Restriction of the Conformational Dynamics of the Cyclic Acyldepsipeptide Antibiotics Improves Their Antibacterial Activity

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Restriction of the Conformational Dynamics of the Cyclic Acyldepsipeptide Antibiotics Improves Their Antibacterial Activity

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ABSTRACT: The cyclic acyldepsipeptide (ADEP) antibiotics are a new class of antibacterial agents that kill bacteria via a mechanism that is distinct from all clinically used drugs. These molecules bind and dysregulate the activity of the ClpP peptidase. The potential of these antibiotics as antibacterial drugs has been enhanced by the elimination of pharmacological liabilities through medicinal chemistry efforts. Here, we demonstrate that the ADEP deformation observed in the ADEP−ClpP crystal structure is fortified by transannular hydrogen bonding and can be further stabilized by judicious replacement of constituent amino acids within the peptidolactone core structure with more conformationally constrained counterparts. Evidence supporting constraint of the molecule into the bioactive conformer was obtained by measurements of deuterium-exchange kinetics of hydrogens that were proposed to be engaged in transannular hydrogen bonds. We show that the rigidified ADEP analogs bind and activate ClpP at lower concentrations in vitro. Remarkably, these compounds have up to 1200-fold enhanced antibacterial activity when compared to those with the peptidolactone core structure common to two ADEP natural products. This study compellingly demonstrates how rational modulation of conformational dynamics may be used to improve the bioactivities of natural products.

INTRODUCTION

Among the most interesting antibacterial drug targets to emerge in the past decade is the proteolytic complex formed by ClpP (caseinolytic peptidase) and its AAA+ partners (ATPases associated with diverse cellular activities). ClpP is a highly conserved peptidase that is involved in the turnover of a wide variety of cellular proteins, including transcription factors that regulate virulence-factor production and stress responses.1–3 These accessory ATPases play critical roles in regulating the activity of ClpP. Genetic studies have established that the clpP gene and genes encoding the AAA+ partners are essential for virulence in some pathogenic bacteria (e.g., Staphylococcus aureus, Listeria monocytogenes, and Streptococcus pneumoniae) and for viability in others (e.g., Mycobacterium tuberculosis).13–20 Although no drugs that target ClpP have been introduced into the clinic yet, the critical physiological roles of ClpP make it an attractive target for the development of antibacterial agents.

Several molecules reported to perturb ClpP activity have been discovered in high-throughput screens or in mechanistic investigations of natural products with antibacterial activity.13,14,23 These compounds are classified as either activators or inhibitors of ClpP. Treatment of bacteria with inhibitors of ClpP phenocopies the effects of clpP null mutations (i.e., compromised virulence or viability),21,22 whereas bacteria are killed upon exposure to ClpP activators.23 The first ClpP activators to be reported were the cyclic acyldepsipeptide antibiotics (ADEPs).24,25 The representative members of this group of antibiotics are "AS4556A and B" produced by Streptomyces hawaiensis24 and enopeptins A and B produced by Streptomyces sp. RK-1051 (Figure 1).25 Collectively, the ADEPs have been reported to exhibit potent activity against a broad range of Gram-positive bacterial pathogens, including S. aureus, S. pneumoniae, Enterococci, and M. tuberculosis.24–26 As reflected by the fact that no clinically used antibacterial drugs target ClpP, the ADEPs have activity against multidrug resistant,
pathogenic bacteria observed in clinical and community settings.26,29

Structural studies indicate that the ADEPs bind at the subunit interfaces of the ClpP tetradecamer, which also serve as docking sites for the accessory ATPases.30,31 A consequence of this competitive binding is expansion of ClpP’s axial pores.26,30–32 Remarkably, these ADEP-induced changes in ClpP’s quaternary structure enable it to degrade oligopeptides and unstructured or nascent proteins without the intervention of the accessory ATPases. The indiscriminate degradation of cellular proteins like the essential cell-division protein, FtsZ, by the ADEPs has a N-methylalanine residue within the core macrocycle, whereas ADEP-4 has a cyclic amino acid, pipecolate (a five-membered ring) in place of N-methylalanine.34 In addition to potent activity in vitro against S. aureus, S. pneumoniae, and Enterococci,29 ADEP-4 close analogs are reported to be toxic to M. tuberculosis in vitro, particularly in combination with efflux pump inhibitors.28 Importantly, ADEP-4 has impressive activity in vivo. In fact, mice with potentially lethal infections of S. aureus were cured by via intravenous administration of ADEP-4.29 Recently, it has been reported to completely eradicate S. aureus biofilms in vitro and in mouse models of chronic infection when coadministered with the antibacterial drug rifampicin.35

In a previous study,27 we investigated the consequences of replacing amino acids in the ADEP macrocycle with more conformationally constrained residues. One of the compounds that we prepared was an analog of ADEP-4 with 4-methylpipecolate in place of the pipecolate. The methyl substituent was predicted to further restrict the conformational flexibility of the pipecolate and by extension that of the peptidolactone. The compound with 4-methyl pipecolate was 2- and 4-fold more potent than ADEP-4 against clinical isolates of methicillin-resistant S. aureus and vancomycin-resistant Enterococcus faecalis, respectively.27 Motivated by these initial findings, we sought to study the phenomenon of restricting peptidolactone conformation and define its impact on the ADEPs’ binding to and activation of ClpP and on their bioactivity. We utilized deuterium exchange experiments with 1H NMR to empirically measure the effects of various structural modifications on peptidolactone conformational dynamics. In
addition, enzymatic assays were used to measure the capacity of the ADEP analogs to bind and activate ClpP. Finally, we used bioassays to assess the toxicities of the compounds to three species of pathogenic bacteria. It is noteworthy that some of the rigidified ADEPs bind and activate ClpP at substantially lower concentrations in vitro and have up to 1200-fold enhanced antibacterial activity.

■ RESULTS AND DISCUSSION
Assessment of the Conformationally Biasing Hydrogen Bonds in the ADEPs. Numerous noncovalent interactions between the ADEPs and ClpP are observed in crystal structures of the complex.30,31 The ADEPs themselves adopt a compact conformation that appears to be enforced by two transannular hydrogen bonds between the peptidolactone and the appendant side chain (Figure 2A, Table S1). Interestingly, a similar conformation is observed in crystals of free ADEP, where analogous hydrogen bonding between the peptidolactone and the side chain has been predicted.29 The similarities suggest that the free ADEPs may be predisposed to adopt a conformation that is compatible with ClpP binding.

To test the prediction that free ADEPs exhibit intramolecular hydrogen-bonding in solution, we performed experiments in which 1H NMR was used to measure deuteration exchange rates of amide hydrogen atoms predicted to participate in the bonds (Figure 2B). Hydrogen−deuterium exchange rates have been shown to be dependent upon the presence and strength of intramolecular hydrogen bonds in peptides.36 Accordingly, we anticipated that deuterium exchange rates at the amides engaged in hydrogen bonds would be markedly slower than at non-hydrogen-bonded amides. Given the limited solubility of the ADEPs in water, we selected deutero-methanol (CD3OD) as the solvent for the deuterium exchange experiments, accepting the possibility that the molecules’ conformations could differ in organic and aqueous solvents. Immediately after preparation of a dilute solution of ADEP 1a in CD3OD (Figures 2B and 3), we monitored attenuation of the amide proton resonances by 1H NMR over a period of hours at 25 °C. As expected, the hydrogen atoms of the three secondary amides in the ADEP exchanged with deuterium at markedly different rates (Figure 2B,C). The amide hydrogen of the serine residue, which does not participate in a transannular hydrogen bond, exchanged completely in CD3OD within several seconds and could never be observed in a 1H NMR spectrum.

By comparison, the amide hydrogen of the side chain difluorophenylalanine residue required several minutes to completely exchange with deuterium; whereas, that of the alanine amide within the macrocycle exchanged over the course of two hours (Figure 2C). These observations are consistent with the existence of transannular hydrogen bonds that are analogous to those inferred from the crystal structures of both free ADEP and ADEP in complex with ClpP.29−31

Chemical Syntheses of ADEPs with Conformationally Constrained Peptidolactones. We hypothesized that the bioactive conformation of the ADEPs could be stabilized by judicious replacement of particular amino acid constituents of the peptidolactone with more conformationally constrained analogs. First, we envisioned substituting the N-methylalanine residue with pipecolate residues bearing C-4 substituents of varying size. Cyclic amino acids are typically more conformationally constrained than their acyclic counterparts, and ring substituents tend to limit ring conformational dynamics by imposing high energetic penalties to certain conformations
macrocycle and thus served as a point of comparison for the other ADEPs.

Using deuterium-exchange experiments, we systematically assessed the conformational consequences of replacing the N-methylalanine and serine residues in the ADEP macrocycle with conformationally constrained pipecolate and allo-threonine residues, respectively (Figure 4). Interestingly, the pipecolate moiety does not fortify both of the transannular bonds that are apparent in compounds containing N-methylalanine (compounds 1a and 1e). Indeed, we found that the amide hydrogen of the difluorophenylalanine of compound 1b exchanged completely within seconds in CD$_3$OD, whereas the analogous hydrogen in compound 1a had a half-life of 3.87 min (see Supporting Information). Apparently, with the pipecolate residue in the macrocycle, the potential donor and acceptor atoms of the hydrogen bond are either too far apart or do not have appropriate trajectories for bonding. In contrast, the transannular hydrogen bond in which the alanine residue is the donor is retained in all of the ADEPs and strengthened by the presence of conformationally constrained amino acids within the macrocycle. For instance, compound 1b harboring a pipecolate residue in the peptidolactone had a slower rate of deuterium exchange rate than 1a. Further, we found that the deuterium-exchange rate decreased as the steric bulk of the C4 substituent on the pipecolate increased (see data for compounds 1b, 1c, and 1d in Figure 4). Replacement of the native serine residue in the ADEP peptidolactones with allo-threonine profoundly slowed the deuterium-exchange rate. For instance, the half-lives of the alanine amide hydrogens in compound 1a, which has serine, and compound 1e, which has allo-threonine, are ~100-fold different. Likewise, the deuterium exchange rate of the hydrogen atom of the difluorophenylalanine moieties in compounds 1a and 1e differed by 2.8-fold. As expected, inclusion of both pipecolate and allo-threonine (1f) into the peptidolactone had a synergistic effect on deuterium exchange. Interestingly, the apparent relationship between rigidifying structural features and deuterium exchange rate was not completely conserved when 4-methylpipecolate and allo-threonine were present together in the peptidolactone (1g). This compound had a faster rate of deuterium exchange than compounds with allo-threonine and either N-methylalanine (1e) or pipecolate (1f) in the peptidolactone. A reasonable explanation for this observation is that substituted pipecolate residues and allo-threonine each stabilize slightly different low energy conformers. Accordingly, the opposing forces could prevent a single, low energy conformer from being reached. As expected, the rate of deuterium exchange increased for all compounds at an elevated temperature (i.e., 40 °C), whereas the trend for relative rates of deuterium exchange remained the same (see Supporting Information), suggesting that the observed effects are the result of entropic factors. Overall, the general trend represented by these data supports our hypothesis that the incorporation of conformationally constrained residues in the peptidolactone has a rigidifying effect.

Figure 4. ADEP hydrogen–deuterium exchange in CD$_3$OD. Deuterium exchange rates were measured for 2 mM solutions of each ADEP under pseudo-first order conditions in deuterated methanol at 25 °C. The exchange rates for the hydrogen atoms of the alanine residues within the peptidolactone are shown.

**In Vitro Assessment of ClpP Binding and Activation by the Rigidified ADEPs.** Binding of either the ADEPs or the regulatory ATPases to ClpP stabilizes an open conformation of the peptidase core and stimulates degradation of oligopeptides. On the basis of predictions that the entropic costs of ClpP binding would be lower for the conformationally constrained ClpP derivatives, we expected that ADEP modifications that enhance macrocycle rigidity would improve ClpP binding and activation in a commensurate fashion. To test this hypothesis, we assayed ClpP catalyzed hydrolysis of an internally quenched fluorogenic decapeptide in the absence and presence of ADEPs (Figure 5). Using deuterium-exchange experiments, we systematically assessed the conformational consequences of replacing the N-methylalanine and serine residues in the ADEP macrocycle with conformationally constrained pipecolate and allo-threonine residues, respectively (Figure 4). Interestingly, the pipecolate moiety does not fortify both of the transannular bonds that are apparent in compounds containing N-methylalanine (compounds 1a and 1e). Indeed, we found that the amide hydrogen of the difluorophenylalanine of compound 1b exchanged completely within seconds in CD$_3$OD, whereas the analogous hydrogen in compound 1a had a half-life of 3.87 min (see Supporting Information). Apparently, with the pipecolate residue in the macrocycle, the potential donor and acceptor atoms of the hydrogen bond are either too far apart or do not have appropriate trajectories for bonding. In contrast, the transannular hydrogen bond in which the alanine residue is the donor is retained in all of the ADEPs and strengthened by the presence of conformationally constrained amino acids within the macrocycle. For instance, compound 1b harboring a pipecolate residue in the peptidolactone had a slower rate of deuterium exchange rate than 1a. Further, we found that the deuterium-exchange rate decreased as the steric bulk of the C4 substituent on the pipecolate increased (see data for compounds 1b, 1c, and 1d in Figure 4). Replacement of the native serine residue in the ADEP peptidolactones with allo-threonine profoundly slowed the deuterium-exchange rate. For instance, the half-lives of the alanine amide hydrogens in compound 1a, which has serine, and compound 1e, which has allo-threonine, are ~100-fold different. Likewise, the deuterium exchange rate of the hydrogen atom of the difluorophenylalanine moieties in compounds 1a and 1e differed by 2.8-fold. As expected, inclusion of both pipecolate and allo-threonine (1f) into the peptidolactone had a synergistic effect on deuterium exchange. Interestingly, the apparent relationship between rigidifying structural features and deuterium exchange rate was not completely conserved when 4-methylpipecolate and allo-threonine were present together in the peptidolactone (1g). This compound had a faster rate of deuterium exchange than compounds with allo-threonine and either N-methylalanine (1e) or pipecolate (1f) in the peptidolactone. A reasonable explanation for this observation is that substituted pipecolate residues and allo-threonine each stabilize slightly different low energy conformers. Accordingly, the opposing forces could prevent a single, low energy conformer from being reached. As expected, the rate of deuterium exchange increased for all compounds at an elevated temperature (i.e., 40 °C), whereas the trend for relative rates of deuterium exchange remained the same (see Supporting Information), suggesting that the observed effects are the result of entropic factors. Overall, the general trend represented by these data supports our hypothesis that the incorporation of conformationally constrained residues in the peptidolactone has a rigidifying effect.

![Figure 4](image-url)
the presence of the ADEP derivatives. Cleavage between an aminobenzoic acid fluorophore and 2-nitrotyrosine quencher in this substrate relieves quenching, resulting in increased fluorescence that serves as a readout of peptidase activity. The capacities of each of the compounds to activate ClpP were assessed across a range of concentrations, and the resulting activities were fit to yield apparent dissociation constants (Figure S5A, Table 1). As expected, we found a generally strong and positive correlation between the potency of the compounds as activators of ClpP and their rigidity as assessed in the deuterium exchange experiments. 

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<th>compound</th>
<th>D exchange $t_{1/2}$ (min)</th>
<th>ClpP Activation $K_{app}$ (μM)</th>
<th>ClpX Competition $IC_{50}$ (μM)</th>
<th>S. aureus (μg/mL)</th>
<th>S. pneumoniae (μg/mL)</th>
<th>E. faecalis (μg/mL)</th>
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<td>26.1</td>
<td>7.5 ± 0.34</td>
<td>53 ± 39</td>
<td>0.78</td>
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<td>1b</td>
<td>61.6</td>
<td>2.9 ± 0.077</td>
<td>33 ± 6.5</td>
<td>0.39</td>
<td>0.006</td>
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<tr>
<td>1c</td>
<td>115</td>
<td>3.0 ± 0.10</td>
<td>18 ± 4.2</td>
<td>0.39</td>
<td>0.012</td>
<td>0.003</td>
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<tr>
<td>1d</td>
<td>191</td>
<td>7.4 ± 0.31</td>
<td>no fit</td>
<td>1.16</td>
<td>0.098</td>
<td>0.098</td>
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<tr>
<td>1e</td>
<td>2500</td>
<td>1.3 ± 0.10</td>
<td>9.8 ± 3.9</td>
<td>0.098</td>
<td>0.003</td>
<td>0.00076</td>
</tr>
<tr>
<td>1f</td>
<td>10000</td>
<td>1.3 ± 0.067</td>
<td>2.9 ± 1.0</td>
<td>0.098</td>
<td>&lt;0.00002</td>
<td>&lt;0.00002</td>
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<tr>
<td>1g</td>
<td>1180</td>
<td>1.1 ± 0.060</td>
<td>4.7 ± 1.3</td>
<td>0.024</td>
<td>&lt;0.00002</td>
<td>&lt;0.00002</td>
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The bacterial concentrations (colony forming units/mL) in each well of the dilution antimicrobial susceptibility tests were as follows: S. aureus ($1.20 \times 10^6$), E. faecalis ($8.5 \times 10^3$), and S. pneumoniae ($3.65 \times 10^3$). The deuterium exchange rates for the hydrogen atoms of the alanine residues in the macrocycles are shown.

Table 1. Comparison of in Vitro and in Vitro ADEP Properties

■ CONCLUSION

The cyclic acyldepsipeptide antibiotics are a promising class of antibacterial agents that act by binding and dysregulating the activity of the ClpP peptidase. Reports by our group and others state that the activities of these compounds can be dramatically improved by replacing certain amino acid constituents of the peptidolactone core structure with more conformationally constrained counterparts. It has been proposed that improvements in bioactivity are a consequence of these amino acids’ capacity to stabilize a bioactive conformation of the ADEPs, which incurs a lower entropic cost upon binding to ClpP. While compelling, this proposal had very little experimental support. Herein, we present data indicating that replacement of selected constituent amino acids in the ADEP peptidolactone core does indeed stabilize a bioactive conformation. Specifically, analyses of ADEPs harboring conformationally constrained amino acids via deuterium exchange experiments revealed that they exhibit the same hydrogen bonds in solution that are inferred from the crystal structures of an ADEP in complex with ClpP. Our finding that replacement of the N-methylalanine moiety of the ADEP natural products...
with a pipecolate attenuates deuterium exchange of only one of the two donors (i.e., amide hydrogen of alanine) in the hydrogen bonds, whereas the substitution of the natural serine with \textit{allo}-threonine suppresses deuterium exchange rates of both hydrogen bond donors indicates the position of the conformationally constrained amino acid within the macrocycle has important effects on molecular conformation. In molecules with either one or two hydrogen bonds, the inverse correlations between the number of constrained amino acids constituting the peptidolactone and the rates of deuterium exchange indicated that the amino acid substitutions lock the ADEPs into a conformation that is compatible with ClpP binding. The apparent enhancements of the conformationally constrained ADEPs’ capacities to both activate ClpP and compete with its binding to the accessory ATPase ClpX corroborate the proposal that a bioactive conformation has been fortified. These improvements are also consistent with the proposal that there is a lower entropic cost in the binding of the rigidified ADEPs to ClpP. The latter point is of particular interest because the commonly held view that rigid ligands suffer a lower entropic cost in receptor binding than flexible ones has recently been challenged by cases wherein there are entropic penalties for ligand preorganization in receptor–ligand interactions. In any case, the finding that ClpP activation by the ADEPs was enhanced by up to 7-fold via the introduction of conformational constraints, while these same changes enhanced antibacterial activity by up to 1200-fold indicates that there are other factors involved. A likely explanation is that the constrained compounds are more cell-permeable. Indeed, peptides with enforced transannular hydrogen bonds exhibit dramatically enhanced cell-permeability and oral bioavailability because the bonding reduces the energetic costs of desolvation that accompanies membrane penetration in aqueous environments. Apparently, the conformational constraints that we have introduced enhance the ADEPs’ intrinsic transannular hydrogen bonding interactions that predispose them for both ClpP binding and membrane penetration.

It is well-known that the conformational constraints of macrocyclic molecules can be further enhanced by judicious introduction of substituents on the ring. In this case, it is notable that installation of small methyl substituents profoundly enhances the affinity of a large macrocycle for its biomolecular receptor and the molecules’ bioactivities. Although replacement of hydrogen atoms with methyl groups is common in structure–activity relationship (SAR) studies and medicinal chemistry optimization programs, the inclusion of a methyl group on a ligand typically is deleterious or minimally improves receptor binding. Indeed, a recent analysis of published SAR studies by Jørgensen and co-workers states that in 8% of cases the inclusion of a methyl group enhances bioactivity 10-fold or better. In only 0.4% of cases did molecules with an additional methyl group have 100-fold enhanced bioactivity. Their analysis also revealed that significant improvements in bioactivity are usually the result of the methyl group’s capacity to fill a hydrophobic environment in the receptor and to influence the conformation of the ligand. Interestingly, the substituent effect strategy exploited in medicinal chemistry is mirrored in the ADEP natural products themselves. Specifically, enopeptin A, which has a 4-methylproline residue in its macrocycle, has a 2-fold lower MIC against \textit{S. aureus}, \textit{S. pneumoniae}, \textit{E. faecalis}, and \textit{E. faecium} than enopeptin B which has an unsubstituted proline residue at the same position. In this study, we found that the position of the methyl substituent on the ADEP peptidolactone is very important. When comparing the ADEPs lacking methyl substituents (compounds 1a and 1b) to analogs harboring either 4-methyl pipecolate (compound 1c) or \textit{allo}-threonine (compound 1e), we find that the \textit{allo}-threonine residue exerts the strongest influence over conformational dynamics, ClpP affinity, and bioactivity. Furthermore, it should be noted that inclusion of \textit{allo}-threonine in the ADEP peptidolactone improves the MIC 10-fold, while inclusion of 4-methylproline improves the antibacterial activity only 2-fold (as evidenced in the reported MICs of enopeptin A and enopeptin B).

In this case, the unique characteristics of both the small molecule ligands and their receptor facilitated in-depth studies of a receptor–ligand interaction. Observations and modulations of the conformational dynamics of ADEPs were accompanied by measurements of their affinity for ClpP and antibacterial activity. A distinguishing feature of our multifaceted study was the use of deuterium-exchange \textsuperscript{1}H NMR experiments to assess relative differences in conformational rigidities of the ADEPs. We have shown that in such cases, hydrogen bonding can be exploited to study the effects of structural modification on conformational rigidity. We anticipate that this approach to small molecule dynamics could be applied to studies of many ligand–receptor interactions because many small molecules that interact with biological macromolecules exhibit transannular hydrogen bonds (especially peptides). It is a much simpler alternative to sophisticated multidimensional NMR experiments wherein \textsuperscript{15}N- and \textsuperscript{13}C-labeled compounds are used to assess the dynamics of small molecules.

The ADEP analogs reported herein constituted by the conformationally constrained amino acids \textit{allo}-threonine and 4-methylpipecolate have some of the lowest MICs ever reported for antibacterial agents. The most potent ADEP reported prior to this work, ADEP-4, was reported to cure \textit{S. aureus} infections in mice and \textit{S. pneumoniae} infections in rats with even greater efficacy than linezolid, a clinically used drug. Given that our optimized analogs have MICs against \textit{S. pneumoniae} and \textit{E. faecalis} that are 200-fold lower than those reported for ADEP-4, it is tempting to speculate that a dramatically lower and potentially safer dose of our most potent compound could be efficacious in the treatment of infections caused by \textit{Streptococci}, \textit{Enterococci}, and potentially other Gram-positive pathogens. An added advantage of the optimized compounds reported here with respect to drug development is that the key \textit{allo}-threonine residue is much less expensive and easier to prepare than the 4-methylproline constituent of ADEP-4. The promise of these molecules is further enhanced by the observations that peptides with strong transannular hydrogen bonds have enhanced oral bioavailability. Testing of these compounds in animal models of infection is currently underway in our laboratories. In total, our findings provide a compelling illustration of how the pharmacological properties of natural products can be improved by rational design.

\section*{EXPERIMENTAL SECTION}

\section*{H-D Exchange Kinetics.} NMR samples were prepared by dissolving thoroughly dried ADEP in ampule sealed CD\textsubscript{3}OD at a concentration of 2 mM. The ADEP in CD\textsubscript{3}OD was promptly transferred to a clean NMR tube, purged with an argon atmosphere, then capped and sealed with parafilm before being placed into the NMR spectrometer. Standard proton NMR spectra were acquired periodically over the course of several hours. The integration of the exchanging amide signal of interest was calibrated to a nonexchanging reference peak. Each data set was normalized such that the integral of
the amide signal of interest in the first spectrum acquired was equal to 1.00 and designated as $t_c$. Data sets were plotted in Microsoft Excel as normalized integrals vs time. Plotted data sets were fit with exponential curves with $Y$ intercepts set to 1. Exponential half-lives were calculated from the exponential function.

**Protein Expression and Purification.** *E. coli* ClpP bearing a C-terminal His$_6$ tag and single-chain pseudohexamer *E. coli* ClpP$_{X^{3N}}$ (amino acids 62–424) were expressed and purified by metal affinity, anion exchange, and gel-filtration chromatography as described. 39,47

**Activity and Competition Assays.** In *vitro* assays were performed at 30 °C in PD buffer (25 mM HEPES, pH 7.5, 100 mM KCl, 5 mM MgCl$_2$, 1 mM DTT, 10% (w/v) glycerol, 10% (v/v) DMSO) using a SpectraMax M5 microplate reader (Molecular Devices). Peptidase activation was measured by incubating 25 nM of ClpP tetradecamer and each ADEP analog with 15 μM of an internally quenched fluorogenic peptide substrate, Abz-KASPVSLGY NO$_2$D, 39,44a incorporating a 2-aminoxybenzoic acid (Abz) fluorophore and 3-nitrotyrosine (Y$_{NO_2}$) quencher. Peptide hydrolysis by ClpP was monitored by following the increase in 420 nm fluorescence upon 320 nm excitation. Initial analysis of peptidase data showed negligible cooperativity; thus, data were fit to a quadratic form of a noncooperative binding equation, assuming 14 equivalent ADEP cooperativity; thus, data were

**Devices.** Peptidase activation was measured by incubating 25 nM of ClpP tetradecamer, 10 nM of ClpX$_{X^{3N}}$ pseudohexamer, 0–100 μM of ADEP and 2.5 μM of ATP were incubated with an NADH-coupled ATP regeneration system. 49 ATP hydrolysis was monitored by following the coupled disappearance of NADH, via decrease in 340 nm absorbance. Pseudohexameric ClpP$_{X^{3N}}$ is functionally identical to monomERICally encoded ClpP$_{X^{3N}}$ 39,44a and was used to ensure hexamer stability at low ClpX concentrations. ATPase data were fit as above, assuming two ClpX binding sites per ClpP tetradecamer.

**MIC Determinations.** MIC determinations were performed in BSL2+ conditions at the New England Center for Research Excellence (NERCE) in Biodense at Harvard Medical School following standard dilution antimicrobial susceptibility testing protocols. 39,44a Following incubation with the bacteria and the compound, each well was visually examined for growth with the unaided eye. The MIC is determined to be the first set of replicate wells of the dilution series exhibiting no growth when compared to the growth control wells.

**REFERENCES**
