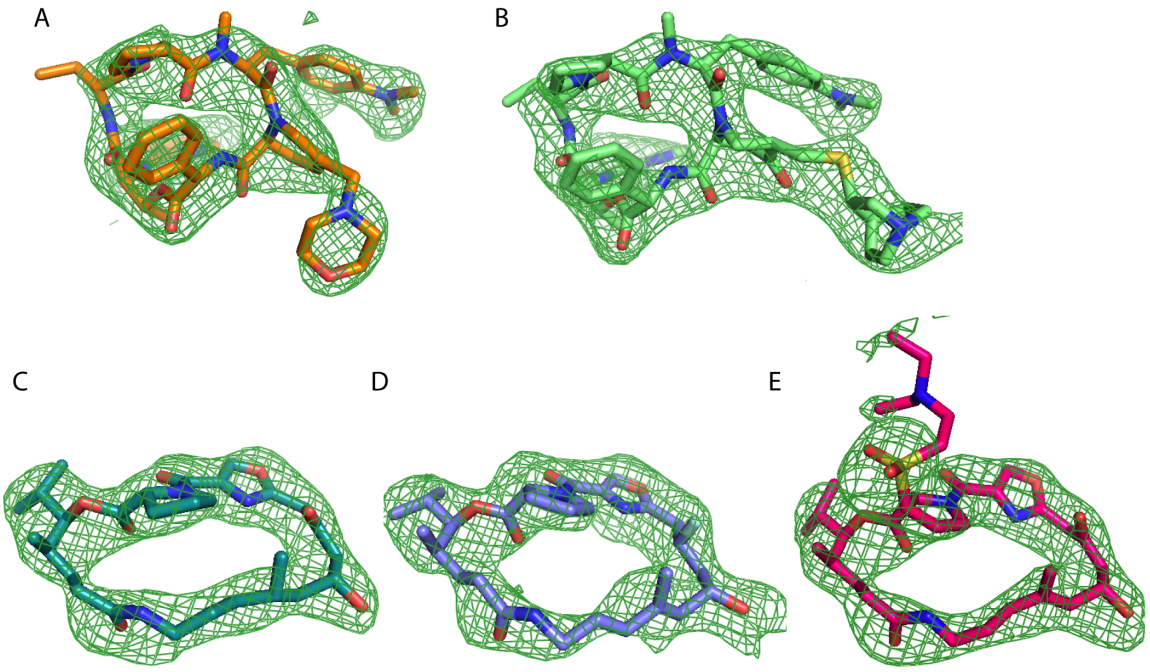


FIG S1. Unbiased difference electron density for streptogramin antibiotics with only one compound at a time bound to the ribosome. Unbiased F_{obs}-F_{calc} difference electron density contoured at 3.0 standard deviations from the mean is shown for A) linopristin, B) quinupristin, C) dalfopristin under hydrolyzing conditions, D) flopristin, and E) dalfopristin.

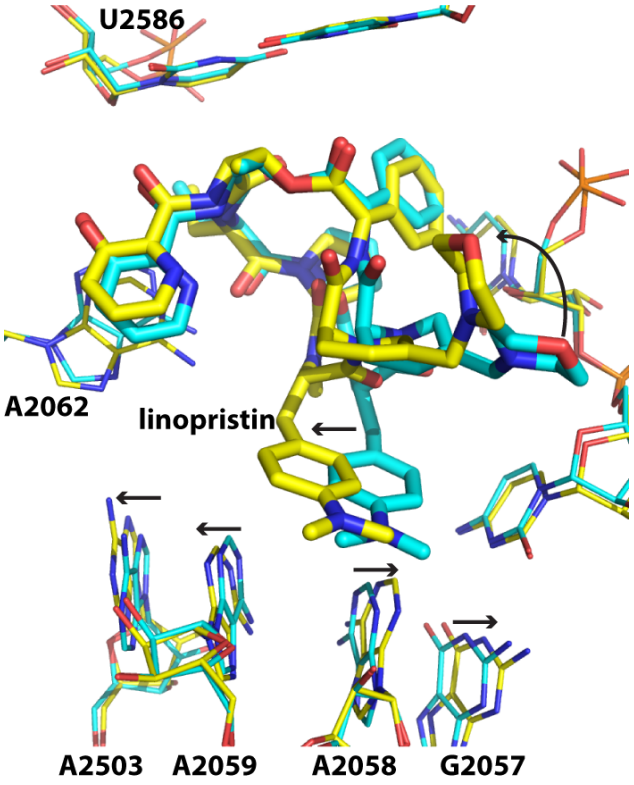


FIG S2. Binding of linopristin to the ribosome either alone or in the presence of flopristin. Structures of the 70S ribosome bound to either linopristin alone (yellow) or to the streptogramin combination NXL 103 (cyan) are shown. The differences between both structures are indicated by arrows.

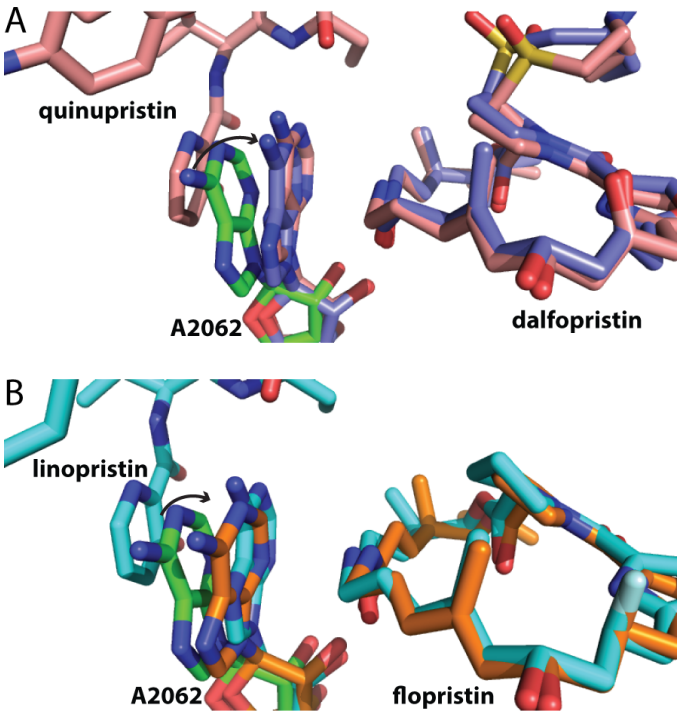
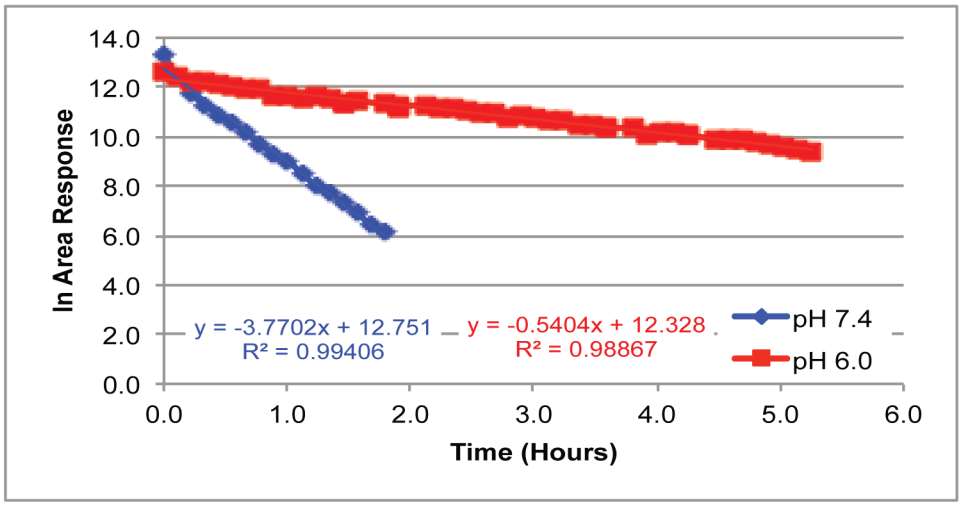
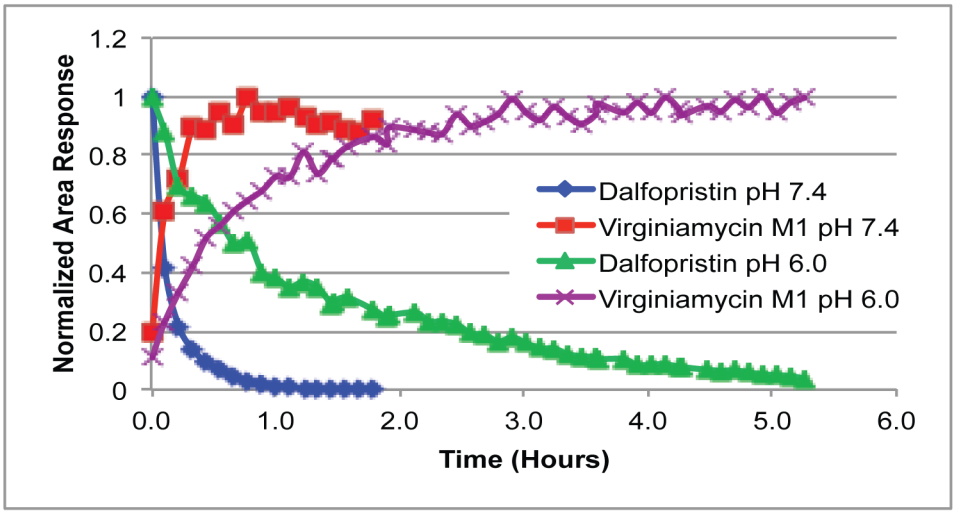


FIG S3. A pre-formed binding pocket for streptogramin B due to streptogramin A binding. A2062 is shown for the vacant ribosome (green), when ribosomes are bound to streptogramin A only, and when the combination of streptogramin A + B is bound. A) dalfopristin only (purple), dalfopristin + quinupristin (salmon). B) flopristin only (orange), flopristin + linopristin (cyan).

A



B



C

	$t_{1/2}$ [hours]	% deviation
Dalfopristin at pH 6.0	1.283	3.181
Dalfopristin at pH 7.4	0.184	4.263
Virginiamycin M at pH 7.4	44.917	16.129

FIG S4. Hydrolysis of dalfopristin as a function of pH. A) Natural log of the area responses plotted against time for dalfopristin in pH 6.0 CAMH II media and pH 7.4 phosphate buffer. B) Disappearance of dalfopristin and the formation of virginiamycin M, the normalized area responses of dalfopristin and virginiamycin M are plotted against time for pH 7.4 and pH 6.0. C) First-order derived half-lifes ($t_{1/2}$) based on the data in (A).

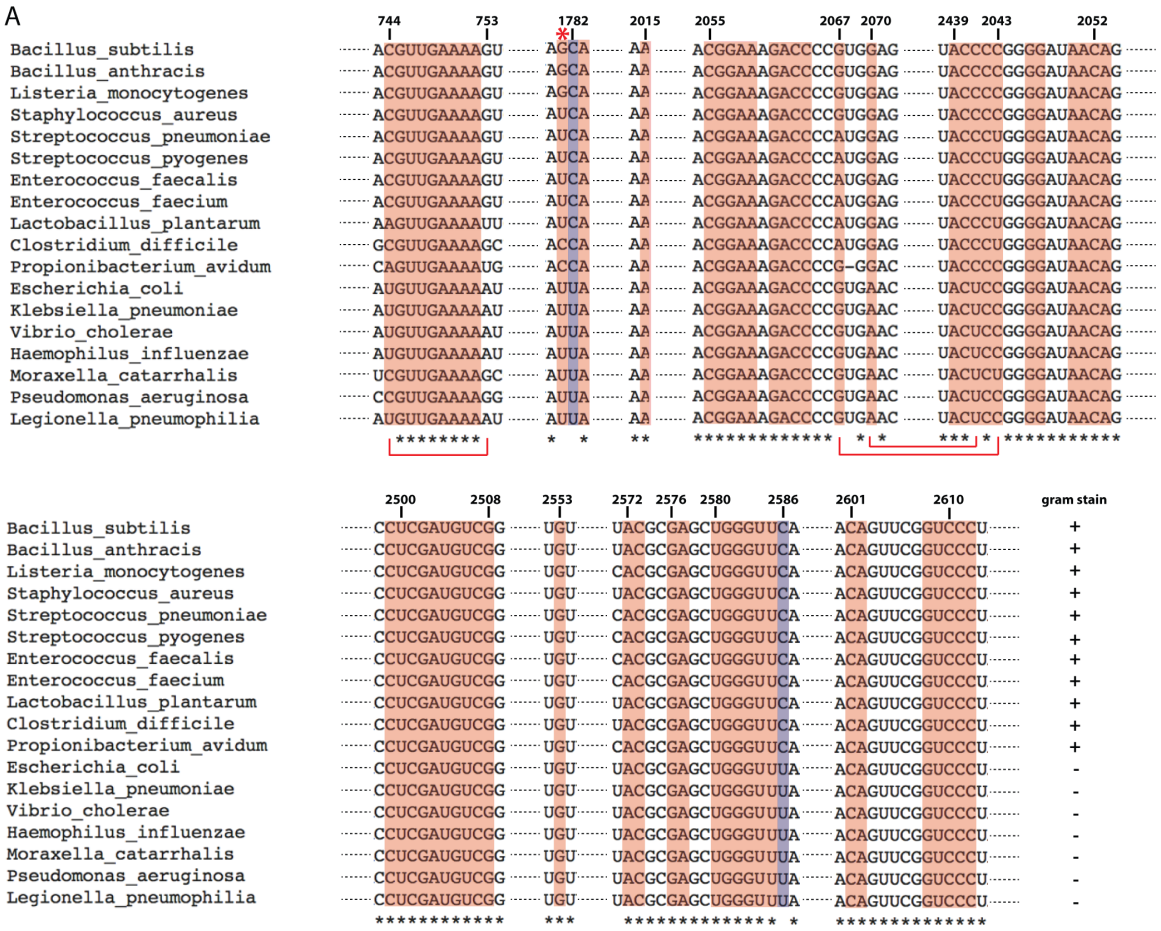

A
744
753
1782
2015
2055
2067
2070
2439
2043
2052
Bacillus_subtilis
Bacillus_anthracis
Listeria_monocytogenes
Staphylococcus_aureus
Streptococcus_pneumoniae
Streptococcus_pyogenes
Enterococcus_faecalis
Enterococcus_faecium
Lactobacillus_plantarum
Clostridium_difficile
Propionibacterium_avidum
Escherichia_coli
Klebsiella_pneumoniae
Vibrio_cholerae
Haemophilus_influenzae
Moraxella_catarrhalis
Pseudomonas_aeruginosa
Legionella_pneumophilia
2500
2508
2553
2572
2576
2580
2586
2601
2610
gram stain
B
2070
2441
1781
2586
1782
2067
2443
753
744

FIG S5. Sequence alignment of 23S rRNA of various Gram-positive and Gram-negative pathogens. A) Residues within 10Å of either streptogramin A or B component are shaded in red. Positions 1782-2586 (shaded in blue) form a base pair that lines the binding site of streptogramin B. Watson-Crick base pairs that are not conserved are indicated by red lines. The red asterisk indicates position 1781. B) Structure of Synercid (green sticks) with all residues shown that are within 10 Å of either streptogramin component. U1782-U2586 base pair is indicated by magenta sticks. Watson-Crick base pairs are shown in cyan sticks. Nucleotide 1781 is shown in yellow sticks.

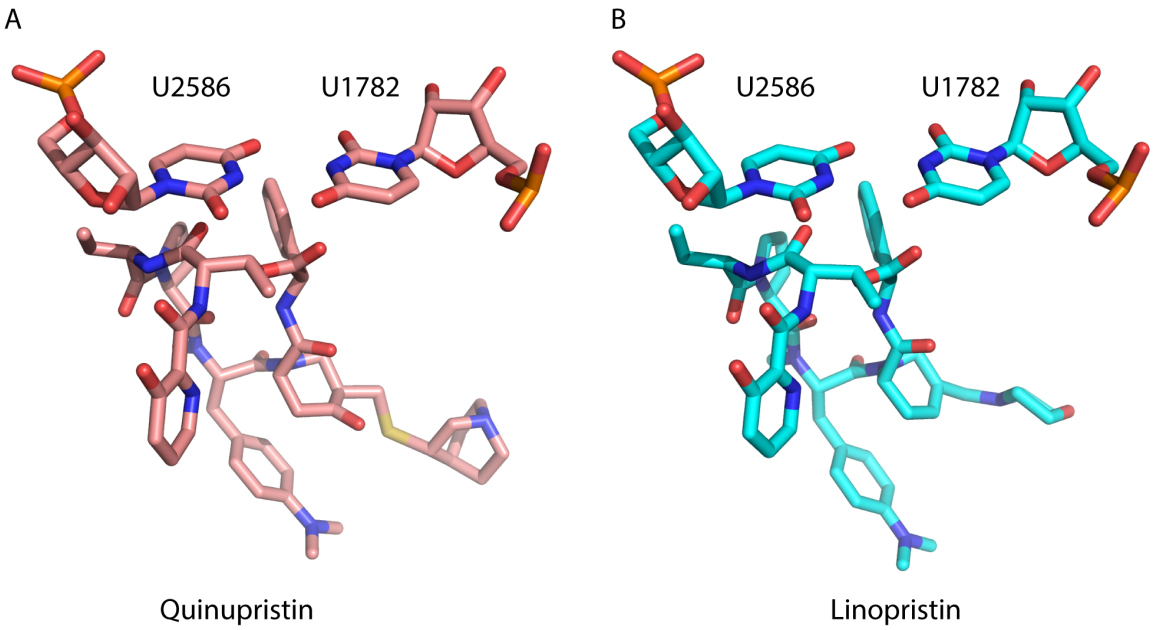


FIG S6. U-U base pair in the streptogramin B binding site. Structure of Synercid (A) and NXL 103 (B) with the U1782-U2586 base pair lining the binding site of the streptogramin B binding site.

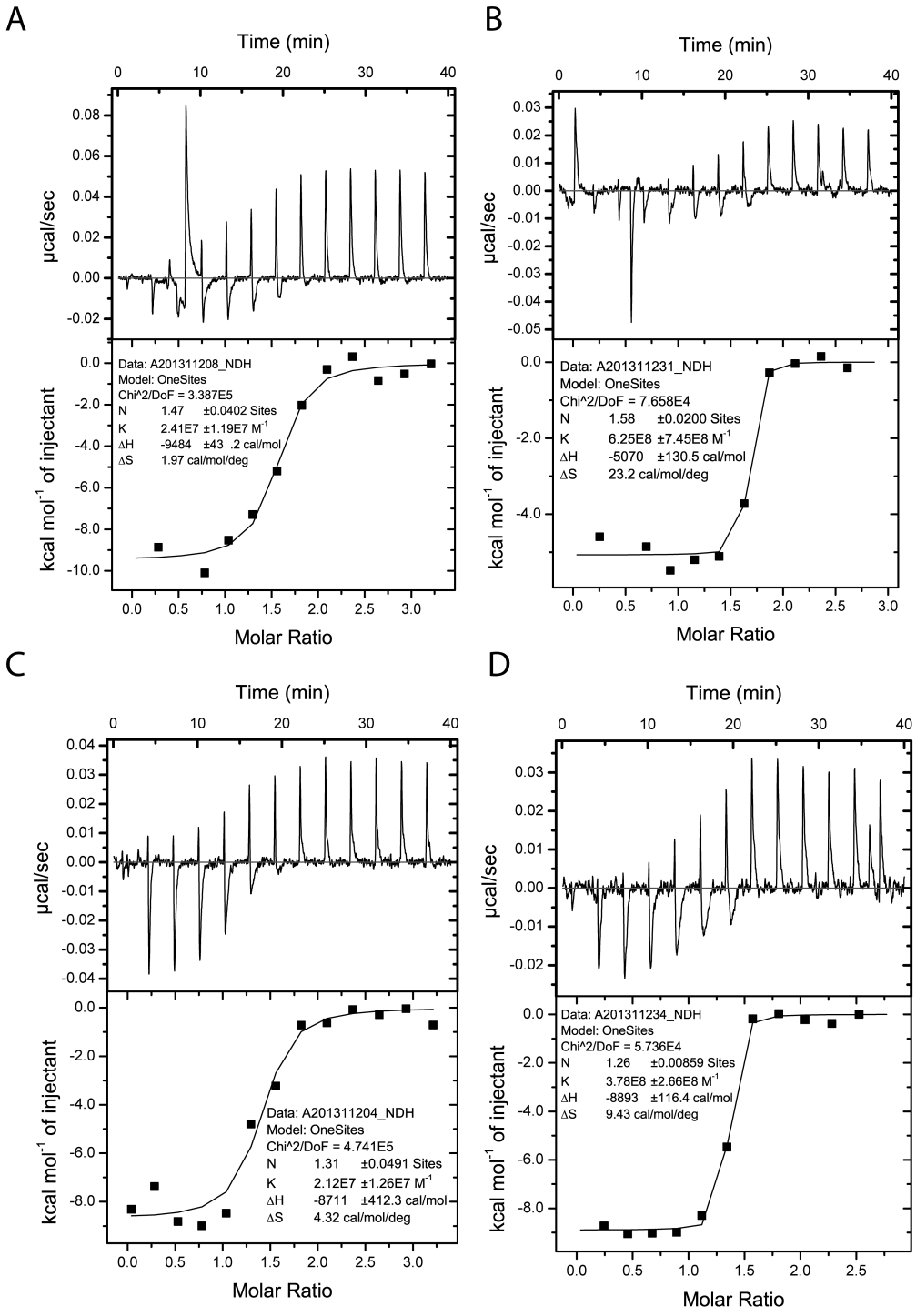


FIG S7. Isothermal titration calorimetry of ribosomes with streptogramins. A) Titration of the 70S ribosome with quinupristin. B) Titration of the 70S ribosome pre-bound to dalfopristin with quinupristin. C) Titration of the 70S ribosome with linopristin. D) Titration of the 70S ribosome pre-bound to flopristin with linopristin.

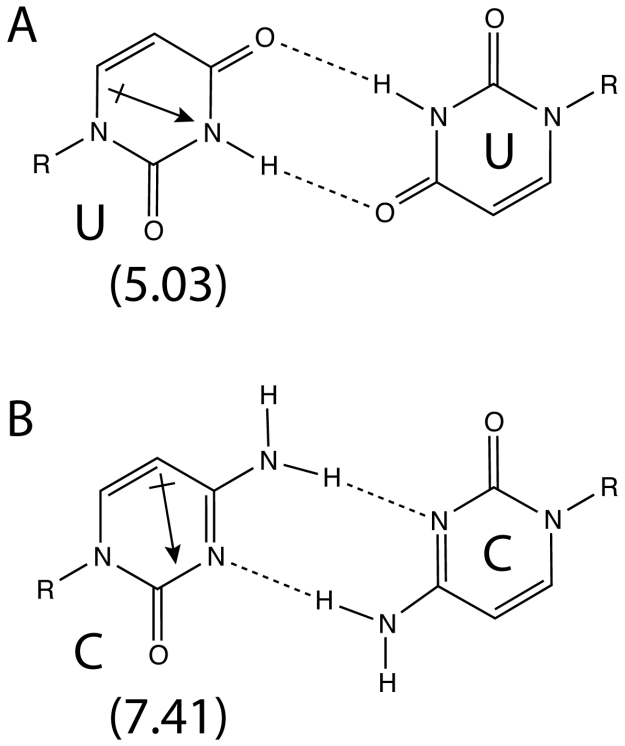


FIG S8. Chemical properties of U-U and C-C base pairs. The base pairing geometry and hydrogen bonding pattern is retained between a U-U (A) and a C-C (B) base pair however, the orientation of dipole moment vectors and their magnitude changes between a uracil (A) and a cytosine (B). Dipole moment vectors are indicated by an arrow and the magnitude of the dipole moment vector is shown in Debyes in parentheses.[34]

Table S1. Crystallographic data and refinement statistics

Antibiotic	NXL 103	Synercid	flopristin	lino-pristin	dalfo-pristin	quinu-pristin	dalfo-pristin hyd.
	Crystallographic statistics						
Space group	P212121	P212121	P212121	P212121	P212121	P212121	P212121
Unit cell dimensions ($Å^3$)	212.0 x 434.7 x 623.9	211.3 x 432.3 x 621.4	211.8 x 433.1 x 623.9	211.5 x 433.9 x 621.8	210.2 x 433.0 x 619.2	211.1 x 432.7 x 631.9	210.6 x 434.6 x 625.0
Resolution (Å)[a]	70 – 3.00 (2.90 – 2.80)	70 – 3.00 (2.90 – 2.80)	70 – 3.05 (3.00 - 2.90)	70 – 3.15 (3.10 – 3.00)	70 – 3.05 (3.00 – 2.90)	70 – 3.05 (3.05 – 2.95)	70 – 3.10 (3.00 - 2.90)
R_{meas} (%)[a]	15.5 (107.6)	15.9 (143.2)	15.7 (76.6)	16.0 (107.6)	14.7 (93.5)	14.3 (81.4)	19.7 (99.6)
I / σ (I)[a]	5.38 (0.56)	6.62 (0.56)	4.39 (0.72)	4.76 (0.68)	6.77 (0.63)	5.04 (0.75)	3.9 0.55)
Completeness (%)[a]	89.2 (86.8)	94.1 (87.6)	87.4 (75.3)	90.0 (86.2)	94.3 (87.1)	93.3 (88.6)	90.1 (80.6)
Measurement redundancy[a]	2.6 (2.0)	3.2 (2.5)	2.1 (2.0)	2.8 (2.2)	3.0 (2.13)	1.8 (1.5)	2.1 (1.9)
CC(1/2) (%)[a]	99.2 (36.9)	99.4 (29.6)	98.9 (57.0)	99.5 (39.8)	99.3 (44.4)	99.1 (37.2)	98.4 (39.1)
	Refinement statistics						
Resolution (Å)	70 – 2.8	70 – 2.8	70 – 2.9	70 – 3.0	70 – 2.9	70 – 2.95	70 – 2.9
No. Reflections	1,244,949	1,296,566	1,093,642	1,017,015	1,169,356	1,118,451	1,126,727
R/R_{free} (%)	21.5 / 26.0	22.5 / 27.1	23.5 / 27.9	22.5 / 27.4	22.0 / 26.4	24.9 / 28.2	22.3 / 26.5
No. Atoms	288,396	288,423	288,258	288,320	288,277	288,328	288,258
RMSD Bond Length (Å)	0.005	0.004	0.006	0.007	0.006	0.006	0.006
RMSD Angles (°)	0.902	0.872	1.113	1.209	1.069	1.159	1.094

[a]Values in parentheses are given for highest resolution bin.

Table S2. IC50 values for individual streptogramin components determined by cell-free translation assays

	U1782-U2586[a]	C1782-C2586[b]
dalfopristin	509 ± 36	486 ± 15
hydrolyzed dalfopristin	192 ± 19	158 ± 12
virginiamycin M	182 ± 13	169 ± 11
flopristin	130 ± 7	145 ± 9
linopristin	513 ± 43	269 ± 13
quinupristin	279 ± 31	192 ± 6

[a]Wild type *E. coli* cell extract bearing a U1782-U2586 base pair

[b]Mutant *E. coli* cell extract bearing a C1782-C2586 base pair reflecting the streptogramin binding pocket in Gram-positive pathogens.

Mean IC50 values (nM) from a representative experiment performed in triplicate are shown with standard deviation.

Table S3. IC50 values of streptogramin A components in the presence of either linopristin or quinupristin at their respective IC50 values[a] (exact values are indicted)

	U1782-U2586[b]			C1782-C2586[c]		
	single dose	+ 560 nM linopristin	+ 200 nM quinupristin	single dose	+ 280 nM linopristin	+ 200 nM quinupristin
dalfopristin	509 ± 36	137 ± 27	187 ± 27	486 ± 15	303 ± 21	407 ± 24
hydrolyzed dalfopristin	192 ± 19	122 ± 22	118 ± 27	158 ± 12	193 ± 26	259 ± 16
virginiamycin M	182 ± 13	129 ± 14	110 ± 17	169 ± 11	193 ± 14	213 ± 11
flopristin	130 ± 7	92 ± 17	94 ± 21	145 ± 9	159 ± 16	192 ± 16

[a]Mean IC50 values (nM) from a representative experiment performed in triplicate are shown with standard deviation.

[b]wild type *E. coli* cell extract bearing a U1782-U2586 base pair

[c]mutant *E. coli* cell extract bearing a C1782-C2586 base pair reflecting the streptogramin binding pocket in Gram-positive pathogens.

Table S4. IC50 values of streptogramin B components in the presence of streptogramin A components at their respective IC50 value (exact values are indicated)

	U1782-U2586[a]		C1782-C2586[a]	
	linopristin[c]	quinupristin[d]	linopristin[c]	quinupristin[d]
single dose	809 ± 47	304 ± 26	361 ± 17	211 ± 23
+ 500 nM dalfopristin	178 ± 25	147 ± 18	177 ± 12	155 ± 11
+ 150 nM hydrolyzed dalfopristin	504 ± 54	301 ± 35	228 ± 23	255 ± 17
+ 150 nM virginiamycin M	707 ± 45	279 ± 32	349 ± 16	261 ± 13
+ 110 nM flopristin	711 ± 36	314 ± 25	351 ± 16	268 ± 10

[a]wild type *E. coli* cell extract bearing a U1782-U2586 base pair

[b]mutant *E. coli* cell extract bearing a C1782-C2586 base pair reflecting the streptogramin binding pocket in Gram-positive pathogens.

Absolute IC50 values (nM) of either [c]only linopristin or [d]only quinupristin are different compared to Table S1 due to a different pipetting procedure (see Materials and Methods). Mean IC50 values (nM) from a representative experiment performed in triplicate are shown with standard deviation.

Table S5. K_a values measured by isothermal titration calorimetry (ITC)

Injectant	Cell content	K_a [M^{-1}]	K_a error [M^{-1}][a]	K_a error [% of K_a][b]	K_d [nM][c]	K_d error [nM][d]
quinupristin	70S	2.41E+07	1.19E+07	49	41.5	20.5
		4.23E+07	1.63E+07	39	23.6	9.1
		1.52E+07	5.07E+06	33	65.8	21.9
	70 + dalfopristin	8.75E+07	3.26E+07	37	11.4	4.3
		1.31E+08	1.18E+08	90	7.6	6.9
		6.25E+08	7.45E+08	119	1.6	1.9
linopristin	70S	2.78E+07	1.46E+07	53	36.0	19.0
		2.12E+07	1.26E+07	59	47.2	28.0
		2.77E+07	1.07E+07	39	36.1	13.9
	70S + flopristin	6.84E+07	1.12E+08	164	14.6	23.9
		5.90E+07	1.80E+07	31	16.9	5.2
		3.78E+08	2.66E+08	70	2.7	1.9

[a]Error in K_a from ITC experiment.

[b]The error in K_a is given as the percentage of K_a.

[c]K_d is the inverse of K_a.

[d]The error in K_d results from the percentage error in K_a applied to K_d. All experiments were carried out in triplicate.