functional *Erm(41*).

Table S7. eAmSPC retain activity against gram-positive bacteria.

		Compound MIC (μg/mL)					
	Species	**SPC**	**1980**	**2593**	**2694**	**2845**	**2953**
Gram-positive	*S. aureus (MSSA)*	100	12.5	12.5	200	50	>200
	S. aureus (MRSA)	>200	25	25	>200	>200	>200
	B. anthracis	50	12.5	12.5	50	50	>200
	B. subtilis	25	3.1	6.3	25	50	>200
	S. pneumoniae	25	3.1	6.3	25	12.5	>200
	S. pyogenes	25	3.1	1.6	100	25	>200
Gram-negative	*E. coli*	50	12.5	12.5	100	50	>200
	E. coli ΔtolC	6.3	6.3	1.6	25	25	200
	K. pneumoniae	>200	50	200	100	100	>200
	S. maltophilia	50	50	50	25	25	>200
	B. cepacia	50	>200	>200	>200	>200	>200
	P. mirabilis	>200	>200	>200	>200	>200	>200
	P. vulgaris	50	100	100	200	200	>200
	P. aeruginosa	100	>200	200	>200	>200	>200
	A. baumannii	>200	>200	>200	>200	>200	>200

Whole cell antimicrobial activity of lead eAmSPC against a panel of gram-positive and gram-negative bacteria. Strains tested: *Acinetobacter baumannii* ATCC 19606; *B. anthracis* Sterne 34F2; *B. subtilis* ATCC 23857, *Burkholderia cepacia* ATCC 25416; *Escherichia coli* K12; *E. coli* K12 *ΔtolC; Klebsiella pneumoniae* ATCC 33495; *Proteus mirabilis* Hauser ATCC 25933; *Proteus vulgaris* ATCC 33420; *Pseudomonas aeruginosa* PA01; *S. aureus* NRS70 (MRSA); *Staphyloccocus aureus* ATCC 29213 (MSSA); *Stenotrophomonos maltophilia* ATCC 13637; *Streptococcus pneumoniae* R6; *Streptococcus pyogenes* ATCC 700294; Abbreviations: MIC, minimum inhibitory concentration; SPC, spectinomycin. MIC determined by microbroth dilution. Results of a single experiment are presented.

Table S8. eAmSPC display favorable drug-like pharmacokinetic and toxicity profiles.

	In vitro toxicity		*In vivo* pharmacokinetics (treatment at 10 mg/kg IV)					*In vivo* toxicity
Compound	Vero IC_{50} (μM)	HepG2 IC_{50} (μM)	C_0 (mg/L)	AUC_{inf} (h*mg/L)	$t_{1/2}^*$ (h)	CL (L/h/kg)	V_{ss} (L/kg)	MTD (mg/kg)
2592	>100	>100	73.6	14.9	0.30	0.672	0.286	ND
2593	>100	>100	54.1	18.7	0.33	0.536	0.282	50
2694	>100	>100	37.7	15.5	0.29	0.644	0.356	100
2845	>100	>100	19.3	6.95	0.21	1.439	0.862	ND

eAmSPC are minimally toxic to the mammalian cell lines Vero (kidney epithelial cells, ATCC CCL-81) and HepG2 (liver hepatocellular carcinoma cells, ATCC HB-8065). Pharmacological profiling of intravenously injected eAmSPC show that compounds exhibit attractive drug-like properties and are tolerated at doses ≥50 mg/kg. Abbreviations: AUC_{inf}, area under the curve from time 0 to infinity; C_0: initial concentration after administration; CL: clearance; ND, not determined; $t_{1/2}$, terminal half-life; V_{ss}, volume at steady-state. *$t_{1/2}$ is based on decline of plasma concentrations in the therapeutically relevant concentration range.

Table S9. Safety profiling does not indicate significant inhibition or activation of off-target receptors by 2593.

Target	% Inhibition	Species
CYP450, 1A2	0	Human
CYP450, 2C19	6	Human
CYP450, 2C9	2	Human
CYP450, 2D6	5	Human
CYP450, 3A4	-3	Human
Adenosine A_1	-15	Human
Adenosine A_{2A}	6	Human
Adrenergic α_{1A}	24	Rat
Adrenergic α_{1B}	39	Rat
Adrenergic α_{2A}	17	Human
Adrenergic β_1	7	Human
Adrenergic β_2	-8	Human
Calcium Channel L-Type, Dihydropyridine	1	Rat
Cannabinoid CB_1	-1	Human
Dopamine D_1	-5	Human
Dopamine D_{2S}	17	Human
GABAA, Flunitrazepam, Central	-3	Rat
GABAA, Muscimol, Central	1	Rat
Glutamate, NMDA, Phencyclidine	-12	Rat
Histamine H_1	9	Human
Imidazoline I_2, Central	5	Rat
Muscarinic M_2	25	Human
Muscarinic M_3	2	Human
Nicotinic Acetylcholine	-10	Human
Nicotinic Acetylcholine α1, Bungarotoxin	-3	Human
Opiate μ(OP3, MOP)	4	Human
Phorbol Ester	3	mouse
Potassium Channel [K_{ATP}]	-15	hamster
Potassium Channel hERG	-13	Human
Prostanoid EP_4	6	Human
Rolipram	-6	Rat
Serotonin 5-HT_{2B}	9	Human
Sigma σ_1	1	Human
Sodium Channel, Site 2	-7	Rat
Transporter, Norepinephrine (NET)	10	Human

The target, % inhibition by 10 μM 2593, and source species for each assay tested in the Hit Profiling Screen + CYP450 conducted by Eurofins Panlabs.

Table S10. eAmSPC are metabolically stable in liver microsomes across species.

	Mouse (CD1)		Rat (SD)		Human (Pooled)	
Compound (treatment at 0.5 mg/L)	% remaining after 90 min	t ½ [min]	% remaining after 90 min	t ½ [min]	% remaining after 90 min	t ½ [min]
2593	100	-	100	-	100	-
2694	100	-	100	-	100	-
Verapamil	0.17	9.80 ± 0.16	0.13	9.36 ± 0.21	0.02	7.28 ± 0.07

Metabolic stability profiling in liver microsomal preparations from mice, rat and humans indicate no detectable metabolic instability up to 90 min of incubation. t½ degradation half-life.

Table S11. LC-MS/MS parameters used in whole-cell accumulation assay.

Compound	Parent (m/z)	Fragment ion (m/z)	DP (volts)	CE (volts)	CXP (volts)	Retention Time (m)	R^2 of linear Regression (Weighted =1/x^2)
SPC	351.1	207.0	66	31	12	1.14	0.993
1810	469.2	263.2	90	24	16	1.16	0.996
1950	472.3	207.1	90	31	11	1.17	0.994
1980	468.3	207.2	90	30	18	1.17	0.994
2592	482.3	207.1	80	30	12	1.19	0.997
2593	486.901	207.1	72	33	12	1.18	0.994
2694	476.221	207.2	68	33	12	1.17	0.990
2842	474.2	207.1	90	29	13	1.17	0.997
AMK	586.19	264.1	146	35	14	1.15	0.999

List of selected compounds measured in accumulation assay with corresponding precursor ion *m/z*, fragment ion *m/z*, declustering potential (DP), collision energy (CE), collision cell exit potential (CXP), retention time, and correlation coefficient for standard curve.

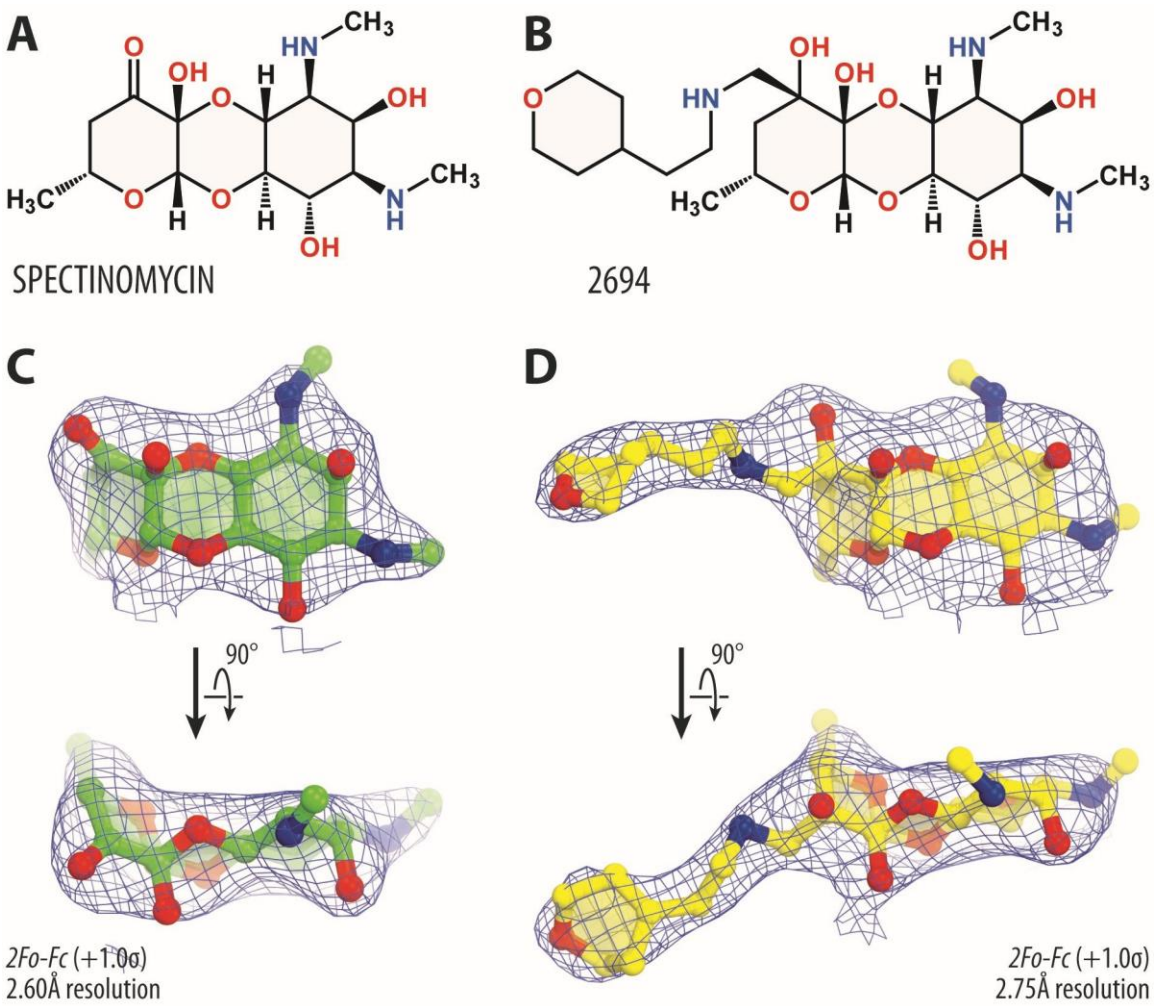


Fig. S1. Chemical structures and electron density maps of ribosome-bound spectinomycin and its derivative 2694. (**A, B**) Chemical structures of spectinomycin (A) and aminomethyl spectinomycin derivative 2694 (B, 2694). (**C, D**) $2F_o$-F_c electron difference Fourier maps (blue mesh) of spectinomycin (C, green) and 2694 (D, yellow) in complex with the *T. thermophilus* 70S ribosome. The refined models of spectinomycin and 2694 are displayed in the electron density maps contoured at 1.0σ. Carbon atoms are colored green for spectinomycin and yellow for 2694; nitrogens are blue; oxygens are red.

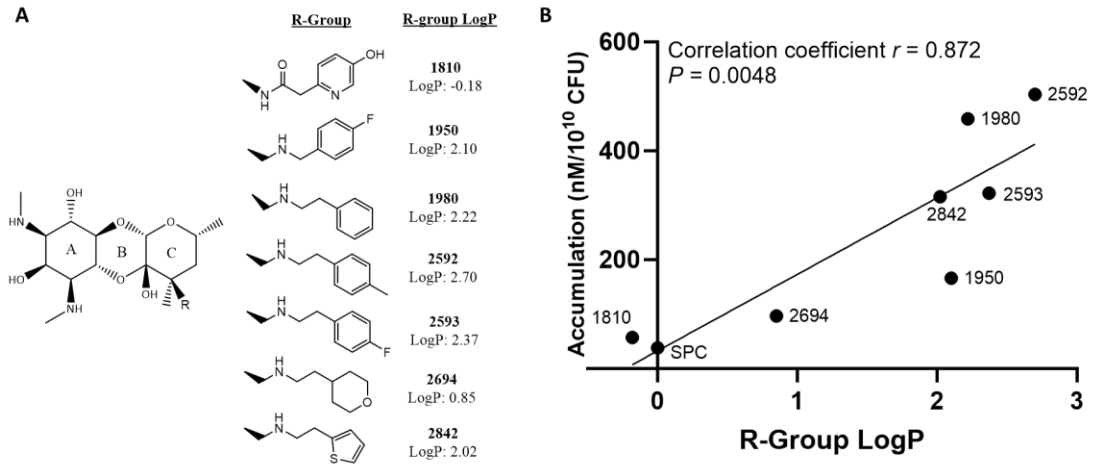


Fig. S2. SPC analog R-group lipophilicity associated with increased accumulation. (A) Substituent R-groups of analogs off the C-ring of the SPC scaffold and their corresponding LogP. **(B)** Pearson correlation coefficient of SPC analogs suggest higher LogP values are associated with higher levels of accumulation (two-sided *P* value = 0.0048).

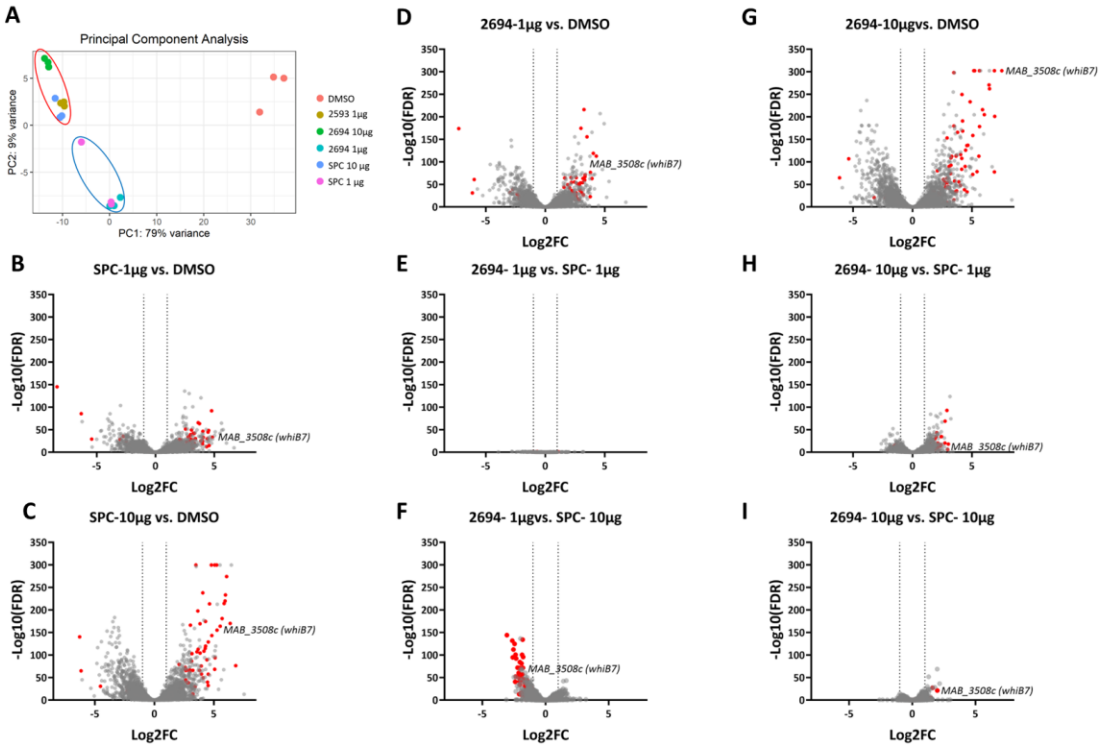


Fig. S3. Differential gene expression analysis of spectinomycins in *M. abscessus*. (A) Unbiased principle-component analysis (PCA) of *M. abscessus* transcriptomic profiles upon treatment with 2593 at 1 µg/mL (2593-1µg), 2694 at 1 µg/mL (2694-1µg) and 10 µg/mL (2694-10µg), SPC at 1µg/mL (SPC-1µg) and 10µg/mL (SPC-10µg), and no treatment (DMSO). PCA reveals that 2593-1µg, 2694-10µg, and SPC-10µg cluster closely together (red oval), as well as 2694-1µg and SPC-1µg (blue oval). **(B-I)** Volcano plots showing differential gene expression analysis (Log2 Fold-Change vs. –Log10 (FDR)) of **(B)** SPC-1µg vs. DMSO, **(D)** SPC-10µg vs. DMSO, **(D)** 2694-1µg vs. DMSO, **(E)** 2694-1µg vs SPC-1µg, **(F)** 2694-1µg vs. SPC-10µg, **(G)** 2694-10µg vs. DMSO, **(H)** 2694-10µg vs. SPC-1µg, and **(I)** 2694-10µg vs. SPC-10µg. For **(B-I)**, circles on to the right side of the plot indicate an upregulation of genes for the treatment group listed first, whereas circles on the left side of the plot indicate a downregulation of genes for the treatment group listed first. Red circles represent whiB7 response genes identified in ref.(5). Transcriptomic data shown are representative of three independent experiments. All concentrations listed are per mL.

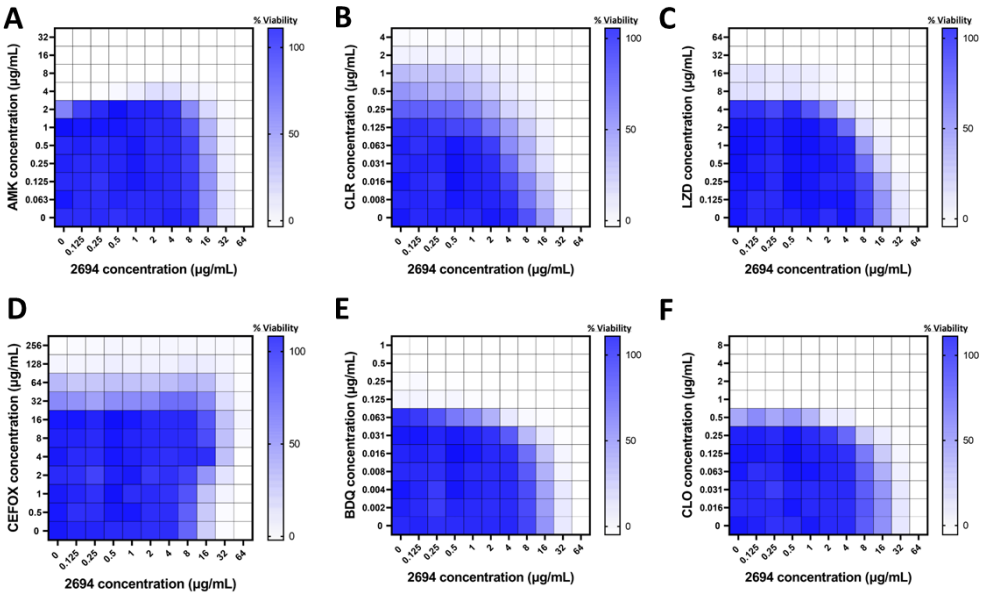


Fig. S4. Heat maps of checkerboard assay results for 2694 in combination with anti-mycobacterial antibiotics. (a-f) Heatmap of individual checkerboard assay results in *M. abscessus* between 2694 and **(a)** AMK, **(b)** CLR, **(c)** LZD, **(d)** CEFOX, **(e)** BDQ, and **(f)** CLO. Abbreviations: AMK, amikacin; BDQ, bedaquiline; CEFOX, cefoxitin; CLO, clofazimine; CLR, clarithromycin; LZD, linezolid.

Materials and Methods

Cytotoxicity Testing

Cytotoxicity of lead compounds against mammalian cell lines, Vero (kidney epithelial cells, ATCC CCL-81) and HepG2 (liver hepatocellular carcinoma cells ATCC HB-8065), was performed as previously described (7). Briefly, after culturing cell and seeding cells (5000 cells/well in flat, bottom 96-well plates [Nunc, cat no. 136101]) in their respective media (Dulbecco's Modified Eagle's Medium [Hyclone: DMEM/ High Glucose] for Vero cells and Eagle's Minimum Essential Medium [EMEM; ATCC 30-2003] each containing 10 % Fetal Bovine Serum [ATCC-30-2020]), experimental compounds 2592, 2593, 2694, and 2845 were solubilized in DMSO and serially diluted (range: 200-0.195 µM) in media. A serial dilution of DMSO only was made to monitor for vehicle toxicity against HepG2 and Vero. Equal volume of media containing compound dilutions was transferred to plates containing seeded cells using Biomek FXP liquid handling robot (Beckman Coulter, CA) and incubated for 72 hours (final concentration range: 100-0.098 µM). After 72 hours of incubation, viability was indirectly measured using the CellTiter-Glo® Luminescent Cell Viability (G7572 Promega) assay. Assay plates were read at peak emission wavelength of 590 nm on a PHERAstar FS Multilabel reader (BMG, Cary,NC). The raw data was normalized to no drug (DMSO only) wells as 100% viability standard. The concentration of test compounds that inhibited growth by 50% (IC_{50} value) was computed using nonlinear regression-based fitting of inhibition curves using [inhibitor] vs. response-variable slope model in GraphPad Prism version 9.5.0 (GraphPad Software, La Jolla California USA). Reported results were from one replicate per cell line.

Pharmacokinetic Studies

Pharmacokinetic profiling was performed by Sai Life Sciences Limited (Pume, India). All procedures were in accordance with the study plan SAIDMPK/PK-15-06-364 and the guidelines of the Institutional Animal Ethics Committee (IAEC). Healthy male BALB/c mice (8-12 weeks old) weighing between 25 to 35 g were procured from In vivo biosciences (Bengaluru, India). Temperature and humidity were maintained at 22 ± 3ºC and 40-70%, respectively, and illumination was controlled to give a sequence of 12-hr light/dark cycle. Temperature and humidity were recorded by auto-controlled data logger system. All the animals were provided laboratory rodent diet (Vetcare India Pvt. Lt; Bengaluru, India). Reverse osmosis water treated with ultraviolet light was provided *ad libitum*. Thirty-six male mice were divided into groups of 9. Animals were administered 10 mg/kg of 2592, 2593, 2694, and 2842 solution formulation in Captisol (30% w/v) intravenously (IV) at 5 mL/kg. Blood samples (~60 µL) were collected from retro orbital plexus under light isoflurane anesthesia at the following time points: at pre-dose, and 0.08, 0.25, 0.5, 1, 2, 4, 8 and 24 hr post-dose. Blood samples were collected from a set of three mice at each time point in labeled micro centrifuge tubes containing K_2EDTA solution (20% K_2EDTA solution) as anticoagulant. Plasma samples were separated by centrifugation of whole blood and stored below -70±10ºC until bioanalysis. All samples were processed for analysis by protein precipitation using acetonitrile and analyzed with a fit for purpose LC-MS/MS method. Pharmacokinetic parameters were calculated using the noncompartmental analysis tool of Phoenix WinNonlin (Version 6.3). Peak concentrations (C_0) and time to reach maximum concentrations (T_{max}) were the observed values. The areas under the concentration time curve (AUC_{last} and AUC_{inf}) were calculated by linear trapezoidal rule. The terminal elimination rate constant, k_e, was determined by regression analysis of the linear terminal portion of the log plasma concentration-time curve. The terminal half-life ($T_{1/2}$), CL, and V_{ss} were estimated by the following equations: $T_{1/2} = 0.693/k_e$, $CL = dose/AUC_{inf}$, and V_{ss}=mean residence time X CL.

Microsomal Metabolic Stability

Pooled human liver, *Sprague-Dawley* rat, and CD1 mouse liver microsomes were purchased from Corning Life Sciences (Oneonta, NY, USA). Microsomes (20 mg/mL) were thawed on ice and diluted using phosphate buffer (100 mM, pH 7.4), resulting in a protein concentration of 1 mg/mL. A final concentration of 0.5 mg/L of 2593, 2694 or the positive control verapamil were used for incubation with the microsomes. NADPH (final concentration: 1 mM) was used as a co-factor. All the above solutions except NADPH were added to individual wells (12-well) in triplicate and were allowed to equilibrate for 5 min at 37°C. NAPDH was then added to each well to initiate the metabolic reaction. 50 µL aliquots in triplicate were drawn from the incubation mixture at 0, 5, 10,

20, 30, 45, 60 and 90 min and immediately the reaction was quenched by addition of ice-cold methanol (4-volumes). Analysis for the remaining 2593, 2694 or verapamil was performed by LC-MS/MS. Negative controls for each compound were prepared and analyzed at 0 and 90 min and were assessed with the addition of equal volumes of water instead of NADPH to account for non-specific binding and any chemical instability. The slope of the natural log-linear regression of the concentration in each incubation well was used to calculate the degradation half-life and remaining percentage for each drug.

Off-target Activity Profiling

In vitro off-target activity of 2593 was tested on a commercial panel of targets (Hit Profiling Screen + CYP450) at Eurofins Panlabs Discovery Services (Taipei, Taiwan). All assays were conducted at a concentration of 10 μM in duplicate with >50% inhibition/stimulation considered significant.

MTD Model.

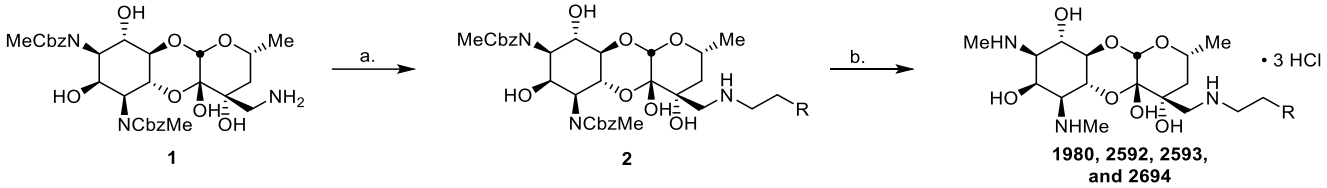
6- to 8-week-old Balb/C female mice were ordered from Charles River Laboratories. Both 2593 and 2694 were prepared in Plasma-lyte, and 3 mice were dosed per compound at 50, 75, and 100 mg/kg twice per day for 3 consecutive days by subcutaneous (SC) injection at 200 μl/mouse. Observations are recorded immediately, at 10 min, 1 hour, 2 hours, 4 hours, and 24 hours after dosing. Any animals that show signs of toxicity are euthanized in accordance with Colorado State University IACUC guidelines.

Chemistry Materials and Methods

General information. All reagents, starting materials, and solvents were purchased from commercial sources and used without further purification. Any moisture or air sensitive reactions were carried out in oven-dried glassware and under an atmosphere of nitrogen unless stated otherwise. All reagent grade solvents for chromatography purposes were purchased from Fisher Scientific (Suwanee, GA) and flash column chromatography silica cartridges were obtained from Biotage, Inc. (Lake Forest, VA). The Biotage FLASH column chromatography system was used to purify mixtures. All ^{1}H NMR spectra were recorded on a Varian INOVA-400 (400 MHz) spectrometer and chemical shifts (δ) are reported in parts per million relative to the residual solvent peak and coupling constants (*J*) are reported in hertz (Hz). Spectral splitting patterns are designated as s, singlet; d, doublet; t, triplet; q, quartet; p, pentet; m, multiplet; comp, complex; app, apparent; hom, higher order multiplet; ABq, AB quartet; os, overlapping singlets; and br, broad. All ^{13}C NMR spectra were recorded on a Bruker Avance500 (126 MHz ^{13}C) spectrometer. Chemical shifts are reported in parts per million. High resolution mass spectra were recorded on an Acquity UPLC-Waters Xevo G2 via electrospray. Gradient conditions M1: solvent A (0.1 % formic acid in water) and solvent B (0.1% formic acid in methanol): 0-1.00 min 95%A, 1.01-3.00 min 5-95% B (linear gradient), 3.01-4.50 min 100% B, 4.6-5.0 min 95% A detection by UV PDA (220-400 nm) and by CAD. Gradient conditions M2: same as M1 except that solvent B is 0.1% formic acid in acetonitrile. The purity of the compounds was determined using Waters Acquity UPLC-SQD and was confirmed to be >95%. Elemental analysis for compounds used in animal studies was performed by Atlantic Microlabs to confirm formula and salt composition.

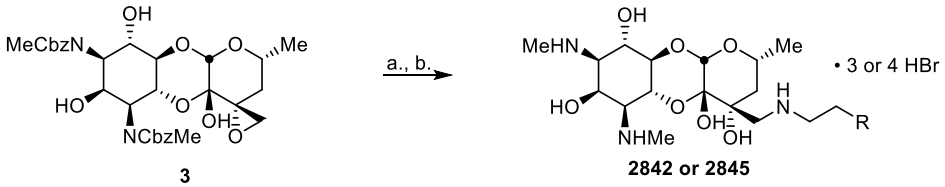
Supplementary Schemes

SS1.Synthesis of R-3'-aminomethyl-3'-hydroxy spectinomycins via reductive amination – 1980, 2592, 2593, and 2694.



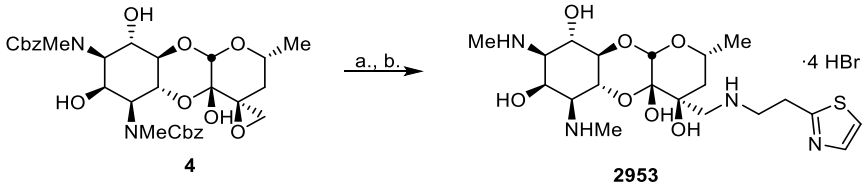
Conditions: a.) RCH_2CHO, 2-picoline borane, AcOH in MeOH (1:10 v/v), 29-56%. e.) H_2, 10% Pd/C, 1M HCl in MeOH, r.t., 30-90 min, 83-89%.

SS2. Synthesis of R-3'-aminomethyl-3'-hydroxy spectinomycins via epoxide opening – 2842 and 2845.



Conditions: a.) $LiClO_4$, $RCH_2CH_2NH_2$, MeOH, 40°C, 24 hrs., 36-54%. b.) 48% HBr in water, 2 hrs, r.t., 55-69%.

SS3. Synthesis of S-3'-aminomethyl-3'-hydroxyspectinomycin 2953.



Conditions: a.) $LiClO_4$, $RCH_2CH_2NH_2$, MeOH, 40°C, 24 hrs., 46%. b.) 48% HBr in water, 2 hrs, r.t., 54%.

Compounds **1**, **3**, and **4** were synthesized according to previously reported procedures.(8, 9)

General procedure for SS1.

Reductive amination (a.): To a stirred solution of **1** (183 mg, 0.290 mmol) in methanol (MeOH): acetic acid (CH_3COOH) (10:1) (2.0 mL) and the selected aldehyde (0.290 mmol) was added 2-picoline borane (0.290 mmol) and stirred at room temperature for 2 hours. The methanol was removed, and the residue partitioned between ethyl acetate (EtOAc) and water and the 2-phase mixture extracted with EtOAc (2 ´ 5 mL). The combined organic layers were dried with sodium sulfate (Na_2SO_4) and concentrated under reduced pressure and purified by column chromatography to afford corresponding protected amine using acetonitrile (CH_3CN)/MeOH gradient eluent system.

Cbz deprotection (b.): To a 2 necked round bottom flask is added palladium on carbon (10% Pd/C, 15.8 mg, 0.0149 mmol). The flask is sealed and evacuated, then purged with nitrogen. The protected amine (0.149 mmol) is added as a solution in 2.5 mL MeOH, followed by 0.5 mL of 1M HCl in MeOH (0.500 mmol). The reaction is stirred until deprotection is complete (1-2 hrs.), the catalyst is filtered off and rinsed with additional methanol, then placed under water for disposal. The filtrate is concentrated *in vacuo*, then triturated with acetone to remove residual solvent and concentrated to afford the target AmSPC.

General procedure for SS2.

Epoxide ring opening (a.): Following a modified procedure by Heydari(10) and Azizi(11). To a stirred solution of **3** (0.325 mmol) and the selected amine (1.627 mmol) in MeOH (10 mL) was added $LiClO_4$ (0.976 mmol). The reaction was heated for 24 hrs at 40°C. The MeOH was removed

and the residue partitioned between $CHCl_3$ and water and the 2-phase mixture extracted with $CHCl_3$ (2 x 10 mL). The combined organic layers were dried with Na_2SO_4 and concentrated under reduced pressure and purified by column chromatography to afford the corresponding protected amine using $CHCl_3$/MeOH gradient eluent system.
Hydrobromic acid (HBr) deprotection (b.): To the amine recovered in (a) was added minimal HBr (48% in water) to dissolve the amine. The reaction was stirred at room temp. for 2 hours, and then the solution was poured into 300 mL of acetone while stirring gently with a glass rod. The acetone solution was decanted and the resultant solid was washed with acetone (3 x 50 mL). The residue was dissolved in MeOH and concentrated under reduced pressure to afford the target AmSPC.

General procedure for SS3.
For the synthesis of 2953, the same epoxide opening and deprotection procedures were used as in **SS2**, with the corresponding S-diastereomer epoxide **4** in the place of **3**.

Compound Characterization

3'-(R)-3'-[(2-phenylethyl)aminomethyl]dihydrospectinomycin trihydro chloride (1980). Compound **1980** was prepared via reductive amination as described above in **SS1** to afford the title compound (520 mg, 57% from **1**) as the trihydro chloride salt. ^{1}H NMR (D_2O, 400 MHz) δ 7.36 (d, *J* = 7.2 Hz, 2H), 7.29 (d, *J* = 8.1 Hz, 3H), 4.82 (s, 1H), 4.21 (t, *J* = 10.4 Hz, 1H), 3.99 (t, *J* = 9.9 Hz, 1H), 3.90 (t, *J* = 10.0 Hz, 1H), 3.76 (q, *J* = 5.1, 4.2 Hz, 1H), 3.52 – 3.41 (m, 2H), 3.40-3.31 (m, 2H), 3.28 (s, 1H), 3.26-3.16 (m, 2H), 3.03 td, *J* = 7.9, 7.5, 3.7 Hz, 2H), 2.77 (s, 6H), 1.82-1.73 (m, 2H), 1.19 (d, *J* = 5.9 Hz, 3H). ^{13}C NMR (D_2O, 125 MHz) δ 181.4, 138.1, 128.9, 128.8, 126.9, 94.1, 92.8, 71.9, 71.0, 67.4, 67.3, 66.4, 62.2, 60.7, 59.0, 51.2, 49.4, 40.5, 33.0, 31.1, 30.8, 23.2, 19.9. High-resolution electrospray ionization mass spectrometry (HRMS-ESI) calculated for $C_{23}H_{37}N_3O_7$ $[M+H]^+$ 468.271, found 468.271.

3'-(R)-3'-[((4-methylphenyl)ethyl)aminomethyl]dihydrospectinomycin trihydro chloride (2592).).
Compound **2592** was prepared via reductive amination as described above in **SS1** to afford the title compound (12.6 mg, 46% from **1**) as the trihydro chloride salt. ^{1}H NMR (D_2O, 500 MHz) δ 7.27-7.09 (m, 4H), 4.87 (s, 1H), 4.79 (s, 1H), 4.27 (t, *J* = 10.5 Hz, 1H), 4.05 (t, *J* = 10.0 Hz, 1H), 3.97 (t, *J* = 10.1 Hz, 1H), 3.85-3.78 (m, 1H), 3.54 (dd, *J* = 11.1, 3.0 Hz, 1H), 3.48 (d, *J* = 13.5 Hz, 1H), 3.38 (dq, *J* = 10.1, 3.6 Hz, 2H), 3.27 (t, *J* = 12.1 Hz, 2H), 3.05 (q, *J* = 7.5, 7.1 Hz, 2H), 2.83 (t, *J* = 3.3 Hz, 6H), 2.33 (s, 3H), 1.92 – 1.79 (m, 2H), 1.25 (d, *J* = 6.2 Hz, 3H).^{13}C NMR (125 MHz, D_2O) δ 137.6, 133.1, 129.7, 128.7, 93.2, 92.4, 72.2, 69.6, 67.4, 65.9, 65.3, 61.5, 59.5, 58.5, 50.4, 49.5, 40.1, 30.7, 30.5, 30.3, 20.0, 19.9. High-resolution electrospray ionization mass spectrometry (HRMS-ESI) calculated for $C_{24}H_{40}N_3O_7$ $[M+H]^+$ 482.2866, found 482.2873.

3'-(R)-3'-[((4-fluorophenyl)ethyl)aminomethyl]dihydrospectinomycin trihydro chloride (2593). Compound **2593** was prepared via reductive amination as described above in **SS1** to afford the title compound (32 mg, 34% from **1**) as the trihydro chloride salt. ^{1}H NMR (D_2O, 500 MHz) δ 7.34-7.32 (dd, *J* = 8.3, 5.3 Hz, 2H), 7.16-7.13 (t, *J* = 8.8 Hz, 2H), 5.00 (s, 1H), 4.36-4.32 (t, *J* = 10.8 Hz, 1H), 4.21-4.18 (ddd, *J* = 10.17, 6.45, 3.29 Hz, 1H), 4.07-4.03 (t, *J* = 9.95 Hz, 1H), 3.99-3.95 (t, *J* = 10.11 Hz, 1H), 3.64-3.61 (d, *J* = 13.37 Hz, 1H), 3.56-3.53 (dd, *J* = 2.67, 11.07 Hz, 1H), 3.38-3.35 (m, 3H), 3.27-3.23 (m, 2H), 3.08-3.05 (t, *J* = 7.89 Hz, 2H), 2.83 (s, 6H), 1.80-1.70 (m, 2H), 1.16 (d, *J* = 6.29 Hz, 3H). ^{13}C NMR (D_2O, 125 MHz) δ 162.7, 160.8, 132.02, 132.00, 130.47, 130.40, 115.7, 115.58, 92.5, 91.7, 71.7, 69.8, 67.0, 65.9, 65.8, 61.3, 60.0, 58.0, 51.2, 49.6, 48.8, 38, 30.7, 30.5, 30.2, 19.5. High-resolution electrospray ionization mass spectrometry (HRMS-ESI) calculated for $C_{23}H_{37}FN_3O_7$ $[M+H]^+$ 486.2616, found 486.2622. Elemental analysis theoretical composition for empirical formula $C_{23}H_{36}FN_3O_7 \cdot 3\ HCl \cdot 2\ H_2O$: C, 43.78; H, 6.87; N, 6.66; Cl, 16.86. Found for formula $C_{23}H_{36}FN_3O_7 \cdot 3\ HCl \cdot 2\ H_2O$: C, 43.53; H, 6.87; N, 6.47; Cl, 16.48.

3'-(R)-3'-[(2-(terahydro-2H-pyran-4-yl)ethyl)aminomethyl]dihydrospectinomycin trihydro chloride (2694). Compound **2694** was prepared via reductive amination as described above in **SS1** to afford the title compound (70 mg, 31% from **1**) as the trihydro chloride salt. ^{1}H NMR (D_2O, 400 MHz) δ 4.87 (s, 1H), 4.26-4.21 (m, 1H), 3.98 (t, *J* = 9.9 Hz, 1H), 3.92-3.86 (m, 3H), 3.81 (m, 1H), 3.48 (dd, *J* = 11.0, 2.8 Hz, 1H), 3.42-3.35 (m, 3H), 3.21-3.17 (m, 2H), 3.10-3.05 (m, 2H), 2.75 (s, 6H), 1.84-1.75 (m, 2H), 1.60 (m, 5H), 1.28-1.20 (m, 3H), 1.19 (d, *J* = 6.0 Hz, 3H). ^{13}C NMR (D_2O, 125 MHz) *δ* 93.2, 92.3, 72.3, 69.6, 67.5 (2C), 67.4, 65.9, 65.3, 61.5, 59.5, 58.5, 50.1, 46.5, 39.8, 31.62 (2C), 31.57, 31.5, 30.5, 30.2, 19.9. High-resolution electrospray ionization mass spectrometry (HRMS-ESI) calculated for $C_{22}H_{41}N_3O_8$ $[M+H]^+$ 476.2972, found 476.2965.

3'-(R)-3'-[((2-thiophen-2-yl)ethyl)aminomethyl]dihydrospectinomycin trihydro bromide (2842).
Compound **2842** was prepared via epoxide ring opening as described above in **SS2** to afford the title compound (29 mg, 24.8% from **3**) as the trihydro bromide salt. ^{1}H NMR (D_2O, 500 Mhz) δ 7.31 (dd, *J* = 4.9, 1.4 Hz, 1H), 7.02 – 6.94 (m, 2H), 4.83 (s, 1H), 4.19 (t, *J* = 10.5 Hz, 1H), 3.98 (t, *J* = 9.9 Hz, 1H), 3.89 (t, *J* = 10.1 Hz, 1H), 3.77 (ddd, *J* = 11.4, 6.1, 2.3 Hz, 1H), 3.71-3.66 (m, 1H), 3.51-

3.42 (m, 2H), 3.39-3.33 (m, 2H), 3.27-3.13 (m, 4H), 2.75 (s, 6H), 1.83-1.71 (m, 2H), 1.18 (d, *J* = 6.1 Hz, 3H). ^{13}C NMR (D_2O, 125 MHz): δ 138.0, 127.6, 126.7, 125.5, 93.2, 92.4, 74.6, 72.1, 69.6, 68.5, 67.3, 65.8, 65.3, 61.4, 59.4, 50.5, 49.4, 40.2, 30.3, 25.4, 19.9. High-resolution electrospray ionization mass spectrometry (HRMS-ESI) calculated for $C_{21}H_{35}N_3O_7S$ $[M+H]^+$ 474.2274, found 474.2271.

3'-(R)-3'-[((2-(thiazol-2-yl)ethyl)aminomethyl]dihydrospectinomycin tetrahydro bromide (2845).

Compound **2845** was prepared via epoxide ring opening as described above in **SS2** to afford the title compound (77 mg, 29.7% from **3**) as the tetrahydro bromide salt. ^{1}H NMR (D_2O, 500 MHz) δ 7.78 (d, *J* = 3.5 Hz, 1H), 7.58 (d, *J* = 3.5 Hz, 1H), 4.89 (s, 1H), 4.22 (dd, *J* = 11.0, 10.1 Hz, 1H), 3.99 (t, *J* = 10.0 Hz, 1H), 3.89 (d, *J* = 10.2 Hz, 1H), 3.81 (ddt, *J* = 12.0, 6.0, 3.0 Hz, 1H), 3.56-3.49 (m, 6H) 3.42 (d, *J* = 7.2 Hz, 1H), 3.30 (d, *J* = 13.5 Hz, 1H), 3.20 (dd, *J* = 10.4, 3.0 Hz, 1H), 2.76 (s, 6H), 1.85-1.76 (m, 2H), 1.19 (d, *J* = 6.1 Hz, 3H). ^{13}C NMR (D_2O, 125 MHz) δ 167.2, 140.0, 121.4, 93.2, 92.4, 72.3, 69.6, 67.3, 65.9, 65.3, 61.5, 59.5, 58.5, 50.6, 47.1, 40.2, 30.6, 30.4, 27.2, 19.9. High-resolution electrospray ionization mass spectrometry (HRMS-ESI) calculated for $C_{20}H_{34}N_4O_7S$ $[M+H]^+$ 475.2226, found 475.2240.

3'-(S)-3'-[((2-(thiazol-2-yl)ethyl)aminomethyl]dihydrospectinomycin tetrahydro bromide (2953).

Compound **2953** was prepared via epoxide ring opening as described above in **SS3** to afford the title compound (97 mg, 24.8% from **4**) as the tetrahydro bromide salt. ^{1}H NMR (D_2O, 500 MHz) δ 7.88 (d, *J* = 5 Hz, 1H), 7.68 (d, *J* = 5 Hz, 1H), 5.01 (s, 1H), 4.37 (t, *J* = 10 Hz, 1H), 4.21 (m, 1H), 4.08 (t, *J* = 10 Hz, 1H), 3.98 (t, *J* = 10 Hz, 1H), 3.70 (d, *J* = 15 Hz, 1H), 3.60 (m, 4 H), 3.29 (m, 2H), 2.86 (s, 6H), 1.79 (m, 2H), 1.28 (d, *J* = 5 Hz, 3H). ^{13}C NMR (D_2O, 125 MHz) δ 167.2, 140.0, 121.5, 92.5, 91.7, 71.8, 69.8, 67.0, 66.0, 65.8, 61.3, 60.0, 58.0, 51.2, 47.2, 38.0, 30.8, 30.5, 27.5, 19.6. High-resolution electrospray ionization mass spectrometry (HRMS-ESI) calculated for $C_{20}H_{34}N_4O_7S$ $[M+H]^+$ 475.2226, found 475.2226.

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