Acyldepsipeptide Probes Facilitate Specific Detection of Caseinolytic Protease P Independent of Its Oligomeric and Activity State

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Introduction

Caseinolytic protease P (ClpP) is a tetradecameric peptidase that assembles with chaperones such as ClpX to gain proteolytic activity. Acyldepsipeptides (ADEPs) are small-molecule mimics of ClpX that bind into hydrophobic pockets on the apical site of the complex, thereby activating ClpP. Detection of ClpP has so far been facilitated with active-site-directed probes which depend on the activity and oligomeric state of the complex. To expand the scope of ClpP labeling, we took a stepwise synthetic approach toward customized ADEP photoprobes. Structure–activity relationship studies with small fragments and ADEP derivatives paired with modeling studies revealed the design principles for suitable probe molecules. The derivatives were tested for activation of ClpP and subsequently applied in labeling studies of the wild-type peptidase as well as enzymes bearing mutations at the active site and an oligomerization sensor. Satisfyingly, the ADEP photoprobes provided a labeling readout of ClpP independent of its activity and oligomeric state.

Figure 1. Structure of tetradecameric SaClpP depicted in the A) side view with marked central helix (red) and in the B) top view, with marked hydrophobic pockets (red). C) SaClpP with the marked amino acids S98 (orange) and R171 (blue) (PDB ID: 3STA).

ClpP, the proteolytic core of the caseinolytic protease, is a barrel-shaped serine peptidase composed of two heptameric rings which assemble into a tetradecameric complex (Figure 1 A, B).[1,2] Several structural studies have shown that the enzyme samples various conformations including an active extended state with an elongated central helix and an aligned catalytic triad as well as a compressed state with a fully kinked helix and a misaligned triad.[3,4] In addition, a compact state representing a transition between the two has been observed.[3,5–7] Several crucial residues within ClpP have been identified which support the oligomeric state and corresponding activity. For example, an oligomerization sensor between Asp170 and Arg171 forms crucial contacts between the heptamer–heptamer interface and ensures a stable tetradecameric complex.[8] Mutations of either of the two residues results in complex disassembly into inactive heptamers. Overall, these previous studies demonstrated that the conformational state of the complex and its corresponding enzymatic activity are directly linked.[9]

ClpP digests small peptide chains, but is unable to act on larger proteins due to the restricted pore size of the proteolytic chamber. For the degradation of these proteins, the complex binds to chaperones such as ClpX which assist in ATP-dependent unfolding and threading the linear peptide chain into the ClpP barrel.[10–13] ClpX uses IGF peptide loops to specifically dock into cognate hydrophobic pockets on the apical site of ClpP.[14] Natural product acyldepsipeptides (ADEPs) represent small-molecule mimics of these IGF loops which address the same binding site and thereby induce opening of the central pore to facilitate access of larger substrates.[15–17] This uncontrolled access and digestion of cellular proteins results in dysregulation of protein homeostasis and corresponding antibiotic resistance.
activity against for example Staphylococcus aureus and its persistent strains.\textsuperscript{[18, 19]}

In addition to activation, natural product derived $\beta$-lactones were shown to acylate the active site and thereby inhibit enzymatic turnover. The consequence of this mechanism is a decrease in $S.\ aureus$ virulence. Alkynylated probes of $\beta$-lactones or other active site inhibitors have been used to readout the activity state of ClpP in various settings including living cells\textsuperscript{[20, 21]} While these probes require an aligned active site for acylation, they solely detect ClpP in its active state, but are blind for other inactive conformations. Despite intensive studies on ClpP labeling, probes to target an activity-independent allosteric site have not yet been introduced. To obtain complementary probes for detecting ClpP in various conformational states, we here exploited IGF loop mimics for binding into the hydrophobic pocket and their readout on different ClpP mutants.

Results and Discussion

ADEX probe design and synthesis

To design novel probes for ClpP affinity-based protein profiling (AfBPP), ADEX derivatives need to be functionalized with an alkyne handle as well as a photoreactive group.\textsuperscript{[22–24]} The photo-crosslinker forms a covalent bond with amino acid residues in the proximity of the binding site upon UV irradiation and subsequent modification of the alkyne with an azide tag via click chemistry allows visualization and detection. Ideally, these moieties need to be introduced at positions that do not affect the compounds’ binding to the hydrophobic pockets of ClpP. Previous structure–activity relationship studies (SAR) with ADEXs revealed that small fragments containing the $N$-acyldifluorophenylalanine moiety retain ClpP activation and antibiotic activity albeit with lower potency than the full-length depsipeptides.\textsuperscript{[25]} Moreover, derivatives lacking an unsaturated bond

Scheme 1. Structures of A) different ADEX fragments, with published structures 01\textsuperscript{[25]} and 05\textsuperscript{[25]} and fragment photoprobe 235 and B) ADEX derivatives 10,\textsuperscript{[26]} ADEX4, and photoprobe 266 and 288.
next to the amide in the alkyl chain were nearly inactive. Several ADEP fragments were designed to decipher if other modifications are tolerated at this moiety. These included variations in the length of the side chain, introduction of an alkyne, the deletion of the double bond and an azide at the phenyl alanine moiety.\(^{[26]}\) Starting from compound 01, previously introduced by Sello and co-workers,\(^{[25]}\) we first tested if an alkyne handle would be accepted at the terminal end of the acyl chain. Most of the fragments, 02, 03, 04, and 06, were easily accessible via an esterification, followed by Boc deprotection and amide coupling (Scheme 1A, Scheme S1B in the Supporting Information). The two larger fragments 05 and 09 were synthesized via a four-step synthetic route, depicted in Scheme S1A (Scheme 1A).

To rank the corresponding activities of all derivatives we performed ClpP activity assays with FITC-casein as a substrate.\(^{[27,28]}\) In the absence of an activator the small ClpP pore prevents access of the substrate, while activator binding opens the channel and allows processing. Compound 01 was used as an internal control. As expected, derivatives 02, 03, 04 and 09 with varying alkyne chain lengths but which lack the crucial amide double bond, were found to be inactive (Figure 3A). To our surprise, even in presence of the crucial double bond, compound 06 did not gain any activity, demonstrating that the introduction of an alkyne at the acyl chain is not tolerated.

To exploit other putative modifications, we prepared aryl azides 07 and 08 starting from Boc-protected phenylalanine with bromide in the para position and nitro group in the meta position, respectively. The introduction of an azide yielded two possible photoprobes (Schemes 1A and S2). Activity tests with these probes revealed that neither the para (07) nor meta (08) positioned azide showed any activating effect, highlighting again the restricted flexibility in this crucial fragment (Figure 3A). Finally, a minimal alkyne chain equipped with a diazirine photo-crosslinker on the western side of the molecule (235) resulted in about 90% activation relative to the starting compound, yielding the first functional fragment probe (Figure 3A, Schemes 1A and S1B).

To further improve the activity, we aimed to design a probe that is based on the whole ADEP scaffold. Here, based on the previous experience with fragments, modifications were deliberately made solely at the macrocyclic core, that is, at the alanine and one of the two proline residues. Starting from the structure of ADEP4, we designed two probes with an alkyne modified alanine and either a photoproline in the southern position (266) or the northern position (288; Scheme 1B). The synthesis of these probes was executed by using solid-phase peptide synthesis with subsequent ring-closure reaction. First, Fmoc-protected methylproline (11), Fmoc-protected photopropoline (12) and Fmoc-protected pipecolic acid (13) were prepared, as depicted in Scheme S3. The first amino acid, methylproline in case of 266 and photopropoline in case of 288, was loaded on the resin, followed by the coupling of Fmoc-protected propargylglycine, pipecolic acid, photopropoline or proline, serine, phenylalanine derivative and heptenoic acid. Ring closure was achieved by lactonization, with the method published by Batey and co-workers in 2015 (Schemes 2 and S4).\(^{[29]}\) Final activity studies of the two ADEP probes revealed that 266 was a significantly better activator of ClpP (150% relative to internal control) than 288, which only achieved around 30% (Figure 3A). ADEP4 and compound 10, a derivative lacking the fluorine atoms at the phenylalanine, the methyl group at the proline and the pipecolic acid moiety, were included as controls. They also achieved maximum activation similar to the most active 266 probe, albeit at much lower concentrations in the case of ADEP4, emphasizing its higher affinity and better fit into the hydrophobic pocket (Figure S1).

Modeling of the probes into the hydrophobic pocket provided a rational explanation for the observed differences in activation. In Figure 2 the modeled positions and orientations of 266 and 288 are shown together with the crystal structures of co-crystallized ADEPs (Figure 2C, D, Scheme S4A). While the over-

![Scheme 2. Synthetic route for the synthesis of ADEP derivatives 10, 266, and 288.](image-url)
all binding mode is conserved for all molecules there are distinctive differences, especially with respect to a signature methylproline moiety (green circle in Figure 2), which was found to in the experimental structures of ADEPs suggesting a similar mechanism of binding and activation (Figure 2A versus C,D). However, in 288 the diazirine moiety, replacing the methyl group, induces a shift of the macrocycle, such that the photocrosslinker points inside the binding site close to for example, Y61, which could explain the observed strong and stable labeling in situ with intact S. aureus cells in the stationary phase. Probes were incubated for short time points of 1 h in order to minimize cell killing. The most prominent band was confirmed to be ClpP via western blot with the respective antibody and the labeling in a clpP knockout strain resulted in a lack of the characteristic band (Figure S2 B, C). Furthermore, ClpP could be identified in an LC–MS/MS-based AfBPP workflow (Figure S5). These results highlight the required specificity of the probe for its target.

Labeling of ClpP in different conformational states
To determine if ClpP can be labeled in different conformational states, we overexpressed and purified an active-site mutant of ClpP (D, PDB ID: 3MT6). Ligands are shown in stick representation, and proteins are illustrated as cartoons. Red arrows mark the position of the α-helix important for propagating structural changes to the β-sheet loops (yellow circle in panel D) at the edge of the pore. Methylproline is highlighted with a green circle. Residues closest to the photoproline moiety (indicated with black arrows) are shown as sticks for 266 and 288. Atom color scheme: nitrogen, dark blue; oxygen, red; fluorine, green; carbon, other.

Figure 2. Most prominent docked poses of ligands A) 266 and B) 288 and X-ray structures of bound ADEP (structure in Figure S3 A) in SoClP (C, PDB ID: 5V22) and ADEP1 in EcClP (D, PDB ID: 3MT6). Ligands are shown in stick representation, and proteins are illustrated as cartoons. Red arrows mark the position of the α-helix important for propagating structural changes to the β-sheet loops (yellow circle in panel D) at the edge of the pore. Methylproline is highlighted with a green circle. Residues closest to the photoproline moiety (indicated with black arrows) are shown as sticks for 266 and 288. Atom color scheme: nitrogen, dark blue; oxygen, red; fluorine, green; carbon, other.

Biological activity and labeling of ClpP in living cells
All ClpP-activating compounds were tested for their antibiotic activities against S. aureus cells and corresponding minimal inhibitory concentrations (MIC) were determined. Compound 01 served again as control with a MIC value of 20.0 μM (± 6.12 μM). While ADEP4 exhibited the highest potency with < 15 nm, compounds 266 and 10 showed, in agreement with ClpP activation, moderate MICs of 3.09 ± 0.06 μM and 4.35 ± 2.33 μM, respectively. This again highlights that their structural perturbations compromised the overall activity; however, they were sufficient to retain antibiotic effects. In contrast 288 was inactive at the concentrations tested, reflecting its rather weak activation of ClpP. Surprisingly, probe 235 was also inactive despite a good activation of ClpP similar to 01 (Table S1).

To investigate if the bioactivity correlates with labeling of ClpP, we performed AfBPP studies with 235, 266, and 288. Purified SoClP was incubated with 10 μM probe, irradiated and clicked to a fluorescence tag. Importantly, as predicted 288 protruded as a strong labeling probe exceeding the performance of 266 (Figure S1B). Further studies with ADEP4 demonstrated, that the 288 probe signal vanishes with increasing concentration of a competitor, suggesting that both compounds address the same binding pocket (Figure 3B). Labeling could also be shown in situ with intact S. aureus cells in the stationary phase. Probes were incubated for short time points of 1 h in order to minimize cell killing. The most prominent band was confirmed to be ClpP via western blot with the respective antibody and the labeling in a clpP knockout strain resulted in a lack of the characteristic band (Figure S2 B, C). Furthermore, ClpP could be identified in an LC–MS/MS-based AfBPP workflow (Figure S5). These results highlight the required specificity of the probe for its target.

Figure 3. A) FITC-casein activity assay of several ADEP fragments and derivatives. Shown is the activity on SoClP at a concentration of 20 μM, normalized to the internal standard 01. Experiments were carried out in triplicates. B) Labeling of recombinant SoClP with photoprobe 288 and ADEP4 and 01 as competitors. Competitor and probe were added at the same time and incubated for one hour. C) Labeling of SoClP wild-type and mutants, S98A and R171, with photoprobe 288 in comparison with β-lactone D3 at a concentration of 10 μM. D) Structure of D3. E) MIC values of ADEP fragments and derivatives in S. aureus NCTC8325.
(S98A) and a disrupted oligomerization mutant (R171A), together with the wild-type enzyme as control (Figure 1C). These proteins were subsequently labeled with the 288 probe and an active-site-directed β-lactone (D3) as benchmark control. Interestingly, while 288 detected wild-type and both mutants, the D3 signal was only obtained for the active wild-type (Figure 3C,D). In line with our hypothesis, these results establish 288 as a conformation-independent probe for selective and universal ClpP detection.

Conclusion
We here introduce a complementary chemical tool for the selective detection of ClpP that goes beyond active site labeling of previously introduced probes. SAR studies not only guided the design of ADEP probes but also provided more general aspects of binding into the hydrophobic pocket which was further elucidated by molecular docking. Overall, only limited structural modifications were tolerated but still resulted in a significant drop of activity which challenged the design of photo-crosslinker probes.

Here the linker either retains higher activity but points outward of the pocket or is inside but clashes with the binding site. However, as 288 serves as a suitable detection tool, the reduced activity is acceptable, and sufficient labeling could still be observed in vitro and in situ, suggesting that the probe has great potential for applications to monitor ClpP, in its active and inactive conformational states.

Experimental Section
Synthesis of fragment photoprobe 235: N-Boc-3,5-difluorophenylalanine (50.0 mg, 166 μmol, 1.0 equiv) and minimal photo-crosslinker 32 (22.9 mg, 166 μmol, 1.0 equiv) were dissolved in CH2Cl2 (1.5 mL) and EDC·HCl (38.2 mg, 199 μmol, 1.2 equiv) and DMAP (1.01 mg, 8.30 μmol, 0.1 equiv) were added. The reaction was stirred at room temperature for 2.5 h, the solvent was removed under reduced pressure. A light-yellow solid (28.6 mg, 43 % over three steps) was obtained after purification with column chromatography (hexane/ethyl acetate 5:1 to 3:1). For analytical data, please see the Supporting Information, Chapter 4.3.

Synthesis of ADEP-photoprobe 266: 2-chlorotoltriyl chloride resin (503 mg) was loaded with Fmoc-proline derivative 11 (126 mg, 359 μmol), to obtain a loading of 75 μmol/g (general procedure D). Following amino acids were coupled to the resin using general procedures E, F and G: Fmoc-progarglycine (158 mg, 473 μmol), Fmoc-pipepecolic acid (170 mg, 473 μmol), Fmoc-photoprobe 12 (124 mg, 341 μmol), Fmoc-serine (154 mg, 473 μmol), Fmoc-3,5-difluorophenylalanine (129 mg, 306 μmol) and E-2-heptenoic acid (63.4 μL, 950 mg mL−1, 473 μmol). The heptapeptide was cleaved of the resin using general procedure H. (yield: 76 % over six coupling steps) The cyclization was performed according to general procedure I, using the resulting heptapeptide (10.0 mg, 11.9 μmol, 1.0 equiv), MNBA (12.3 mg, 35.7 μmol, 3.0 equiv), Dipea (5.68 μL, 742 mg mL−1, 33.4 μmol, 2.8 equiv), DMAP (8.70 mg, 71.5 μmol, 6.0 equiv) and Dy(OTf)3 (7.30 mg, 11.9 μmol, 1.0 equiv). The crude product was purified by reversed phase HPLC (ACN/H2O; ACN 2 % to 98 %). Yield: 8.34 mg (85 %). For general procedures and analytical data, please see the Supporting Information, Chapters 4.5 and 5.6.

Synthesis of ADEP-photoprobe 288: 2-chlorotoltriyl chloride resin (559 mg) was loaded with Fmoc-photoprobe 110 mg, 303 μmol), to obtain a loading of 400 μmol/g (general procedure D). Following amino acids were coupled to the resin using general procedures E, F and G: Fmoc-progarglycine (172 mg, 510 μmol), Fmoc-pipepecolic acid (181 mg, 510 μmol), Fmoc-proline (173 mg, 510 μmol), Fmoc-serine (177 mg, 510 μmol), Fmoc-3,5-difluorophenylalanine (218 mg, 510 μmol) and E-2-heptenoic acid (69.4 μL, 950 mg mL−1, 510 μmol). The heptapeptide was cleaved of the resin using general procedure H. (Yield: 22 % over six coupling steps) The cyclization was performed according to general procedure I, using the resulting heptapeptide (50.0 mg, 60.6 μmol, 1.0 equiv), MNBA (62.6 mg, 182 μmol, 3.0 equiv), Dipea (28.9 μL, 760 mg mL−1, 170 μmol, 2.8 equiv), DMAP (44.4 μL, 364 μmol, 6.0 equiv) and Dy(OTf)3 (37.0 mg, 60.6 μmol, 1.0 equiv). The crude product was purified by reversed-phase HPLC (ACN/H2O; ACN 2 % to 98 %). Yield: 3.05 mg (6 %). For general procedures and analytical data, please see the Supporting Information, Chapters 4.5 and 5.6.

FITC-casein assay:27,28 The aim of this assay was to determine the proteolytic activity of ClpP with different compounds. For the FITC-casein assay, 100 μL sample was prepared in a black 96-well plate, with a concentration of 1 μM ClpP and different final concentrations of the compound. Compound 01 was used as positive control with a concentration of 20 μM and DMSO as a negative control. All set ups were carried out in triplicates; 1 μL DMSO or compound in DMSO was placed in each well and 80 μL ClpP in buffer (12.5 μM, in PBS buffer: 25 mM HEPES, 200 mM KCl, 5 mM MgCl2, 1 mM DTT, 10 % glycerol, pH 7.6) was added. After incubation at 37 °C for 15 min, 20 μL of substrate (2 mM FITC-casein, 10 mM casein, in PBS) was added to each probe. After excitation at 485 nm, the fluorescence emission was measured by a Tecan infinite F200 Pro at 535 nm. Initial slopes over time were evaluated by a linear regression model, and all values were normalized to the 01 control, after subtraction of the DMSO control.

Labeling of recombinant protein: 0.5 μL DMSO or compound (1:100, different concentrations in DMSO) was placed in a transparent 96-well plate and 44 μL SoClP in PBS (final concentration 2 μM) was added. After incubation for one hour in the dark, the mixture was irradiated for 10 min at 365 nm (Philips TL-DLB18W), 5 μL gel-based click reagent mix [1 μL RhN3 (TAMRA azide, 5 mM in DMSO), 1 μL freshly prepared TCEP (50 mM in ddH2O), 3 μL TBA ligand (1.67 mM in 80 % tBuOH and 20 % DMSO)] and 1 μL 50 mM CuSO4 were added and incubated for 1 h in the dark. Then 50 μL...
2 x Laemmli sample buffer (63 mM Tris-HCl, 10% glycerol, 2% SDS, 0.0025% Bromophenol Blue, 5% 2-mercaptoethanol) were added and the samples analyzed via SDS-PAGE (12.5% or 15% agarose gel, 2.5 h, 150 V, 8 µL fluorescent protein standard, fluorescence imaging [GE Healthcare, ImageQuant LAS-4000]).

**Computational studies:** For all modeling studies the X-ray structure PDB ID: 5VZ2 (ClpP from *S. aureus* — acyldepsipeptide) of the SoClpP protein with bound ADEP was used. The modeling procedure applied is based on a molecular dynamics-based docking pipeline, which was previously developed and optimized specifically for the docking of macrocyclic compounds. It contains the following steps: I) Conformational sampling of the compounds in aqueous solution, clustering and extraction of the most prominent conformations of the macrocycle. II) Equilibrium molecular dynamics simulations of the experimental structure of the protein—ligand complex (PDB ID: 5VZ2). III) Fully flexible molecular dynamics-based docking of all compound conformations obtained from (I) into the equilibrated binding site from (II). For the steps (I) and (II) the program Amber17 was used. All molecular docking calculations were performed with the DynaDock program. In addition, to evaluate the performance of the pipeline and optimize its parameters for the molecular system at hand, first re-docking experiments of the co-crystallized ADEP molecule (Figure 2C) into its binding site in SoClpP were performed using the equilibrated PDB ID: 5VZ2 protein—ligand complex. With the docking pipeline presented herein, a binding pose of the ADEP molecule with a root-mean-square deviation (RMSD) of 1.6 Å for all non-hydrogen ligand atoms (data not shown) from those of the equilibrated holo-structure (ClpP5Z2) could be obtained, thereby assuring the suitability of the procedure for docking studies of the SoClpP—ligand system. The so-optimized and validated docking parameters were afterward applied for the prediction of the bound conformations of compounds 266 and 288 into the same SoClpP protein structure. A detailed description of the computational conditions and parameters applied is provided in the Supporting Information, Chapter 3.

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**Conflict of Interest**

The authors declare no conflict of interest.

**Keywords:** acyldepsipeptides · antibiotics · caseinolytic protease P · photoaffinity labeling · virulence

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