Lassomycin, a Ribosomally Synthesized Cyclic Peptide, Kills *Mycobacterium tuberculosis* by Targeting the ATP-Dependent Protease ClpC1P1P2

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**SUMMARY**

Languishing antibiotic discovery and flourishing antibiotic resistance have prompted the development of alternative untapped sources for antibiotic discovery, including previously uncultured bacteria. Here, we screen extracts from uncultured species against *Mycobacterium tuberculosis* and identify lassomycin, an antibiotic that exhibits potent bactericidal activity against both growing and dormant mycobacteria, including drug-resistant forms of *M. tuberculosis*, but little activity against other bacteria or mammalian cells. Lassomycin is a highly basic, ribosomally encoded cyclic peptide with an unusual structural fold that only partially resembles that of other lasso peptides. We show that lassomycin binds to a highly acidic region of the ClpC1 ATPase complex and markedly stimulates its ATPase activity without stimulating ClpP1P2-catalyzed protein breakdown, which is essential for viability of mycobacteria. This mechanism, uncoupling ATPase from proteolytic activity, accounts for the bactericidal activity of lassomycin.

**INTRODUCTION**

The scarcity of suitable lead compounds is now the major bottleneck in the development of novel antimicrobial drugs (Lewis, 2013; Payne et al., 2007). In the absence of new therapeutic agents, the rise and spread of multidrug resistant pathogens will continue unchecked. Most antibiotics in use today resulted from screening of soil actinomycetes for active compounds. However, repeated exploitation of this limited resource has led to diminishing returns, and most current efforts result in rediscovering known compounds (Lewis, 2012). Consequently, there has been a general elimination of natural product-based discovery in most pharmaceutical companies. Using new microbial sources of compounds could reduce the problem of rediscovery and lead to novel antimicrobial agents. Uncultured species of bacteria account for 99% of all microbial diversity and by definition represent an unexploited source of secondary metabolites (Staley and Konopka, 1985). We have developed general methods to grow previously uncultured bacteria, based on cultivation in diffusion chambers in their natural environments (Kaeberlein et al., 2002) and on prolonged incubation in vitro (Buerger et al., 2012). This approach results in cultivation of up to 40% of cells from environmental samples. However, even with this alternate source, most of the effort is expended on rediscovery of known compounds or generally toxic ones. We reasoned that this problem could be addressed by a species-selective approach, whereby compounds with broad antibiotic spectra are eliminated, and only compounds active against a specific species are considered. We chose *Mycobacterium tuberculosis* as a target organism for this approach, because few natural products are known to act specifically against this pathogen, and therefore most of the specific hits obtained should be new agents. There is also a considerable medical need for novel anti-tuberculosis compounds (Sacchettini et al., 2008; Zumla et al., 2013) to stem the spread of extremely and totally drug-resistant strains of the pathogen.

We screened extracts from a collection of soil bacteria, obtained by in situ cultivation and by prolonged incubation, against *Mycobacterium tuberculosis* and counterscreened against *Staphylococcus aureus*. An intriguing antimicrobial, lassomycin, was purified from an extract of *Lentzea kentuckyensis* sp. Lassomycin is a potent bactericidal compound that targets the ClpC1 ATPase, an essential enzyme in mycobacteria, which normally functions in protein degradation together with the ClpP1P2 proteolytic complex (Akopian et al., 2012). This agent is a highly basic lasso peptide antibiotic that is encoded in the genome and is unusual in its specificity for mycobacteria and its mode of action.
Lassomycin Targets the ClpC1P1P2 Protease in Mtb

RESULTS AND DISCUSSION

Isolation of Lassomycin

A library of extracts from soil actinomycetes was screened against *M. tuberculosis*. To shorten the duration of screens, we constructed a strain constitutively expressing mCherry and used bacterial fluorescence as the readout. This method allowed for reliable detection of growth inhibition in 5 days. The screen had a hit rate of 10% against *M. tuberculosis*. A counterscreen against *S. aureus* had a hit rate of 30%, and the hit rate for extracts specifically acting against *M. tuberculosis* was 2%. One of the extracts that acted specifically against *M. tuberculosis* was from isolate IS009804, a *Lentzea kentuckyensis* sp. (99.7% identical to *spectrometry (LC-MS) indicated that a single major compound purification. This fraction was lyophilized, leaving a white powder, which we have named lassomycin, consists of 16 amino acids in which the N-terminal residues form an eight-residue ring through formation of an amide bond between the N-terminal amine and the side chain carboxyl group of Asp8. The overall structure resembles a lasso in which the eight-residue ring forms the loop, and residues 9–16 form the spoke. In addition, the C-terminal carboxyl is converted to a methyl ester (Figure 1A).

Acid hydrolysis of lassomycin, followed by derivatization with Marfey’s reagent, and LC-MS analysis established that all of the residues are L-amino acids.

The three-dimensional solution structure of lassomycin was deduced from the NOE distance restraints obtained from three-dimensional NMR data using CYANA 2.1 (Figure 1B). Surprisingly, the solution structure of lassomycin lacks the characteristic knot structure reported for other homologous lasso peptides such as lariatin A and microcin J25 (Arnison et al., 2013) because the C-terminal end packs tightly against the N-terminal ring instead of passing through the macrolactam (Figure 1B; Figure S1 and Table S1 available online). Lassomycin has four positively charged arginine side chains and no negatively charged groups because the terminal carboxyl is esterified.

Biosynthetic Gene Cluster of Lassomycin

The resulting structure was consistent with the putative biosynthetic genes identified in the producing strain’s genome. The structural gene itself shows highest homology by BLAST to *larA* in the *laraABCDE* operon which codes for lariatin A. This antibiotic is a prototypical member of the lasso peptide family, which is produced by *Rhodococcus jostii* and inhibits cell wall biosynthesis (Iwatsuki et al., 2006). Lasso peptides consist of 16–21 amino acids and feature a macroactam ring formed from a connection between the N terminus and a sidechain carboxyl group. Lasso peptides are produced by both Actinobacteria (*Streptomyces* and *Rhodococcus*) and Proteobacteria (*Escherichia, Burkholderia*). Lariatin A is an 18-amino acid peptide with an 8-residue ring that is formed between the N-terminal glycine amino group and a glutamic acid side chain at position 8. The *larA* gene encodes a precursor peptide that is believed to be cleaved by LarD and enzymatically converted to the mature lasso structure by LarB to produce the active peptide. LarE exports the mature peptide. LarC’s function is unknown, but it is necessary for antibiotic activity (Inokoshi et al., 2012). The lariatin A precursor peptide shares only 53% homology with the predicted lassomycin precursor, LasA. In contrast, four other genes in the putative lassomycin operon (Figure 2), *lasB, lasC, lasD*, and *lasE*, share high (84%–97%) homology with the corresponding genes in the lariatin operon. The last gene product of the lassomycin biosynthetic cluster, LasF, is a putative O-methyltransferase that is likely responsible for the formation of the methyl ester at the C terminus of the mature peptide.

Lassomycin Bioactivity

Lassomycin had a minimum inhibitory concentration (MIC) of 0.8–3 μg/ml, fairly potent for a peptide, against a variety of *M. tuberculosis* strains, including multidrug resistant and extremely drug resistant isolates (Table 1). Lassomycin was discovered in a screen designed to identify compounds acting specifically against *M. tuberculosis*. We
Lassomycin Targets the ClpC1P1P2 Protease in Mtb

Figure 2. The Putative Lassomycin Biosynthetic Operon

Target Identification and Mechanism of Action of Lassomycin

To determine the target of lassomycin, resistant mutants of M. tuberculosis were obtained by selecting colonies on nutrient agar plates containing the compound. Mutants resistant to 16 μg/ml of lassomycin were obtained at a frequency of 3 × 10⁻⁷. Genome sequencing of six resistant mutants (Figure 4) showed mutations in the clpC1 gene, which encodes the subunit of the hexameric ATPase complex, ClpC1. It functions in protein degradation with the two-ring protease complex, ClpP1P2, in mycobacteria (Akopian et al., 2012; Raju et al., 2012). Together, they form the large (26 subunit) ATP-dependent protease complex, ClpC1P1P2 central chamber for proteolysis (Akopian et al., 2012). Because all the resistant mutants were mapped to the clpC1 gene, we tested directly if lassomycin altered either of ClpC1’s enzymatic activities: its ability to hydrolyze ATP and to support ATP-dependent protein breakdown by ClpP1P2 (Akopian et al., 2012; Raju et al., 2012), a novel member of the ClpP family of compartmentalized proteases (Lupas et al., 1997; Yu and Houry, 2007). The cloned His6-tagged M. smegmatis ClpC1 was expressed in M. smegmatis and purified to near homogeneity, as described previously (Akopian et al., 2012). As expected, ClpC1 exhibited ATPase activity and stimulated the ATP-dependent degradation of the model protein substrate, casein, by the pure ClpP1P2 protease complex, prepared as we recently described (Akopian et al., 2012). Thus, ClpC1 catalyzes casein translocation into the proteolytic compartment formed by ClpP1P2. Surprisingly, in the presence of low concentrations of lassomycin, ATP hydrolysis by ClpC1 increased up to 7- to 10-fold. This effect of lassomycin on ATP hydrolysis was highly cooperative and showed a Hill coefficient of 2 (Figure 5A). This strong activation by lassomycin showed an apparent Kₐ of 0.41 μM, which resembles the value of its MIC against M. tuberculosis cells. Because killing seems to result from binding to ClpC1 (see below), there is probably little or no barrier to lassomycin’s entry into the bacteria.

This unexpected stimulation of ClpC1’s ATPase activity initially suggested that lassomycin also activated protein degradation by the ClpC1P1P2 protease. Such a mechanism appeared likely in light of the discovery that the natural product antibiotics, acyldepsipeptides (Kirstein et al., 2009), are bactericidal by binding to ClpP and inducing excessive breakdown of cell proteins. In fact, by activating ClpP these antibiotics can kill persisters and eradicate a chronic biofilm infection (Conlon et al., 2013). Another antibiotic, cyclomarin A, which binds to ClpC1, has also been proposed to cause excessive proteolysis (Schnitt et al., 2011; Vasudevan et al., 2013). Surprisingly, however, we found that lassomycin, while markedly stimulating the ATPase activity of ClpC1, completely eliminated its ability to support ATP-dependent degradation of casein (Figure 5B), and somehow uncoupled the ATPase activity of ClpC1 from proteolysis. The resulting inhibition of proteolysis, while accelerating ATP hydrolysis, represents an interesting mechanism of antibiotic action that should be highly deleterious to the bacteria, because it prevents the regulated selective destruction of key cell proteins by the ClpC1P1P2 complex.

One potential and easily testable mechanism for uncoupling of ATP hydrolysis from protein degradation would be if lassomycin prevented the binding of protein substrates to ClpC1. This possibility could be easily tested, because protein substrates of ClpC1 are known to enhance its ATPase activity 2- to 3-fold (Akopian et al., 2012). To determine whether lassomycin blocks casein binding to ClpC1, we tested whether it decreases the activation of ATP hydrolysis by casein. On the contrary, lassomycin and casein had additive effects on ClpC1’s ATPase activity (Figure 5C), and therefore, they probably bind to ClpC1 in distinct places (see below). Therefore, in the presence of the antibiotic, ClpC1 seems to bind protein substrates normally, but is incapable of translocating them into the proteolytic compartment.

ClpC1 is a member of the large AAA family of ATPases that serve a variety of key functions in animal and bacterial cells.
**Table 1. Lassomycin Spectrum of Activity**

<table>
<thead>
<tr>
<th>Strains</th>
<th>MIC (µg/ml)</th>
<th>MIC (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>M. tuberculosis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H37Rv</td>
<td>0.78–1.56</td>
<td>0.41–0.83</td>
</tr>
<tr>
<td>186, susceptible clinical isolate</td>
<td>1.56</td>
<td>0.83</td>
</tr>
<tr>
<td>83, susceptible clinical isolate</td>
<td>1.56–3.1</td>
<td>0.83–1.65</td>
</tr>
<tr>
<td>84, resistant to INH, STR</td>
<td>1.56–3.1</td>
<td>0.83–1.65</td>
</tr>
<tr>
<td>85, resistant to INH, RIF</td>
<td>1.56–3.1</td>
<td>0.83–1.65</td>
</tr>
<tr>
<td>7, resistant to INH, RIF</td>
<td>1.56</td>
<td>0.83</td>
</tr>
<tr>
<td>86, resistant to INH, RIF, STR</td>
<td>1.56</td>
<td>0.83</td>
</tr>
<tr>
<td>136, resistant to INH, RIF, STR, FQ</td>
<td>0.78</td>
<td>0.41</td>
</tr>
<tr>
<td>133, resistant to INH, RIF, STR, FQ</td>
<td>0.78</td>
<td>0.41</td>
</tr>
<tr>
<td>189, resistant to INH, RIF, STR, FQ</td>
<td>3.1</td>
<td>1.65</td>
</tr>
<tr>
<td>3, resistant to INH, RIF, EMB, FQ</td>
<td>3.1</td>
<td>1.65</td>
</tr>
<tr>
<td>30, resistant to INH, RIF, EMB, PZA, FQ</td>
<td>0.78</td>
<td>0.41</td>
</tr>
<tr>
<td>181, resistant to INH, RIF, EMB, PZA, FQ</td>
<td>0.78</td>
<td>0.41</td>
</tr>
<tr>
<td>183, resistant to INH, RIF, STR, EMB, PZA, FQ</td>
<td>3.1</td>
<td>1.65</td>
</tr>
<tr>
<td>188, resistant to INH, RIF, STR, EMB, PZA, FQ</td>
<td>3.1</td>
<td>1.65</td>
</tr>
<tr>
<td>mc²6020</td>
<td>0.39–0.78</td>
<td>0.21–0.41</td>
</tr>
<tr>
<td><strong>Other mycobacteria</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M. avium subsp. paratuberculosis</td>
<td>0.125–0.25</td>
<td>0.07–0.13</td>
</tr>
<tr>
<td>M. smegmatis</td>
<td>0.78–2</td>
<td>0.41–1.06</td>
</tr>
<tr>
<td><strong>Other actinobacteria</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propionibacterium acnes</td>
<td>12.5–25</td>
<td>6.7–13</td>
</tr>
<tr>
<td>Bifidobacterium longum</td>
<td>25–50</td>
<td>13–27</td>
</tr>
<tr>
<td><strong>Other Gram-positive bacteria</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clostridium difficile</td>
<td>&gt;50</td>
<td>&gt;27</td>
</tr>
<tr>
<td>Clostridium perfringens</td>
<td>&gt;50</td>
<td>&gt;27</td>
</tr>
<tr>
<td>Lactobacillus reuteri</td>
<td>&gt;50</td>
<td>&gt;27</td>
</tr>
<tr>
<td>Lactobacillus casei</td>
<td>&gt;50</td>
<td>&gt;27</td>
</tr>
<tr>
<td>Streptococcus mutans</td>
<td>&gt;50</td>
<td>&gt;27</td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>&gt;50</td>
<td>&gt;27</td>
</tr>
<tr>
<td>Enterococcus faecalis VRE</td>
<td>&gt;50</td>
<td>&gt;27</td>
</tr>
<tr>
<td>Bacillus anthracis Sterne</td>
<td>&gt;50</td>
<td>&gt;27</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>&gt;50</td>
<td>&gt;27</td>
</tr>
<tr>
<td><strong>Gram-negative bacteria</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteroides fragilis</td>
<td>&gt;50</td>
<td>&gt;27</td>
</tr>
<tr>
<td>Escherichia coli K12</td>
<td>&gt;50</td>
<td>&gt;27</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>&gt;50</td>
<td>&gt;27</td>
</tr>
<tr>
<td>INH, isoniazid; Rif, rifampicin; STR, streptomycin; EMB, ethambutol; PZA, pyrazinamide; FQ, resistant to at least one fluoroquinolone.</td>
<td></td>
<td></td>
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</tbody>
</table>

(Erzberger and Berger, 2006; Hanson and Whiteheart, 2005; White and Lauring, 2007). Use of lassomycin as an anti-tuberculosis drug would presumably not be advisable if it activated other such ATPases. To determine whether lassomycin also affected the activities of other AAA ATPases, we tested several purified well-characterized bacterial and mammalian homologs. In M. tuberculosis, ClpX, like ClpC1, supports protein degradation by ClpP1P2; nevertheless, lassomycin had no effect on ATP hydrolysis by ClpX. In addition, no stimulation was observed with the Escherichia coli ClpC1 homolog, ClpA, a component of the E. coli ClpAP protease complex; PAN, the proteasomal activating ATPase from the archaeabacterium, Methanococcus jannaschii; and the mammalian 26S proteasome (Figure 5D). This highly specific activation of ClpC1 is of appreciable mechanistic interest. Somehow, binding of multiple lassomycin molecules must lead to an accelerated ATP-ADP exchange cycle (Smith et al., 2011) and much more rapid ATP hydrolysis by its six subunits.

**Docking of Lassomycin to ClpC1**

To gain further insight into the interaction of lassomycin with ClpC1, an in silico approach was utilized. We focused on the N-terminal region where all the lassomycin-resistant mutations were localized. Recently, the X-ray structure of the ClpC1 N terminus was solved by Vasudevan and colleagues (Vasudevan et al., 2013). Depiction of the mutation sites in this model revealed them to be close to each other on ClpC1 (Figure 6A). Furthermore, they were all located in a highly acidic region, which is likely to be the binding site for lassomycin based on its four positively charged guanidinium groups. Docking (Trott and Olson, 2010) of lassomycin onto this structure for ClpC1’s N terminus showed that eight of the nine obtained binding sites were in the same vicinity (Figures 6A and 6B). Analysis of the residues contributing to binding indicated that Gln17, which was altered in four of six resistant mutants (Figure 6A), is the major interacting residue through hydrogen bonding. In the resistant strains, Gln17 was mutated to an Arg or His, and the resulting reversal of the charge should markedly reduce the tendency for lassomycin binding. The other mutation sites, Arg21 and particularly Pro79 are located, in most of the binding states, on the rim of the surface area contacting the peptide (Figure 6A).

To clarify the mode of binding of lassomycin, models of the resistant mutant forms of ClpC1 were created. Because the actual conformations resulting from the mutations of these various residues are not known, several variants of the models were tested. The docking showed that the Gln17Arg mutation has the largest impact on lassomycin binding, and all of the four Gln17Arg mutant models showed a significant reduction in the number of likely positions for lassomycin (Figure 6D). Two variants of the Pro79Thr and two variants of the Arg21Ser mutation also reduced binding greatly, considering a flexible backbone of lassomycin. Both results confirmed that Gln17 is critical for antibiotic binding, whereas Arg21 and Pro79 appear to be less important and might decrease binding by altering the protein’s tertiary structure. This analysis, while useful, has clear limitations. For example, changes in ClpC1’s tertiary structure upon hexamer formation or ATP hydrolysis are not considered. In fact, AAA ATPases are highly dynamic structures (Smith et al., 2011), and the upper loop in the Corynebacterium glutamicum ClpC N-terminal crystal structure has a large temperature-factor (Debye-Waller) value, indicating a flexible region (Figure 6C). In addition, the conformation of lassomycin on the enzyme and possible influence of water on its binding are not known. Nevertheless, the approach indicates clear differences between wild-type and mutant structures in the likely binding site for lassomycin (Figure 6D). In the future, it will be valuable to analyze the crystal structure of ClpC1 with lassomycin bound to...
understand the basis for the large acceleration of ATP hydrolysis and its inability to support ATP-dependent proteolysis. Even though lassomycin and substrates (casein) both activate ATPase, casein must bind to a different site on ClpC1 because casein could stimulate ClpC1 in the presence of lassomycin (Figure 5C). It is also unclear how lassomycin’s binding to this highly acidic N-terminal region can allosterically regulate ClpC1’s ATPase activity and lead to the inhibition of ClpP1P2-dependent proteolysis. Most likely, the conformational changes induced by lassomycin interfere with ClpC1’s association with ClpP1P2. Because mutations in the binding region prevent killing by lassomycin, this uncoupling of ATP hydrolysis from protein degradation must be responsible for its bactericidal actions.

**SIGNIFICANCE**

In multiple respects, lassomycin is a very unusual bactericidal antibiotic with an intriguing mechanism of action. First, it kills mycobacteria selectively. This specificity is due to targeting ClpC1 without affecting related hexameric AAA ATPases. ClpC1, like ClpP1P2, is essential for viability in mycobacteria but not in other bacteria. Second, its structure is generated by cleavage of a ribosomal product, cyclization, and C-terminal esterification. Third, it is an unusually basic, 16-residue lasso peptide, which even at low concentrations, enters *M. tuberculosis* and binds to an acidic N-terminal pocket on ClpC1. Fourth, it is unusual for an antibiotic to activate rather than inhibit its target enzyme. An important question for future research is to understand how lassomycin’s binding to ClpC1 stimulates its ATPase activity but uncouples it from ClpP1P2-dependent proteolysis. Normally, AAA ATPases, like ClpC, function by an ordered reaction cycle involving each of its six subunits (Smith et al., 2011), which extend loops into specific pockets on ClpP hexamers. Most likely, this coupling mechanism is disrupted by lassomycin. Fifth, although this interaction with ClpC1’s N terminus is critical for lassomycin’s bactericidal action, it is unclear whether cell death results from this prevention of ClpC1-mediated breakdown of key proteins or from the large increase in ClpC1’s activity (e.g., excessive ATP hydrolysis or excessive protein unfolding). Sixth, based on lassomycin’s potency, which resembles or exceeds that of the standard treatments for tuberculosis, agents with similar effects on ClpC1 function represent promising approaches to treat this disease and its drug-resistant forms.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids**

*M. smegmatis* mc²155 was grown at 37°C in Middlebrook 7H9 broth with 0.05% Tween-80 and ADC (0.5% BSA, 0.2% dextrose, 0.085% NaCl, 0.003 g catalase/1 l medium) with hygromycin (50 μg/ml) and anhydrotetracycline (ATc; 100 ng/ml). *M. tuberculosis* strains were grown in Middlebrook 7H9 Broth (Difco) supplemented with 10% oleic acid-albumin-dextrose-catalase (Difco) with additional supplements. The supplements for the auxotrophic strain of *M. tuberculosis* mc²6020 (ΔlysA, ΔpanCD; Sambandamurthy et al., 2005) included 0.5% glycerol, 0.2% casamino acids (Amresco), 0.05% tylxapol, pantothenic acid (24 μg/ml), and lysine (80 μg/ml). The *M. tuberculosis* strain mc²6020 was transformed with a plasmid pBEN (gift from Dr. L. Ramakrishnan, University of Washington). Before transformation, the gene for green fluorescent protein in the pBEN plasmid was replaced with a gene coding for the red fluorescent protein mCherry (Shaner et al., 2004). The supplements for *M. tuberculosis* clinical isolates included 0.2% glycerol and 0.05% Tween-80. The clinical isolates were kindly provided by Dr. Clifton Barry III (NIH).
C-terminally 6XHis-tagged ClpC1 was expressed in M. smegmatis on pTetOR plasmid, which has an inducible tetracycline promoter.

**Long-Term Incubation Setup**

One gram of soil sample was vortexed vigorously in 10 ml of deionized H$_2$O for 10 min in a 50 ml conical tube. This sample was serially diluted and mixed with molten 2% SMS agar (0.125 g casein, 1 g potato starch, 1 g casamino acids, and 0.2 g CaCl$_2$·2H$_2$O per 1 l deionized H$_2$O, pH adjusted to 7 before autoclaving). The samples were concentrated in 50% aqueous DMSO and the mixture was then centrifuged. The hexanes were then decanted, tested for activity, and discarded because there was no activity. The remaining residue was then reconstituted in 50% aqueous DMSO and the sample was fractionated using an HPLC instrument equipped with a reversed-phase C18 column eluting with a gradient of H$_2$O/acetonitrile/0.1% trifluoroacetic acid (TFA) over 45 min. All fractions were tested for activity against M. smegmatis and only one fraction was active. This fraction was lyophilized, leaving a white powder (80 mg). Analysis of this fraction with LC-MS indicated that a major compound was present ([M+H$^+$] = 1,880). Subsequent searches in a natural product database (AntiBase) did not provide in any results.

**Electrospray Ionization-LC-MS Analysis**

Electrospray ionization-LC-MS data were recorded on a MicroMass Q-Tof-2 spectrometer equipped with an Agilent 1100 solvent delivery system and an online diode array detector using a Phenomenex Gemini-C18 reversed phase column (50 × 2.0 mm, 3.0 µm particle size). Elution was performed with a linear gradient using deionized water with 0.1% formic acid and CH$_3$CN with 0.1% formic acid as solvents A and B, respectively, at a flow rate of 0.2 ml/min. The gradient increased from 25% to 100% of solvent B over 10 min followed by an isocratic elution at 100% of solvent B for 7 min. Analytical and semipreparative chromatography were performed on a Zorbax SB-C18 reversed phase column (50 × 2.0 mm, 5.0 µm particle size). Elution was performed with a linear gradient using deionized water with 0.1% formic acid and CH$_3$CN with 0.1% formic acid as solvents A and B, respectively, at a flow rate of 0.2 ml/min. The gradient increased from 25% to 100% of solvent B over 10 min followed by an isocratic elution at 100% of solvent B for 7 min. Analytical and semipreparative chromatography were performed on a Zorbax SB-C18 reversed phase column (250 × 9.4 mm, 5.0 µm particle size) using a Shimadzu SCL-10AVP HPLC system including a SPD-M10AVP diode array detector set at 254 nm. Elution was performed with a linear gradient using deionized water with 0.1% TFA and CH$_3$CN with 0.1% TFA as solvents A and B, respectively, at a flow rate of 3.0 ml/min. The gradient increased from 0% to 100% of solvent B over 20 min followed by an isocratic elution at 100% of solvent B for 8 min.

**Partial Hydrolysis of Lassomycin**

The peptide was partially hydrolyzed using microwave-assisted acid hydrolysis. In a 1.5 ml polypropylene centrifuge tube, the sample solution contained 0.1 µg/µl peptide in 25% TFA or 3 M HCl. The sample tube was placed in the water bath of a CEM microwave chamber (CEM Discover System; CEM) to perform the hydrolysis. Microwave hydrolysis conditions were 80°C, 300W, 32–70 min for TFA hydrolysis and 12 min for HCl hydrolysis. After the microwave hydrolysis, samples were either dried using a SpeedVac vacuum centrifuge (Savant), or purified or diluted 5 to 10 times by water, followed by MS analysis.

**MS/MS Sequencing and Exact Mass Determination of Partially Hydrolyzed Lassomycin**

The partially hydrolyzed lassomycin peptide was diluted five to ten times and directly spotted (0.5 µl) onto a Bruker Daltonics MTP AnchorChip 800/384 target and air-dried; 0.5 µl of a-cyano-4-hydroxy cinnamic acid matrix solution was spotted. The sample was analyzed on the same instrument using the parameters described above.
Lassomycin Targets the ClpC1P1P2 Protease in Mtb

ClpP1P2 (100 nM) and ClpC1 (100 nM) were mixed in 80 μl of reaction buffer containing 50 mM potassium phosphate (pH 7.6), 100 mM KCl, 8 mM MgCl₂, 5% glycerol, 2 mM ATP, and 5 mM Z-Leu-Leu. Enzymatic activity was measured fluorometrically using FITC-casein as a substrate in the presence or absence of 10 μM of lassomycin. The rate of degradation of ClpP1P2 was taken as 100%.

Lassomycin does not interfere with casein binding to ClpC1. ATPase activity of ClpC1 (32 nM) was measured as in Figure 5A in the presence or absence of ClpP1P2 (100 nM) and ClpC1 (100 nM) were mixed in 80 μl of reaction buffer containing 50 mM potassium phosphate (pH 7.6), 100 mM KCl, 8 mM MgCl₂, 5% glycerol, 2 mM ATP, and 5 mM Z-Leu-Leu. Enzymatic activity was measured fluorometrically using FITC-casein as a substrate in the presence or absence of 10 μM of lassomycin. The rate of degradation of ClpP1P2 was taken as 100%.

The absence of lassomycin was taken as 100%.

Similar results were obtained when the ATPase activity in the absence of lassomycin was taken as 100%. The apparent K₅ and Hill coefficient for lassomycin activation of ClpC1 ATPase were determined using curve fitting with classic Hill-kinetic through a scaled Levenberg-Marquardt algorithm; tolerance 0.0001. (B) ClpC1 does not activate degradation of casein by ClpP1P2 in the presence of lassomycin.

(A) Lassomycin stimulates ATPase activity of ClpC1. 32 nM of pure ClpC1 were mixed with 100 μl of the assay buffer (50 mM Tris/HCl pH 7.8; 100 mM KCl; 10% glycerol; 1 mM phosphoenolpyruvate; 1 mM NADH; 2 units of pyruvate kinase/lactic dehydrogenase [Sigma]; 4 mM MgCl₂, and 1 mM ATP) and the ATPase activity of ClpC1 was followed by measuring the coupled oxidation of NADH to NAD⁺ spectrophotometrically at 340 nm. The rate of ATPase activity in the absence of lassomycin was taken as 100%. The apparent K₅ and Hill coefficient for lassomycin activation of ClpC1 ATPase were determined using curve fitting with classic Hill-kinetic through a scaled Levenberg-Marquardt algorithm; tolerance 0.0001. (B) ClpC1 does not activate degradation of casein by ClpP1P2 in the presence of lassomycin.

(B) Stimulation of ClpC1 ATPase activity by lassomycin is highly specific. The activities of purified ATPases from bacteria (E. coli ClpX, E. coli ClpP, ClpB, and GroEL), archaea (PAN), and mouse (26S proteasome) were measured in the presence and absence of lassomycin (10 μM). ATPase activity of each ATPase in the presence of lassomycin was taken as 100%.

In addition, LC-MS/MS was performed as follows: the partially hydrolyzed peptide was ZipTip (Millipore) purified and 5 μl of the resultant peptide solution was loaded onto a Waters nanoAcquity UPLC system (Waters) using a peptide trap (180 μm × 20 mm, Symmetry C18 nanoAcquity column; Waters) and an analytical column (75 μm × 150 mm, Atlantis dC18 nanoAcquity column; Waters). Desalting on the peptide trap was achieved by flushing the trap with 2% acetonitrile and 0.1% formic acid at 10 μl/min for 3 min. Peptides were separated with a gradient of 2%–60% solvent B (acetonitrile and 0.1% formic acid) over 35 min at 350 nl/min. The column was connected to a Waters O-TOF Premier (Waters) for electrospray ionization-MS/MS analysis. Data interpretation was completed manually to propose a proposed peptide sequence.

NMR Spectroscopy of [13C, 15N]Lassomycin

NMR data were acquired and processed as previously described (Rea et al., 2010; Sit et al., 2011). A Varian Inova 800-MHz spectrometer with a triple-resonance HCN cold probe and pulsed field gradients was used to record spectra. [13C, 15N]Lassomycin was dissolved in dimethyl sulfoxide-d₆ (Cambridge Isotope Laboratories), and the sample was heated to 40 °C for data collection.

Compound Spectrum, Activity

Potency was determined by measuring MIC with broth microdilution. Strains were grown to an optical density of 0.3 at 650 nm and diluted 1:100 into fresh media. Antibiotics were serially diluted 2-fold in 96-well plates and an equal volume of diluted bacterial culture was added to each well. The plates were sealed with Breathe-Easy (3M) and incubated at 37 °C for 14 days after which growth was visually inspected. The MIC was the concentration of compound..
that resulted in no growth compared to controls wells. MBC was determined by plating cells from wells containing 2×, 4×, and 8× MIC concentrations of compound. The plates were incubated for 3–4 weeks at 37°C. The MBC was the concentration of compound that decreased colony count by ≥99.9%.

To monitor in vitro cytotoxicity, exponentially growing NIH 3T3 and HepG2 cells in media supplemented with 10% FBS were harvested and seeded at 5,000 cells per well in a 96-well flat bottom plate (Smeet et al., 2002). After 24 hr, the medium was replaced with fresh medium containing compounds added at a 2-fold serial dilution, in a process similar to an MIC assay. After 24 and 48 hr of incubation, cell viability was measured with the CellTiter assay, according to manufacturer’s recommendations.

**HighThroughput Screen**

A frozen aliquot of *M. tuberculosis* mc²6020 pBEN mCherry used to inoculate (0.5%–1% inoculum) 50 ml of 7H9 with OADC, glycerol, casamino acids, talcopol, panthothenic acid, lysin, and 50 µg/ml of kanamycin. The culture was incubated at 37°C with shaking until the optical density 600 reached 0.4–0.5. The culture was then diluted to have approximately 10³ colony-forming units per milliliter, and transferred to black, clear-bottom 96-well microplates (Corning), and 1%–2% of the crude extracts were added to the tested wells. Rifampicin 1 µg/ml (Sigma) was used as a positive control. The last column of the plate was used as negative control (cell with 2 µl of DMSO). Plates were sealed with Breathe-Easy Sealing Membrane (RP1) and were incubated with shaking at 37°C for 5–7 days. After incubation, fluorescence was measured from the bottom using SynergyMX plate reader (BioTek) with excitation 580 nm and emission 610 nm.

An auxotrophic strain of *M. tuberculosis* mc²6020 (lysAΔ,panCD; Sambandamurthy et al., 2003) expressing the red fluorescent protein mCherry was screened against 25,600 crude extracts (3,200 strains, four fermentation media, two time points). All crude extracts were counterscreened against *S. aureus*. The extracts showing activity against *S. aureus* were excluded from the screening steps, and the resulting hit rate was 2%. To evaluate the quality of our assay, the Z’-factor was calculated for several growth conditions. A Z’ factor between 1 and 0.9 is considered to be an excellent assay, between 0.9 and 0.7 is good, and between 0.7 and 0.5 will benefit significantly from any improvement (Zhang et al., 1999). The highest Z’ factor (0.8 ± 0.06) was obtained for the plates containing 150 µl of cell culture after 5 days of incubation with shaking, so we proceeded with these conditions for the high-throughput screening.

**Whole-Genome Sequencing**

Genomic DNA from *M. tuberculosis* mc²6020 (lysAΔ panCD) wild-type and resistance mutants were isolated using a previously described protocol (Kieser et al., 2000). An Illumina single-end library was constructed and 50 base pair sequencing was conducted on an illumina HiSeq2000 following the manufacturer’s instructions at Biopolymers facility (Harvard Medical School, Boston, MA). Genomes were assembled and mapped using the reference genome of H37Rv with CLC Genomics Workbench (CLC bio). Genomes of resistance mutants were aligned with the genome of the wild-type to detect single base-pair polymorphisms (SNPs).

The results were filtered to exclude any SNPs that had low confidence after assembly. The remaining SNPs were verified with PCR amplification and Sanger sequencing.

**Purification of *M. tuberculosis* ClpC1**

C-terminally 6XHis-tagged *M. tuberculosis* ClpC1 subunits were overexpressed in *E. coli* using an Atc inducible expression system. After overnight induction with Atc (100 ng/ml), cells were resuspended in buffer A (50 mM TrisHCl pH 7.8, 100 mM KCl, 10% glycerol, 1 mM ATP, and 4 mM MgCl2), lysed by French press, and lysates were centrifuged for 1 hr at 100,000 × g. ClpC1 was isolated from the supernatant by Ni-NTA affinity chromatography (QIAGEN). Eluted fractions containing ClpC1 protein were pooled and further purified by size-exclusion chromatography on Sephacryl S-300 column in buffer A. Fractions containing ClpC1 protein were concentrated and used in the assays of ATPase activity and protein degradation.

**Measurement of ATPase Activity**

Two micrograms of pure ClpC1 was mixed with 100 µl of the assay buffer (50 mM TrisHCl pH 7.8; 100 mM KCl; 10% glycerol; 1 mM phosphoenolpyruvate; 1 mM NaH2PO4; 2 µl pyruvate kinase/lactic dehydrogenase [Sigma]; 4 mM MgCl2, and 1 mM ATP) and the ATPase activity of ClpC1 was followed by measuring the coupled oxidation of NADH to NAD⁺ spectrometrically at 340 nm.

**Measurement of Casein Degradation**

Assay of proteolytic activity was performed at 37°C in 96 wells plate using Plate Reader SpectraMax M5 (Molecular Devices). Wells contained 3 µg ClpC1P12, 6 µg ClpC1, 5 mM Z-Leu-Leu, and 2–5 µg of fluorescein isothiocyanate (FITC)-casein in 80 µl of 50 mM phosphate buffer (pH 7.6) with 5% glycerol, 100 mM KCl, 8 mM MgCl2, and 2 mM ATP. FITC-casein hydrolysis was continuously monitored at 518 nm (Ex at 492 nm). Deviations in the measurements of FITC-casein were less than 10%.
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**SUPPLEMENTAL INFORMATION**

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**AUTHOR CONTRIBUTIONS**


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**protein structure**

The PyMOL Molecular Graphics System, version 1.5.0.1 (Schrödinger), was used for protein depiction.

**Molecule Docking**

AutoDock Vina v1.1.2 was used to perform the docking runs (Trott and Olson, 2010). The structure of the ClpC1 N-domain was obtained through the Protein Data Bank (PDB: http://www.rcsb.org; Berman et al., 2000; PDB entry 3WDB) and prepared using PyMol. Solvents and molecules other than the ClpC1 N-domain were removed, and in silico mutations were introduced. All structure files were validated with MolProbity 4.02 (Chen et al., 2010). Only rotamers resulting in a clash score close to the wild-type model (clash score <6; wild-type was 5.2) were chosen for the docking. The structure files for the ligand and the receptor were prepared with AutoDockTools v1.5.6 (Morris et al., 2009). Preparation of the receptor molecules (M. tuberculosis ClpC1 N-domain model and mutants) included the following steps: addition of all hydrogens, computation of Gasteiger charges, and merging of nonpolar hydrogens. To account for the fact that the bioactive conformation of lassomycin is not known, its structure file was converted with AutoDockTools in two variants: (1) all bonds were assumed rotatable, except the closed lasso ring backbone; and (2) only side chains were rotatable, but the whole peptide backbone remains rigid. The search space was restricted to the acidic half of the ClpC1 N-domain and the exhaustiveness set to 100 (default is 8) to limit the generation of local minima in the optimization function. Computations of the docking runs were conducted on the Harvard University Faculty of Arts and Science Research Computing Cluster “Odyssey.” Vina calculates a binding energy for each position it finds based on the number of rotatable bonds allowed for the ligand and the environment on the receptor (Trott and Olson, 2010). Hence, in the docking approach here, a comparison of the binding energies between different receptor models is not appropriate because the mutant models have a different protein sequence. Instead, the results were scored by the position of the docked ligand on the receptor molecule. Human error in this evaluation was eliminated by the fact that positive and negative positions showed a great distance from each other.

**ACCESSION NUMBERS**

The PDB accession number for the lassomycin solution structure reported in this paper is 2MAI. The BioMagResBank accession number for the lassomycin chemical shift assignments reported in this paper is 19355.


