Supplementary Information

Cinnamic aldehyde derived probes for the active site labelling of pathogenesis associated enzymes

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Materials
All chemicals exhibited reagent grade or better and were used without further purification. Chemicals and solvents were purchased from Sigma Aldrich or Acros Organics. For all reactions, only commercially available solvents of highest purity, dried over molecular sieve and stored under Argon atmosphere were used. Solvents for chromatography and workup purposes were generally of reagent grade and purified before use by distillation. In all reactions, temperatures were measured externally. All experiments were carried out under argon atmosphere in flame-dried glassware.

Column chromatography was performed on Merck silica gel (Acros Organics 0.035 – 0.070 mm, mesh 60 Å).

Recombinant PTP1B was purchased from Merck.

$^1$H NMR spectra were recorded on a Varian Mercury 200 (200 MHz), a Varian NMR-System-600 (600 MHz) or a Varian NMR-System 300 (300 MHz) and referenced to the residual proton of the deuterated solvent.

ESI spectra were recorded with a Thermo Finnigan LTQ FT. HPLC analysis was accomplished with a Waters 2695 separations module, a X-Bridge™ BEH130 C18 column (4.6x100 mm) and a Waters 2996 PDA detector. Mobile phase (HPLC grade): A: 0.1 % (v/v) TFA in H$_2$O; B: 0.1 % TFA in Acetonitrile; Gradient: 95 % A → 25 % A in 40 min. Flow (anal.): 0.5 ml/min. Flow (prep.): 5 ml/min.
1) Synthesis of the Cinnamic-acid derivatives

(E)-4-(3-tert-butoxy-3-oxoprop-1-enyl)benzoic acid (A)
A 25-ml, three-necked, round-bottomed flask was charged with a solution of 4-iodbenzoic acid (496 mg; 2 mmol) in DMF (2 ml). Tert-butylacrylat (320 µl; 2.2 mmol; 1,1 eq) and triethylamin (560µl; 4 mmol; 2,0 eq) were added and the resulting mixture was stirred for several minutes at room temperature. Finally P(O-Tol)₃ (24 mg; 0,08 mmol; 4 mol%) and Pd(OAc)₂ (4,5 mg; 0,02 mmol; 1 mol%) were added. The yellow solution was refluxed at 110 °C for 2 h under argon atmosphere. The solvent was removed in vacuo. The brown solid was purified by flash-chromatography (i-hexane/ethylacetate; 1:1; 1 % AcOH). The product was obtained as a lightly yellow crystalline solid (320 mg, 64 %). The compound has been characterized previously.[1]

¹H-NMR (200 MHz, CDCl₃) δ = 8.11 (dd, J = 8.4, 2H), 7.64 (d, J = 16.5, 2H), 7.58 (s, 1H), 6.47 (d, J = 16.0, 1H), 1.54 (s, 9H).

(E)-4-(3-oxoprop-1-enyl)benzoic acid (B)
A 25-ml, three-necked, round-bottomed flask was charged with a solution of 4-iodbenzoic acid (496 mg; 2 mmol) in DMF (8 ml). 3,3-Diethoxy-1-propene (915 µl; 6,00 mmol) was added under argon atmosphere. The resulting solution was stirred under room temperature for several minutes. Finally potassium acetate (393 mg; 4,00 mmol), potassium carbonate (415 mg; 3 mmol) and tetra-butyl-ammonium chloride (556 mg; 2 mmol) were dried under reduced pressure for 30 min and added to the solution. The colourless suspension was stirred for about ten minutes at room temperature. Pd(OAc)₂ (13 mg; 0,06 mmol) was added and the yellow suspension was refluxed at 100 °C. After 2 h the black suspension was slowly quenched with an excess of 2 M aq. HCl which caused a slight evolution of gas. The grey suspension was extracted with diethyl ether (3x 25 ml) and the organic phases were combined, washed with brine and dried over anhydrous MgSO₄. After filtration the solvent was evaporated in vacuo. Purification by flash-chromatography (i-hexane/ ethylacetate; 9:1; 1 % AcOH) gave the product (176 mg, 50 %) as a light yellow solid. The compound has been characterized previously.[2]
$^1$H NMR (200 MHz, DMSO) $\delta = 9.70$ (d, $J=7.7$, 1H), 7.91 (dd, $J=8.4$, 26.5, 4H), 7.80 (d, $J=15.9$, 1H), 6.93 (dd, $J=7.7$, 16.1, 1H).

**Solid Phase synthesis of the tripeptides**

All solid phase reactions were carried out in a size of 100 µmol. The rink-amide resin (139 mg, 1,00 eq) was swelled in dry DCM (2 ml) for 20 min and further equilibrated in dry DMF (2 ml) for 10 min. The cleavage of the FMOC group was accomplished by the addition of a 20 % (v/v) solution of piperidine in DMF (2 ml) and incubation for 10 min on a shaker. To ensure complete cleavage another 2 ml of 20 % piperidine in DMF was added followed by 10 min incubation. The resin was washed four times with DMF (2 ml) and three times with DCM (2 ml). The filtrate was discarded. The resin was equilibrated with dry DMF (2 ml) for 10 min. The first amino acid (0,3 mmol; 3,00 eq) was dissolved in dry DMF (700 µl). To this solution, HOBt (0,29 mmol; 2,90 eq) and DIC (0,3 mmol; 3,00 eq) were added. The solution was incubated for 10 min at room temperature to form the reactive ester. Finally, the reactive ester was added to the resin. After 2 h reaction time at ambient temperature the supernatant was discarded and the resin was washed two times with DMF (2 ml) and three times with DCM (2 ml).

The cleavage and coupling steps were repeated for every following amino acid. After every step the outcome of the reaction was monitored via the Kaiser test.

**Coupling of the Michael acceptor on the tripeptides**

The resin was equilibrated in dry DMF for 10 min. The FMOC group was cleaved by the addition of a 20 % (v/v) solution of piperidine in DMF (2 ml) and incubation for 10 min on a shaker. To ensure completeness of the reaction another 2 ml of 20 % (v/v) piperidine in DMF was added, followed by 10 min incubation. The resin was washed four times with DMF (2 ml) and three times with DCM (2 ml). The resin was equilibrated in dry DMF (2 ml) for 10 min.

A or B (0,3 mmol, 3 eq) was dissolved in dry DMF (500 µl). To this solution HOBt (0,29 mmol; 2,90 eq) and DIC (0,3 mmol; 3,00 eq) were added. After incubation for 10 min at room temperature the solution was added to a resin containing the deprotected tripeptide. After 2 h reaction time at ambient temperature the supernatant was discarded and the resin was washed two times with DMF (2 ml) and three times with DCM (2 ml).
The cleavage of the final compound from the solid phase was accomplished by the addition of TFA (95% with 5% Water) and incubation for 1 hour at room temperature on a shaker. The yellow solution was added dropwise to ice cold diethylether (50 ml). Depending on the sequence of the tripeptide, a white or red precipitate formed immediately. Removal of the solvent in vacuo resulted in a viscous brown oil (48-91 % yield). The product was analyzed by ESI-MS and analytical HPLC. If side products could be detected, they were removed via preparative HPLC (for compound 10).

<table>
<thead>
<tr>
<th></th>
<th>calc.:</th>
<th>ESI-MS (m/z):</th>
<th>[M+H]$^+$</th>
<th>Rt(anal.):</th>
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<tr>
<td>12</td>
<td>545.1645</td>
<td>546.1723 [M+H]$^+$</td>
<td></td>
<td>1,8-2,3 min</td>
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<tr>
<td>13</td>
<td>401.1223</td>
<td>402.1306 [M+H]$^+$</td>
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<td>386.1347 [M+H]$^+$</td>
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<td>456.2248 [M+H]$^+$</td>
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<td>10,4-10,9 min</td>
</tr>
<tr>
<td>3</td>
<td>474.1903</td>
<td>475.1984 [M+H]$^+$</td>
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<td>15,2-15,6 min</td>
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<td>541.2536</td>
<td>542.2620 [M+H]$^+$</td>
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</table>

Supporting Table 1.

2) Preparation of proteomes

The proteome of *Staphylococcus aureus* Mu50 was prepared from 1 l liquid culture harvested 1 h after transition in the stationary phase by centrifugation at 13,000 rpm. The strain was grown in BHB (brain heart broth) medium. *Staphylococcus aureus* cell pellets were lysed by sonication with a Bandelin Sonopuls with 15 x 20 sec. pulsed at 100% max. power under ice cooling.
3) Labelling of bacterial proteomes

Proteome samples were adjusted to a final concentration of 1 mg protein/ml by dilution in PBS prior to probe labelling. Experiments for visualization by 1D SDS-PAGE were carried out in 43 μL total volume and those for affinity enrichment in 1894 μL total volume, such that once CC reagents were added, the total reaction volume was 50 μL and 2 ml, respectively. Reactions were initiated by addition of the probe and allowed to incubate for 60 min at room temperature. For heat controls the proteome was denatured with 2 μL of 21.5% SDS at 99 °C for 10 min and cooled to room temperature before the probe was applied. Following incubation, reporter tagged-azide reagents (13 μM rhodamine-azide for analytical or 20 μM rhodaminebiotin-azide for preparative scale) were added followed by 1 mM TCEP and 100 μM ligand. Samples were gently vortexed and the cycloaddition initiated by the addition of 1 mM CuSO₄. The reactions were incubated at room temperature for 1 h.

For analytical gel electrophoresis, 50 μl 2x SDS loading buffer were added and 50 μL applied on the gel. Fluorescence was recorded in a Fujifilm Las-3000 Fluoreszenz Darkbox with a Fujinon VRF 43LMD Lens, 605DF40 filter and 520 nm EPI excitation wavelength.

Reactions for enrichment were carried out together with a control lacking the probe, to compare the results of the biotin-avidin enriched samples with the background of unspecific protein binding on avidin-agarose beads. After CC, proteins were precipitated using an equal volume of pre-chilled acetone. Samples were stored on ice for 20 min and centrifuged at 13,000 rpm for 10 min. The supernatant was discarded and the pellet washed two times with 400 μl of pre-chilled methanol and resuspended by sonication. Subsequently, the pellet was dissolved in 1 ml PBS with 0.4% SDS by sonication and incubated under gentle mixing with 50 μl of avidin-agarose beads (Sigma-Aldrich) for 1 h at room temperature. The beads were washed three times with 1 ml of 0.4% SDS in PBS, twice with 1 ml of 6 M urea and three times with 1 ml PBS. 50 μl of 2x SDS loading buffer were added and the proteins released for preparative SDS-PAGE by 6 min incubation at 95 °C. Gel bands were isolated, washed and tryptically digested as described previously.[3]
4) Labelling of recombinant enzymes
SsaA2, PTPA and B and the point mutants of PTPA and SsaA2 were labelled in the membrane fraction of *E. coli* proteome. The proteome was adjusted to a concentration of 1 mg/ml prior to probe labelling. For the labelling of PTP1B 250 ng in PBS were used.

5) Competitive labelling with N-ethylmaleimide and cinnamic acids
In competitive assays, a 40fold excess of a cinnamic acid (12) or a 100fold excess of N-ethylmaleimide was added to the proteome 10 min prior to the addition of the cinnamic aldehyde probes.

6) IC50 Determination
PTP mediated degradation of the *p*-nitrophenyl-phosphate leads to the release of *p*-nitrophenole which can be monitored at 410 nm. Various concentrations of cinnamic aldehyde 10 were added to a buffer (100 mM sodiumcitrate, 1 mM EDTA, pH = 6.2) containing the substrate (40 mM) and enzyme (230 nM).[^4] Reactions were started by addition of the enzyme and subsequently monitored for 10 min at 37 °C. The mean average slopes of absorption (out of three independent experiments) versus aldehyde concentration were plotted and the concentration of 50% inhibition (IC50) was extrapolated. While solubility was no problem for the probes 12 and 14, the hydrophobic compounds 10 and 4 were insoluble in the aqueous activity buffer above a concentration of about 100 µM, which reduced inhibition at higher concentrations.

7) Mass spectrometry and bio-informatics
Tryptic peptides were loaded onto a Dionex C18 Nano Trap Column (100 µm) and subsequently eluted and separated by a Dionex C18 PepMap 100 (3 µm) column for analysis by tandem MS followed by high resolution MS using a coupled Dionex Ultimate 3000 LC-ThermoFinnegan Orbitrap XL MS system. The mass spectrometry data were searched using the SEQUEST algorithm against the corresponding databases via the software “bioworks”. The search was limited to only tryptic peptides, two missed cleavage sites, monoisotopic precursor ions and a peptide tolerance of <10 ppm. Filters were set to further refine the search results. The Xcorr vs. charge state filter was set to Xcorr values of 1.5, 2.0 and 2.5 for charge
states +1, +2 and +3, respectively. The number of different peptides has to be \( \geq 2 \) and the peptide probability filter was set to < 0.001. Maximum P-values and Xcorr values of each run as well as the total number of obtained peptides are reported in Table S2.

<table>
<thead>
<tr>
<th>Species</th>
<th>Protein</th>
<th>Protein ID</th>
<th>Calc. MW (NCBI)</th>
<th>R</th>
<th>min. p Value</th>
<th>max. Xcorr</th>
<th>NP</th>
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<tr>
<td><em>S. aureus</em> MU 50</td>
<td>Staphylococcal secretory antigen precursor ssaA2</td>
<td>NP_372823</td>
<td>29309</td>
<td>1</td>
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<td>6.93</td>
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<td>2</td>
<td>1.5 \times 10^{-13}</td>
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<td>3</td>
<td>1 \times 10^{-30}</td>
<td>7.21</td>
<td>7</td>
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**Table S2.** This list shows Protein ID, molecular weight (MW) of the protein, the replicates (R) in which the proteins have been identified, the maximum p values, maximum Xcorr and the number of peptides (NP) found in each replicate.

**MS/MS data:**

<table>
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<th>Scan(s)</th>
<th>Peptide</th>
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<td>1568 - 1570</td>
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<tr>
<td>2477 - 2479</td>
<td>R.TGGLGASYSTSSNVQVTTL*APSSNGR.S</td>
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<tr>
<td>2701</td>
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<td>3148.26880</td>
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<td>3257</td>
<td>R.TISASQAAGYNIH.-</td>
<td>1479.72266</td>
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<td>3434</td>
<td>K.IGSTWGNASNWANA.A</td>
<td>1746.83069</td>
<td>2</td>
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</table>

**8) Recombinant expression**

SsaA2 of the *Staphylococcus aureus* (Mu50) proteome as well as two low-molecular weight phosphatases (PTPA and B) from *Staphylococcus aureus* (Mu50) were recombinantly expressed in *E. coli* by using the Invitrogen™ Gateway® Technology. Target genes were amplified from the corresponding genomes by PCR with an AccuPrime™ *Pfx* DNA Polymerase kit with 38 ng of genomic DNA, prepared by standard protocols. *attB1* forward primer and *attB2* reverse primer were designed to yield *attB*-PCR products needed for the Gateway® Technology:
PTPA, *Staphylococcus aureus* Mu50
forward primer: 5’-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTA CAT GGT AGA TGT AGC ATT TGT CTG
reverse primer: 5’-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTG CTA CCC CTC TTT CAA ATT TGC AT

PTPB, *Staphylococcus aureus* Mu50
forward primer: 5’-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTA CAT GAA GAT TTT ATT CGT TTG TA
reverse primer: 5’-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTG CTA GCA AAT AAT ATC TTT TAA TTT T

SsaA2, *Staphylococcus aureus* Mu50
forward primer: 5’-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTA CAT GAA GAA AAT CGC TAC AGC T
reverse primer: 5’-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTG TAA TTT TTC GAA CTG CGG GTG GCT CCA GTG AAT GAA GTT ATA ACC AGC

PCR products were identified on agarose gels via UV detection and gel bands were isolated and extracted with an E.Z.N.A.™ MicroElute™ Gel Extraction Kit. Concentrations of DNA were measured by a NanoDrop Spectrophotometer ND-1000. 100 fmol of purified attB-PCR product and 100 fmol of attP-containing donor vector pDONR™201 in TE buffer (pH=8,00) were used for *in vitro* BP recombination reaction with BP Clonase™ II enzyme mix to yield the appropriate attL-containing entry clone. After transformation in chemically competent One Shot® TOP10 *E. coli* (Invitrogen), cells were plated on LB agar plates containing 25 μg/ml kanamycin. Clones of transformed cells were selected and grown in kanamycin-LB medium. Cells were harvested and plasmids were isolated using the E.Z.N.A.™ Plasmid Mini Kit. The identity of the gene was confirmed by sequence analysis. The corresponding attB containing expression clone was generated by *in vitro* LR recombination reaction of approx. 50 fmol of the attL-containing entry clone and 50 fmol of the attR-containing destination vector pDest using LR Clonase™ II enzyme mix in TE buffer. The expression clone was transformed in chemically competent BL21 *E. coli* cells (Novagen) and selected on LB agar plates containing 100 μg/ml carbenicillin.
Validity of the clones was confirmed by plasmid sequence analysis. Recombinant clones were grown in carbenicillin LB medium and target gene expression was induced with anhydrotetracyclin (0.01 % v/v in relation the medium).

After expression for 2 hours at 37 °C the cells were centrifuged at 4000 rpm for 10 min at 4 °C followed by lysis via French press in PBS. Unlysed cells were removed by centrifugation at 4000 rpm for 10 min. The supernatant was transferred to a new vial and centrifuged at 18 000 rpm for 45 min to pellet the membrane fraction. The pellet was resuspended in 2% Triton X-100 in PBS via sonication and incubated for 15 min on ice. Strep-Beads (Iba GmbH) were added to the suspension and incubated for 1h on ice with gentle mixing. The beads were washed two times with 2% Triton X-100 in PBS, and five times with PBS. Elution from the beads was accomplished with desthiobiotin in PBS.

9) Point mutations
The three cysteine residues of PTPA (8, 13 and 138) were mutated to alanine. 100 ng of the wildtype donor vector were used as a template for a mismatch PCR with a Pfu Ultra Polymerase.

PTPA C8 mutation, wildtype gene: *Staphylococcus aureus Mu50*
forward primer: 5’-TAA TGG TAG ATG TAG CAT TTG TCG CTC TTG GCA ATA TAT GTC GTT CTC
reverse primer: 5’-GAG AAC GAC ATA TAT TGC CAA GAG CGA CAA ATG CTA CAT CTA CCA TTA

PTPA C13 mutation, wildtype gene: *Staphylococcus aureus Mu50*
forward primer: 5’-GTA GCA TTT GT C TGT CTT GGC AAT ATA GCA CGT TCT CCA ATG G
reverse primer: 5’-CCA TTG GAG AAC GTG CTA TAT TGC CAA GAC AGA CAA ATG CTA C

PTPA C138 mutation, wildtype gene: *Staphylococcus aureus Mu50*
forward primer: 5’-TTT TGA AGG TGT ATA CGA CAT GGT ATT ATC ATC TGC TGA TAA TTT AAT AGA CTA CAT C
The cysteine residue of SsaA2 (Cys171) was mutated to alanine. 100 ng of the wildtype donor vector were used as a template for a missmatch PCR with a Pfu Ultra Polymerase.

SSA1 Protein missmatch cysteine 171

forward primer: 5’-gacgtaacttatacctctgggtcaagctacatatctgatttgatcgtg

reverse primer: 5’-cacgatcaaatatcagtatagctgaccaagtgtataagttacgtc

PCR products were identified on agarose gels via UV detection and gel bands were isolated and extracted with an E.Z.N.A.™ MicroElute™ Gel Extraction Kit. Concentrations of DNA were measured by a NanoDrop Spectrophotometer ND-1000. The extracted DNA was digested with DpnI for one hour at 37 °C to remove the methylated wildtype DNA. The remaining DNA was transformed into chemically competent One Shot® TOP10 E. coli (Invitrogen). They were plated on LB agar plates containing 25 µg/ml kanamycin. From here on the vectors were used as described above. The identity of the point-mutations were confirmed by sequence analysis of the Dest vector.
Figure S1: Synthetic procedure and structures of all compounds. A) CA probes. B) CAC probes.

Figure S2: Heat controls of PTPA and PTPB.
Figure S3: IC50 values of PTPA inhibition.

References: