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Activators of Cylindrical Proteases as Antimicrobials: Identification and Development of Small Molecule Activators of ClpP Protease

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SUMMARY

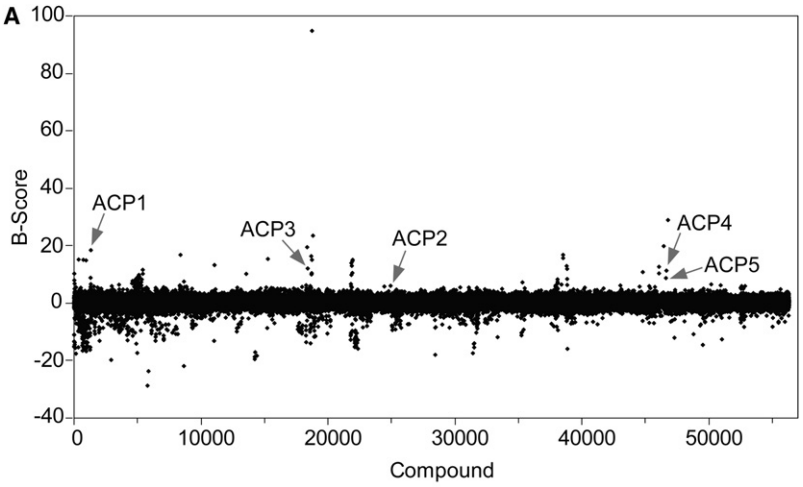
ClpP is a cylindrical serine protease whose ability to degrade proteins is regulated by the unfoldase ATP-dependent chaperones. ClpP on its own can only degrade small peptides. Here, we used ClpP as a target in a high-throughput screen for compounds, which activate the protease and allow it to degrade larger proteins, hence, abolishing the specificity arising from the ATP-dependent chaperones. Our screen resulted in five distinct compounds, which we designate as Activators of Self-Compartmentalizing Proteases 1 to 5 (ACP1 to 5). The compounds are found to stabilize the ClpP double-ring structure. The ACP1 chemical structure was considered to have drug-like characteristics and was further optimized to give analogs with bactericidal activity. Hence, the ACPs represent classes of compounds that can activate ClpP and that can be developed as potential novel antibiotics.

INTRODUCTION

In recent years, there has been an alarming trend of increased bacterial infections caused by strains resistant to most known antibiotics. As a result, diseases that were thought to be controlled by currently available drugs are re-emerging not only in developing countries but also in industrialized nations, especially in clinical settings such as hospitals. Therefore, there is an urgent need for the development of new types of antibiotics that can be used to effectively treat multidrug-resistant bacteria. The development of new drugs with novel mechanisms of action is clearly needed to avert an impending crisis.

Recently, a novel antibacterial target was identified when Brötz-Oesterhelt et al. (2005) discovered that the caseinolytic protease P, ClpP, is activated by acyldepsipeptides, ADEPs, a class of compounds that were first reported to have antibiotic properties in 1985 (Michel and Kastner, 1985). The ADEPs were later chemically optimized to address issues related to potency and aqueous solubility (Hinzen et al., 2006). The protein target of the ADEPs, ClpP protease, is a tetradecameric serine protease comprised of two stacked heptameric rings, which, in *Escherichia coli*, can form complexes with the AAA+ ATPase chaperones ClpX or ClpA (Katayama et al., 1988; Maurizi and Xia, 2004). ClpX and ClpA are hexameric chaperones that bind on one or both ends of the ClpP protease. The chaperones bind to target proteins, unfold them, and then thread them into the ClpP proteolytic chamber through axial pores lined by axial loops for degradation. These activities require ATP (Gottesman et al., 1997). In the absence of the ATPase components, ClpP alone can efficiently degrade small peptides of up to about 30 amino acids (Gottesman et al., 1997) and can also degrade unstructured proteins albeit with much lower efficiency when compared with ClpXP or ClpAP (Jennings et al., 2008; Bewley et al., 2009). ADEPs enhance the efficiency of ClpP-dependent degradation of unstructured proteins by opening up the ClpP axial pores (Lee et al., 2010; Li et al., 2010).

The aforementioned studies demonstrated that ClpP is an attractive target for developing new antibiotics with a novel mode of action. In this study, we have identified, by using a high-throughput screening approach, several structurally



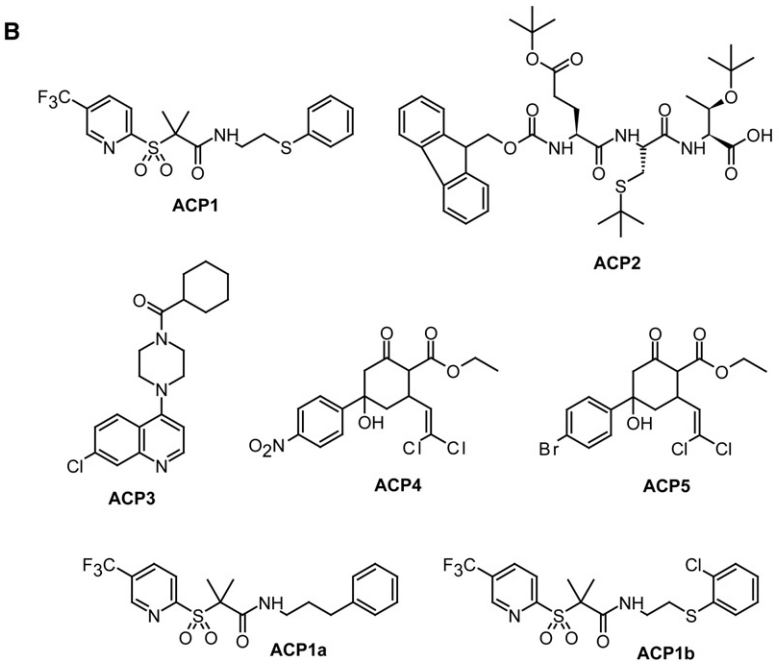


Figure 1. Results of the High-Throughput Compound Screen for Activators of ClpP

(A) About 60,000 compounds were screened to find activators of *E. coli* ClpP. B-score values were calculated for each compound from the increase in fluorescence intensity after a 6 hour incubation of casein-FITC with ClpP and compound. Compounds confirmed as hits, designated ACP1 through ACP5, are indicated by the arrows (see also Figure S1). Other compounds with high B-scores were false hits.

(B) Shown are chemical structures of ACP1-ACP5 as well as of ACP1a and ACP1b. ACP1 is N-1-[2-(phenylthio) ethyl]-2-methyl-2-{[5-(trifluoromethyl)-2-pyridyl]sulfonyl}propanamide, ACP2 is 3-(tertbutoxy)-2-{[2-[(5-(tertbutoxy)-2-{[(9-H-9-fluorenylmethoxy)carbonyl]amino}-5-oxopentanoyl) amino]-3-(tertbutylsulfanyl)propanoyl]amino}butanoic acid, ACP3 is [4-(7-chloroquinolin-4-yl)piperazino](cyclohexyl) methanone, ACP4 is ethyl 2-(2,2-dichlorovinyl)-4-hydroxy-4-(3-nitrophenyl)-6-oxocyclohexanecarboxylate, and ACP5 is ethyl 4-(4-bromophenyl)-2-(2,2-dichlorovinyl)-4-hydroxy-6-oxocyclohexanecarboxylate.

diverse non-ADEP compounds that activate the ClpP proteolytic core to degrade protein substrates in the absence of the ClpX or ClpA ATPases. These newly identified compounds have been termed Activators of Self-Compartmentalizing Proteases (ACP). Their chemical structures differ significantly from the structures of the previously identified ADEPs. The chemical optimization of ACP1 resulted in analogs having improved bioactivity and bactericidal effects. Hence, our study provides the basis for the development of novel antibiotics based on the activation and dysregulation of ClpP activity using different structural scaffolds.

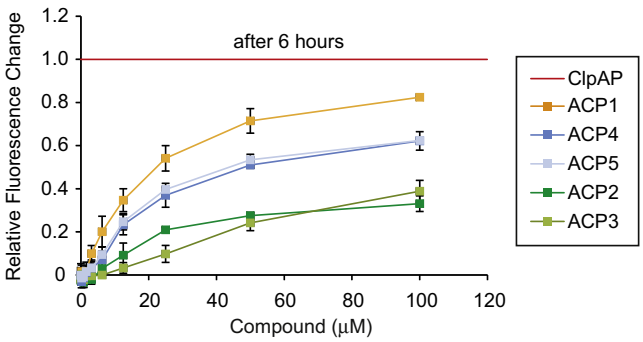
RESULTS

High-Throughput Screen for ClpP Activators

To identify compounds that activate ClpP, we developed a high-throughput screening assay with a fluorescence-based readout. The assay employed fluorescein isothiocyanate-labeled casein (casein-FITC) as the proteolytic target of the *E. coli* ClpP protease. When casein-FITC is intact, FITC fluorescence is quenched; protease-catalyzed hydrolysis of casein-FITC relieves this quenching, yielding highly fluorescent dye-labeled peptides. The principle of the screen was to select for compounds that result in increased fluorescence upon incubation of casein-FITC with ClpP. Preliminary tests performed in the presence and absence of the unfoldase chaperone ClpA revealed a 5-fold dynamic range after 30 min incubation and intra- and interassay variability, expressed as coefficient of variation, of 2% and 5%, respectively. In light of ClpP stability over several hours at 37°C, reactions were typically monitored every 15 min for 6 hr to rule out time-dependent effects.

With the initial intent of exploring drug-repositioning opportunities, we employed 4500 chemicals composed of biologically and pharmacologically active entities, of which approximately 45% were marketed drugs or drug candidates evaluated in clinical trial stages. Notably, none of these compounds were active at a concentration of 10 μM, suggesting the necessity to significantly broaden chemical diversity to explore ClpP druggability and the likelihood to activate ClpP using small molecules. Thus, we expanded the screening campaign to include additional ~60,000 highly diverse, drug-like chemicals. This undertaking was carried out using a final compound concentration of 20 μM. Results were normalized and corrected for systematic errors using the B-score method (Brideau et al., 2003) (Figure 1A) and positive hits were defined as the compounds whose signals were at least three standard deviations (99.73% confidence interval) from the mean of the general sample population. An excellent quality of the screening setup was shown by the dimensionless parameters Z′- and Z-factors (Zhang et al.,

A



B

Compound	RD25	SD
ACP1	0.53	0.04
ACP2	0.20	0.01
ACP3	0.10	0.04
ACP4	0.37	0.05
ACP5	0.39	0.04

Figure 2. Relative Degradation Index

(A) Shown is the effect of compound concentration on casein-FITC degradation by ClpP after 6 hour incubation. Data are the average of three repeats. Error bars represent standard deviations.

(B) Comparison of the RD25 values for the ACP compounds. Data shown represent the average of three repeats. SD is standard deviation.

1999), which were consistently in the 0.7 range throughout the entire screening campaign, thereby, indicating an effective combination of dynamic range, variability, and hit rate.

This chemical screen led to the selection of five confirmed hits (Figure 1B), that were designated ACP1 to 5. ACP1, ACP2, and ACP3 were hits from the Maybridge library, while ACP4 and ACP5 were hits from the Chembridge library. Interestingly, ACP4 and ACP5 were analogous molecules, with identical structures that only differed in the modification of an aromatic group (Figure 1B).

Characterization of ACP-Mediated ClpP Activation

To assess and compare the potency of ACPs, dose-response analyses were carried out following the degradation of casein-FITC for 6 hours (Figure 2A). The results were evaluated using a quantitative measure, which we developed and named the relative degradation index (RD), defined as follows:

$$\mathbf{RD} = \frac{\left(\Delta\varphi_{\mathrm{ClpP+compound}}\right)_{\text{after 6 hrs}} - \left(\Delta\varphi_{\mathrm{ClpP}}\right)_{\text{after 6 hrs}}}{\left(\Delta\varphi_{\textit{E.coli}\ \mathrm{ClpAP}}\right)_{\text{after 6 hrs}} - \left(\Delta\varphi_{\textit{E.coli}\ \mathrm{ClpP}}\right)_{\text{after 6 hrs}}}. \quad (1)$$

$\Delta\varphi$ is the change in fluorescence after 6 hr of starting the reaction (see Experimental Procedures) measured using 485 nm excitation and 535 nm emission, which primarily detects the signal from casein-FITC. *E. coli* ClpAP was used as a benchmark for maximum ClpP proteolytic activity. The ClpP in the numerator can be from any other organism. Based on RD25 measurements, which refers to the measurement in the presence of 25 μM compound, the ranking of the activators from strongest to weakest is as follows: ACP1, ACP5, ACP4, ACP2, and ACP3 (Figure 2B). It should be emphasized that the RD value reflects how much casein is degraded after 6 hr and, in theory, need not directly correlate with the K_d for the binding of a given compound to casein or with degradation rates, although, in practice, we observe a general correspondence between these parameters.

The activation of ClpP by the various ACPs was further confirmed by direct observation of unlabeled casein degradation on SDS-PAGE gels (see Figure S1A available online), which also indicated that the rank order of compound-mediated ClpP activation was consistent with the observations obtained from fluorometric determinations.

In order to verify that the activators were acting on ClpP rather than on the casein substrate directly, the ability of compound-activated ClpP to degrade proteins from various organisms was tested using a variety of model substrates and unrelated proteins (Figures S1B and S1C). The stronger activators induced ClpP to degrade a wider variety of protein substrates than the weaker activators with no apparent specificity. However, the proteins subject to extensive proteolytic cleavage, such as Casein (Creamer et al., 1981), Tah1 (Zhao et al., 2005), CFTR R-region (Ostedgaard et al., 2001), reduced carboxymethylated α-lactalbumin (RCMLa), λN protein (Mogridge et al., 1998), and α-synuclein (Tompa, 2009) are either considered to be unstable or disordered proteins or to have disordered regions. Conversely, well-folded substrates, such as GFP-SsrA and creatine kinase, were degraded to a lesser extent or not at all, likely due to the stability of their structures. Proteins that were only clipped, but not completely degraded, by compound-activated ClpP (α-M protein, ClpA, and ClpX) were all larger sized proteins.

Chemical Optimization of ACP1

When tested for bactericidal properties against ten different bacteria, several of the ACPs showed minimum bactericidal concentrations (MBC in μg/ml) at relatively low concentrations (Table 1). Although certain compounds were more effective against some bacteria compared with others, it was generally observed that gram-negative bacteria were more sensitive to these compounds than gram-positives (Table 1).

To determine whether the effects observed were due to the interaction of the ACPs with ClpP in the bacteria, we constructed *N. meningitidis* H44/76 strain (Nme H44/76, a genetically tractable strain) in which the *clpP* gene was disrupted as described in Experimental Procedures. The disruption of *clpP* was verified by PCR (Figure S2A). The susceptibility of WT and Δ*clpP* Nme H44/76 strains to the different compounds was assessed using either the disk diffusion plate assay or the liquid culture MBC assay. A differential effect was observed for ADEP1A and ADEP1B (Figure S2B) in addition to ACP1 and, to a weaker extent, ACP4 (not shown). For ACP1, the MBC for WT Nme H44/76 was 128 μg/ml, while for Δ*clpP* it was 64 μg/ml. The Nme H44/76 Δ*clpP* strain was more resistant to the effect of the compounds compared with WT as would be expected if the compounds are primarily targeting the bacterial ClpP. These observations prompted us to implement medicinal chemistry rationales aimed at the refinement and optimization of primary hits.

The chemical structures of ACP1–5 (Figure 1B) do not show any obvious structural similarities with each other, except for ACP4 and 5, or with the ADEPs (Figure S2C). ACP4 and 5 were considered to be unsuitable for further optimization as they do

Table 1. Minimum Bactericidal Concentration of Compounds

	ADEP1A		ACP1		ACP2		ACP3		ACP4		ACP5		ACP1a		ACP1b	
PMBN	+	–	+	–	+	–	+	–	+	–	+	–	+	–	+	–
N. gonorrhoeae (N.279)	0.125[a]	0.125[a]	>256	>256	32[b]	32[b]	>256	>256	4[a]	4[a]	>256	>256	128	>256	>256	>256
N. meningitidis (MC58)	0.25[a]	0.25[a]	64[b]	64[b]	>256	64[b]	>256	>256	32[b]	32[b]	>256	>256	64[b]	64[b]	16[b]	16[b]
H. influenzae (H2192)	8[a]	128	64[b]	>256	8[a]	>256	>256	>256	4[a]	128	32[b]	>256	32[b]	>256	8[a]	>256
E. coli (DH5α)	16[b]	>256	>256	>256	8[a]	>256	>256	>256	16[b]	>256	16[b]	>256	>256	>256	>256	>256
S. typhimurium (SL1344)	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256
P. aeruginosa (PAO1)	16[b]	>256	>256	>256	8[a]	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256
S. aureus (ATCC 29213)	N/A	4[a]	N/A	>256	N/A	>256	N/A	>256	N/A	>256	N/A	>256	N/A	>256	N/A	>256
S. pneumoniae (ATCC 49619)	N/A	16[b]	N/A	>256	N/A	16[b]	N/A	>256	N/A	8[a]	N/A	>256	N/A	N/A	N/A	N/A
L. monocytogenes (EGD)	N/A	0.125[a]	N/A	>256	N/A	32[b]	N/A	>256	N/A	>256	N/A	>256	N/A	N/A	N/A	N/A
M. smegmatis (mc^2155)	N/A	>256	N/A	>256	N/A	>256	N/A	>256	N/A	128	N/A	256	N/A	>256	N/A	>256

128 or more except where indicated with a footnote. The membrane permeabilizing agent polymyxin B nonapeptide, PMBN, was added at 120 μg/ml where indicated.
[a] 0.125–8.
[b] 16–64.

not have drug-like structures and would be more challenging to assess in a structure activity relationship (SAR) studies (Keller et al., 2006). ACP2 would be relatively straightforward to evaluate in an SAR study, but the protected tripeptide derivative framework was not considered optimal as a potential small-molecule lead. Instead, ACP1 and 3 both display drug-like structures amenable to further optimization, synthesis, and SAR studies. In light of the higher RD25 score for ACP1 (Figure 2B) and the fact that it seems to indeed target ClpP in the cell as described above, we decided to explore chemical diversity around the ACP1 molecule.

ACP1 satisfies Lipinski's rule of five (Lipinski et al., 2001) and has a topological polar surface area of 75.6, calculated molar refractivity of 10.4, and Clog P of 3.98. The structure of ACP1 consists of a central β-amido sulfone core appended with a western electron deficient pyridyl ring and an eastern hydrophobic tail incorporating a phenylthioether group (Figures 1B and 3A). The natural synthetic disconnection point chosen for the synthesis of ACP1 analogs was the amide linkage (Figure 3A). Analogs were synthesized using a late-stage amide-bond forming reaction between the eastern amine and the western β-sulfonyl carboxylic acid (Figure 3B) by applying standard synthetic protocols (see Experimental Procedures) and, subsequently, evaluated by measuring their RD25 values. We used the natural products ADEP1 factor A and B (Figure S2C) isolated from *Streptomyces hawaiiensis* (see Experimental Procedures) as a reference. ADEP1A is a better ClpP activator than ADEP1B (Figure 3C).

Of more than 70 analogs generated, one compound, which we termed ACP1b (Figure 1B), was found to exhibit an RD25 value slightly higher than that of ADEP1A (Figure 3C). For comparison, another analog, which we termed ACP1a (shown in Figure 1B), displayed a lower RD25 value than that of ACP1b but comparable to that of ADEP1B (Figure 3C). ACP1a incorporates a sulfur to methylene substitution in the eastern tail, and ACP1b has an *ortho*-chloro substituent in the arylthioether ring (Figure 1B).

The extent of casein degradation upon activation of ClpP by these compounds is shown in Figure 3D and is generally consistent with the RD25 results. It has previously been shown that ADEPs activate *E. coli* ClpP to degrade casein with reduced processivity (Brötz-Oesterhelt et al., 2005; Kirstein et al., 2009). This was also true for ACP1, ACP1a, and ACP1b (Figure 3E). The patterns of appearance and disappearance of the degradation intermediates suggests similarities between the general mechanisms of activation by the different compounds.

Notably, among the different bacterial species tested, ACP1b showed an MBC value against *H. influenzae* that was comparable to that of ADEP1A and much lower than that of ACP1 and ACP1a (Table 1). ACP1b also had improved MBC value against *N. meningitidis* MC58 compared with ACP1 and ACP1a, but not ADEP1A (Table 1). The MBC values for ACP1b were 32 and 64 μg/ml for WT and Δ*clpP* Nme H44/76 described above, respectively. Hence, while the WT is more sensitive to ACP1b than the Δ*clpP* strain indicating that ClpP is indeed a target for the compound in the cell, ACP1b might have other cellular targets and further optimization will have to concentrate on increasing specificity.

Although ACP1b was optimized against *E. coli* ClpP, *E. coli* DH5α was resistant to this compound (Table 1), which is consistent with earlier results (Brötz-Oesterhelt et al., 2005).

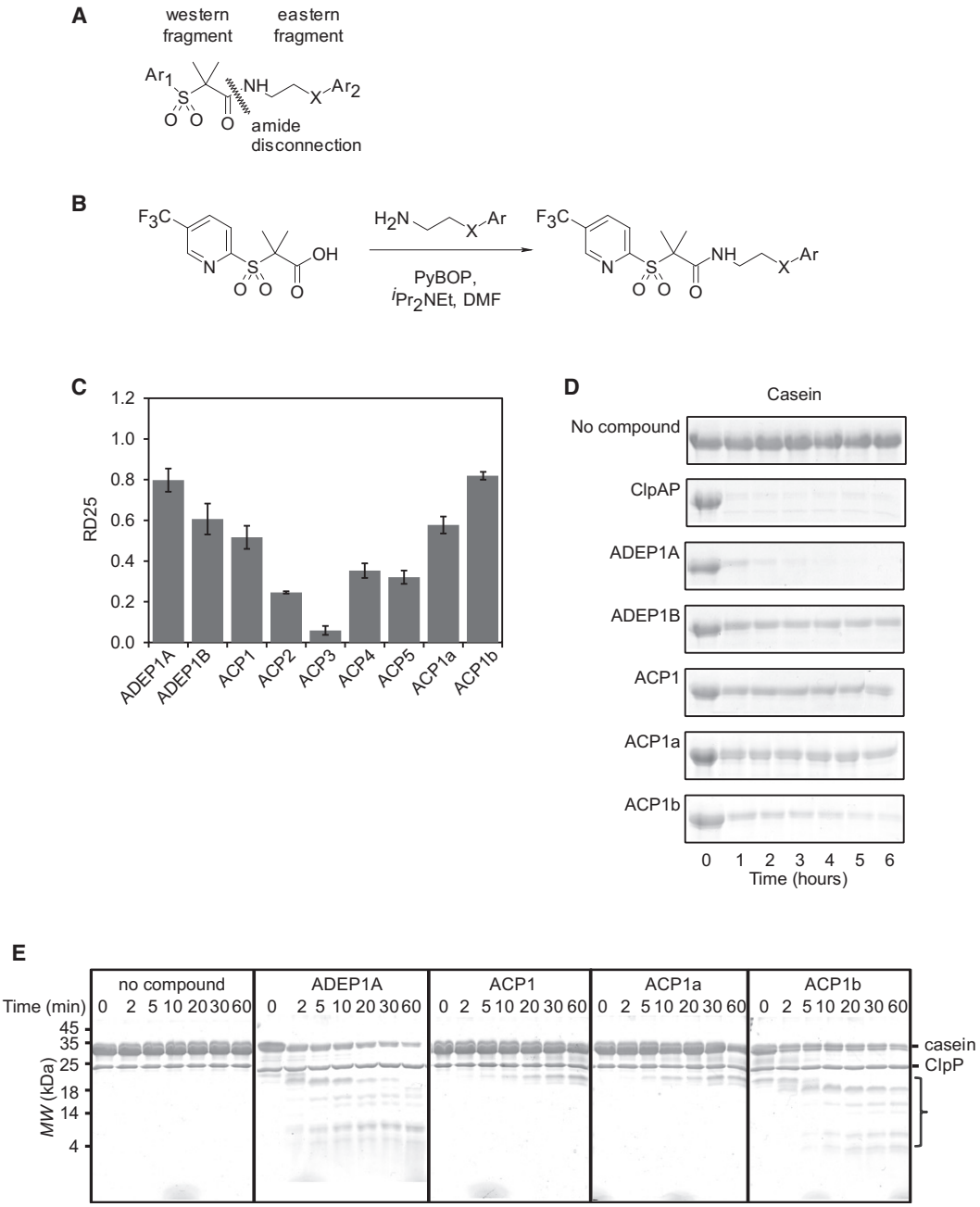


Figure 3. Chemical Optimization of ACP1

(A) General chemical structure of the ACP1 analogs.

(B) A schematic of the chemical reaction to synthesize the ACP1 analogs using PyBoP mediated amide bond formation between 2-methyl-2-((5-(trifluoromethyl) pyridin-2-yl)thio)propanoic acid and primary amines.

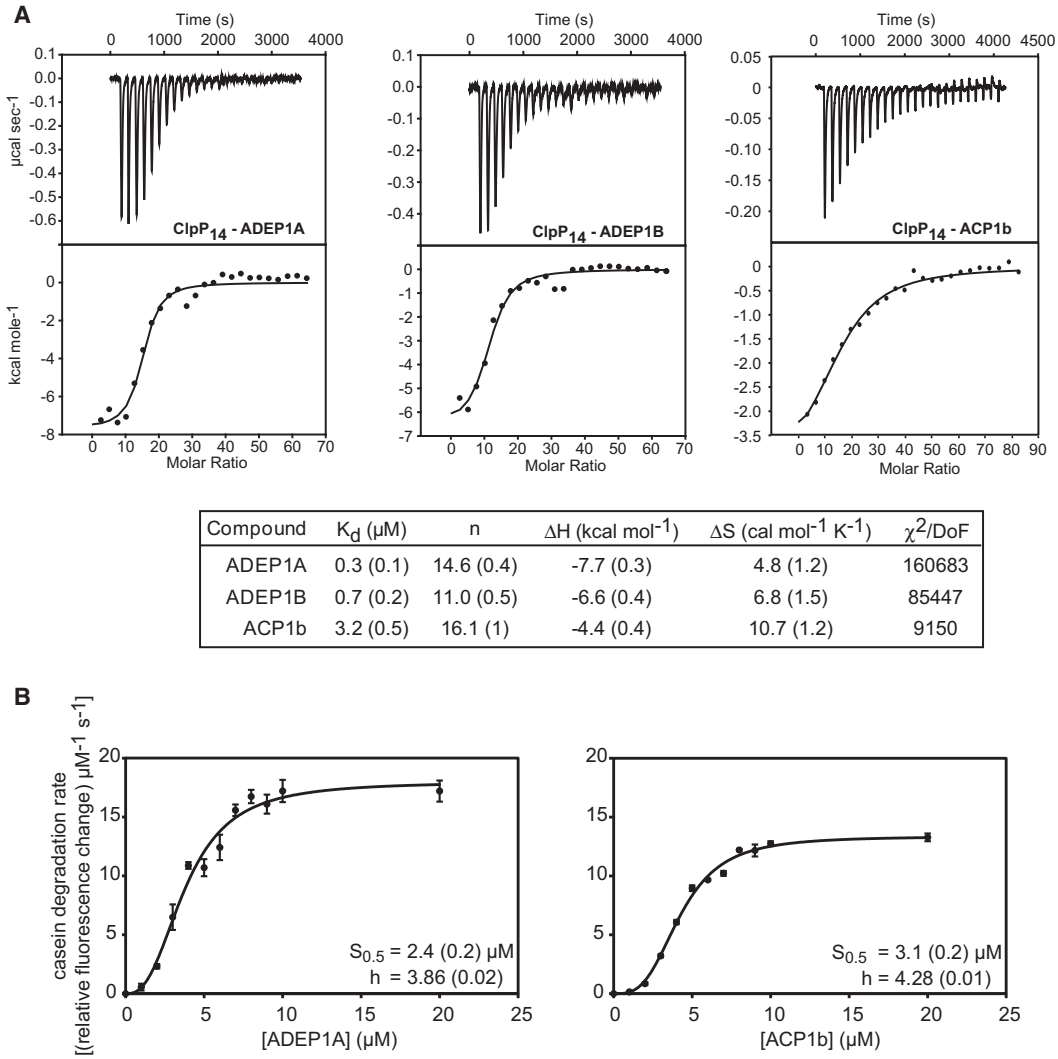
(C) RD25 values of ADEP1A, ADEP1B, ACP1–5, ACP1a, and ACP1b (see also Figure S2). Error bars represent the standard deviations from the average of three repeats.

(D) Shown is the degradation of unlabeled casein by compound-activated ClpP followed on SDS-PAGE gels.

(E) The formation of intermediates during casein degradation by compound-activated ClpP.

Intermediate species, indicated by the parenthesis, resulting from casein degradation were resolved on 18% SDS-PAGE gels.

Brötz-Oesterhelt et al. (2005) did not see an effect of ADEPs on WT *E. coli*, but rather on an *E. coli* strain deleted of the multidrug efflux pump AcrA in the presence of the outer membrane permeabilizer polymyxin B nonapeptide (PMBN). Hence, we combined ACP1b with several other known drugs to enhance sensitivity. Using this approach, ACP1b was found to affect cell growth of *E. coli* MC4100 strain in the presence of 20 μM of the uncoupler ionophore carbonyl cyanide 3-chlorophenylhydrazone (CCCP)



Compound	K_d (μM)	n	ΔH (kcal mol^{-1})	ΔS (cal mol^{-1} K^{-1})	χ^2/DoF
ADEP1A	0.3 (0.1)	14.6 (0.4)	-7.7 (0.3)	4.8 (1.2)	160683
ADEP1B	0.7 (0.2)	11.0 (0.5)	-6.6 (0.4)	6.8 (1.5)	85447
ACP1b	3.2 (0.5)	16.1 (1)	-4.4 (0.4)	10.7 (1.2)	9150

Figure 4. Determination of Binding Affinity of Compounds to ClpP

(A) ITC binding curves for ClpP-ADEP1A, ADEP1B, or ACP1b interaction are shown. Results for the fit of the data to a one set of identical independent binding site model is given in the table. Numbers in parentheses refer to standard deviations. χ^2/DoF refers to chi-square divided by the degrees of freedom and indicates the quality of the fit (see also Figure S3).

(B) Cooperativity of binding of ADEP1A and ACP1b to ClpP was determined by measuring the change in casein degradation rate by compound-activated ClpP as a function of compound concentration. Error bars represent the standard deviations from the average of three repeats.

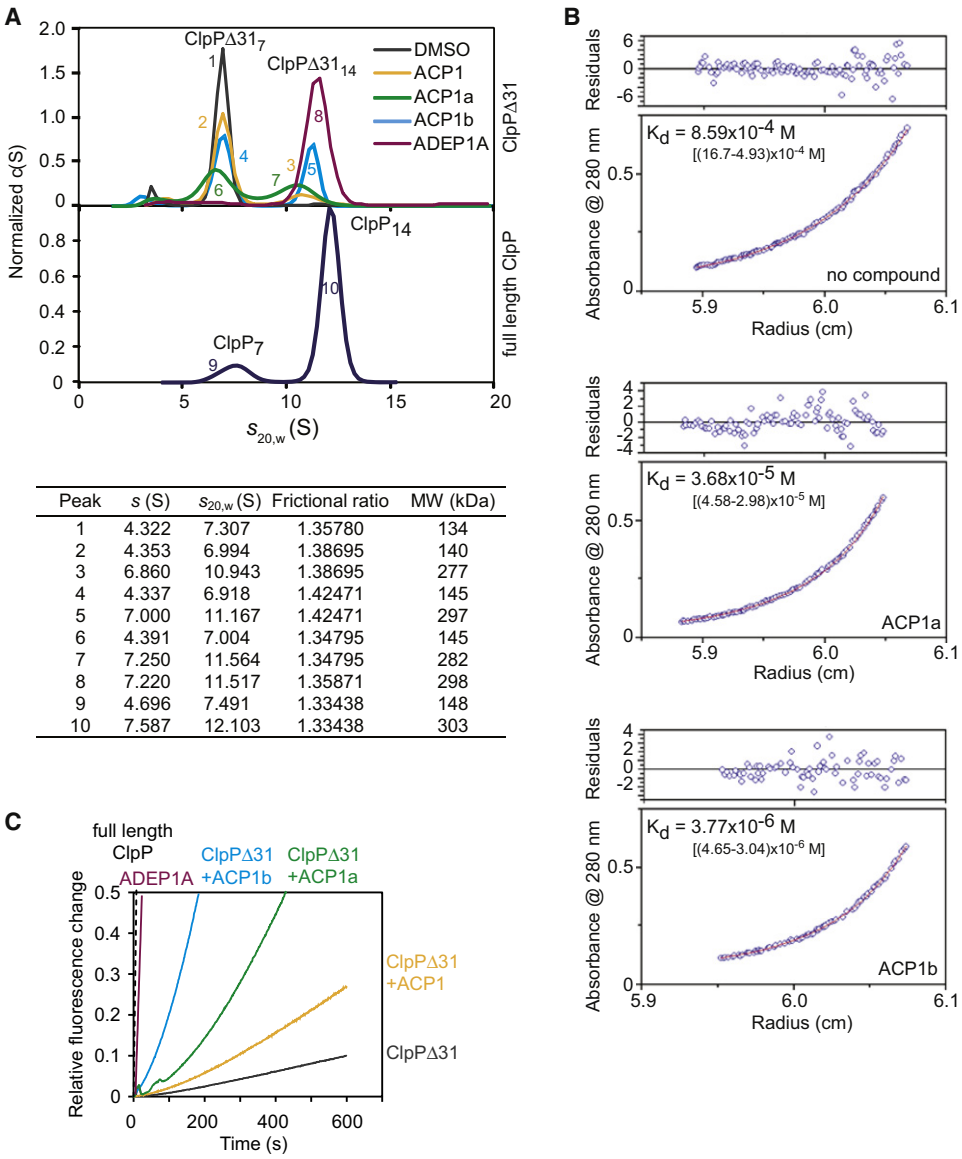
(Figure S2D). This sensitivity was not observed for MC4100 Δ*clpP* strain consistent with ClpP being a target for ACP1B in the cell.

The above observations indicate that the antibacterial properties of ACP1 can be improved through relatively simple chemical modifications and that further efforts will have to concentrate on enhancing specificity.

Determination of Binding Affinity and Stoichiometry of ACP1b Interaction with ClpP

The dissociation constant (K_d) for the binding of ACP1b to ClpP was measured using isothermal titration calorimetry (ITC) and was found to be about 3.2 ± 0.5 μM, which is comparable to, albeit slightly higher, than that of ADEP1A (0.3 ± 0.1 μM) and ADEP1B (0.7 ± 0.2 μM) (Figure 4A). The ITC data were fit by allowing the number of binding sites (n) to vary (Figure 4A), by fixing n to a whole number (Figure S3A), or by fixing n to 14 (Figure S3B). The results suggest that there are 14 binding sites for the ADEPs and ACP1b on $ClpP_{14}$. In comparison, ACP1 has a dissociation constant for ClpP of about 130 μM as determined by Surface Plasmon Resonance (SPR) measurements (Figure S3C), whereas ITC was unsuitable to measure ACP1 affinity for ClpP due to weak binding. The similar K_d values obtained for the ADEPs by SPR (Figure S3C) and ITC (Figure 4A) clearly indicate that ACP1 has lower affinity than the ADEPs for ClpP. SPR experiments were also carried out for ACP2–5, and the K_d values obtained were much weaker (>150 μM) than that of ACP1 as expected (data not shown).

Measurement of casein degradation rate by compound-activated ClpP as a function of ADEP1A or ACP1b concentration resulted in a sigmoidal saturation curve with $S_{0.5}$ of 2–3 μM and



Peak	s (S)	$s_{20,w}$ (S)	Frictional ratio	MW (kDa)
1	4.322	7.307	1.35780	134
2	4.353	6.994	1.38695	140
3	6.860	10.943	1.38695	277
4	4.337	6.918	1.42471	145
5	7.000	11.167	1.42471	297
6	4.391	7.004	1.34795	145
7	7.250	11.564	1.34795	282
8	7.220	11.517	1.35871	298
9	4.696	7.491	1.33438	148
10	7.587	12.103	1.33438	303

Figure 5. Effect of ACP on ClpP Oligomeric Stability

(A) Sedimentation velocity analytical ultracentrifugation of full length ClpP (46 μM) and ClpPΔ31 (51 μM) at 4°C shown using the continuous distribution model c(S) versus $s_{20,w}$, scaled to initial absorbance. ACP1, ACP1a, ACP1b, and ADEP1A (at 100 μM) promote the tetradecamerization of ClpPΔ31 to different extents. The table lists the sedimentation coefficients, frictional ratios, and molecular weights corresponding to the various peaks.

(B) Sedimentation equilibrium profiles and the corresponding distribution of residuals for 51 μM ClpPΔ31 in the absence of compound (top), or in the presence of 100 μM ACP1a (middle) or 100 μM ACP1b (bottom). The solid lines represent the best fit to a monomer-dimer model, with the heptameric ClpP (MW of 138,544 Da) treated as the monomer. The resulting dissociation constants are given for each data set. The numbers in brackets give the range of K_d values for the 95% confidence interval.

(C) The effect of compounds (at 100 μM) on the peptidase activity of ClpPΔ31 (1 μM) is shown. The defective peptidase activity of this mutant is partially recovered by the presence of the ACP compounds.

a Hill coefficient, h, of about 4 (Figure 4B). The data suggest that these compounds act on ClpP in a similar manner and bind to the protease cooperatively or promote cooperative allosteric transitions within the protease structure.

The ClpP Tetradecamer Is Stabilized by ACP Binding

Thermal melt experiments indicated that the ACP compounds enhanced ClpP stability (not shown). Hence, we asked whether the compounds affect ring-ring interactions within the ClpP double-ring tetradecamer. An N-terminal truncation mutant of *E. coli* ClpP was originally constructed to open the central pore of the protease in an attempt to mimic a proposed mechanism of compound activation. However, this mutant, ClpPΔ31, mainly eluted as a heptameric single ring upon size exclusion chromatography and had no significant peptidase activity. In order to verify the oligomeric state of this protein, we employed sedimentation velocity analytical ultracentrifugation (Figure 5A). The results indicate that the ClpPΔ31 mutant has a sedimentation

coefficient corresponding to that of a heptamer; by comparison, WT ClpP has a sedimentation coefficient corresponding to a tetradecamer with a small proportion of heptamers (Figure 5A).

Addition of the different compounds shifts the oligomeric state of ClpPΔ31 to that of a tetradecamer (Figure 5A). The proportion of ClpPΔ31 tetradecamers formed correlates with the binding affinity of the compounds. For example, ACP1b led to the formation of a higher proportion of tetradecamers than ACP1, while ADEP1A led to the formation of the ClpPΔ31 tetradecamer exclusively. It should be noted that integration of the normalized c(S) signal over the s-values, which cover the range of sedimenting material, shows that the total "loading signal" is similar for the different curves with an average total loading signal of 0.52 (±0.12). The differences in the normalized c(S) peak heights is mainly because some peaks are broader than others for the various treatments of ClpPΔ31. Slight shifts in alignment of the ClpPΔ31 tetradecamer sedimentation coefficient in the presence of the various compounds might indicate some disassembly of the tetradecamer under the sedimentation velocity nonequilibrium conditions or may be attributed to different hydrodynamic properties of the tetradecamers formed. We further verified the oligomeric state of the ClpPΔ31 in the presence of ACP1a or ACP1b using sedimentation equilibrium experiments (Figure 5B). Fits of the data to monomer-dimer equilibrium, assuming the monomer is heptameric ClpPΔ31 with a molecular weight of 138,544 Da, clearly indicate that the presence of ACP1a or ACP1b decreases the dissociation constant of the ClpP tetradecamer with ACP1a having a lower K_d than ACP1b, which, in turn, is lower than that in the absence of the compound (Figure 5B).

Without the addition of compound, ClpPΔ31 had little protease activity (Figure 5C). Addition of ACP1, ACP1a, ACP1b, or ADEP1A promoted the formation of tetradecameric ClpPΔ31 (Figures 5A and 5B) and resulted in a catalytically active ClpPΔ31 (Figure 5C). These results strongly suggest that the ACP compounds stabilize ClpP by promoting the formation of the double-ring structure.

ACP Binding Sites on ClpP

The ADEPs have been found to bind in a hydrophobic pocket (H pocket) on the ClpP apical surface in which the IGF loop of the ClpX/ClpA ATPases also binds (Lee et al., 2010; Li et al., 2010). As a result, the presence of the ADEPs inhibited or reduced the ClpXP/ClpAP-mediated degradation of GFP-ssrA by interfering with the binding of ClpX/ClpA to ClpP. As shown in Figure 6A and Figure S4A, a similar effect was also seen for the ACPs suggesting that these compounds either bind to or allosterically modulate the H pocket of ClpP.

Two binding pockets on ClpP of equal probability were predicted by computational procedures implemented in DOCK6.3 software (Lang et al., 2009) (see Experimental Procedures; Figure 6B; Table S1), namely, the H pocket and a separate pocket, which we have named the C pocket, featuring a larger number of charged residues. The H pocket is composed of residues V42, F44, L62, Y74, Y76, I104, F126, L203, and R206 from one subunit, and L37 and F96 from the neighboring subunit (using Swiss-Prot numbering). The C pocket comprises residues Y90, M94, Q95, D100, V101, and H170 of one subunit and residues H205 and N207 of the neighboring subunit. Interestingly, the H and C pockets are separated by residues at the very C terminus of ClpP, corresponding to amino acids 203–207. While ADEPs cocrystallized with ClpP were found to be bound to the H pocket (Lee et al., 2010; Li et al., 2010), all the ACP compounds were docked well to both the H and C pockets (Figure 6B), and their docking scores differed little between the two pockets (Table S1).

To further validate the results of the docking analyses, we generated and tested mutants of the H pocket (Y76W [Lee et al., 2010], F126A [Bewley et al., 2006], and L203E), C pocket (Q95A, D100A, and H170A), as well as both the H and C pockets (Figure 6B). Peptidase assays were first performed to assess whether the mutations affected ClpP active sites. Except for Y76W, H170A, and Q95A/F126A, the mutants exhibited peptidase activity similar to that of the wild-type (WT) protein (Figure S4B). Intriguingly, we observed that the effects of stronger ClpP activators, such as ADEP1A and ACP1b, markedly decreased in the presence of mutations confined to the H pocket, whereas weaker activators appeared to be affected by mutations in both H and C pockets (Figure 6B). These findings, taken together, suggest that a successful strategy aimed at developing stronger ClpP activators is that of developing small molecules interacting with ClpP within the H pocket.

DISCUSSION

In our study, we have successfully identified five new compounds representing four different structural classes (Figure 1B) that activate ClpP protease and have bactericidal properties. With the exception of ACP4 and ACP5, these compounds have no apparent structural similarities to each other or to the previously reported ADEPs (Brötz-Oesterhelt et al., 2005), dramatically increasing the repertoire of compounds activating this protease. The optimization of ACP1 resulted in compounds that had in vitro ClpP activation properties close to that of the natural product ADEPs (Figure 3C). Importantly, these optimized compounds have good bactericidal properties (Table 1), suggesting potential applications as therapeutics. In particular, our data indicate that these compounds may be dramatically improved through a much tighter affinity for ClpP and specific targeting within the ClpP H pocket.

Based on the results of Figures 4–6, we propose that the identified ACP compounds share a similar mechanism of ClpP activation as the ADEPs. They all stabilize ClpP and promote the formation of the double-ring structure, albeit through a yet unknown mechanism. The apparent lack of substrate preference (Figures S1B and S1C) that the compounds confer on ClpP in addition to the similarities in degradation patterns (Figure 3E) is also indicative of similarities in mechanism of ClpP activation. The compounds prevent the binding of ClpP to its associated unfoldase (Figure 6A; Figure S4A) and, at the same time, activate nonspecific proteolysis probably through the opening of the axial pores.

The recently solved cocrystal structures of ADEP bound to *B. subtilis* and *E. coli* ClpP (Lee et al., 2010; Li et al., 2010) show ADEP binding to the H pocket of the protease. Our results are in general agreement with these findings; however, we propose the presence of an additional pocket, the C pocket, that is more charged (Figure 6B) and, as suggested by our

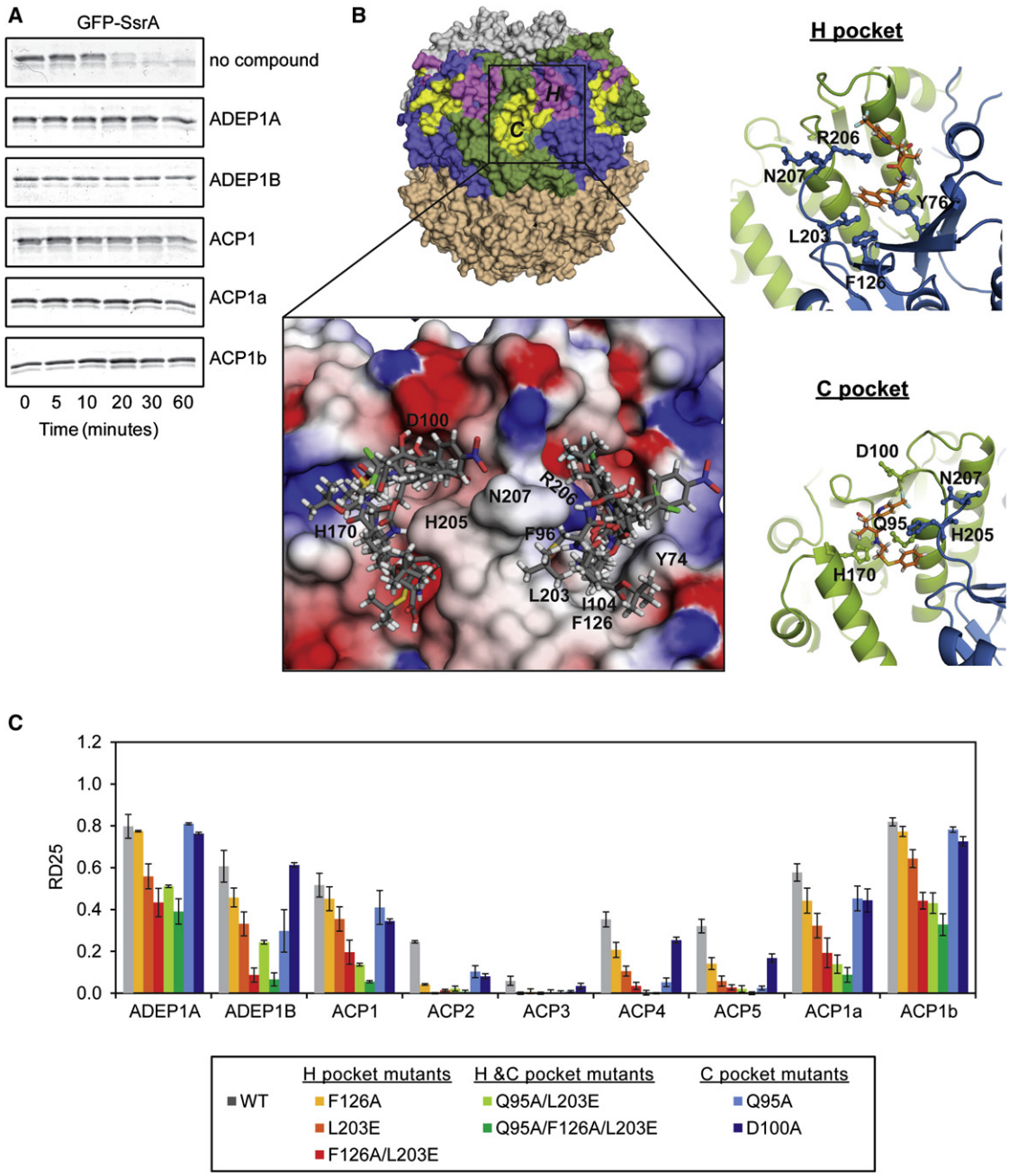


Figure 6. ACP Binding Sites

(A) Shown is the inhibition of ClpXP-mediated GFP-ssrA degradation by ACPs and ADEPs added at 100 μM monitored on SDS-PAGE gels. ClpP was preincubated with compound before the addition of ClpX (see also Figure S4A).

(B) Surface model of ClpP is shown on the top left. Four neighboring subunits are colored in alternating blue and green. The H pockets are colored in purple, while the C pockets are colored in yellow. The bottom left panel shows a close up view of the predicted compound binding conformations in the two ClpP pockets. The five ACP compounds are overlaid in the binding pockets (see also Table S1). ClpP is shown as a surface model and the compounds are shown as stick models. C, N, O, S, F, Cl, Br, and H in the compounds are colored in gray, blue, red, yellow, cyan, green, purple, and white, respectively. The ClpP surface is colored according to the electrostatic surface potential and ranges from red (potential of −4kT) to blue (potential of +4kT) calculated using DelPhi (Rocchia et al., 2002). Also shown on the right panels are stick models of ACP1 docked into the H and C pockets of ClpP drawn as ribbons colored by chain. For ACP1, C, N, O, S, F, and H are colored in orange, blue, red, yellow, cyan, and white, respectively. All molecular graphics figures were prepared using the program PyMOL.

(C) Effect of mutations in the H and C pockets on ClpP activation by compounds measured using RD25. Data shown represent the average of three repeats and error bars give the standard deviations on the measurements (see also Figure S4B).

mutational analysis, involved in compound binding (Figure 6C). It is likely that the binding of compounds in the H pocket mimics the binding of ATPase chaperones and causes conformational changes in the ClpP axial pore, which normally occur upon binding of the Clp ATPase. The ADEP compounds, being much larger and bulkier, make more contacts in the ClpP binding pocket than the ACPs. The ACP compounds make fewer contacts, yet still induce ClpP activation. How these dissimilar compounds can cause the same effects warrants further investigation. Cocrystallization trials are currently under way to gain further understanding of the interaction of these compounds with ClpP.

In summary, our study identified new activators of ClpP that we term ACPs. These activators can be purchased relatively cheaply from regular vendors allowing any group to study this phenomenon without the need to rely on difficult purification procedures of natural products or complicated chemical synthesis methods. We expect that activators of self-compartmentalizing proteases will be important players in future drug development efforts.

SIGNIFICANCE

With the rise of antibiotic-resistant bacteria, there is an urgent need for the development of new compounds having novel mode of function. The ability to dysregulate ClpP serine protease activity represents a novel approach for the development of new antibacterials. ClpP activity is tightly regulated by bound ATPase chaperones. These ATPases typically form hexameric rings that bind on one or both ends of the ClpP double-ring cylinder. The ATPases select target substrates, which are then unfolded and threaded through the ATPase ring and into the ClpP cylinder for degradation. Threading into the ClpP proteolytic chamber occurs through narrow axial pores that do not allow structured proteins to pass, hence, the requirement for protein unfolding. Acyldepsipeptides, ADEPs, were recently found to bind ClpP and open the axial pores of the protease allowing ClpP to degrade folded proteins independent of its chaperone resulting in unregulated degradation of protein substrates. ADEPs were found to have antibacterial activity and were originally purified from *Streptomyces*. However, the chemical synthesis of these compounds is quite challenging. In order to identify novel compounds that activate ClpP, we used a high-throughput screening approach with a fluorescence-based readout. The assay employed fluorescein isothiocyanate-labeled casein as the proteolytic target of the *Escherichia coli* ClpP protease. Five structurally diverse compounds were identified to activate ClpP that we named Activators of Self-Compartmentalizing Proteases 1 to 5 (ACP1–5). Their chemical structures differ significantly from the structures of the ADEPs. The chemical optimization of ACP1 resulted in analogs having improved bioactivity and bactericidal effects. The compounds were found to stabilize the ClpP double-ring structure and to bind in pockets on the ClpP apical surface. Hence, our study provides the basis for the development of novel antibiotics based on the activation and dysregulation of ClpP activity using different structural scaffolds.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification

All proteins were expressed from IPTG inducible promoters. ClpP constructs were expressed in BL21(DE3)1146D strain, which lacks the gene for chromosomal *E. coli* ClpP. All other constructs were expressed in BL21(DE3)Gold (Stratagene). Untagged WT and mutant *E. coli* ClpP, ClpX, and GFP-SsrA were expressed and purified as previously described (Wojtyra et al., 2003). ClpA was expressed and purified as described in Lo et al. (2001). All His-tagged proteins were purified on Ni-NTA agarose resin (QIAGEN) according to the manufacturer's protocols. If possible, the tag was removed using the tobacco etch virus (TEV) protease. Protein concentrations were determined by absorbance at 280 nm with extinction coefficients calculated using ProtParam (http://ca.expasy.org/tools/protparam.html).

Tah1 (Zhao et al., 2008) and λO (Wojtyra et al., 2003) were purified as previously described. pHF010 plasmid encoding H_6-λN was a kind gift from Dr. Irene Lee (Case Western Reserve University) and the protein was purified according to published protocols (Patterson-Ward et al., 2009). CFTR R-domain and α-synuclein were gifts from Dr. Julie Forman-Kay (University of Toronto). The Neisseria α-M protein was from Mr. Shekeb Khan from EFP's group. Reduced carboxymethylated α-lactalbumin was a gift from Dr. John Glover (University of Toronto). Creatine kinase, α-casein, casein fluorescein isothiocyanate (casein-FITC, type II, 20–50 μg FITC/mg, catalog number C3777), and N-Succinyl-Leu-Tyr-7-amido-4-methylcoumarin (Suc-LY-AMC) were purchased from Sigma-Aldrich.

Chemical Libraries and High-Throughput Screening

The libraries employed for the screening campaigns were composed of experimental bioactives, pharmacologically active chemicals and natural products, off-patent marketed drugs, and small molecules with drug-like properties. Samples were obtained from the following, commercially available collections: LOPAC 1280 (Sigma, 1280 samples), Prestwick Chemical library (Prestwick Chemical, France, 1120 samples), SPECTRUM collection (MicroSource, 2000 samples), Maybridge Screening collection (Maybridge-Thermo Fisher Scientific, UK, 50,000 samples), and Chembridge DIVERSet (ChemBridge Corp, 10,000 samples). In all instances, samples stored in 384-well plates as 1 or 5 mM solutions in 100% DMSO were transferred to assay plates in a fixed volume of 200 nl by a pin-tool (V&P Scientific). Screens were conducted using a fully automated procedure run on a DIM4 flipmover platform (Thermo Electron Corp) equipped with a Biomek FX liquid handler (Beckman, USA) and a PHERAStar detection system (BMG Labtech, Germany). The reaction for the screening assay contained 20 μM compound, 3.6 μM ClpP, and 4.5 μM casein-FITC in buffer A (25 mM Tris HCl [pH 7.5], and 100 mM KCl) at 37°C.

Compounds that were identified and confirmed to be hits were obtained from the following companies: ACP1, ACP2, and ACP3 are from Maybridge (Maybridge, Thermo-Fisher Scientific, catalog number BTB09142, JFD02943, and KM11066, respectively); ACP4 and ACP5 are from Chembridge (ChemBridge Corporation, catalog number 5107477 and 5107473, respectively).

Activity Assays

To measure the RD index, each reaction consisted of 3.6 μM ClpP and a specified amount of compound in buffer A. The reactions were preincubated for 10 min at 37°C before 4.5 μM casein-FITC and 15.5 μM unlabeled casein were added. ClpAP-dependent degradation of the same casein-FITC substrate was used as a control. Each ClpAP reaction contained 3.6 μM ClpP, 3 μM ClpA, and 0.3 mM ATP in buffer B (25 mM HEPES [pH 7.5], 20 mM $MgCl_2$, 30 mM KCl, 0.03% Tween 20, and 10% glycerol), and an ATP-regenerating system (13 units/ml of creatine kinase and 16 mM creatine phosphate). The reaction was started by adding casein-FITC. Reactions were incubated at 37°C and the fluorescence (485 nm excitation, 535 nm emission) was monitored every 15 min for 6 hr on a PHERAStar detection system. Casein-FITC degradation by compound-activated ClpP after 6 hr at 37°C was compared with the ClpAP-dependent casein-FITC degradation after 6 hr at 37°C, using Equation 1.

The effects of the compounds on the degradation of GFP-ssrA by ClpXP were also analyzed on SDS-PAGE gels. Each reaction contained 1.2 μM ClpP, 3.9 μM GFP-ssrA, 3 mM ATP, and 100 μM compound in buffer C (25 mM HEPES [pH 7.5], 5 mM $MgCl_2$, 5 mM KCl, 0.03% Tween 20, and

10% glycerol), and an ATP-regenerating system. The reaction mixtures were preincubated for 3 min at 37°C before 1 μM ClpX was added. Samples were taken at various time points, stopped by boiling in 2% SDS, and resolved on 12% SDS-PAGE gels.

Peptidase activity of ClpP was measured by the ability of ClpP to cleave the dipeptide Suc-LY-AMC. Each reaction contained 1 μM ClpP in buffer D (50 mM Tris HCl [pH 8], 200 mM KCl, and 1 mM DTT). ClpP was incubated for 3 min at 37°C before Suc-LY-AMC was added to a final concentration 0.5 mM. Fluorescence (350 nm excitation, 460 nm emission) of the released AMC was detected on the PHERAStar system.

For the Hill plot analysis of Figure 4B, each reaction consisted of 3.6 μM ClpP and specified amount of compound in buffer A. The reactions were pre-incubated for 10 min at 37°C before 4.5 μM casein-FITC was added. Reactions were incubated at 37°C and the fluorescence (485 nm excitation, 535 nm emission) was monitored every 3 s for 5 min on an EnSpire Multilabel Plate Reader (PerkinElmer).

N. meningitidis H44/76 *clpP* Insertional Mutagenesis

To construct the *N. meningitidis* H44/76 *clpP* insertional mutant vector, designated Nme-clpP-ery, primers f2 and r2 shown in Figure S2A were used to amplify a fragment of the genome that contained the *clpP* gene and the resulting PCR fragment was cloned into the pTrc99A vector. Using the f3 and r1 primers (Figure S2A), an erythromycin cassette with EagI ends was amplified from the pFLOB4300 vector (Litt et al., 2000) and the resulting PCR fragment was cloned into the EagI restriction site located at the 5′ end of the *clpP* gene within the Nme-clpP vector. The *N. meningitidis* H44/76 *clpP* insertional mutant was generated by transforming WT *N. meningitidis* H44/76 with the Nme-clpP-ery vector using an electroporation protocol adapted from (Dillard, 2006). PCR verification of the mutant strain is shown in Figure S2A.

Determination of Minimum Bactericidal Concentrations

Bacterial strains used included both gram-negative and gram-positive bacteria: *Escherichia coli* DH5α, *Salmonella typhimurium* SL1344, *Pseudomonas aeruginosa* PAO1, *Haemophilus influenzae* H2192, *Neisseria gonorrhoeae* N.279, *Neisseria meningitidis* H44/76, *Neisseria meningitidis* MC58, *Staphylococcus aureus* ATCC 29213, *Streptococcus pneumoniae* ATCC 49619, and *Listeria monocytogenes* EGD. Due to the low water solubility of the ADEP and ACP compounds, minimum bactericidal concentrations (MBC) values were determined by plating compound-treated bacteria on agar plates without compound. All compounds were diluted in Brain Heart Infusion (BHI) medium with 1% Isovitale X (Becton Dickinson). All bacteria were also inoculated into BHI. A 2-fold dilution series of each compound was created in 96-well plates, with and without 120 μg/ml of the membrane permeabilizing agent polymyxin B nonapeptide, PMBN (Sigma-Aldrich) (Ofek et al., 1994; Tsubery et al., 2002). All bacterial suspensions were pelleted, resuspended in BHI, and added to the compound containing media. *H. influenzae*, *N. gonorrhoeae*, and *N. meningitidis* were incubated 18–20 hr, while the remaining strains were incubated 12 to 16 hr. *H. influenzae*, *N. gonorrhoeae*, *N. meningitidis*, and *S. pneumoniae* were incubated in the presence of 5% CO_2. 2 μl of culture from the incubations were then plated onto compound-free agar plates (*H. influenzae*, *N. gonorrhoeae*, *N. meningitidis* for 18–20 hr; remaining strains for 12–16 hr) to determine the bactericidal activity. *H. influenzae* was grown on chocolate agar; *N. gonorrhoeae* on GC agar base; *N. meningitidis*, *S. aureus*, and *L. monocytogenes* on BHI agar; *S. pneumoniae* on 5% sheep blood agar; and *E. coli*, *S. typhimurium*, and *P. aeruginosa* on LB agar. The lowest concentration of compound at which no bacterial growth was seen was designated as the minimum bactericidal concentration.

Antimycobacterial activities of the ClpP activating compounds were investigated using the 96-well plate broth microdilution method modified from Wallace et al. (1986), followed by plating for viable bacteria. Compounds were dissolved in 100% DMSO. *M. smegmatis* mc^2155 cultures were grown to an OD_{600} of 1.0–1.3 in Middlebrook 7H9 broth (Difco, BD Biosciences) supplemented with 2% oleic acid, albumin, dextrose, and catalase (OADC enrichment, BD Biosciences) as well as 0.2% glycerol and 0.5% Tween 80 to avoid clumping of bacteria. Cultures were then diluted 10-fold and 5 μl were used to inoculate 100 μl of a 2-fold dilution series of compounds in the range of 1 μg/ml to 256 μg/ml in 7H9 broth supplemented with OADC and 0.2% glycerol in the presence or absence of 12.5 μg/ml polymyxin B. Polymyxin B is known to enhance mycobacterial permeability to hydrophobic compounds (Korycka-Machala et al., 2001). The solvent control, DMSO at 2% or less, showed no inhibitory effects on *M. smegmatis* growth. Plates were incubated in a CO_2 incubator for 8 days. Following incubation, dilutions of sample aliquots were spread on Middlebrook 7H11 plates (Difco, BD Biosciences) supplemented with 2% OADC and 0.5% glycerol for determination of bacterial viability.

Monitoring of *E. coli* Growth in the Presence of ACP Compounds

The *E. coli* strains used for growth curves shown in Figure S2D, MC4100 and MC4100 Δ*clpP*::*cat*, were grown overnight in LB and then diluted into fresh LB to OD_{600} of 0.02. One hundred microliters of the diluted cell culture was added to 100 μl of LB containing ACP1b and CCCP (carbonyl cyanide 3-chlorophenylhydrazone) (Sigma-Aldrich). Final concentrations were 128 μg/ml ACP1b and/or 20 μM CCCP. The solvent control cultures contained 20 μM CCCP and 2.2% DMSO in place of ACP1b. Growth was monitored overnight at 30°C in a Bioscreen-C incubator system (Growth Curves, USA).

Other Procedures

Additional methods are given in the Supplemental Experimental Procedures, namely, subcloning and mutagenesis, surface plasmon resonance measurements, isothermal titration calorimetry measurements, analytical ultracentrifugation measurements, thermal denaturation of ClpP, isolation of ADEP1A and ADEP1B from *Streptomyces hawaiiensis* NRRL 15010, docking procedure, and chemical synthesis of ACP1 and analogs.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, one table, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.chembiol.2011.07.023.

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