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Supporting Information

Tailored Phenyl Esters Inhibit ClpXP and Attenuate *Staphylococcus aureus* α-Hemolysin Secretion

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Supplementary Scheme 1: Modular synthesis of phenyl esters via Steglich esterification of various carboxylic acids and phenyl alcohols

Supplementary Figure 1: (A) Chemical structure of peptide Ac-Ala-hArg-2-Aoc-ACC (ACC) for peptidase assays. Cleavage of ACC substrate can be monitored via fluorescence measurements (380 nm/440 nm). (B) Chemical structure of compounds MAS-17 and MAS-20 that have been tested in the peptidase assay.
**Supplementary Figure 2:** Investigation of degradation of a fluorogenic substrate (ACC, final concentration 200 µM) at 37 °C. Remaining ClpP-activity is shown upon incubation of SaClpP (final ClpP14-concentration: 10 nM) with 100 nM of corresponding compounds compared to a DMSO control (100 % activity). The experiment was performed in at least 2 independent occasions (n = 2) in triplicates.

**Supplementary Figure 3:** Investigation of inhibitory activity of MAS-50 and MAS-83 upon incubation with detergents compared to MAS-19 by monitoring degradation of ssrA-tagged eGFP (final concentration 300 nM). ClpXP was pre-incubated with respective compounds at 100 µM at 32 °C with and without added detergents. Relative ClpXP-activity is shown upon incubation of SaClpXP (final concentrations: 100 nM ClpP14, 200 nM ClpX6) with 0.1% NP-40 (dark blue), 0.1% Tween-20 (petrol) and without addition of detergent (light blue) of corresponding compounds compared to a DMSO control. The experiment was performed in at least two independent occasions (n = 2) in triplicates, the bars represent the mean of all 6 replicates, the error bars are SEM.
II Methodology

1 Chemistry

1.1 General Methods

All air- and moisture sensitive reactions were performed in oven-dried glassware and under an argon atmosphere. Solutions and dry solvents were handled in previously argon flushed plastic syringes. Solids were added in argon counter stream.

The reactions were heated with paraffin baths, where the temperature was set and monitored with adjustable contact thermometers.

Solvents and Reagents

All chemicals and reagents were purchased from commercial sources (Sigma-Aldrich Co. LLC, Thermo Fisher Scientific Inc., Merck KGaA, ABCR GmbH & Co.KG, TCI Europe GmbH, Johnson Matthey Plc).

Anhydrous solvents were prepared according to processes known from literature or obtained from commercial sources (Sigma-Aldrich Co. LLC, Thermo Fisher Scientific Inc., VWR International, LLC).

Chromatography

Analytical thin layer chromatography (TLC) was performed on silica gel plates (Merck KGaA). Short wavelength UV light (254 nm), potassium permanganate and ninhydrin solutions were used to visualize the components.

For the flash column chromatography silica gel (silica gel 60, 40-63 mm, Merck KGaA) was used.

Preparative reversed-phase HPLC separation was performed on a Waters 2695 quaternary gradient module, equipped with an X-BridgeTM Prep C18 5 µm OBDM (30 x 150 mm) column, a Waters 2998 PDA detector and a Waters Fraction Collector III. Eluents for analytical and preparative RP-HPLC were 0.1% (v/v) TFA in water (buffer A) and 0.1% (v/v) TFA in acetonitrile (buffer B).
NMR-Spectroscopy

$^1$H-NMR and $^{13}$C-NMR experiments were measured on Bruker AV-HD300, AV-HD400 and AV-HD500 spectrometers with CDCl$_3$ and $d_6$-DMSO as solvents at 300 K. The signals refer to the residual proton signals of the deuterated solvent:

- CDCl$_3$: $\delta$ ($^1$H) = 7.26 ppm, $\delta$ ($^{13}$C) = 77.16 ppm
- $d_6$-DMSO: $\delta$ ($^1$H) = 2.50 ppm, $\delta$ ($^{13}$C) = 39.52 ppm
- CD$_3$OD: $\delta$ ($^1$H) = 3.31, 4.87 ppm, $\delta$ ($^{13}$C) = 49.00 ppm

The chemical shifts are given in $\delta$ -values in parts per million (ppm), the coupling constants $J$ in Hertz (Hz). The NMR spectra were processed using MestReNova7. The following abbreviations were used to describe the multiplicities of the signals: s (singlet), d (doublet), t (triplet), q (quartet), p (quintet), m (multiplet).

Mass spectrometry

Mass spectra for reaction controls were recorded on an MSQ PlusTM coupled to an Dionex Ultimate 3000 HPLC system (Thermo Fisher Scientific Inc.). MS data were evaluated with Thermo Xcalibur 2.1.

Mass spectra for reaction controls and compound stability assays in mouse plasma were recorded on an LCQ-Fleet coupled to an Dionex Ultimate 3000 HPLC system (Thermo Fisher Scientific Inc.). MS data were evaluated with Thermo Xcalibur 2.1.

Reversed-phase HPLC-HR-ESI-MS, HPLC-HR-APCI-MS mass spectra were recorded on a LTQ-Orbitrap XL mass spectrometer (Thermo Scientific) coupled to a Dionex UltiMate 3000 HPLC system. A Waters XBridge C18 column (3.5 µm, 4.6 x 100 mm, flow rate = 1.1 ml/min) was used for separation of analytes. The column temperature was maintained at 30 °C. The mobile phase for elution consisted of a gradient mixture of 0.1% (v/v) formic acid in water (buffer A) and 0.1% (v/v) formic acid in acetonitrile:water 90:10 (buffer B). MS data were evaluated with Thermo Xcalibur 2.1. Reversed-phase HPLC-HR-ESI-MS, HPLC-HR-APCI-MS mass spectra for MAS-26 and MAS-83 were recorded on SynaptXS mass spectrometer (Waters) coupled to a ACQUITY Premier HPLC system (Waters). A Waters XBridge C18 column (3.5 µm, 4.6 x 100 mm, flow rate = 0.3 ml/min) was used for separation of analytes. The column temperature was maintained at 60 °C. The mobile phase for elution consisted of a
gradient mixture of 0.1% (v/v) formic acid in water (buffer A) and 0.1% (v/v) formic acid in acetonitrile (buffer B). MS data were evaluated with Waters Masslynx V4.2.

1.2 Synthesis

Steglich Esterification

General Procedure 1

Steglich esterifications are performed according to general procedure 1 (GP 1). Here, each acid (1.5 eq.) is mixed with the respective alcohol (1.0 eq.). DMAP (0.1 eq.) is added and the educts are dissolved in DCM to a final concentration of 250 mM before EDC*HCl (1.0 eq.) is added. The reaction mixture is stirred overnight at room temperature before it is diluted with DCM (10 mL) and washed with water (3 × 5 mL) and brine (1 × 5 mL). The organic phase is dried over Na$_2$SO$_4$ and the solvent is removed under reduced pressure. The crude product is purified by column chromatography.

General Procedure 2

Steglich esterifications are performed according to general procedure 2 (GP 2). Here, each alcohol (1.5 eq.) is mixed with the respective acid (1.0 eq.). DMAP (0.1 eq.) is added and the educts are dissolved in DCM to a final concentration of 250 mM before EDC*HCl (1.0 eq.) is added. The reaction mixture is stirred overnight at room temperature before it is diluted with DCM (10 mL) and washed with water (3 × 5 mL) and brine (1 × 5 mL). The organic phase is dried over Na$_2$SO$_4$ and the solvent is removed under reduced pressure. The crude product is purified by column chromatography.
1.2.1 Synthesis of isopropyl 4-(benzoyloxy)benzoate (MAS-14)

MAS-14 was synthesized based on GP 1, starting with 101 mg (830 µmol) of benzoic acid (10) and 230 mg (1.25 mmol) isopropyl-4-hydroxybenzoate (11). MAS-14 is isolated after purification by column chromatography (0-2% EtOAc/Hexane) with a yield of 87% (721 µmol, 205 mg) as a white solid.

$^1$H-NMR (400 MHz, CDCl$_3$, 300 K): $\delta$ [ppm] = 8.25 – 8.17 (m, 2H), 8.16 – 8.09 (m, 2H), 7.70 – 7.62 (m, 1H), 7.57 – 7.48 (m, 2H), 7.34 – 7.27 (m, 2H), 5.27 (hept, $^3$J = 6.2 Hz, 1H), 1.38 (d, $^3$J = 6.2 Hz, 6H).

$^{13}$C-NMR (101 MHz, CDCl$_3$, 300 K): $\delta$ [ppm] = 165.3, 164.7, 154.5, 133.8, 133.8, 131.1, 131.1, 130.3, 130.3, 129.2, 128.7, 128.7, 121.6, 121.6, 68.5, 22.0, 22.0.

TLC $R_f$ = 0.26 (2% EtOAc/Hexane) [UV/KMnO$_4$].

HRMS for C$_{17}$H$_{16}$O$_4$ [M+H]$^+$ calcd. 285.1121, found 285.1121.
1.2.2 Synthesis of 4-(isopropoxycarbonyl)phenyl 4-methylbenzoate (MAS-15)

MAS-15 was synthesized based on GP 1, starting with 113 mg (830 µmol) of 4-methylbenzoic acid (12) and 230 mg (1.25 mmol) isopropyl-4-hydroxybenzoate (11). MAS-15 is isolated after purification by column chromatography (0-2% EtOAc/Hexane) with a yield of 84% (208 mg, 697 µmol) of a white solid.

$^1$H-NMR (400 MHz, CDCl$_3$, 300 K): δ [ppm] = 8.19 – 7.99 (m, 4H), 7.36 – 7.27 (m, 4H), 5.26 (hept, $^3$J = 6.3 Hz, 1H), 2.46 (s, 3H), 1.38 (d, $^3$J = 6.2 Hz, 6H).

$^{13}$C-NMR (101 MHz, CDCl$_3$, 300 K): δ [ppm] = 165.5, 164.9, 154.7, 144.9, 131.3, 131.3, 130.4, 130.4, 129.5, 129.5, 128.6, 126.5, 121.8, 121.8, 68.7, 22.1, 22.1, 21.9.

TLC $R_f$ = 0.26 (2% EtOAc/Hexane) [UV/KMnO$_4$].

HRMS for C$_{18}$H$_{18}$O$_4$ [M+H]$^+$ calcd. 299.1278, found 299.1273.
1.2.3 Synthesis of 4-(isopropoxycarbonyl)phenyl 4-chlorobenzoate (MAS-16)

MAS-16 was synthesized based on GP 1, starting with 130 mg (830 µmol) of 4-chlorobenzoic acid (13) and 230 mg (1.25 mmol) isopropyl-4-hydroxybenzoate (11). MAS-16 is isolated after purification by column chromatography (0-2% EtOAc/Hexane) with a yield of 87% (230 mg 722 µmol) of a white solid.

$\text{C}_{17}\text{H}_{15}\text{ClO}_4$ $\text{M}^+ = 319.07$ Da

$\text{MW} = 318.75$ Da

$1^H\text{NMR}$ (300 MHz, CDCl$_3$, 300 K): $\delta$ [ppm] = 8.18 – 8.08 (m, 4H), 7.53 – 7.46 (m, 2H), 7.33 – 7.26 (m, 2H), 5.27 (hept, $^3J = 6.2$ Hz, 1H), 1.38 (d, $^3J = 6.2$ Hz, 6H).

$13^C\text{NMR}$ (75 MHz, CDCl$_3$, 300 K): $\delta$ [ppm] = 165.4, 164.0, 154.4, 140.6, 131.8, 131.8, 131.3, 131.3, 129.2, 129.2, 128.9, 127.8, 121.7, 121.7, 68.8, 22.1, 22.1.

$\text{TLC}$ $R_f = 0.29$ (2% EtOAc/Hexane) [UV/KMnO$_4$].

$\text{HRMS}$ for $\text{C}_{17}\text{H}_{15}\text{ClO}_4$ [M+H]$^+$ 319.0732 calcd, found 319.0727.
1.2.4 Synthesis of isopropyl 4-((3-methylbutanoyl)oxy)benzoate (MAS-17)

MAS-17 was synthesized based on GP 1, starting with 85.0 mg (94.0 µL, 830 µmol) of isopentanoic acid (14) and 230 mg (1.25 mmol) isopropyl-4-hydroxybenzoate (11). MAS-17 is isolated after purification by column chromatography (0-2% EtOAc/Hexane) with a yield of 67% (147 mg, 557 µmol) of a clear oil.

$^1$H-NMR (400 MHz, CDCl$_3$, 300 K): $\delta$ [ppm] = 8.10 – 8.03 (m, 2H), 7.19 – 7.11 (m, 2H), 5.25 (hept, $^3$J = 6.3 Hz, 1H), 2.45 (d, $^3$J = 7.1 Hz, 2H), 2.25 (m, 1H), 1.36 (d, $^3$J = 6.2 Hz, 6H), 1.06 (d, $^3$J = 6.6 Hz, 6H).

$^{13}$C-NMR (101 MHz, CDCl$_3$, 300 K): $\delta$ [ppm] = 171.0, 165.3, 154.2, 131.1, 131.1, 128.4, 121.5, 121.5, 68.5, 43.3, 25.8, 22.4, 22.4, 22.0, 22.0.

TLC $R_f$ = 0.25 (2% EtOAc/Hexane) [UV/KMnO$_4$].

HRMS for C$_{15}$H$_{20}$O$_4$ [M+H]$^+$ calcd 265.1434, found 265.1435.
1.2.5 Synthesis of 1-(tert-butyl) 4-(4-(isopropoxycarbonyl)phenyl) piperidine-1,4-dicarboxylate (MAS-19)

**MAS-19** was synthesized based on GP 2, starting with 287 mg (1.25 mmol) of Boc-Inp-OH (15) and 150 mg (830 µmol) isopropyl-4-hydroxybenzoate (11). **MAS-19** is isolated after purification by column chromatography (2-10% EtOAc/Hexane) with a yield of 84% (273 mg, 697 µmol) of a white solid.

**1H-NMR** (400 MHz, CDCl₃, 300 K): δ [ppm] = 8.12 – 8.02 (m, 2H), 7.17 – 7.08 (m, 2H), 5.24 (hept, 3J = 6.3 Hz, 1H), 4.17 – 4.03 (m, 2H), 3.00 – 2.85 (m, 2H), 2.80 – 2.64 (m, 1H), 2.12 – 1.97 (m, 2H), 1.86 – 1.70 (m, 2H), 1.47 (s, 9H), 1.36 (d, 3J = 6.2 Hz, 6H).

**13C-NMR** (101 MHz, CDCl₃, 300 K): δ [ppm] = 172.6, 165.3, 154.7, 154.1, 131.1, 131.1, 128.6, 121.4, 121.4, 79.8, 68.6, 43.0, 42.0, 41.3, 28.5, 28.5, 27.9, 27.9, 27.9, 21.9, 21.9.

**TLC** Rf = 0.25 (10% EtOAc/Hexane) [UV/KMnO₄].

**HRMS** for C₂₁H₂₉NO₆ [M-Boc+H]+ calcd. 292.1543, found 292.1541.
1.2.6 Synthesis of isopropyl 4-(propionyloxy)benzoate (MAS-20)

MAS-20 was synthesized based on GP 1, starting with 62.0 mg (62 µL, 830 µmol) of propanoic acid (16) and 230 mg (1.25 mmol) isopropyl-4-hydroxybenzoate (11). MAS-20 is isolated after purification by column chromatography (0-3% EtOAc/Hexane) with a yield of 85% (166 mg, 703 µmol, 85%) of a colorless liquid.

\[\text{\textsuperscript{1}H-NMR} \text{ (400 MHz, CDCl\textsubscript{3}, 300 K): } \delta [\text{ppm}] = \text{8.11 - 8.00 (m, 2H), 7.20 - 7.10 (m, 2H), 5.24 (hept, } J = 6.3 \text{ Hz, 1H), 2.61 (q, } J = 7.5 \text{ Hz, 2H), 1.36 (d, } J = 6.2 \text{ Hz, 6H), 1.27 (t, } J = 7.5 \text{ Hz, 3H).}\]

\[\text{\textsuperscript{13}C-NMR} \text{ (101 MHz, CDCl\textsubscript{3}, 300 K): } \delta [\text{ppm}] = \text{172.4, 165.3, 154.3, 131.1, 131.1, 128.4, 121.5, 121.5, 68.5, 27.8, 22.0, 22.0, 9.0.}\]

\[\text{TLC } R_f = 0.3 \text{ (3% EtOAc/Hexane) [UV/KMnO}_4\text{].}\]

\[\text{HRMS for } C_{13}H_{16}O_4 [M+H]^+ \text{ calcd. 237.1121, found 237.1117.}\]

1.2.7 Synthesis of 4-(isopropoxycarbonyl)phenyl 3-(fluorosulfonyl)benzoate (MAS-21)

MAS-21 was synthesized based on GP 1, starting with 170 mg (830 µmol) of 3-(fluorosulfonyl)-benzoic acid (17) and 230 mg (1.25 mmol) isopropyl-4-hydroxybenzoate (11). MAS-21 is isolated after purification by column chromatography (0-15% EtOAc/Hexane) with a yield of 72% (219 mg, 598 µmol) of a white solid.

\[ \text{MAS-21} \]

**1H-NMR** (400 MHz, CDCl₃, 300 K): \( \delta [\text{ppm}] = 8.84 \ (t, \ ^{4}J = 1.5 \text{ Hz}, \ 1\text{H}), 8.60 \ (dt, \ ^{3}J = 7.9, \ ^{4}J = 1.5 \text{ Hz}, \ 1\text{H}), 8.30 \ (dt, \ ^{3}J = 7.9, \ ^{4}J = 1.5 \text{ Hz}, \ 1\text{H}), 8.22 - 8.07 \ (m, \ 2\text{H}), 7.84 \ (t, \ ^{3}J = 7.9 \text{ Hz}, \ 1\text{H}), 7.35 - 7.29 \ (m, \ 2\text{H}), 5.27 \ (\text{hept, } ^{3}J = 6.2 \text{ Hz}, \ 1\text{H}), 1.39 \ (d, \ ^{3}J = 6.2 \text{ Hz}, \ 6\text{H}).

\[ \text{13C-NMR} \] (101 MHz, CDCl₃, 300 K): \( \delta [\text{ppm}] = 165.3, 162.5, 153.9, 137.0, 134.4, 134.2, 133.1, 131.5, 131.4, 130.5, 130.3, 129.4, 121.5, 121.5, 68.9, 22.1, 22.1.

**TLC** \( R_{f} = 0.42 \) (15% EtOAc/Hexane) [UV/KMnO₄].

**HRMS** for C₁₁H₁₅O₆S [M+H]⁺ calcd. 367.0646, found 367.0650.
1.2.8 Synthesis of tert-butyl(hexyloxy)dimethylsilane (MAS-25)

Hexan-1-ol (3) (511 mg, 623 µL, 5.00 mmol, 1.0 eq.), TBDMSCl (904 mg, 6.00 mmol, 1.2 eq.) and imidazole (851 mg, 12.5 mmol, 2.5 eq.) are dissolved in 2 mL MeCN and stirred overnight at room temperature. The solvent is removed under reduced pressure and the crude product is redissolved in pentane (5 mL). Non-soluble solids are removed, the organic phase is washed with HCl (1 M, 3 × 5 mL) as well as water (1 × 5 mL) and dried over MgSO₄. The crude product is purified by column chromatography (0-2% EtOAc/hexane). **MAS-25** is isolated with a yield of 48% (522 mg, 2.41 mmol) of a colorless oil.

**¹H-NMR** (400 MHz, CDCl₃, 300 K): δ [ppm] = 3.60 (t, $^3J = 6.6$ Hz, 2H), 1.57 – 1.46 (m, 2H), 1.36 – 1.24 (m, 6H), 0.89 (s, 9H), 0.89 (s, 3H), 0.05 (s, 6H).

**¹³C-NMR** (101 MHz, CDCl₃, 300 K): δ [ppm] = 63.5, 33.0, 31.8, 26.2, 26.2, 25.6, 22.8, 18.6, 14.2, -5.1, -5.1, -5.1.

**TLC** $R_f = 0.86$ (2% EtOAc/Hexane) [UV/KMnO₄].
**1.2.9 Synthesis of 4-(isopropoxycarbonyl)phenyl 3-((hexyloxy)sulfonyl)benzoate (MAS-26)**

**MAS-21** (73.0 mg, 200 µmol, 1.0 eq.) and **MAS-25** (44.0 mg, 300 µmol, 1.5 eq.) are dissolved in MeCN (1 mL) at 0 °C before 60 µL DBU (61.0 mg, 400 mmol, 2.0 eq.) are added dropwise. The reaction mixture is stirred for 3 h at RT before 10 mL water are added. The aqueous solution is extracted with EtOAc (3 × 5 mL) and the combined organic phases are washed with brine and dried over Na$_2$SO$_4$. The solvent is removed under reduced pressure and the crude product is purified by column chromatography (0-9% EtOAc/hexane). **MAS-26** is isolated with a yield of 25% (22 mg, 50 µmol) of a colorless oil.

**1H-NMR** (400 MHz, CDCl$_3$, 300 K): δ [ppm] = 8.52 (t, $^4J = 1.8$ Hz, 1H), 8.34 (dt, $^3J = 7.8$, $^4J = 1.4$ Hz, 1H), 8.06 – 7.92 (m, 3H), 7.63 (t, $^3J = 7.9$ Hz, 1H), 7.15 – 7.02 (m, 2H), 5.22 (hept, $^3J = 6.3$ Hz, 1H), 4.35 (t, $^3J = 6.8$ Hz, 2H), 1.77 (p, $^3J = 6.9$ Hz, 2H), 1.47 – 1.38 (m, 2H), 1.37 – 1.30 (m, 4H), 1.35 (d, $^3J = 6.3$ Hz, 6H), 0.95 – 0.86 (m, 3H).

**13C-NMR** (101 MHz, CDCl$_3$, 300 K): δ [ppm] = 165.0, 164.6, 152.6, 136.0, 135.4, 132.3, 132.2, 131.5, 130.1, 129.7, 129.5, 122.2, 77.5, 77.2, 76.84, 69.1, 66.3, 31.5, 28.7, 25.8, 22.7, 22.0, 14.1.

**TLC** $R_f = 0.22$ (7% EtOAc/Hexane) [UV/KMnO$_4$].

**HRMS** for C$_{23}$H$_{28}$O$_7$S [M+H]$^+$ calcd. 449.1629, found 449.1631.
1.2.10 Synthesis of 4-(isopropoxycarbonyl)phenyl piperidine-4-carboxylate (MAS-27)

**MAS-19** (30.0 mg, 7.70 µmol, 1.0 eq.) is solved in a 50:50-mixture of TFA and DCM (380 µL each). The reaction mixture is stirred at RT for 45 minutes before the solvent and TFA is removed under reduced pressure. The crude product is purified by HPLC using the following gradient ($t_R = 6.8$ min, $\lambda = 234$ nm).

![HPLC gradient](image)

| t [min] | %-H$_2$O | %-ACN |
|---------|-----------|-------|
| 0       | 98        | 2     |
| 1       | 90        | 10    |
| 12      | 2         | 98    |
| 14      | 2         | 98    |
| 15      | 98        | 2     |
| 17      | 98        | 2     |

The acetonitrile/water mixture is lyophilized to yield 80% (20.2 mg, 6.93 µmol,) of a fluffy white solid.

**$^1$H-NMR** (400 MHz, CDCl$_3$, 300 K): $\delta$ [ppm] = 8.12 – 8.04 (m, 2H), 7.18 – 7.11 (m, 2H), 5.25 (hept, $^3J = 6.3$ Hz, 1H), 3.67 – 3.43 (m, 2H), 3.26 – 3.06 (m, 2H), 2.99 – 2.89 (m, 1H), 2.46 – 2.09 (m, 4H), 1.93 (m, 1H), 1.37 (d, $^3J = 6.3$, 1.0 Hz, 6H).

**$^{13}$C-NMR** (101 MHz, CDCl$_3$, 300 K): $\delta$ [ppm] = 170.5, 165.3, 158.5, 131.3, 131.2, 128.9, 121.3, 121.2, 68.9, 43.1, 43.1, 40.6, 38.2, 38.2, 21.9, 21.9.

**HRMS** for C$_{16}$H$_{21}$NO$_4$ [M+H]$^+$ calcd. 292.1543, found 292.1536.
1.2.11 Synthesis of 1-benzyl 4-(4-(isopropoxycarbonyl)phenyl) piperidine-1,4-dicarboxylate (MAS-30)

MAS-27 (120 mg, 690 µmol, 1.0 eq.) and Na$_2$CO$_3$ (44.0 mg, 690 mmol, 1.0 eq.) are dissolved in DCM (2 mL) and cooled to 0 °C. CbzCl (77.0 mg, 65 µL, 760 µmol, 1.2 eq.) is solved in DCM (1 mL) and added dropwise before the reaction mixture is stirred at RT overnight and filtered through celite. The solvent is removed under reduced pressure and the crude product is dissolved in Et$_2$O, washed with HCl (2 M), dried over Na$_2$SO$_4$, and purified by column chromatography (10-20% EtOAc/hexane). MAS-30 is isolated with a yield of 22% (64 mg, 150 µmol) of a colorless oil.

$^1$H-NMR (400 MHz, CDCl$_3$, 300 K): δ [ppm] = 8.10 – 8.03 (m, 2H), 7.40 – 7.30 (m, 5H), 7.16 – 7.09 (m, 2H), 5.24 (hept, $^3$J = 6.2 Hz, 1H), 5.15 (s, 2H), 4.26 – 4.11 (m, 2H), 3.17 – 2.94 (m, 2H), 2.80 – 2.69 (m, 1H), 2.13 – 2.00 (m, 2H), 1.89 – 1.72 (m, 2H), 1.36 (d, $^3$J = 6.2 Hz, 6H).

$^{13}$C-NMR (101 MHz, CDCl$_3$, 300 K): δ [ppm] = 172.4, 165.2, 157.7, 154.0, 136.7, 131.1, 131.1, 128.5, 128.5, 128.1, 128.1, 128.0, 127.9, 127.9, 121.3, 121.3, 68.6, 67.3, 43.2, 43.2, 41.1, 27.8, 21.9, 21.9.

TLC $R_f$ = 0.24 (20% EtOAc/Hexane) [UV/KMnO$_4$].

HRMS for C$_{24}$H$_{27}$NO$_6$ [M+H]$^+$ calcd. 426.1911, found 426.1906.
MAS-33 was synthesized based on GP1, starting with 72.0 mg (320 µmol) of Boc-Inp-OH (15) and 28 mg (210 mmol) of MAS-32. MAS-33 is isolated after purification by column chromatography (0-10% EtOAc/Hexane) with a yield of 42% (47.0 mg, 135 µmol) of a white solid.

\[ \text{1H-NMR (400 MHz, CDCl}_3, 300 K): \delta [ppm] = 6.98 – 6.88 (m, 2H), 6.81 – 6.69 (m, 2H), 4.16 – 3.99 (m, 2H), 2.93 (s, 6H), 2.91 – 2.84 (m, 2H), 2.67 (tt, \ J = 10.9, 3J =3.9 Hz, 1H), 2.08 – 1.95 (m, 2H), 1.84 – 1.68 (m, 2H), 1.46 (s, 9H). \]

\[ \text{13C-NMR (75 MHz, CDCl}_3, 300 K): \delta [ppm] = 173.7, 154.8, 148.3, 142.5, 121.9, 121.9, 114.0, 114.0, 79.7, 43.2, 43.1, 41.4, 41.3, 41.3, 28.6, 28.6, 28.6, 28.6, 28.1, 28.1. \]

\[ \text{TLC } R_f = 0.26 \text{ (10% EtOAc/Hexane) [UV/KMnO}_4]. \]

\[ \text{HRMS for C}_{19}\text{H}_{28}\text{N}_{2}\text{O}_4 [M+H]^+ \text{ calcd. 349.2122, found 349.2118.} \]
1.2.13 Synthesis of 1-(tert-butyl) 4-(4-(2-oxopyrrolidin-1-yl)phenyl) piperidine-1,4-dicarboxylate (MAS-50)

MAS-50 was synthesized based on GP1, starting with 35.0 mg (150 µmol) of Boc-Inp-OH (15) and 18.0 mg (100 µmol) of MAS-48. MAS-50 is isolated after purification by column chromatography (0-1% MeOH/DCM) with a yield of 90% (35.0 mg, 90 µmol) of a white solid.

\(^1\)H-NMR (400 MHz, CDCl\(_3\), 300 K): \(\delta [ppm] = 7.66 – 7.57 (m, 2H), 7.09 – 7.00 (m, 2H), 4.15 – 3.98 (m, 2H), 3.82 (t, \(^3J = 7.0\) Hz, 2H), 2.96 – 2.82 (m, 2H), 2.74 – 2.62 (m, 1H), 2.58 (t, \(^3J = 8.0\) Hz, 2H), 2.13 (p, \(^3J = 7.6\) Hz, 2H), 2.05 – 1.95 (m, 2H), 1.82 – 1.67 (m, 2H), 1.45 (s, 9H).

\(^13\)C-NMR (101 MHz, CDCl\(_3\), 300 K): \(\delta [ppm] = 174.3, 173.2, 154.7, 147.0, 137.2, 121.7, 121.73, 120.8, 120.8, 79.7, 48.9, 43.1, 41.2, 41.2, 32.7, 28.5, 28.5, 28.5, 28.0, 28.0, 18.0.

TLC \(R_f = 0.49\) (1% MeOH/DCM) [UV/KMnO\(_4\)].

HRMS for C\(_{21}\)H\(_{28}\)N\(_2\)O\(_5\) [M-Boc+H]\(^+\) calcd. 289.1547, found 289.1543.
1.2.14 Synthesis of 4-(dimethylamino)benzoyl chloride (MAS-51)

4-Dimethylamino benzoic acid (9) (109 mg, 660 µmol, 1.0 eq.) is suspended in DCM (2 mL) and cooled to 0 °C. 63 µL oxalyl chloride (93 mg, 730 µmol, 1.1 eq.) is added dropwise and the reaction mixture is stirred for 2.5 hours at 0 °C, before the solvent is removed under reduced pressure. Due to high reactivity, full conversion is assumed, and MAS-51 is converted in the next step without any further purification.
1.2.15 Synthesis of 4-(2-methoxy-2-oxoethyl)phenyl 4-(dimethylamino)-benzoate (MAS-53)

**MAS-52** (85.0 mg, 510 µmol, 1.0 eq.) and 105 µL NEt$_3$ (77.0 mg, 760 µmol, 1.5 eq.) are solved in DCM (600 µL) before **MAS-51** (103 mg, 560 µmol, 1.1 eq.) is added dropwise. The reaction mixture is stirred at RT overnight before the solvent is removed under reduced pressure. The crude product is purified by column chromatography (5-20% EtOAc/hexane). **MAS-53** is isolated with a yield of 33% (52.0 mg, 166 µmol) of a white solid.

**1H-NMR** (400 MHz, CDCl$_3$, 300 K): δ [ppm] = 8.16 – 8.07 (m, 2H), 7.36 – 7.30 (m, 2H), 7.19 – 7.13 (m, 2H), 7.05 – 6.94 (m, 2H), 3.70 (s, 3H), 3.64 (s, 2H), 3.12 (s, 6H).

**13C-NMR** (101 MHz, CDCl$_3$, 300 K): δ [ppm] = 172.0, 165.0, 155.6, 150.4, 132.3, 130.6, 130.6, 130.4, 130.4, 122.1, 122.1, 115.6, 114.3, 114.3, 52.3, 40.8, 40.8, 40.4.

**TLC** $R_f$ = 0.24 (20% EtOAc/hexane) [UV/KMnO$_4$].

**HRMS** for C$_{18}$H$_{19}$NO$_4$ [M+H]$^+$ calcd. 314.1387, found 314.1386.
1.2.16 Synthesis of isopropyl 4-((cyclohexanecarbonyl)oxy)benzoate (MAS-55)

MAS-55 was synthesized based on GP1, starting with 35.0 mg (150 µmol) of Boc-Inp-OH (11) and 18.0 mg (100 µmol) of MAS-54. MAS-55 is isolated after purification by column chromatography (1-4% EtOAc/hexane) with a yield of 42% (35.0 mg, 90 µmol) of a white solid.

$^1$H-NMR (300 MHz, CDCl$_3$, 300 K): δ [ppm] = 8.10 – 8.01 (m, 2H), 7.17 – 7.08 (m, 2H), 5.24 (hept, $^3$J = 6.3 Hz, 1H), 2.64 – 2.48 (m, 1H), 2.12 – 2.01 (m, 2H), 1.90 – 1.77 (m, 2H), 1.74 – 1.51 (m, 4H), 1.45 – 1.27 (m, 2H), 1.36 (d, $^3$J = 6.2 Hz, 6H).

$^{13}$C-NMR (75 MHz, CDCl$_3$, 300 K): δ [ppm] = 174.2, 165.5, 154.6, 131.2, 128.5, 121.6, 68.6, 43.4, 29.1, 25.8, 25.5, 22.1.

TLC $R_f$ = 0.20 (3% EtOAc/hexane) [UV/KMnO$_4$].

HRMS for C$_{17}$H$_{22}$O$_4$[M+H]$^+$ calcd. 291.1591, found 291.1583.
1.2.17 Synthesis of 1-(tert-butyl) 4-(4-(methoxycarbonyl)phenyl) piperidine-1,4-dicarboxylate (MAS-66)

MAS-66 was synthesized based on GP1, starting with 100 mg (440 µmol) of Boc-Inp-OH (15) and 100 mg (650 µmol) of methyl-4-hydroxybenzoate (18). MAS-66 is isolated after purification by column chromatography (5-15% EtOAc/hexane) with a yield of 68% (108 mg, 297 µmol) of a white solid.

$^1$H-NMR (300 MHz, CDCl$_3$, 300 K): $\delta$ [ppm] = 8.12 – 8.02 (m, 2H), 7.20 – 7.09 (m, 2H), 4.16 – 4.04 (m, 2H), 3.92 (s, 3H), 2.99 – 2.86 (m, 2H), 2.79 – 2.66 (m, 1H), 2.09 – 1.98 (m, 2H), 1.87 – 1.69 (m, 2H), 1.47 (s, 9H).

$^{13}$C-NMR (75 MHz, CDCl$_3$, 300 K): $\delta$ [ppm] = 172.5, 166.3, 154.7, 154.3, 131.2, 127.8, 121.5, 79.8, 77.4, 77.0, 76.6, 52.2, 43.0, 41.3, 28.4, 27.9.

TLC $R_f$ = 0.10 (15% EtOAc/hexane) [UV/KMnO$_4$].

HRMS for C$_{19}$H$_{25}$NO$_6$ [M-Boc+H]$^+$ calcd. 264.1230, found 264.1228.
1.2.18 Synthesis of 1-(tert-butyl) 4-(4-(trifluoromethyl)phenyl) piperidine-1,4-dicarboxylate (MAS-72)

MAS-72 was synthesized based on GP1, starting with 212 mg (930 µmol) of Boc-Inp-OH (15) and 100 mg (620 µmol) of 4-(trifluoromethyl)phenol (19). MAS-72 is isolated after purification by column chromatography (2-15% EtOAc/hexane) with a yield of 68% (108 mg, 297 µmol) of a white solid.

$^1$H-NMR (400 MHz, CDCl$_3$, 300 K): $\delta$ [ppm] = 7.65 (d, $^3J = 8.7$ Hz, 2H), 7.20 (d, $^3J = 8.4$ Hz, 2H), 4.14 – 4.05 (m, 2H), 2.98 – 2.86 (m, 2H), 2.78 – 2.67 (m, 1H), 2.10 – 1.98 (m, 2H), 1.85 – 1.70 (m, 2H), 1.47 (s, 9H).

$^{13}$C-NMR (101 MHz, CDCl$_3$, 300 K): $\delta$ [ppm] = 172.7, 154.8, 153.3, 128.0, 127.0, 127.0, 125.3, 122.1, 80.0, 43.1, 41.4, 28.6, 28.0.

TLC $R_f = 0.26$ (10% EtOAc/hexane) [UV/KMnO$_4$].

HRMS for C$_{18}$H$_{22}$F$_3$NO$_4$ [M-Boc+H]$^+$ calcd. 274.1049, found 274.1047.
1.2.19 Synthesis of 3-(methylsulfonyl)benzoic acid (MAS-80)

Na$_2$SO$_3$ (257 mg, 2.04 mmol, 3.0 eq) is solved in 500 µL H$_2$O at 80 °C. 150 mg chlorosulfonyl benzoic acid (20) (680 µmol, 1.0 eq) are solved in 500 µL aceton and the aqueous Na$_2$SO$_3$-solution is added dropwise. NaHCO$_3$ (aq) is added until the solution is basic. The reaction mixture is stirred for 90 minutes at 80 °C before aceton is removed under reduced pressure, the aqueous solution is acidified using HCl (aq) and extracted with EtOAc (3 × 5 mL). The organic solvent is removed under reduced pressure, the white solid is dissolved in 500 µL EtOH and NaHCO$_3$ (aq) is added until the pH is basic. 106 mg (750 µmol, 1.1 eq) MeI are added and the reaction mixture is stirred under reflux for 2h before NaOH (2M, 1 mL) is added. The solution is acidified using HCl (2M) and extracted with EtOAc (3 × 5 mL). The solvent is removed under reduced pressure and the crude product is purified by HPLC using the following gradient (t$_R$ = 4.4 min, λ = 210 nm).

Table 2: HPLC gradient without TFA on a P1 column.

| t [min] | %-H$_2$O | %-ACN |
|---------|----------|--------|
| 0       | 98       | 2      |
| 1       | 90       | 10     |
| 12      | 2        | 98     |
| 14      | 2        | 98     |
| 15      | 98       | 2      |
| 17      | 98       | 2      |

The acetonitrile/water mixture is lyophilized to yield 35% (36.0 mg, 180 µmol,) of a fluffy white solid.

$^1$H-NMR (300 MHz, Acetone, 300 K): δ [ppm] = 8.55 (s, 1H), 8.35 (d, $^3$J = 7.1 Hz, 1H), 8.21 (d, $^3$J = 7.85 Hz, 1H), 7.83 (t, $^3$J = 8.2 Hz, 1H), 3.21 (s, 3H).

$^{13}$C-NMR (75 MHz, Acetone, 300 K): δ [ppm] = 166.2, 143.0, 135.1, 132.7, 132.4, 130.8, 129.8, 129.2, 126.1, 44.2.

HRMS for C$_8$H$_8$O$_4$S [M-H]$^-$ calcd. 199.0071, found 199.0062.
**1.2.20 Synthesis of 4-(isopropoxycarbonyl)phenyl 3-(methylsulfonyl)benzoate (MAS-83)**

MAS-83 was synthesized based on GP1, starting with 11.0 mg (55.0 µmol) of MAS-80 and 15.0 mg (620 µmol) of isopropyl-4-hydroxybutyrate (11). MAS-83 is isolated after purification by HPLC using the following gradient (t_R = 11.5 min, λ = 234 nm).

Table 3: HPLC gradient without TFA on a P1 column.

| t [min] | %-H_O | %-ACN |
|---------|--------|-------|
| 0       | 98     | 2     |
| 1       | 90     | 10    |
| 12      | 2      | 98    |
| 14      | 2      | 98    |
| 15      | 98     | 2     |
| 17      | 98     | 2     |

The acetonitrile/water mixture is lyophilized to yield 75% (15.0 mg, 41.0 µmol,) of a fluffy white solid.

**^1H-NMR** (400 MHz, CDCl₃, 300 K): δ [ppm] = 8.78 (s, 1H), 8.49 (d, ^3^J = 8.0 Hz, 1H), 8.24 (d, ^3^J = 7.8 Hz, 1H), 8.19 – 8.11 (m, 2H), 7.78 (t, ^3^J = 7.8 Hz, 1H), 7.36 – 7.27 (m, 2H), 5.27 (hept, ^3^J = 6.3 Hz, 1H), 3.13 (s, 3H), 1.39 (d, ^3^J = 6.2 Hz, 6H).

**^13C-NMR** (126 MHz, CDCl₃, 300 K): δ [ppm] = 165.3, 163.0, 154.0, 141.6, 135.3, 132.4, 131.4, 130.8, 130.2, 129.3, 129.1, 121.6, 68.8, 44.6, 22.0.

**TLC** R_f = 0.40 (5% EtOAc/DCM) [UV/KMnO_4].

**HRMS** for C_8H_8O_4S [M+H]^+ calcd. 363.0897, found 363.0908.
II Methodology

2 Biochemistry

2.1.1 Protein Purification

2.1.1.1 Purification of SaClpP

SaClpP was expressed and purified as described previously.\textsuperscript{1,2} In short, the sequence was cloned in pET301 expression vectors with a C-terminal Strep-tag II and expressed in \textit{E. coli} BL21 (DE3) cells at 25 °C overnight after induction at OD\textsubscript{600} of 0.6 with 500 µM IPTG. The cells were harvested (6000 × g, 5 min, 4 °C; rotor SLA-3000; Sorvall RC 6+, \textit{Thermo Scientific}, USA), washed with PBS and resuspended in ClpP lysis buffer (100 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA). The cells were lysed by sonication in two cycles with alternating 7 min 30% and 3 min 80% intensity using a Sonolus HD 2070 (\textit{Bandelin}, GER) under constant cooling with ice and centrifuged (38720 × g, 30 min, 4 °C) before the lysate was separated from the membrane fraction.

Purification was performed at an ÄKTA purifier 10 FPLC System using a StrepTrap column (StrepTrap HP 5 mL, \textit{GE Healthcare}, UK) and a subsequent preparative size exclusion column (HiLoad 16/60 Superdex 200 pg, \textit{GE Healthcare}, UK). The column was pre-equilibrated with lysis buffer before the lysate was loaded onto the StrepTrap column using a Superloop (\textit{GE Healthcare}, UK) with a flow rate of 5 mL/min. Non-specifically bound proteins were removed by washing with 10 column volumes (CV) STREP binding buffer. Bound proteins were eluted by applying 4 CV elution buffer (lysis buffer + 2.5 mM desthiobiotin). The eluate was concentrated using centrifugal filters (\textit{Millipore}, USA, 50 MWCO; 3000 × g, 4 °C). The protein was loaded onto a pre-equilibrated (ClpP storage buffer, 20 mM HEPES, pH 7.0, 100 mM NaCl) size exclusion column (HiLoad 16/60 Superdex 200 pg (\textit{GE Healthcare}, UK)) and eluted with 5 CV ClpP storage buffer. The identity and purity of the protein was validated by intact-protein mass spectrometry (IP-MS) and SDS-PAGE. Fractions containing ClpP were pooled, concentrated, split into aliquots and snap-frozen in liquid nitrogen to be stored at -80 °C.
2.1.1.2 Expression and Purification of SaClpX

SaClpX was expressed and purified as described previously.\(^3\)\(^,\)\(^4\) The protein was kindly provided by Dr. Christian Fetzer. The sequence was cloned in pET300 expression vectors with N-terminal His\(_6\) tag and TEV cleavage site, expressed in E. coli BL21(DE3) cells at 25 °C overnight after induction at OD\(_{600}\) of 0.6 with 500 µM IPTG. The cells were harvested (6000 × g, 5 min, 4 °C; rotor SLA-3000; Sorvall RC 6+, Thermo Scientific, USA), washed with PBS and resuspended in ClpX lysis buffer (25 mM HEPES, pH 7.6, 200 mM KCl, 0.5 mM ATP, 1 mM DTT, 5 mM MgCl\(_2\), 5% glycerol). The cells were lysed by sonication in two cycles with alternating 7 min 30% and 3 min 80% intensity using a Sonolus HD 2070 (Bandelin, GER) under constant cooling with ice and centrifuged (38720 × g, 30 min, 4 °C) before the lysate was separated from the membrane fraction.

SaClpX purification was performed using an Äkta purifier 10 FPLC System using a pre-equilibrated HisTrap HP 5 mL column (GE Healthcare, UK). After the protein was loaded onto the column with a Superloop (GE Healthcare, UK), non-specifically bound proteins were removed with 10 CV lysis buffer + 40 mM imidazole before bound proteins were eluted with elution buffer (lysis buffer + 500 mM imidazole, 4 CV). Cleavage of the His\(_6\)-tag was performed by incubation with EDTA (1 mM) and TEV-protease (ratio 1:50 w/w) at 10 °C overnight. Full cleavage of the purification tag was verified by IP-MS, the protein solution was concentrated using centrifugal filters (Millipore, USA, 50 MWCO) to a final volume of 4.5 mL (3000 × g, 4 °C). The concentrated protein was loaded onto a size exclusion column (HiLoad 16/60 Superdex 200 pg (GE Healthcare, UK)) pre-equilibrated in ClpX storage buffer (25 mM HEPES, pH 7.6, 200 mM KCl, 5 mM MgCl\(_2\), 1 mM DTT, 0.5 M ATP, 5 % glycerol) and eluted with 5 CV ClpX storage buffer. The identity and purity of the protein was validated by IP-MS and SDS-PAGE. Fractions containing ClpX were pooled, concentrated, split into aliquots and snap frozen in liquid nitrogen to be stored at -80 °C.
2.1.1.3 Purification of eGFP-ssrA

eGFP-ssrA was expressed and purified as described previously. The protein was kindly provided by Dr. Markus Lakemeyer. The sequence was cloned in pET300 expression vectors with N-terminal His tag and TEV cleavage site, expressed in E. coli SG1146a cells at 37 °C for 6 h after induction at OD$_{600}$ of 0.6 with 1 mM IPTG. The cells were harvested (6000 × g, 5 min, 4 °C; rotor SLA-3000; Sorvall RC 6+, Thermo Scientific, USA), washed with PBS and resuspended in GFP lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10 mM imidazole, 2 mM β-mercaptoethanol) + 0.2% NP-40). The cells were lysed by sonication in two cycles with alternating 7 min 30% and 3 min 80% intensity using a Sonolus HD 2070 (Bandelin, GER) under constant cooling with ice and centrifuged (38720 × g, 30 min, 4 °C) before the lysate was separated from the membrane fraction.

The protein was purified using an ÄKTA purifier 10 FPLC system (GE Healthcare, UK) with a HisTrap HP 5 mL column (GE Healthcare, UK) pre-equilibrated in lysis buffer. The lysate was loaded onto the column using a Superloop (GE Healthcare, UK) with a flow rate of 5 mL/min. Non-specifically bound proteins were removed by washing with 8 CV lysis-buffer, 8 CV lysis-buffer + 850 mM NaCl (total 1 M NaCl), 8 CV lysis-buffer + 10 mM imidazole (total 20 mM imidazole). The His$_6$-tagged proteins were eluted by applying 5 CV elution buffer (lysis buffer + 490 mM imidazole (total 500 mM imidazole)). Appropriate fractions were identified by IP-MS, pooled and concentrated using centrifugal filters (Millipore, USA, 10 MWCO) to a final volume of 4.5 mL (3000 × g, 4 °C). The concentrated protein was loaded onto a size exclusion column (HiLoad, 16/60 Superdex 200 pg (GE Healthcare, UK)) preequilibrated in eGFP-ssrA storage buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10% glycerol) and eluted with 5 CV eGFP-ssrA storage buffer. The identity and purity of the protein was validated by IP-MS and SDS-PAGE. Fractions containing eGFP-ssrA were pooled, concentrated, split into aliquots and snap frozen in liquid nitrogen to be stored at -80 °C.
II Methodology

2.1.2 Toxicity Assay (MTT)

HeLa cells were grown in DMEM high glucose medium (Sigma Aldrich, USA) with 10% FBS (Sigma Aldrich, USA) and 2 mM glutamine (Sigma Aldrich, USA) in a 5% CO₂-atmosphere at 37 °C.

For splitting, the medium was removed, and the cells were washed with 10 mL of PBS before 2 mL of Accutase® (Sigma Aldrich, USA) were added. The flask is incubated at 37 °C until cells were fully detached before 8 mL of medium was added. The cell-solution was mixed thoroughly, centrifuged (500 U/min, 3 min) and the medium was removed. The cells were resuspended in 10 mL medium, and 1 mL of the cell-solution was diluted with 19 mL medium. The procedure was repeated 2-3 times per week.

Evaluation of cytotoxicity was performed by harvesting 4 000 cells per well on a 96-well plate (Thermo Fisher Scientific, USA) and subsequent incubation overnight. The medium was removed and the respective compound in DMSO or DMSO (1% final DMSO concentration each) was added in medium before it was incubated for 24 h. 20 µL Thiazolyl blue tetrazolium bromide (MTT)-reagent (5 mg/mL in PBS, Sigma Aldrich, USA) were added and the plate is incubated for 2 h in a 5% CO₂-atmosphere at 37 °C in order to be metabolized. The medium is removed by suction and 200 µL DMSO/well were added to dissolve the formed formazan. The plate was put on a shaker for 10 min at 300 rpm, the optical density at 570 nm and the background at 630 nm were determined and subtracted using an Infinite M200Pro plate reader (Tecan, Austria). Absorption values were normalized to DMSO control and IC₅₀ values were calculated using a non-linear regression fit variable slope (four parameters) in GraphpadPrism 5. The experiment was performed in at least two independent occasions (n = 2) in triplicates.
2.1.3 MIC Determination

Overnight cultures were diluted 1:10,000 into medium and directly used for the tests. Various dilutions of the compounds in DMSO were prepared and 1 µL thereof was pipetted in triplicates in 96 well-plates (transparent Nunc 96-well flat bottom, *Thermo Fisher Scientific*, USA), including DMSO only, which served as growth control. Then, 99 µL of the diluted bacterial suspension were transferred to each well and a sterile control containing only medium was included. Bacteria were incubated for 24 h (37°C, 200 rpm) and the dilution series was analyzed for microbial growth, indicated by turbidity. The lowest concentration in the dilution series at which no growth of bacteria could be observed by eye was defined as the minimum inhibitory concentration (MIC) of the compound. MIC values were determined by three independent experiments.
III Bibliography

1. Gersch, M.; List, A.; Groll, M.; Sieber, S. A., Insights into structural network responsible for oligomerization and activity of bacterial virulence regulator caseinolytic protease P (ClpP) protein. *The Journal of biological chemistry* **2012**, *287* (12), 9484–9494.

2. Hackl, M. W.; Lakemeyer, M.; Dahmen, M.; Glaser, M.; Pahl, A.; Lorenz-Baath, K.; Menzel, T.; Sievers, S.; Böttcher, T.; Antes, I.; Waldmann, H.; Sieber, S. A., Phenyl Esters Are Potent Inhibitors of Caseinolytic Protease P and Reveal a Stereogenic Switch for Deoligomerization. *Journal of the American Chemical Society* **2015**, *137* (26), 8475–8483.

3. Gersch, M.; Famulla, K.; Dahmen, M.; Gobl, C.; Malik, I.; Richter, K.; Korotkov, V. S.; Sass, P.; Rubsam-Schaeff, H.; Madl, T.; Brotz-Oesterhelt, H.; Sieber, S. A., AAA+ chaperones and acyldepsipeptides activate the ClpP protease via conformational control. *Nat Commun* **2015**, *6*, 6320.

4. Fetzer, C.; Korotkov, V. S.; Thanert, R.; Lee, K. M.; Neuenschwander, M.; von Kries, J. P.; Medina, E.; Sieber, S. A., A Chemical Disruptor of the ClpX Chaperone Complex Attenuates the Virulence of Multidrug-Resistant Staphylococcus aureus. *Angew Chem Int Ed Engl* **2017**, *56* (49), 15746-15750.
## IV List of Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| ACC          | Acetylalanyl-homoarginyl-2-aminoctanoyl-7-amino-4-carbamoylmethylcoumarin (Ac-Ala-hArg-2Aoc-ACC) |
| Boc          | tert-Butoxy carbonyl |
| Cbz          | Benzyloxy carbonyl |
| ClpP         | Caseinolytic protease P |
| ClpX         | Caseinolytic protease X |
| CV           | column volumes |
| d            | days |
| DBU          | 1,8-Diazabicyclo[5.4.0]undec-7-ene |
| DCM          | Dichloromethane |
| DIPEA        | N,N-Diisopropylethylamine |
| DMAP         | 4-Dimethylaminopyridine |
| DMF          | N,N-Dimethylformamid |
| DMSO         | Dimethylsulfoxide |
| EDC          | 1-Ethyl-3-(3-deimethylaminopropyl-)carbodiimide |
| eq.          | Equivalents |
| GFP          | Green fluorescent protein |
| h            | Hours |
| HTS          | High-throughput screening |
| MeCN         | Acetonitrile |
| min          | Minutes |
| MRSA         | Methicillin-resistant *Staphylococcus aureus* |
| MS           | Mass spectrometry |
| NMR          | Nuclear magnetic resonance |
| PBS          | Phosphate-buffered saline |
| ppm          | Parts per million |
| RT           | Room temperature |
| SAR          | Structure-activity relationship |
| *S. aureus*  | *Staphylococcus aureus* |
| SDS-PAGE     | Sodium dodecyl sulphate–polyacrylamide gel electrophoresis |
| Abbreviation | Description                                      |
|--------------|--------------------------------------------------|
| TBDMSCl      | tert-Butyldimethylsilyl chloride                |
| TFA          | Trifluoro acetic acid                           |
| THF          | Tetrahydrofuran                                 |
| TLC          | Thin layer chromatography                       |
| UV           | Ultraviolet                                     |
V. Appendix
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