Accepted Article

This manuscript has been accepted after peer review and appears as an Accepted Article online prior to editing, proofing, and formal publication of the final Version of Record (VoR). This work is currently citable by using the Digital Object Identifier (DOI) given below. The VoR will be published online in Early View as soon as possible and may be different to this Accepted Article as a result of editing. Readers should obtain the VoR from the journal website shown below when it is published to ensure accuracy of information. The authors are responsible for the content of this Accepted Article.

WILEY-VCH

WILEY-VCH

COMMUNICATION

Tailored peptide phenyl esters block ClpXP proteolysis by an unusual breakdown into a heptamer-hexamer assembly

Dr. Markus Lakemeyer, Eva Bertosin, Dr. Friederike Möller, Dóra Balogh, Dr. Ralf Strasser, Prof. Dr. Hendrik Dietz, Prof. Dr. Stephan A. Sieber*

Abstract: *The proteolytic complex ClpXP is fundamental to bacterial homeostasis and pathogenesis. Due to its conformational flexibility, the development of potent ClpXP inhibitors is challenging and novel tools to decipher its intricate regulation are urgently needed. Here, we present amino acid-based phenyl esters as molecular probes to study the activity and oligomerization of the ClpXP complex of S. aureus. Systematic screening of (R)- and (S)-amino acids led to compounds showing potent inhibition, as well as stimulation of ClpXP-mediated proteolysis. Sub-stoichiometric binding of probes arrested ClpXP in an unprecedented heptamer-hexamer assembly, in which the two heptameric ClpP rings are dissociated from each other. At the same time the affinity between ClpX and ClpP increased, leading to inhibition of both enzymes. This conformational arrest is beneficial for the consolidated shutdown of ClpXP, as well as for the study of the oligomeric state during its catalytic cycle.*

ClpXP is a key regulator of bacterial virulence in a variety of pathogens including *Staphylococcus aureus*.[1] The proteolytic complex consists of a peptidolytic core, formed by 14 subunits of caseinolytic peptidase (ClpP), flanked by apical AAA+ chaperones, such as hexameric ClpX. $ClpP_{14}$ exhibits only moderate peptidolytic activity towards short peptides and requires binding to $ClpX_6$ to one or both apical sites of the barrel for full proteolytic activity. Within this complex, $ClpX_6$ recognizes and unfolds substrate proteins for subsequent digestion by $ClpP_{14}$.[2] Previous research identified various small molecule inhibitors of ClpP.[3-7] Among those, reversible oxazole compounds displayed excellent potency on the ClpP peptidase but suffered from a ClpX-mediated repulsion based on conformational selection, thus preventing inhibition of ClpXP.[8] Irreversible β-lactone inhibitors are limited by compound stability, but have been effective in the reduction of virulence.[6] For a subset of these compounds, dissociation of $ClpP_{14}$ into catalytically inactive heptameric species was observed.[9,10] It yet remains unresolved, if $ClpP_7$ is an artifact of inhibition with synthetic molecules, or if this state has a biological function, *e.g.* during proteolysis. Thus, a detailed understanding of the communication within the proteolytic ClpXP complex and how it can efficiently be modulated by molecular probes remains elusive.

[*] Dr. M. Lakemeyer, D. Balogh, Prof. Dr. S. A. Sieber
Center for Integrated Protein Science (CIPS^M) at the Department of Chemistry, Technische Universität München, Lichtenbergstrasse 4, 85747 Garching (Germany)
Email: stephan.sieber@tum.de

E. Bertosin, Prof. Dr. H. Dietz
Physics Department and Institute for Advanced Study, Technische Universität München, Am Coulombwall 4a, 85748 Garching (Germany)

Dr. F. Möller, Dr. R. Strasser
Dynamic Biosensors GmbH, Lochhamerstr. 15, 82152 Planegg (Germany)

Supporting information for this article is available on the WWW under http://dx.doi.org/

Phenyl esters have been identified as a new class of covalent ClpXP inhibitors that surpass previous β-lactone inhibitors in potency and show a stereogenic methyl-switch for de-oligomerization of ClpP.[7] The stereochemistry of the methyl-group in the α-position to the ester determines, if inhibition occurs while maintaining the $ClpP_{14}$ state ((*S*)-**ML90**), or if de-oligomerization into inactive heptameric species is induced ((*R*)-**ML89**) (Figure 1a).[7] As the stereogenic α-methyl switch in **ML89**/**ML90** resembles an amino group in structurally related aromatic amino acids, we here present customized peptide phenyl esters as mimics of native substrates for in-depth investigation of the catalytic activity and oligomerization of the full ClpXP complex.

In the phenyl ester enantiomers **ML89**/**ML90** the prominent 3,4,5-trimethoxyphenyl moiety was shown to be critical for binding based on extensive SAR studies.[7] We therefore selected this motif as side chain decoration of the first amino acid phenyl esters, yielding the unnatural amino acid 3,4,5-trimethoxy-phenylalanine (Tmo). Both enantiomers of the respective compounds, (*R*)-**11** and (*S*)-**12**, were prepared following the Erlenmeyer-synthesis route, starting from 3,4,5-trimethoxybenzaldehyde (Scheme 1). For the synthesis of N-terminally modified and dipeptide phenyl esters, the compounds were subjected to standard solution-phase peptide chemistry (Scheme 1 and supplementary information).

All phenyl esters were initially tested at three concentrations (100, 10, 1 µM) using two different assay setups: 1) inhibition of ClpP's peptidolytic activity towards the fluorogenic peptide Ac-Ala-hArg-2-Aoc-ACC (Figure S1),[11] and 2) inhibition of ClpXP-mediated proteolysis of green-fluorescent protein (GFP) that was tagged with a short SsrA degradation signal.[12]

From the first set of eight N-terminally modified Tmo phenyl esters, the previously described stereo-preference of ClpP was confirmed, meaning that (*R*)-phenyl esters were generally better inhibitors than the respective (*S*)-enantiomers (Figure 1b). While
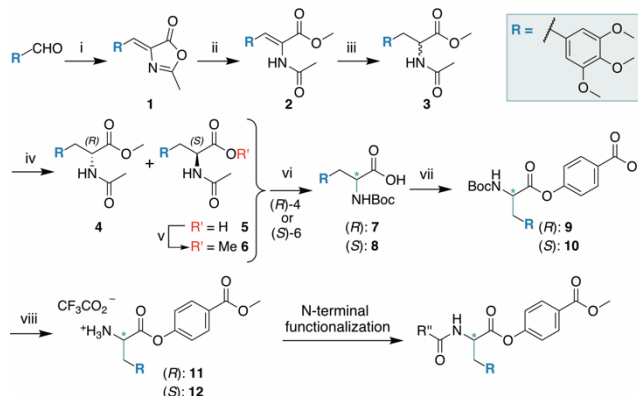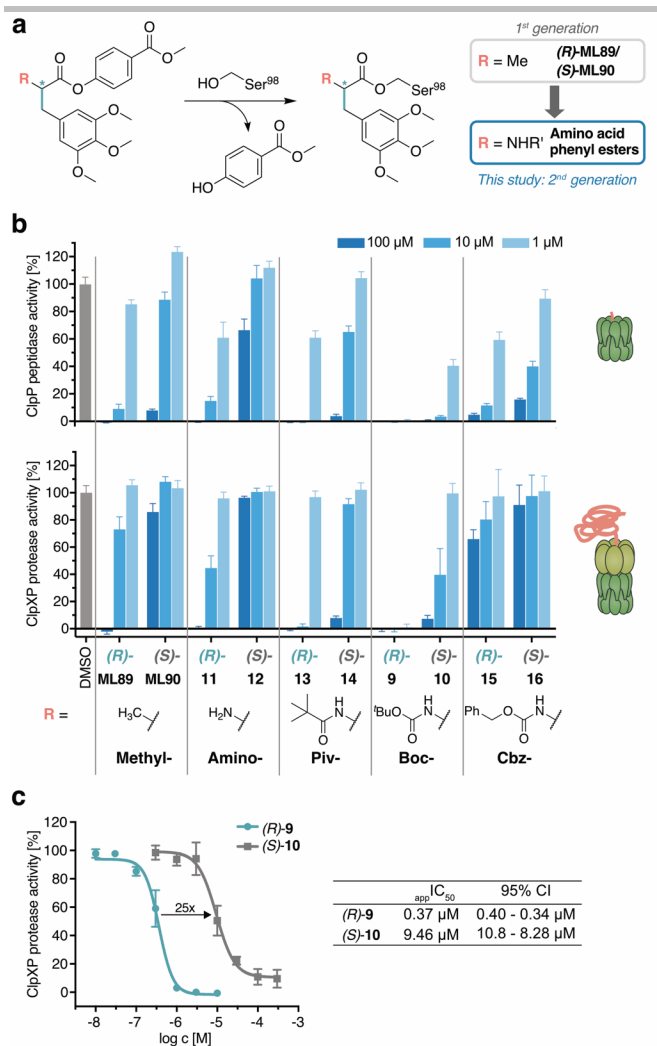

Scheme 1. Stereoselective synthesis of Tmo phenyl ester derivatives. *Reagents and conditions*: i) *N*-acetylglycine, NaOAc, Ac₂O, 125 °C, 5h; ii) NaOMe, MeOH, rt, 1.5 h; iii) H₂, Pd/C, MeOH, rt, 16 h; iv) Alcalase, aq. NaHCO₃, rt, 6 h; v) *p*-TsOH, MeOH, 80 °C, 6 h; vi) Boc₂O, DMAP, 75 °C, 3 h, then aq. LiOH, rt, 3 h; vii) methyl-4-hydroxybenzoate, EDC • HCl, HOBt, DIPEA, DCM, 0 °C → rt, 18 h; viii) TFA, DCM, 0 °C, 5 h.

COMMUNICATION

Figure 1. Biochemical evaluation of N-terminally capped Tmo phenyl esters. a) Reaction of phenyl esters with the active site Ser[98] traps ClpP in the acyl-enzyme state. b) Inhibition of *S. aureus* ClpP peptidase and ClpXP protease activity by the respective enantiomers. c) Apparent IC_{50} of both enantiomers of Boc-Tmp-OAr in the protease assay. Data are normalized to the DMSO control (100% activity). Data depict mean and standard deviation (SD) (n≥6).

the α-methyl compounds (*R*)-**ML89**/(*S*)-**ML90** and the unmodified Tmo phenyl esters (*R*)-**11**/(*S*)-**12** only showed moderate inhibition in both assays, capping the amine with a pivaloyl- (**13** and **14**) or Boc- (**9** and **10**) group led to a significant improvement in inhibition. (*R*)-Boc-Tmo-OAr (**9**) had an apparent IC_{50} of 0.37 μM in the protease assay, which distinguishes it as the most potent ClpXP inhibitor reported to date. Contrarily, its (*S*)-enantiomer **10** is a 25-times weaker binder (Figure 1c) that arrests the protein in the acyl-enzyme state (Figure S2a-d). Importantly, the average grade of active site modification for **9** at the IC_{50} concentration was only ≈10% (Figure S2e). Hence, sub-stoichiometric binding of **9** facilitates an overall conformational rearrangement that leads to the inactivation of all remaining active sites. The sterically more demanding Cbz-group was only well-tolerated for the (*R*)-enantiomer **15** in the peptidase assay, but failed to inhibit the full ClpXP complex (Figure 1b). In general, effective inhibition of ClpP's peptidolytic activity did not necessarily correlate with efficient inhibition of the proteolytic ClpXP complex. This finding is in accordance with previous studies, confirming that ClpX exerts conformational control over ClpP and is able to boost peptidolysis and revoke inhibitor binding.[8,10] We thus focused on

the biologically more relevant ClpXP protease system for subsequent compound evaluation.

Next, we investigated, whether inhibition by (*R*)-amino acid phenyl esters is a general feature or if it is an exclusive trait of the unnatural amino acid Tmo. A collection of thirteen diverse (*R*)-, *i.e.* D-amino acid phenyl esters was synthesized and evaluated for ClpXP inhibition (Figure 2a). Almost all derivatives were inactive, including phenyl esters of nonpolar alanine (**20**) and methionine (**22**), or polar serine (**27**) and glutamate (**29**). The aromatic phenylalanine (**23**) and tyrosine (**24**) derivatives, structurally related to **9**, also did not affect proteolysis. Solely leucine (**21**) and tryptophan (**25**) phenyl esters showed inhibition at high and medium concentrations, respectively.

Previous studies revealed that ClpP contains a large hydrophobic S1 pocket tailored for the accommodation of long alkyl chains.[13] Correspondingly, β-lactones with alkyl chains of various lengths,[6,13] as well as fluorogenic peptide substrates, containing the unnatural amino acid (*S*)-2-aminooctanoic acid (2-Aoc) in P1 position,[11] are good inhibitors and substrates, respectively. This preference was not seen for phenyl esters, as the Boc-(*R*)-2-Aoc analog **31** did not inhibit ClpXP. Likewise, the amide analog of phenyl ester **9**, compound **17,** as well as an activated tetrafluoroanilide (**18**), did not affect ClpXP-mediated proteolysis (Figure S3a). Overall, these results emphasize the importance of (*R*)-Tmo phenyl esters at the C-terminal position.

To further converge to natural peptide substrates of ClpXP, dipeptide Tmo-phenyl esters were synthesized. First, the stereo-preference for the P2 position was evaluated by testing all four diastereomers of Boc-Met-Tmo-OAr and Boc-Leu-Tmo-OAr. The (*S*,*S*)-dipeptides (**32**, **36**) as well as the (*S*,*R*)- and (*R*,*S*)-diastereomers (**33**, **34**, **37**, **38**) failed to efficiently inhibit proteolysis (Figure S3b). Conversely, the (*R*,*R*)-phenyl esters **35** and **39** were potent inhibitors, with **35** showing full inhibition at 10 μM (Figure S3b). Having identified the (*R*)-stereo-preference of ClpXP for the P2-position, a representative set of Boc-(*R*,*R*)-dipeptide Tmo-phenyl esters was synthesized and tested in the GFP degradation assay (Figure 2b). In contrast to the P1 position, ClpXP tolerated various amino acid side chains in P2, ranging from hydrophobic groups, such as alanine (**41**) or methionine (**35**), to polar side chains including serine (**44**), glutamate (**45**) or arginine (**48**). This finding is in accordance with the lack of a well-defined S2 pocket, which allows ClpP to digest peptides with diverse amino acids in the P2 position.[11] However, as Boc-Gly-Tmo-OAr (**40**) did not inhibit ClpXP, a certain degree of sidechain interaction in the correct conformation is required for efficient binding to the protein.

We also identified structurally diverse compounds that did not inhibit, but rather stimulated the GFP-degradation by ClpXP. While the dipeptide Boc-(*S*)-Met-(*R*)-Tmo-OAr (**34**) led to a moderate increase of proteolysis up to a maximum of 150% at 10 μM, the acetylated (*R*)-Tmo phenyl ester **49** and Boc-(*R*)-Glu(OBn)-(*R*)-Tmo-OAr **50** activated the ClpXP complex to about 250% at 100 μM and 10 μM, respectively (Figure 2c). Interestingly, compound **51**, the *S*-enantiomer of **49**, hardly affected ClpXP (Figure S3c), and a benzyl-deprotected derivative of **50** (compound **45**) was a potent inhibitor of ClpXP (Figure 2b). In stark contrast to the activation of proteolysis, the three compounds did not stimulate the peptidase activity of ClpP alone, but rather showed moderate-to-good inhibition (depending on the buffer system used, compare Figure S3d-f), thus differentiating them from non-covalent peptide agonists of *Mycobacterium*

COMMUNICATION
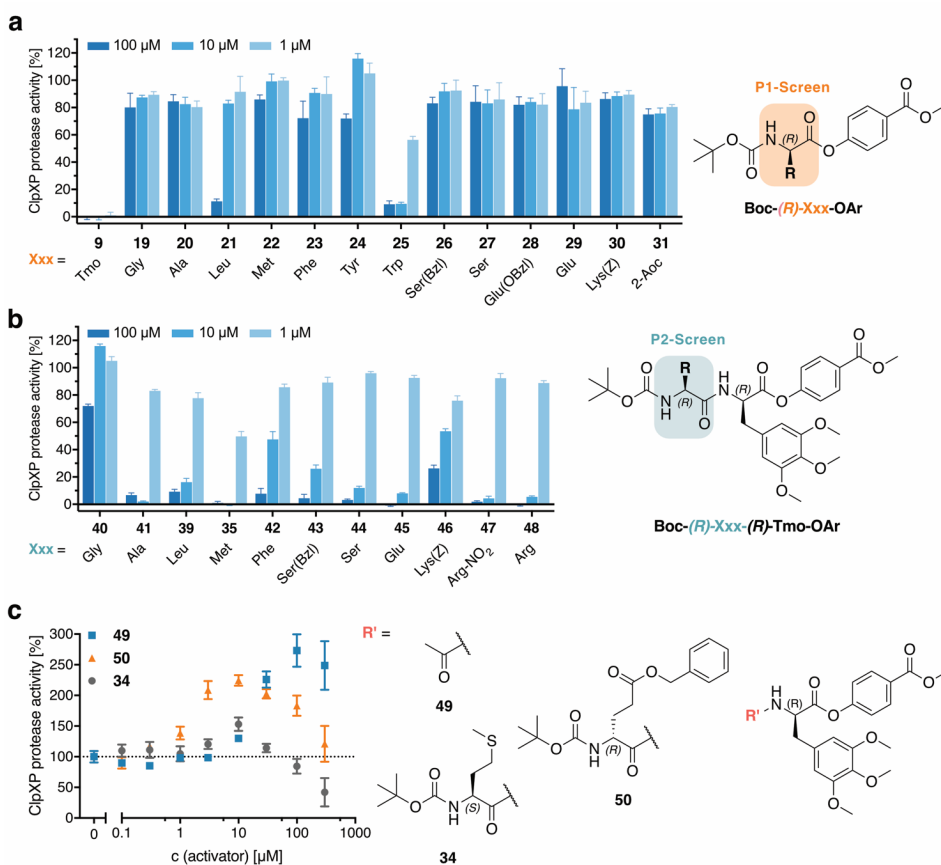
Figure 2. Inhibition of ClpXP activity by a set of Boc-protected (R)-amino acid- (a), and (R,R)-dipeptide- (b) phenyl esters. c) Stimulation of ClpXP-mediated proteolysis by selected phenyl ester compounds (mean and SD, n≥6).

formation of the $ClpX_6P_7$ complex (Figure S4b,d). This indicates that breakdown of the ClpXP complex is indispensable for full proteolytic arrest, providing important principles for inhibitor design.

To gain further insights into the origin of $ClpX_6P_7$ complex formation, we investigated the affinity between ClpP and ClpX. So far, apparent dissociation constants determined via activity-based assays have been the standard procedure for ClpP complexes.[15,17] All our efforts to directly quantify the interaction between ClpX and ClpP by isothermal titration calorimetry (ITC) and surface plasmon resonance (SPR) failed. We thus applied the switchSENSE® technology.[18] For the study of the ClpP-ClpX interaction, $ClpP_{14}$ was conjugated to a short DNA-strand and immobilized on a biochip. ClpX was titrated to the complex and the switching dynamics upon application of alternating current potentials were measured (Figure 3d). Applying this technology allowed, for the first time, the direct determination of the K_d-value of the ClpX and ClpP interaction (19.5 ± 3.2 µM, Figure 3e). This rather low affinity becomes reasonable when considering that in the bacterial cell ClpP is required to dynamically exchange between various cognate chaperones (in S. aureus ClpC and ClpX). Accordingly, the related HslUV protease complex, which does not have additional chaperone partners, has a lower K_d-value of 1 µM.[19] In line with compound-triggered formation of a stable $ClpX_6P_7$ complex, its corresponding dissociation constant was five-times lower compared to the native complex (K_d = 3.9 ± 0.4 µM) (Figure 3e). We next studied if the increased affinity between ClpP and ClpX influences the ATPase activity of the chaperone. Previous comprehensive studies showed that the ATPase activity of E. coli ClpX (EcClpX) is markedly reduced upon binding of ClpP.[17,20] We, however, found that S. aureus ClpX displays an intrinsically low activity, which is drastically enhanced in the presence of increasing equivalents of ClpP (Figure 3f). Hence, the catalytic activities of EcClpX and SaClpX are inversely regulated. Incubation of ClpXP with the inhibitor **35** reduced the ATPase activity of ClpX in a concentration-dependent manner, converging to the rate of ClpX alone (Figure 3g). Vice versa, compound **50**, a stimulator of ClpXP proteolysis, activated the ATPase activity of ClpX within the ClpXP complex in a similar way (Figure 3g). Both compound-induced alterations in ATP-turnover were solely mediated by binding to ClpP, as the compounds did not have an effect on ClpX alone (Figure S6). Accordingly, the ClpXP complex inhibited by **35** did not have any residual GFP-unfolding activity beyond the background level, as compared to the proteolytically inactive complex of the active site mutant ClpP(S98A) and ClpX
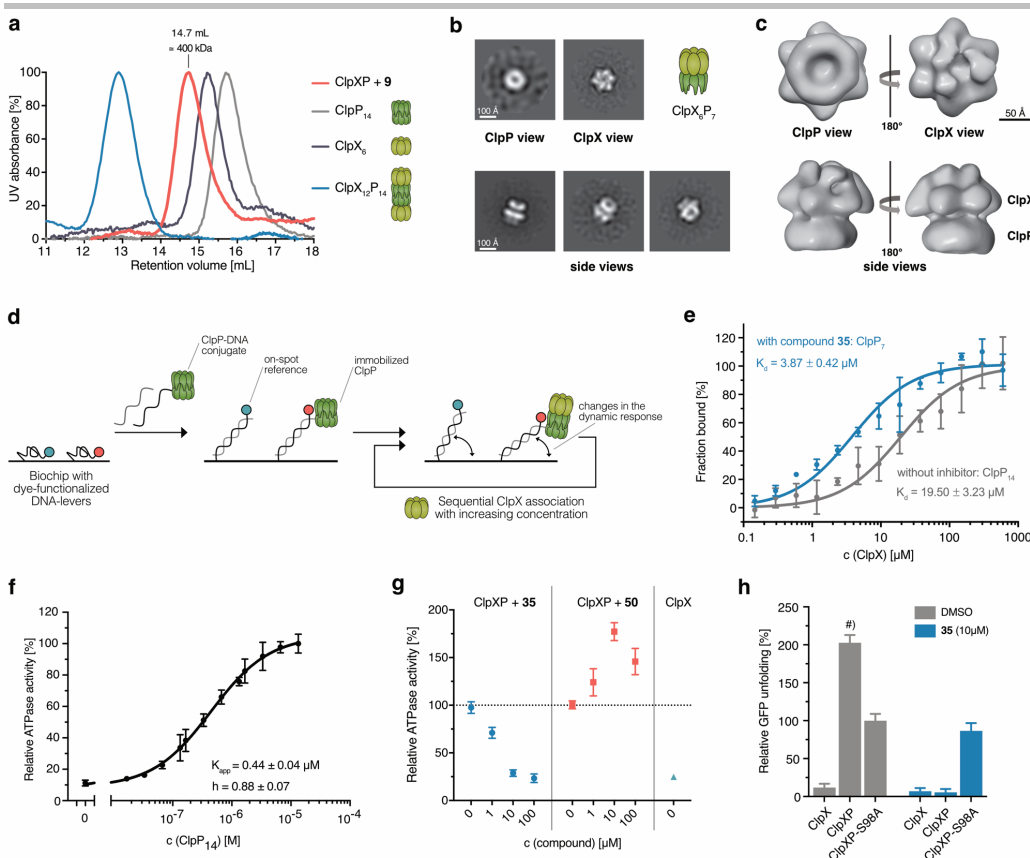
tuberculosis ClpP1P2.[14] This again highlights the conformational control of ClpX on the catalytic activity of ClpP. Of note, a similar small molecule-induced enhancement solely of proteolysis was previously described for the ClpXP2 complex from *Listeria monocytogenes*.[15]

As binding of compounds to ClpP can either retain the tetradecameric state or to induce disassembly into heptameric species, we determined the oligomeric state of the full ClpXP complex upon incubation with phenyl esters. Analytical size-exclusion chromatography (SEC) with ClpXP composed of an ATP-hydrolysis deficient ClpX(E183Q) mutant (to facilitate a stable complex assembly)[16] revealed that the strongly activating compounds **49** and **50** retain the native complex stoichiometry, namely $ClpX_{12}P_{14}$ (Figure S4a,c). In contrast, an unprecedented peak corresponding to a molecular weight of about 400 kDa was detected after incubation with saturating concentrations of inhibitor **9** (Figure 3a). Intact protein mass spectrometry (MS) of the peak fraction revealed the presence of ClpP modified by compound **9** and ClpX (Figure S5). Incubation of ClpP alone with compound **9** led to the formation of heptameric ClpP species (Figure S4d). Hypothesizing that the new complex might correspond to an unusual $ClpX_6P_7$ stoichiometry with a molecular weight of 435 kDa, we visualized the protein species of the peak fraction by negative-stain electron microscopy (EM). Indeed, 2D classification and 3D-reconstruction of the particles unambiguously revealed the presence of an unprecedented complex that consisted of one ClpX hexamer stacked on top of a catalytically inactive ClpP heptamer (Figure 3b,c). The disruption of both the peptidolytic and proteolytic system was observed for all potent amino acid phenyl esters tested, including the (S)-Boc-Tmo-OAr (**10**), which - although 25 times weaker than its (R)-enantiomer **9** - also induced heptamerization of ClpP and partial

COMMUNICATION

Figure 3. Investigation of an unprecedented $ClpX_6P_7$ complex. a) SEC revealed a novel complex upon incubation of $ClpX(E183Q)_{12}P_{14}$ with **9**. Negative-stain EM images of selected 2D class averages (b) and 3D reconstruction (c) verified the $ClpX_6P_7$ complex. d) Schematic representation of the switchSENSE® setup to determine the dissociation constants of ClpX and ClpP. e) Dissociation constants of ClpX and unmodified ClpP in its 14-mer, as well as in its **35**-treated heptameric state (mean and SD, n=5). f) Stimulation of the ATPase activity of *S. aureus* $ClpX_6$ (0.33 µM) upon titration of ClpP. g) Influence of inhibitor **35** as well as activator **50** on ATP turnover by ClpXP. Data for f-g: mean and SD, n=6. h) Residual GFP-unfolding activity of **35**-treated ClpXP as compared to unfolding by the proteolytically inactive ClpXP-S98A mutant (100% GFP unfolding).
#) Increased value due to GFP-degradation by proteolysis, not solely unfolding.

(Figure 3h). As a result, covalent binding of phenyl esters corrupts the interaction of the heptameric ClpP rings, inhibits proteolysis and at the same time strengthens the ClpP-ClpX interaction, while impairing chaperone activity.

Intrigued by the conformational arrest of ClpXP by compounds that are related to its natural substrates, we hypothesized that the trapped $ClpX_6P_7$ complex could resemble a state of biological relevance. While the pathway of substrate delivery is well understood, the mechanism by which cleaved peptide fragments are liberated from ClpXP is still unclear. Two main hypotheses for egress have been proposed based on the temporal kinking of the central E-helices of ClpP: 1) formation of equatorial pores, or 2) opening of the sequestered ClpP barrel by the collapse of the ClpXP complex.[21-24] The detection of the novel $ClpX_6P_7$ state demonstrates that disassembly of the protease into $ClpX_6P_7$-species, and not full disruption, would be sufficient for release. ClpX could thus remain bound to each heptamer, which would facilitate re-assembly of the catalytically active $ClpX_{12}P_{14}$ complex. With tools in hand that facilitate a controlled ClpXP disassembly (and slow re-assembly *via* hydrolysis of the acyl-enzyme intermediates), we established a pulldown-assay to determine, if the catalytic activity of ClpP or ClpXP was associated with a temporal collapse of the $ClpP_7$-$ClpP_7$ interaction. In this assay, we used two different $ClpP_{14}$ complexes either exclusively consisting of Strep-tagged ClpP (Strep-ClpP) or untagged ClpP. A possible exchange of both ClpP forms was analyzed by Strep-Tactin-based affinity purification and subsequent intact protein MS (Figure 4a). After demonstrating that mixed ClpP complexes can be formed by compound **9** and detected (Figure S7), experiments were conducted on the potential temporal de-oligomerization of 1) ClpP upon incubation with the peptide substrate Ac-Ala- hArg-2-Aoc-ACC and 2) ClpXP during

proteolysis of SsrA-tagged GFP. Untagged ClpP did not co-elute with Strep-ClpP, neither under the peptidase (Figure 4b) nor the protease assay (Figure 4c) conditions, suggesting that a temporal collapse of Clp(X)P in the context of its catalytic activity can most likely be excluded.

In conclusion, this work identified tailored chemical tools that efficiently probe the activity and oligomerization of the *S. aureus* ClpXP protease and unraveled a novel mechanism of inhibition. Screening of a comprehensive amino acid- and dipeptide phenyl ester library revealed potent inhibitors which possess a mirror-inverted stereochemistry as compared to ClpXP's natural protein substrates. In addition, we identified compounds that activate proteolytic turnover by binding to the catalytic site, which differentiates them from acyldepsipeptide(ADEP) activators that bind into apical hydrophobic pockets.[25] In-depth biochemical investigations revealed that inhibitor-binding induces the formation of an unprecedented $ClpX_6P_7$ complex. Within this complex, active site modification of ClpP is linked to an abrogation of the $ClpP_7$-$ClpP_7$ interaction and, more importantly, to an increased affinity between ClpP and ClpX. Significantly, binding of phenyl esters to ClpP subunits within $ClpX_6P_7$ not only stalls protein degradation, but also suppresses ClpX-mediated turnover of ATP (Figure 4d). This simultaneous inhibition of both enzyme activities leads to a sustainable blockage of the whole complex, surpassing previous strategies and guiding future design. While a biological function of the $ClpX_6P_7$ complex, including its hypothesized existence as an intermediate during proteolysis, could not be verified within the scope of this work, it is intriguing to speculate that bacterial metabolites regulate the activity of ClpXP in a similar manner as phenyl esters.

WILEY-VCH

COMMUNICATION
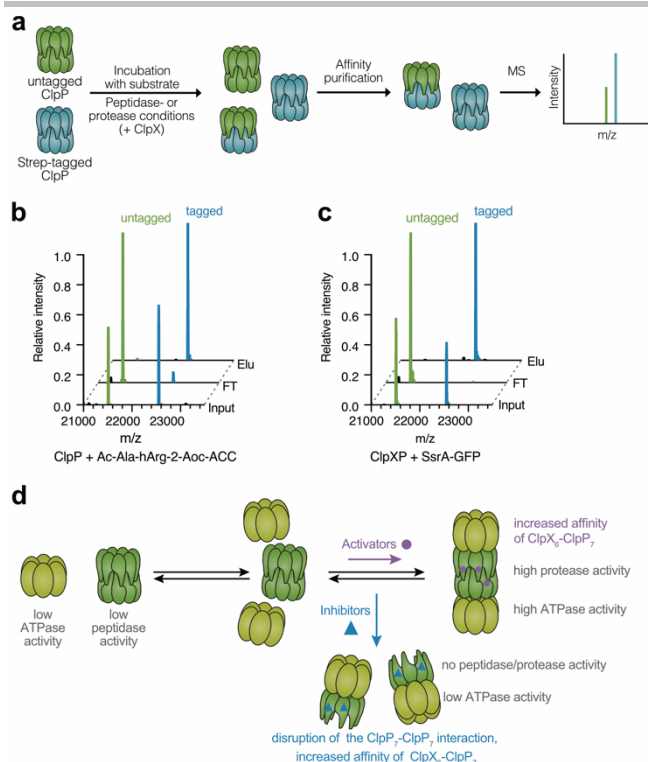
Figure 4. Putative temporal collapse of ClpP$_{14}$ and ClpX$_{12}$P$_{14}$ during peptidase and protease activity, respectively. a) Schematic representation of the experimental setup. b-c) Relative MS-intensities of untagged and tagged ClpP forms for respective fractions after affinity purification. For each experiment, one representative dataset from at least two replicates is shown. FT: Flow-through, Elu: Elution. d) Summary of the phenyl ester-induced structural and catalytic alterations of ClpXP.

Acknowledgements

[1] D. Frees, U. Gerth, H. Ingmer, *Int. J. Med. Microbiol.* **2014**, *304*, 142–149.
[2] T. A. Baker, R. T. Sauer, *Biochim. Biophys. Acta* **2012**, *1823*, 15–28.
[3] V. Bhandari, K. S. Wong, J. L. Zhou, M. F. Mabanglo, R. A. Batey, W. A. Houry, *ACS Chem. Biol.* **2018**, *13*, 1413–1425.
[4] C. Moreno-Cinos, E. Sassetti, I. G. Salado, G. Witt, S. Benramdane, L. Reinhardt, C. Durante Cruz, J. Joossens, P. Van der Veken, H. Brötz-Oesterhelt, et al., *J. Med. Chem.* **2018**, acs.jmedchem.8b01466.
[5] P. Gao, P. L. Ho, B. Yan, K. H. Sze, J. Davies, R. Y. T. Kao, *Proc. Natl. Acad. Sci. U.S.A.* **2018**, *115*, 8003–8008.
[6] T. Böttcher, S. A. Sieber, *J. Am. Chem. Soc.* **2008**, *130*, 14400–14401.
[7] M. W. Hackl, M. Lakemeyer, M. Dahmen, M. Glaser, A. Pahl, K. Lorenz-Baath, T. Menzel, S. Sievers, T. Böttcher, I. Antes, et al., *J. Am. Chem. Soc.* **2015**, *137*, 8475–8483.
[8] A. Pahl, M. Lakemeyer, M. T. Vielberg, M. W. Hackl, J. Vomacka, V. S. Korotkov, M. L. Stein, C. Fetzer, K. Lorenz-Baath, K. Richter, et al., *Angew. Chem. Int. Ed. Engl.* **2015**, *54*, 15892–15896.
[9] M. Gersch, R. Kolb, F. Alte, M. Groll, S. A. Sieber, *J. Am. Chem. Soc.* **2014**, *136*, 1360–1366.
[10] M. Gersch, K. Famulla, M. Dahmen, C. Göbl, I. Malik, K. Richter, V. S. Korotkov, P. Sass, H. Rübsamen-Schaeff, T. Madl, et al., *Nat. Commun.* **2015**, *6*, 6320.
[11] M. Gersch, M. Stahl, M. Poreba, M. Dahmen, A. Dziedzic, M. Drag, S. A. Sieber, *ACS Chem. Biol.* **2016**, *11*, 389–399.
[12] S. Gottesman, E. Roche, Y. Zhou, R. T. Sauer, *Genes Dev.* **1998**, *12*, 1338–1347.
[13] M. Gersch, F. Gut, V. S. Korotkov, J. Lehmann, T. Böttcher, M. Rusch, C. Hedberg, H. Waldmann, G. Klebe, S. A. Sieber, *Angew. Chem. Int. Ed. Engl.* **2013**, *52*, 3009–3014.
[14] K. R. Schmitz, R. T. Sauer, *Mol. Microbiol.* **2014**, *93*, 617–628.
[15] D. Balogh, M. Dahmen, M. Stahl, M. Poreba, M. Gersch, M. Drag, S. A. Sieber, *Chem. Sci.* **2017**, *8*, 1592–1600.
[16] G. L. Hersch, R. E. Burton, D. N. Bolon, T. A. Baker, R. T. Sauer, *Cell* **2005**, *121*, 1017–1027.
[17] R. E. Burton, T. A. Baker, R. T. Sauer, *Protein Sci.* **2003**, *12*, 893–902.
[18] A. Langer, P. A. Hampel, W. Kaiser, J. Knezevic, T. Welte, V. Villa, M. Maruyama, M. Svejda, S. Jähner, F. Fischer, et al., *Nat. Commun.* **2013**, *4*, 2099.
[19] M. K. Azim, W. Goehring, H. K. Song, R. Ramachandran, M. Bochtler, P. Goettig, *Protein Sci.* **2005**, *14*, 1357–1362.
[20] S. A. Joshi, G. L. Hersch, T. A. Baker, R. T. Sauer, *Nat. Struct. Mol. Biol.* **2004**, *11*, 404–411.
[21] M. S. Kimber, A. Y. H. Yu, M. Borg, E. Leung, H. S. Chan, W. A. Houry, *Structure* **2010**, *18*, 798–808.
[22] R. Sprangers, A. Gribun, P. M. Hwang, W. A. Houry, L. E. Kay, *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 16678–16683.
[23] M. Gersch, A. List, M. Groll, S. A. Sieber, *J. Biol. Chem* **2012**, *287*, 9484–9494.
[24] K. R. Schmitz, D. W. Carney, J. K. Sello, R. T. Sauer, *Proc. Natl. Acad. Sci. U.S.A.* **2014**, *111*, E4587–95.
[25] B.-G. Lee, E. Y. Park, K.-E. Lee, H. Jeon, K. H. Sung, H. Paulsen, H. Rübsamen-Schaeff, H. Brötz-Oesterhelt, H. K. Song, *Nat. Struct. Mol. Biol.* **2010**, *17*, 471–478.

WILEY-VCH

COMMUNICATION

Entry for the Table of Contents

COMMUNICATION

Tight and loose: Covalent (*R*)-amino acid modifiers are novel tools for probing the activity and oligomerization of the bacterial ClpXP protease. Sub-stochiometric binding strengthens the ClpX-ClpP interaction. Depending on the compound's substitution, proteolysis is either stimulated, or efficiently inhibited by formation of an unprecedented complex assembly.
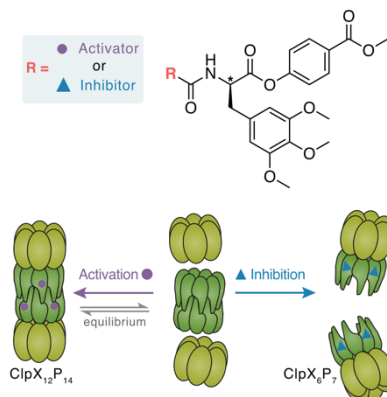
Dr. Markus Lakemeyer, Eva Bertosin, Dr. Friederike Möller, Dóra Balogh, Dr. Ralf Strasser, Prof. Dr. Hendrik Dietz, Prof. Dr. Stephan A. Sieber

Page No. – Page No.

Tailored peptide phenyl esters block ClpXP proteolysis by an unusual breakdown into a heptamer-hexamer assembly