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Examination of a Structural Model of Peptidomimicry by Cyclic Acyldepsipeptide Antibiotics in their Interaction with the ClpP Peptidase

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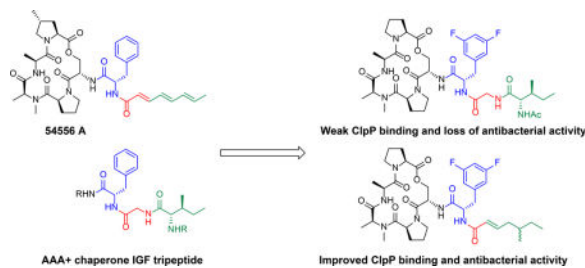
Abstract

The cyclic acyldepsipeptide (ADEP) antibiotics act by binding the ClpP peptidase and dysregulating its activity. Their exocyclic N-acylphenylalanine is thought to structurally mimic the ClpP-binding, (I/L)GF tripeptide loop of the peptidase's accessory ATPases. We found that ADEP analogs with exocyclic N-acyl tripeptides or dipeptides resembling the (I/L)GF motif were weak ClpP activators and had no bioactivity. In contrast, ADEP analogs possessing difluorophenylalanine N-capped with acyl groups having methyl branching like the side chains of residues in the (I/L)GF motifs were superior to the parent ADEP with respect to both ClpP activation and bioactivity. We contend that the ADEP's N-acylphenylalanine moiety is not simply a stand-in for the ATPases' (I/L)GF motif; it likely has physicochemical properties that are better suited for ClpP binding. Further, our finding that the methyl-branching on the acyl group of the ADEPs improves activity opens new avenues for optimization.

Graphical abstract

Antibiotic Mimics a Partner of a Protein-Protein Interaction: The ADEPs' exocyclic N-acylphenylalanine is thought to mimic a tripeptide motif of chaperones that mediates their binding to ClpP. We found that ADEP analogs with greater peptide character in the exocyclic moiety weakly bind ClpP and lack bioactivity. In contrast, those having acyl chains with methyl branching, like particular residues of the tripeptide motif, are superior ClpP activators and antibacterials.

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54556 A · Weak ClpP binding and loss of antibacterial activity · AAA+ chaperone IGF tripeptide · Improved ClpP binding and antibacterial activity

Keywords

ADEP; ClpP; antibacterials; peptidomimicry; proteolysis

Cyclic acyldepsipeptides (ADEPs) are a mechanistically novel class of antibacterial agents that exhibit potent activity against a broad range of Gram-positive bacterial pathogens, including several multidrug-resistant strains of *Staphylococcus aureus*, *Enterococci*, and *Streptococcus pneumoniae*.[1] ADEPs are characterized by a peptidolactone core to which is appended a N-acylphenylalanine side chain.[1a–b] Both the peptidolactone and the side chain of the naturally occurring ADEPs have been extensively modified, yielding analogs that exhibit outstanding efficacy in animal models of bacterial infection.[1c, 2] The cellular target of the ADEPs is ClpP, a peptidase which functions in collaboration with AAA+ (ATPases Associated with Diverse Cellular Activities) partners such as ClpX or ClpA to effect proteolysis of incompletely synthesized proteins or other proteins bearing a degron marker.[2c, 3, 4] ClpP is considered a self-compartmentalized peptidase by virtue of its quaternary structure, which is constituted by heptameric rings that are stacked face-to-face. The barrel-shaped tetradecamer has an interior chamber that is decorated with fourteen serine protease active sites. Consistent with ClpP's capacity to only degrade small peptides, access to the hydrolytic chamber is limited by narrow substrate entry pores located at both ends of the tetradecamer.[5] Accordingly, the proteolytic activity of ClpP is virtually contigent on AAA+ partners that recognize, unfold, and translocate substrates through the entry pores and into the ClpP hydrolytic chamber.[4] The quaternary structure and substrate selectivity of ClpP are significantly affected by ADEP binding. Apparently, ADEP binding to ClpP recapitulates some consequences of AAA+ partner binding, including conformational changes in the tetradecamer that are accompanied by opening of the substrate entry pores and reorganization of the catalytic residues.[4d,6] With its more accessible hydrolytic chamber, the ADEP-bound ClpP indiscriminately and lethally degrades large peptides, unstructured proteins, and nascent proteins as they emerge from the ribosome.[3, 6, 7]

Structural studies have provided a wealth of information about the binding of ADEPs to ClpP and its consequences.[6] In crystal structures, ADEPs bind at the interfaces between ClpP monomers, which are also the putative docking sites of the AAA+ partners (Figure 1A).[6, 8] Hence, the binding of ADEPs to ClpP precludes its association with AAA+ partners.3 Close inspection of the ADEP-ClpP binding interaction reveals that the ADEP peptidolactone binds the surface of ClpP and is significantly solvent exposed, while the exocyclic N-acylphenylalanine moiety binds in an extended channel formed between

adjacent ClpP monomers.[6] The side chain of the phenylalanine residue occupies a deep hydrophobic pocket. Differences in the ways in which the peptidolactone and the N-acylphenylalanine moiety bind ClpP suggest that the latter plays a disproportionately important role in the protein-ligand interaction. Indeed, we have recently reported that the N-acylphenylalanine moiety of the ADEP is both necessary and sufficient for ClpP activation and antibacterial activity.[9] From molecular docking analyses, Li and co-workers have proposed that the ADEPs' N-acylphenylalanine moiety structurally mimics the highly conserved IGF/LGF loop motif of ClpX, an AAA+ partner of ClpP (Figure 1B).[6a] This motif is part of a flexible loop thought to dock into hydrophobic pockets at subunit interfaces on each ClpP heptamer. Although structures of the ClpX-ClpP complex have not been reported, genetic analyses in the IGF/LGF loop of ClpX indicate that this motif is essential for binding to ClpP.[8]

The peptidomimicry proposals by Li *et al*. are quite intriguing, but lack experimental evidence. We reasoned that if the N-acylphenylalanine moiety does indeed mimic the IGF/LGF loop motif, then an ADEP analog with a dipeptide (either N-acetyl Gly-Leu or N-acetyl Gly-Ile) in place of the heptenoyl group would have equal or greater biological activity than the parent compound. Validation of this hypothesis would provide a basis for the structure-based optimization of ADEPs and design of related ClpP activators. We chose compound **1**, a potent and conveniently prepared synthetic ADEP, as a point of comparison for the proposed structural modifications.[10] We envisioned replacing the N-terminal E-2-heptenoyl acyl group with groups that have varying degrees of the IGF/LGF motif's peptide character (Scheme 1). First, difluorophenylalanyl peptidolactone **2** was acylated with either N-acetyl-Leu-Gly-OH or N-acetyl-Ile-Gly-OH. The resulting ADEPs (**3a, 3b**) represent analogs with maximal peptide character in the side chains. Next, we acylated **2** with either 5-methylvaleryl-Gly-OH or 4-methylvaleryl-Gly-OH. These ADEP analogs (**4a, 4b**) have less peptide character in their side chains; they have termini that resemble leucine (**4a**) and isoleucine (**4b**) with the exception of the α-amino groups. Finally, we acylated **2** with α,β-unsaturated carboxylic acids having methyl branches. The corresponding ADEP analogs (**5a–c**) possess minimal peptide character in their side chains. We purposefully selected acyl moieties with methyl substituents that were positionally analogous to those in the side chains of leucine (**5a, 5b**) and isoleucine (**5c**). We do note that the **5b** and **5c** have one less methylene than the IGF/LGF tripeptides.

The compounds were tested for antibacterial activity against *B. subtilis*, a non-pathogenic Gram-positive bacterium. Additionally, we used a ClpP activation assay to measure apparent binding constants for each ADEP analog to the *B. subtilis* ClpP (Figure 2). Intriguingly, neither **3a–b** nor **4a–b** exhibited any appreciable antibacterial activity. We were surprised to observe this inverse correlation between the degree of peptide character in the side chain and the bioactivity of the ADEP. Nevertheless, **3a–b** and **4a–b** did activate ClpP *in vitro*, albeit at much higher concentrations than the parent ADEP (**1**). In contrast, compounds **5a–c** all exhibited outstanding antibacterial activity and ClpP activation *in vitro*. In fact, compound **5c** (MIC = 0.008 μg/mL, K_{app} = 5.1±0.1 nM) is a two-fold more potent antibacterial agent and a notably tighter ClpP binder than the unbranched compound **1** (MIC = 0.016 μg/mL, Kapp 8.3±0.1 nM). All of the active ADEPs (**1, 5a–c**) had measured Hill coefficients

ranging from 1.72 to 2.4, indicating a slight cooperativity in ClpP binding. The relatively high bioactivity of compound **5c** in antibacterial and ClpP activation assays indicated that anteiso methyl branching (isoleucine-like) of the acyl moiety is advantageous. Presumably, by virtue of its position in the chain, the methyl group reduces the conformational flexibility of the acyl moiety in a manner that reduces the entropic costs of ClpP binding. Specifically, the steric bulk of the methyl group further limits rotation about the Cγ-Cδ bond, which is already constrained by allylic strain.

The improved activity of compound **5c** relative to compound **1** prompted us to synthesize fully optimized ADEPs that combined our most potent conformationally constrained peptidolactone **10** with carbon branched acyl chains. In the same manner as compounds **5a–c** were synthesized, a straight chain and two different methyl-branched carboxylic acids were coupled to a difluorophenylalanine-functionalized peptidolactone containing conformationally constrained 4-methylpipecolate and *allo*-threonine residues.[10] These optimized ADEPs were tested for ClpP activation *in vitro* and for antibacterial activity against *B. subtilis*, *S. aureus*, *S. pneumoniae*, and *E. faecalis* (Figure 3). Unexpectedly, the methyl branching on the acyl group did not improve the antibacterial activity of an ADEP with a conformationally constrained peptidolactone. Compounds **6a–c** all exhibited similar ClpP binding *in vitro* and antibacterial activity in assays with *B. subtilis*. Nevertheless, the activities of optimized compounds **6a–c** against *S. pneumoniae* and *E. faecalis* were extraordinary, with MIC   0.00002 μg/mL. Apparently, the substituents that restrict the conformation of the macrocycle have a greater effect on bioactivity than those that limit rotational freedom of the ADEPs' acyclic side chain.

Structural studies of the ADEP-ClpP binding interaction led to the proposal that the N-acylphenylalanine moiety of the ADEPs mimics the IGF/LGF loop motifs of the AAA+ partners of ClpP like ClpX.[6a] By extension, an ADEP to which is appended an IGF or LGF tripeptide would retain bioactivity or be more potent than one with N-acylphenylalanine. In this study, we found that ADEP analogs bearing exocyclic tripeptides or dipeptides analogous to the IGF and LGF motifs were weakly active in ClpP activation assays and lacked antibacterial activity. In contrast, ADEP analogs possessing difluorophenylalanine N-capped with acyl groups having methyl branching like side chains of residues in the (I/L)GF motifs were superior to the parent ADEP with respect to both ClpP activation and bioactivity. One could argue that the (I/L)GF motif mimics on the ADEP scaffold are linear and not constrained in a ClpP binding conformation enforced by the rest of the AAA+ protein. Therefore, the N-acylphenylalanine moities of the ADEPs could be considered surrogates of the IGF or LGF motifs in AAA+ partners with enhanced physicochemical properties (*e.g.*, reduced polarity) for ClpP binding. Indeed, the ADEPs and the motifs of the AAA+ partners are the products of distinct evolutionary pressures that would influence their affinity for ClpP. The selective pressure for ADEP evolution was tight binding to ClpP and toxicity. In contrast, the IGF or LGF motifs of the AAA+ partners are products of an evolutionary pathway directed towards weak interactions with ClpP such that dissociation for either partner exchange or regulation is facile. In any case, we were intrigued to find here and in a previously published report[11] that ADEPs bearing acyl moieties with a methyl substitution pattern reminiscent of the isoleucine side chain were better ClpP activators than

those with an unbranched acyl chain. Apparently, substituents that limit the conformations of either the cyclic or acyclic groups on the ADEPs can translate to improved activities in both enzymatic and antibacterial assays.[9, 10] Further exploitation of this design strategy is underway in these laboratories. Beyond their implications for the further development of ADEPs, our findings are an important addition to the large bodies of literature on peptidomimcry by small molecules and on disruption of protein-protein interactions.[12]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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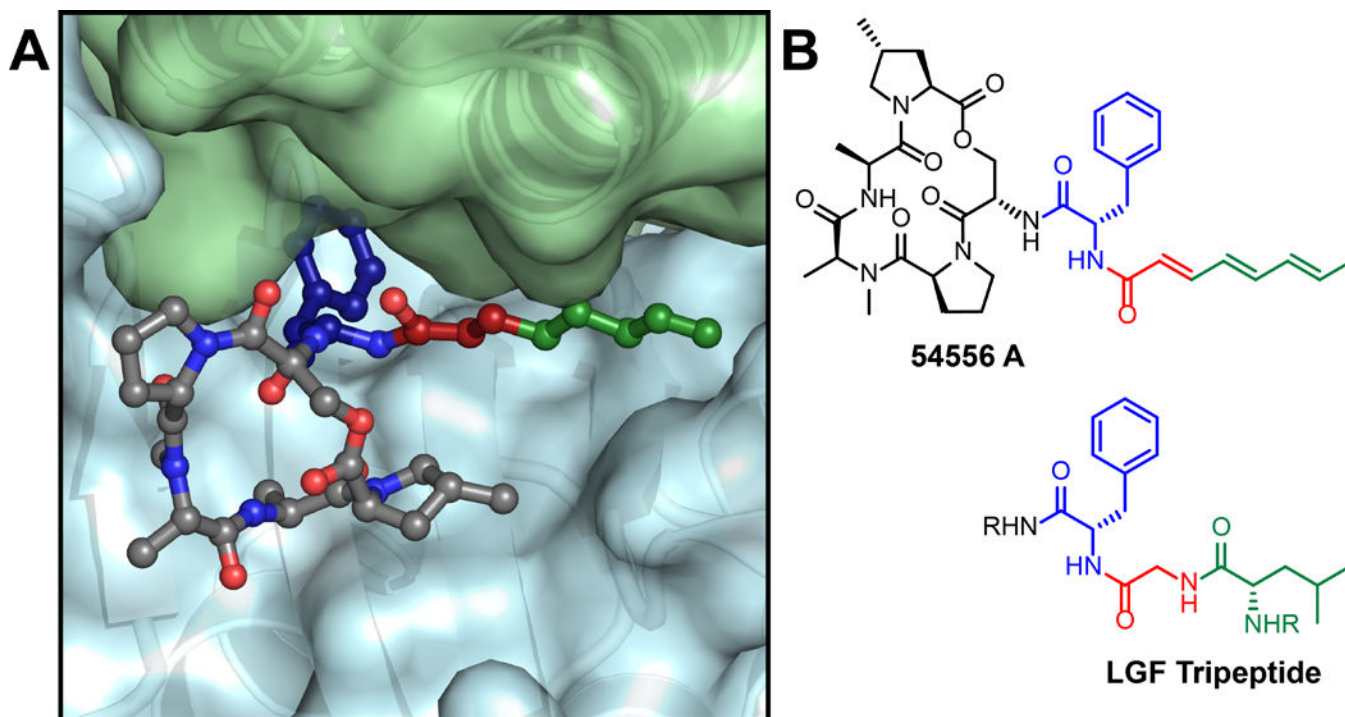
Figure 1.
A) Magnified view of ADEP-1 bound to *B. subtilis* ClpP showing the N-acylphenylalanine moiety occupying a deep channel at the interface between ClpP monomers.[6a] The adjacent ClpP monomers are shown in light green and light blue. The ADEP molecule is shown as sticks, with portions of the N-acylphenylalanine side chain that mimic the (I/G)LF motif coloured dark blue, red, and green. B) Comparison of the structures of an ADEP natural product and a LGF tripeptide (similarities are highlighted with dark blue, red, and green colours) which suggests that the ADEP exocyclic N-acylphenylalanine is a natural peptidomimetic of a AAA+ partner of ClpP.
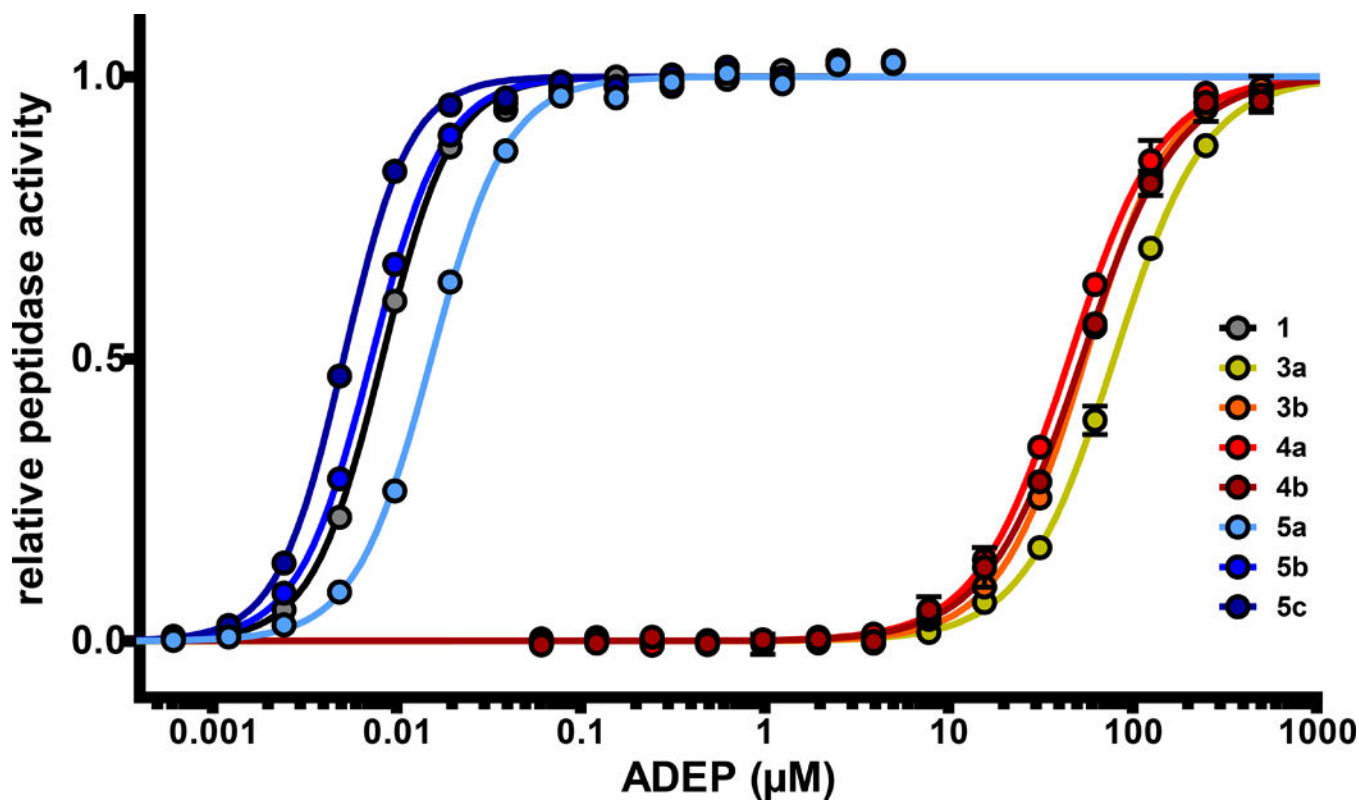
Figure 2.
Biological activity of ADEPs with exocyclic moieties mimicking IGF/LGF tripeptides. Minimum inhibitory concentrations were determined against wild type *B. subtilis* strain AG174 by the agar dilution method. Hydrolysis of a fluorogenic decapeptide substrate (15 μM) by *B. subtilis* ClpP (25 nM) was assayed in the presence of increasing concentrations of ADEP fragments, and activity was fit to a cooperative binding model (solid lines) to determine apparent binding constants (K_{app}) and Hill coefficients. Error bars represent the standard error of the binding model fit.
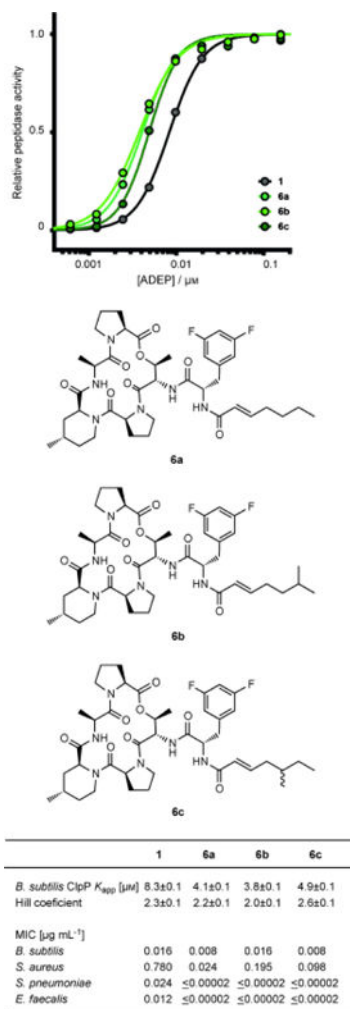
Figure 3.
Biological activity of optimized ADEPs. Minimum inhibitory concentrations were determined against wild type *B. subtilis* strain, AG174 by the agar dilution method. Minimum inhibitory concentrations were determined against *S. aureus* ATCC 29213, *S. pneumoniae* ATCC 49619, and *E. faecalis* ATCC 29212 by the broth dilution method. Hydrolysis of a fluorogenic decapeptide substrate (15 μM) by *B. subtilis* ClpP (25 nM) was assayed in the presence of increasing concentrations of ADEP fragments, and activity was fit to a cooperative binding model (solid lines) to determine apparent binding constants (K_{app}) and Hill coefficients. Error bars represent the standard error of the binding model fit.
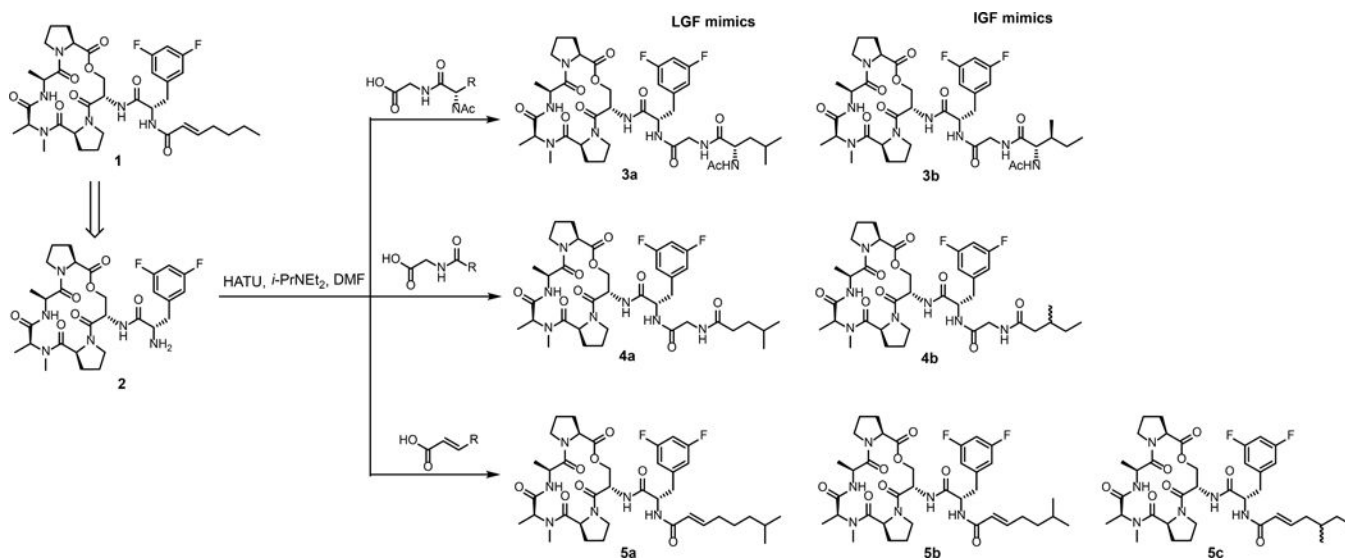
Scheme 1.
Synthesis of ADEPs with LGF and IGF mimetic side chains