

Supporting Information

Auerbach et al. 10.1073/pnas.0914100107

SI Text

Materials and Methods. Crystallography. Crystals of D50S that were grown as in (1) were soaked in solutions containing 0.025 mM of lankacidin for 20 h at 20°C, transferred into cryo-buffer and shock-frozen in liquid nitrogen. X-ray data were collected at 85–100 K from shock-frozen crystals at wavelength of 1.0 and 0.837 Å, at crystal to detector distance of 430 mm, using an oscillation range of 0.3° with synchrotron radiation beam at 19ID at the Advanced Photon Source/Argonne National Laboratory and at ID23-2 at the European Synchrotron Radiation Facility (ESRF), respectively. Data were recorded on charge-coupled device and processed with HKL2000 (2). Complete x-ray data sets were obtained from two crystals. The structure of D50S was refined against the structure factor amplitudes of the antibiotic complex D50S-LC using rigid body refinement as implemented in CNS 1.2 (3, 4). For free R-factor calculation, random 5% of the data were omitted during refinement. To obtain an unbiased electron density map a composite omit map of the entire unit cell was calculated. Further refinement was carried out using CNS 1.2 minimization combined with various CCP4 (5) programs, exploiting the available crystal structure of Bundlin-A (lankacidin) (6).

Finally, the complete molecules were subjected to restraint minimization and grouped B factor refinement with CNS. The ribosome-antibiotic interactions were determined with LigPlot (7) and LPC (8). Images were generated using PyMol (9).

Antibiotic binding. *D. radiodurans* 50S subunits (0.1 μM) were preincubated 15 min at 37°C with 0.1 μM of ¹⁴C-erythromycin (48.8 mCi/mmol, Perkin Elmer) in 100 μl binding buffer (20 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 250 mM NH₄Cl, 6 mM β-mercaptoethanol). LC was then added at varying concentrations (0–2 μM) and incubation continued for 30 min. The ribosome-antibiotic complexes were purified by gel filtration in BioGel P30 spin columns as described (10) and the amount of ribosome-bound radioactivity was measured by scintillation counting.

Inhibition of cell-free protein synthesis by LC and LM. The *E. coli* cell-free transcription-translation system for circular DNA (Promega) was pre-incubated with varying concentrations of antibiotics for 5 min at 20°C. The reactions (10 μl final volume) were initiated by adding 0.64 μg of pBestLuc plasmid DNA (Promega). Reactions were incubated for 40 min at 20°C and stopped by chilling on ice. The activity of firefly luciferase synthesized in the reaction was determined in 96-well plates using Bright-Glo Luciferase Assay System (Promega) as recommended by the manufacturer.

Inhibition of the peptidyl transferase reaction by LC In the reaction catalyzed by large ribosomal subunits, *D. radiodurans* 50S subunits (200 nM final concentration) were combined in 50 μl of the

reaction buffer (20 mM Tris-HCl, pH 8.0, 20 mM MgCl₂, 400 mM KCl) with 28 nM [³H]-fMet-tRNA, 1 mM puromycin at varying concentrations of LC. Reactions were initiated by addition of 25 μl methanol and incubated on ice for 30 min. Reactions were stopped and analyzed as described (11). In the ribosome-based puromycin assay, 70S ribosomes of *S. aureus* (at a final concentration of 200 nM) were preincubated for 15 min at 37°C in 50 μl of polyamine buffer (12) (20 mM HEPES-KOH, pH 7.6, 6 mM Mg-acetate, 150 mM NH₄Cl, 4 mM β-mercaptoethanol, 2 mM spermidine, 0.05 mM spermine) with 600 nM mRNA AAGGAG-AUAAACAAUGGGU and 28 nM [³H]-fMet-tRNA. After addition of varying concentrations of LC, puromycin was added to the final concentration of 0.5 mM and reactions were incubated for 15 min at 37°C. Reactions were stopped and processed as in (11).

RNA probing. RNA probing was carried out essentially as described (13, 14) using *D. radiodurans* 50S ribosomal subunits at 200 nM concentration and antibiotics at the following concentrations: LC–50 μM, LM–500 μM, ERY–50 μM. Prior to addition of the modifying reagents, ribosomal subunits were pre-incubated with the drug 10 min at 37°C and then 10 min at 20°C.

Cell-free translation system used for detecting synergism in vitro. Inhibition of cell-free protein synthesis by LC and LM was measured using the *E. coli* cell-free transcription-translation assay (Fig. S1). Cell free extract was prepared from *Escherichia coli* (Strain BL21-DE3) in a manner similar to that previously reported (15). An aqueous solution containing amino acid mix (1.3 mM for each amino acid), magnesium acetate (20 mM), NTP mix (0.9 mM), ATP (0.4 mM), potassium glutamate (150 mM), *E. coli* tRNA mixture (0.17 mg/mL), DTT (1.8 mM), folinic acid (35 μg/mL), cAMP (0.65 mM), NH₄OAc (28 mM), creatine phosphate (80 mM), HEPES (pH equals 7.5 at 37°C, 140 mM), 9.5% w/v PEG-8000, tyrosine (0.4 mg/mL), creatine kinase (0.35 mg/mL) and S12 cell free extract (12% v/v) was prepared. PIVEX.6D plasmid encoding wild-type GFP (1 ng/μL) was added to this solution commencing the transcription-translation reaction.

This solution was immediately added to a 96 well plate containing serial dilutions of lankamycin and lankacidin. After incubation for 1 h at 37°C, 50 μL of erythromycin (8 μM) was added to each well to completely stop the translation reaction. The fluorescence data were collected on a TECAN SpectraFluor Plus™ 96-well plate reader ($\lambda_{excite} = 485$ nm, $\lambda_{emit} = 535$ nm). The data were fitted using the least-squared regression analysis package in Igor Pro™.

A control experiment was performed in the absence of the antibiotics. This experiment was also used for the determination of the time window required for maximum GFP production before the reaction was quenched.

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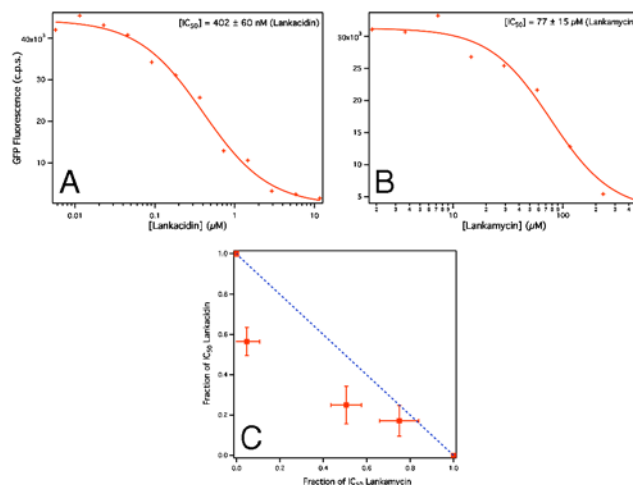


Fig. S1. (A and B): Binding curves for LC and LM respectively. The production of green fluorescent protein in an in vitro transcription-translation reaction was used to determine inhibition. (C): Synergy experiment showing the fractional contributions towards the observed C50 by LC and LM. Points below the dashed blue line indicate synergism.

Table S1. Crystallographic data for the D50S-lankacidin complex

Parameters	
Space group	I222
Resolution (Å)	40-3.5 (3.63-3.5)
R _{sym} (%)	16.3 (82.7)
Completeness (%)	92.4 (91.8)
Redundancy	5.3 (4.6)
I/σ(I)	7.7 (1.5)
Unit Cell (Å)	a = 169.8 b = 410.3 c = 694.4
R/R _{free} (%)	26.8/32.4
Bond length (Å)	0.006
Bond angles (degrees)	1.185