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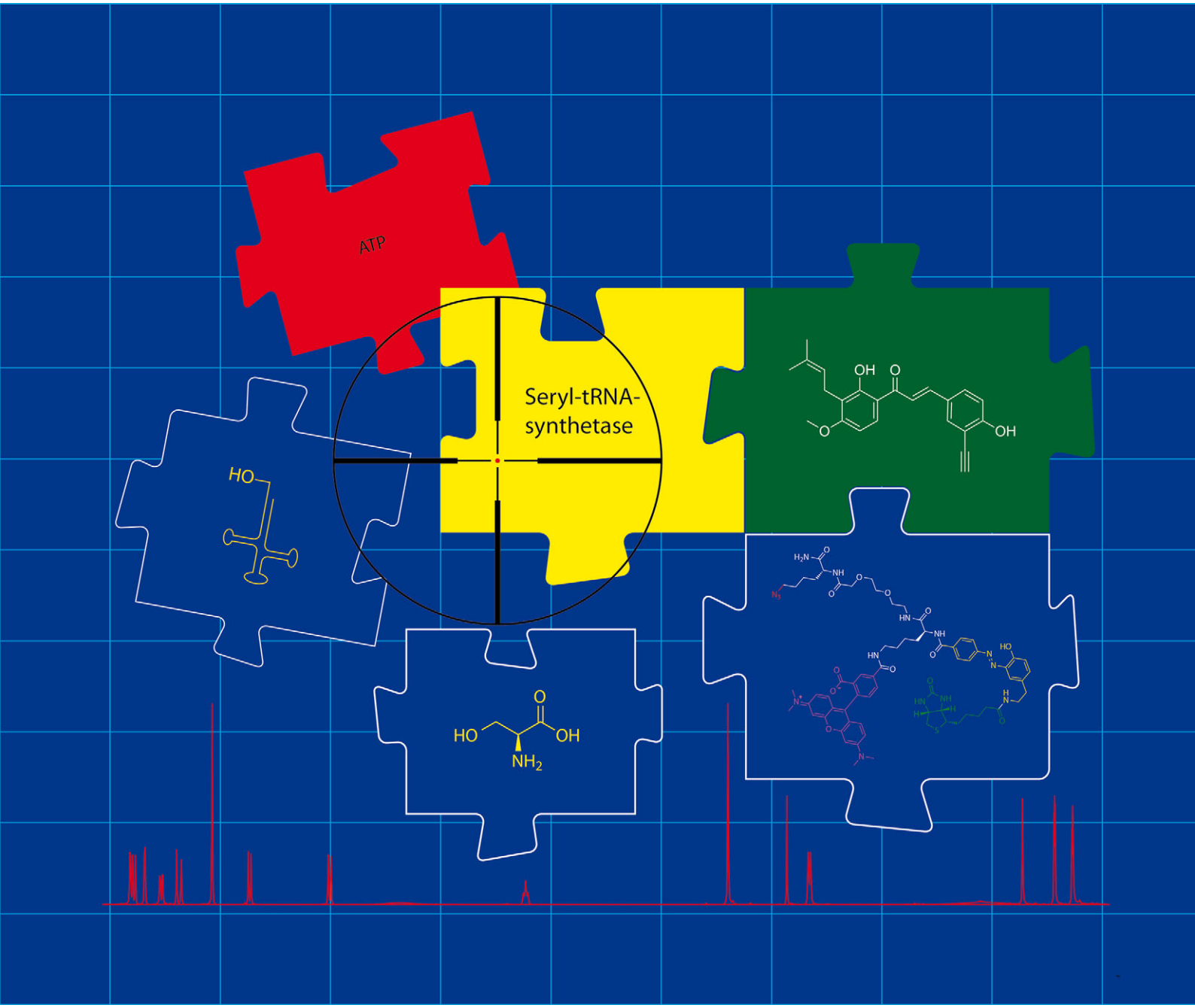

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Target profiling of 4-hydroxyderricin in *S. aureus* reveals seryl-tRNA synthetase binding and inhibition by covalent modification†

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4-Hydroxyderricin is a heat labile bioactive chalcone isolated from the plant *Angelica keiskei*. It received attention due to its antibiotic potency against several strains of bacteria including pathogens such as *Staphylococcus aureus*. Despite these promising pharmacological properties, the exact mode of action or the biological targets are still unknown. Here we report the synthesis and the application of a 4-hydroxyderricin probe for activity-based protein profiling (ABPP) in *S. aureus*. Due to the heat sensitivity of the natural product we utilize a chemical tool for the mild and selective enrichment of labile probe–protein conjugates and report seryl-tRNA synthetase (STS) to be covalently modified by our probe. This modification results in inhibition of the amino acylation of tRNAs catalyzed by *S. aureus* STS which is an essential enzymatic pathway for bacterial viability.

Introduction

Amino-acyl-tRNA synthetases (AARS) are essential enzymes for the biosynthesis of proteins by catalyzing the specific condensation of a single amino acid (AA) with its corresponding tRNA. This conjugation reaction proceeds by a reaction sequence that is mandatory for all ribosomal peptide synthesis: recognition of the correct AA, activation by ATP-hydrolysis (formation of an aminoacyl-adenylate) and transfer to the 3′ end of the cognate tRNA (Fig. 1).[1]

Inhibition of this important cellular function leads to the accumulation of uncharged tRNAs at the ribosome and finally results in the interruption of protein biosynthesis.[2] Although a variety of reported inhibitors for AARS (Fig. 2) in different organisms demonstrate the potential of these enzymes as interesting antimicrobial targets, so far just one compound has found general application in clinical practice.[1–7] This compound, Mupirocin, is the only approved topical antibacterial agent with potent activity against gram-positive pathogens including MRSA by the selective inhibition of bacterial isoleucyl-tRNA synthetase. However, the widespread clinical use of Mupirocin is accompanied by the emergence of resistance mechanisms and accentuates the need for the identification of new natural or synthetic compounds that inhibit amino-acyl-tRNA synthetases as validated antibacterial targets.[8]

4-Hydroxyderricin (**1**, 4-HD, Fig. 3A) is an antibacterial chalcone that was isolated as a main component from *Angelica keiskei*, a hardy perennial herb from the Pacific coast of Japan.[9] Since the publication of its total synthesis in 2008, the interest in the antibacterial potential of this compound has been growing.[10,11] Structure–activity relationship (SAR) studies of 31 antibacterial chalcones revealed that compound **1** exhibits the highest activity against *S. aureus* with an MIC of 23 μM and a cytotoxicity against human cancer cell lines with an IC_{50} of 5.5 μM.[12,13] However, as with many natural products the biological targets and mode of action in bacterial systems of **1** are still unknown. The electrophilic character of the central Michael acceptor and the reported reactivity of **1** towards free thiols led us to believe that the biological activities of **1** might arise from the covalent modification of its target-structures.[14,15] In order to elucidate the binding mode as well as the corresponding bacterial targets we designed molecular probe **2** bearing an alkyne handle for downstream target identification *via* activity based protein profiling (ABPP) and MS identification (Fig. 3B).[16,17] The position of the alkyne was selected based on the available SAR data for the individual building blocks of **1**.[18]

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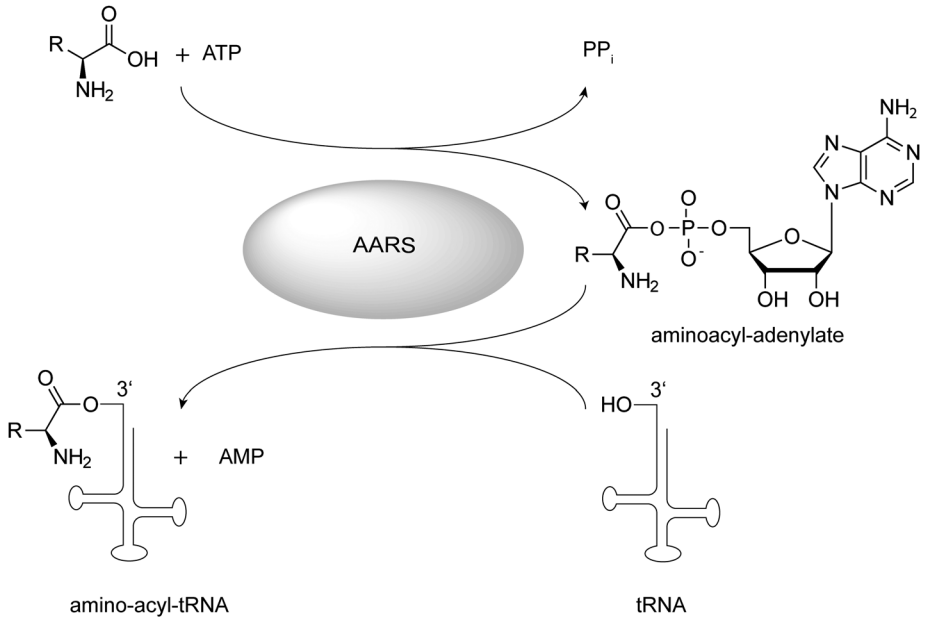


Fig. 1 Enzymatic mechanism of AARS.

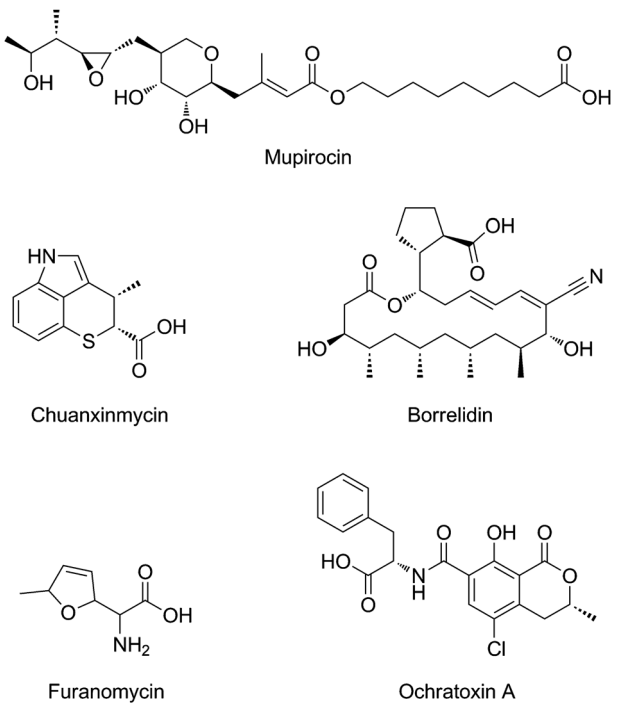


Fig. 2 Molecular structures of selected AARS-inhibitors.[1,2]

In this paper we characterize 4-HD and its derived probe **2** for target interactions by an in-depth chemical biology approach in the bacterial pathogen *S. aureus*. Our studies show that seryl-tRNA synthetase (STS) is inhibited by **2** and provides first insight into a possible antibiotic mode of action. Moreover, we introduce a novel chemical tool for the mild and selective detachment of labile compound–protein conjugates from the solid support – a critical step in proteome target applications.

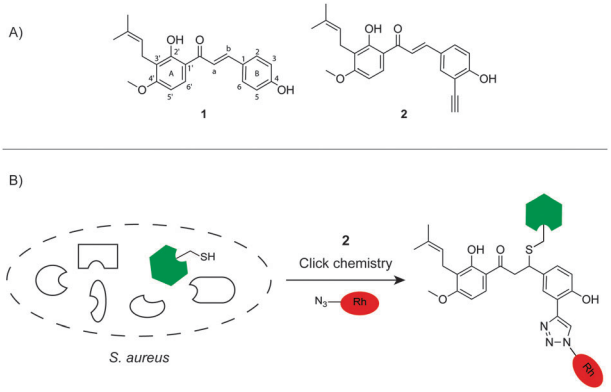
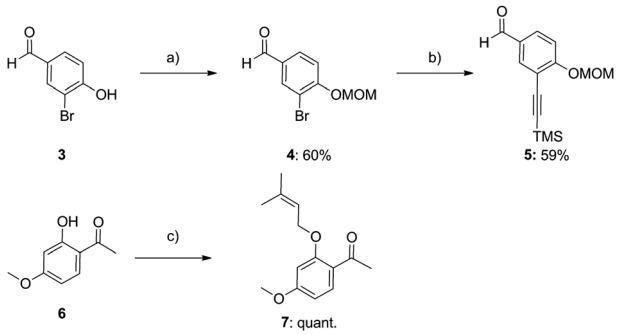


Fig. 3 (A) Structure and numbering of 4-HD (**1**) and probe **2**. (B) Conjugation of a rhodamine tag to probe modified proteins by click chemistry.

Results and discussion

In order to investigate the target preferences of 4-HD in proteome studies we applied synthetic protocols derived from published procedures and prepared the natural product as well as a probe derivative.[11,19] The synthesis of probe **2** represents a modification of the total synthesis of 4-HD (**1**) with the introduction of an alkyne in the 5-position (Fig. 3). The central reaction step for the synthesis of the chalcone **2** was a Claisen–Schmidt condensation of the intermediates **5** and **7**. Aldehyde **5** was prepared by the MOM protection of the commercially available alcohol **3**[20] followed by a Sonogashira cross-coupling with trimethylsilylacetylene (Scheme 1).

The reaction of alcohol **6** with prenyl chloride yielded ketone **7** for use in the subsequent condensation reaction.[19] An aldol condensation of both building blocks with 3 M NaOH in EtOH gave chalcone **8** with the simultaneous removal of the TMS alkyne

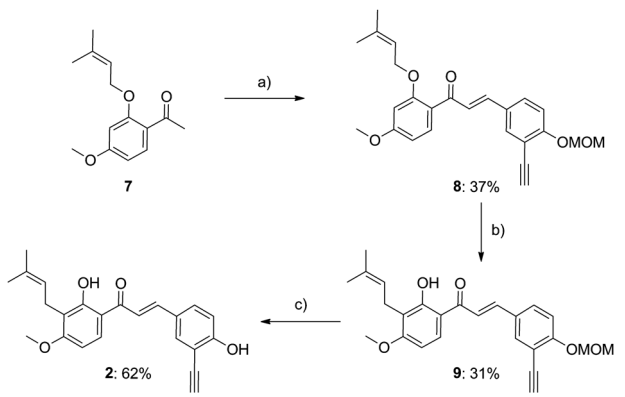


Scheme 1 Synthesis of chalcone building blocks **5** and **7**. (a) MOMCl, K_2CO_3, acetone, Δ; (b) trimethylsilylacetylene, $PdCl_2$, CuI, PPh_3, piperidine, Δ; (c) prenyl chloride, K_2CO_3, acetone, Δ.

protecting group (Scheme 2). Rearrangement of the prenyl group in the presence of the solid acid catalyst montmorillonite K10 followed by MOM deprotection with *p*-toluenesulfonic acid finally yielded the 4-HD-probe **2** (Scheme 2).

Our intention with the design of **2** was to keep the final probe structurally as similar to the natural product as possible without changing the biological activity. To confirm that the slight modification retained the biological effect we compared the antibacterial potential of the natural product **1** with probe **2** by measuring the growth inhibition of *S. aureus* NCTC 8325 (Fig. S1, ESI†). To our satisfaction both compounds exhibited a minimal inhibitory concentration (MIC) below 5 μM demonstrating that the modification of **1** by addition of an alkyne tag did not change the antibacterial properties in *S. aureus.* The reported value is even slightly lower than reported in the literature (5 *vs.* 23 μM) and confirms the potent antibacterial effect of 4-HD.[13]

Based on the hypothesis that the antibacterial activities of **1** and **2** might rely on a covalent modification of the cellular target we applied probe **2** in a *S. aureus* NCTC 8325 proteome labeling experiment. Soluble fractions of cell lysate were incubated with different concentrations of **2** ranging from 5–100 μM. After 1 h, probe modified proteins were reacted with a fluorescent dye (RhN_3, Fig. S2, ESI†) through an alkyne–azide cycloaddition protocol (click chemistry),[21–23] separated based on their molecular weight *via* SDS-gel electrophoresis and analyzed by in-gel fluorescence scanning. Labeling of *S. aureus* lysate with probe **2** revealed several protein bands in the region of about 50 kDa which exhibited stronger labeling intensities with increasing probe concentrations (Fig. 4A, Fig. S3, ESI†). Heat denaturation of the proteome prior to probe labeling resulted in an attenuation of the labeling pattern which shows the selectivity of the probe for correctly folded protein structures (Fig. 4A). In order to confirm that the natural product and the probe share the same protein binding preferences the *S. aureus* proteome was pre-treated with a 1×, 5×, 10× and 50× excess of unmodified **1** for 20 minutes, and subsequently incubated with 100 μM of probe **2**. Fluorescent SDS analysis of this sample revealed a strong reduction of protein labeling at 5-fold excess emphasizing a direct competition between the probe and the natural product for the same binding sites (Fig. 4B).



Scheme 2 Synthesis of 4-HD probe **2**. (a) **5**, NaOH, EtOH; (b) montmorillonite K10, CH_2Cl_2, 0 °C; (c) *p*-TsOH, MeOH, 30 °C.

To identify the molecular targets that are covalently modified by **2** we attempted an enrichment procedure with a trifunctional rhodamine–biotin–azide linker (Rh–biotin–N_3, Fig. S2, ESI†).[24] This tag was reacted with the probe-modified proteome *via* click chemistry. Labeled proteins were subsequently enriched *via* biotin–avidin binding and finally released from biotin beads *via* heat treatment at 99 °C. To our surprise only weak protein bands could be visualized based on fluorescent SDS gel analysis (Fig. 4C). A closer inspection on the heat stability of probe **2** revealed a likely explanation for this result. At a temperature of 99 °C **2** decomposes within 10 minutes as determined by HPLC experiments and therefore results in a detachment of the fluorescent label from the modified protein (Fig. S4, ESI†). In order to avoid decomposition of **2** and subsequent loss of labeled protein, we designed and synthesized a diazobenzene based chemical cleavable linker (Rh–biotin–diazo–N_3, **13**) that allows specific cleavage of biotin bound proteins upon treatment with sodium hydrosulfite (Scheme 3).[25] The synthesis of **13** was performed on a Rink resin according to previously published procedures, which was elongated by Fmoc–SPPS with Fmoc–Lys(N_3)–OH, a PEG spacer, and Fmoc–Lys(Boc)–OH, respectively, to form resin-bound intermediate **10**, which was then coupled to the diazo-linker building block **11**.[26] Fmoc-deprotection followed by coupling of biotin and subsequent cleavage from the resin with TFA/TIS/H_2O gave linker **12**. Compound **12** was finally reacted with Rh–NHS to yield the tetra-functional Rh–biotin–diazo–N_3 linker **13**.

Using mild reducing conditions for the elution of affinity bound proteins instead of harsh thermal disruption of the avidin–biotin interaction resulted in the selective enrichment of one intense protein band (Fig. 4C). Isolation and in-gel-digestion of this protein band followed by mass spectrometric analysis (LC-MS/MS) revealed peptide fragments that were analyzed *via* the SEQUEST algorithm to reveal the probabilities for major protein hits. One prominent hit among the listed proteins (Table S1, ESI†) was seryl-tRNA synthetase (STS), the single enzyme in *S. aureus* that is responsible for the loading reaction of seryl-tRNAs with the correct AA. Selective inhibition of this enzyme by **2** would not just reveal the first small molecule inhibitor of STS in *S. aureus* but also offer a plausible explanation of the strong antibacterial activity of this compound.

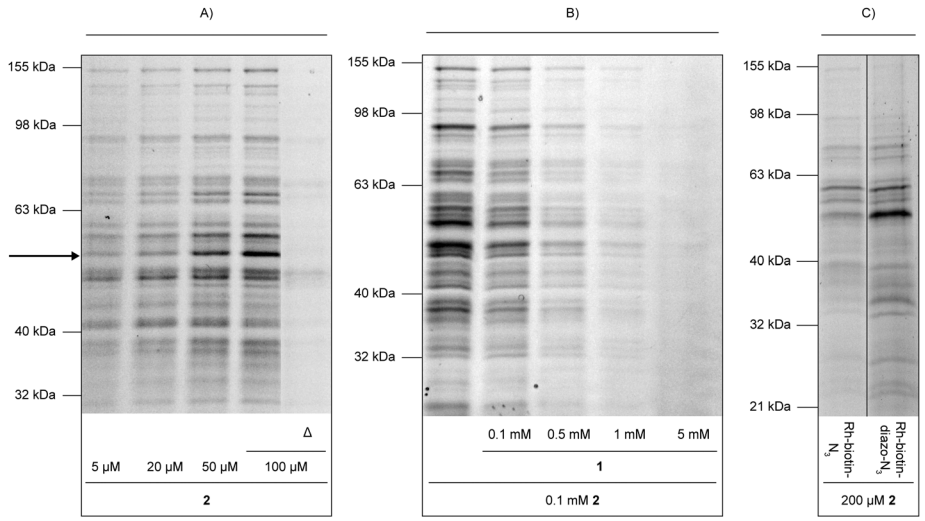
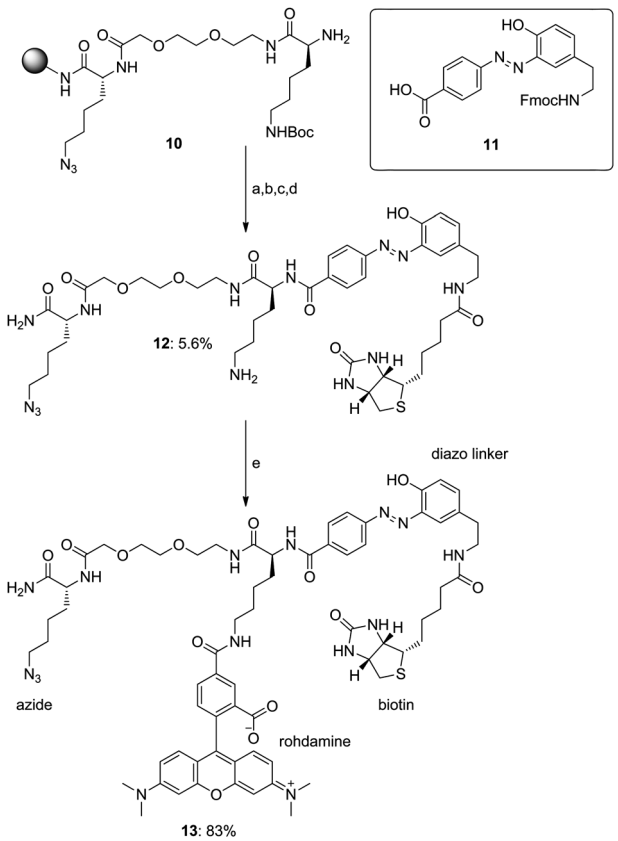


Fig. 4 Labeling experiments of *S. aureus* NCTC 8325 proteome with probe **2.** (A) Concentration dependent labeling (Δ: labeling with heat denatured proteome). (B) Competitive labeling with 1×, 5×, 10× and 50× excess of **1.** (C) Comparative enrichment with Rh–biotin–N_3 and Rh–biotin–diazo–N_3.



Scheme 3 Synthesis of Rh–biotin–diazo–N_3 (**13**). (a) **12**, HBTU, DIEA, DMF; (b) 20% piperidine in DMF; (c) biotin, DIC–HOBt, DMF; (d) TFA, TIS, H_2O; (e) Rh–NHS, DIEA, DMF.

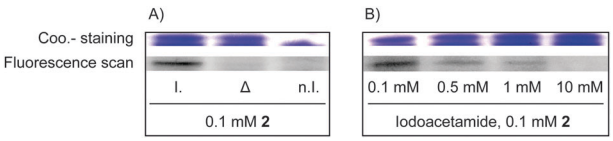


Fig. 5 Fluorescence labeling with **2** and Coomassie (coo.) staining of recombinant seryl-tRNA-synthetase in *E. coli* proteome. (A) Labeling with induced (I) and non-induced (nI) proteome. (Δ: labeling with heat denatured proteome). (B) Competitive labeling with 1×, 5×, 10× and 100× excess of iodoacetamide.

In order to confirm the binding of **1** and **2** to STS as well as to validate their corresponding target inhibition we overexpressed and purified *S. aureus* STS in *E. coli* cells. The recombinant protein was labeled with probe **2** in *E. coli* lysates which revealed a strong fluorescent band only in the induced sample. Heat denaturation of the lysate prior to probe addition again led to the disappearance of the signal and thus confirmed the specificity of probe interaction with the active and folded target (Fig. 5A, Fig. S5, ESI†).

STS is a homodimeric class II AARS that specifically charges cognate tRNAs with serine. Classification is based on the architecture of AARS catalytic domains.[1] Class I synthetases share a characteristic Rossman dinucleotide binding fold with two short consensus AA motifs (HIGH and KMSKS) that are involved in ATP binding and amino acid activation.[27] In comparison class II AARS catalytic domains have an unusual antiparallel β-sheet and three consensus motifs for ATP, AA and tRNA recognition and dimerization.[28] To verify if labeling of STS is accompanied by inhibition of the catalytic activity in seryl-tRNA-synthetase we determined the IC_{50} value for compound **2.** Several assay procedures are reported to monitor catalytic activities of AARS.[29–32] One common approach is based on the radioactive ATP–AMP turnover. Incubation of STS with serine, [α-^{32}P]-ATP and *E. coli* total tRNA results in the formation of [^{32}P]-AMP that can be quantified following TLC separation. A commercial [^{32}P]-AMP sample was used as a standard to localize and identify the reaction product AMP on the TLC plate (Fig. S6 and S7, ESI†). As shown in the dose–response curve in Fig. 6 the addition of **2** inhibits the catalytic activity of STS with an IC_{50} of 23.8 μM.

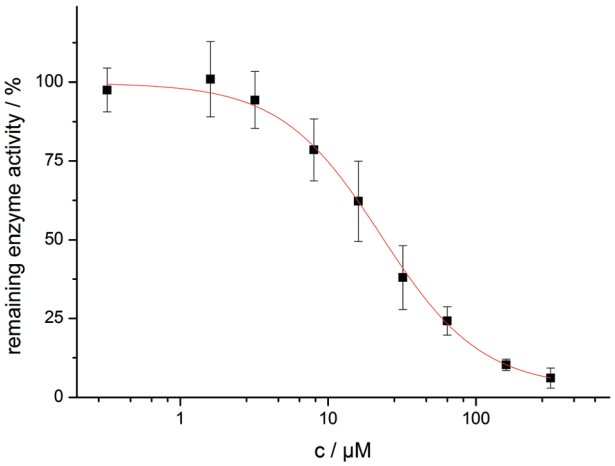


Fig. 6 Dose–response curve for **2** against *S. aureus* seryl-tRNA-synthetase. The data points and the error bars show the means and the standard deviations of four independent experiments.

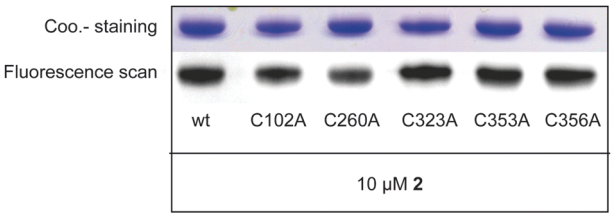


Fig. 7 Fluorescence labeling of purified C to A mutants and wt with probe **2** (wt: wild type).

To gain more information about the nature of the covalent interaction between **2** and STS we performed several downstream experiments and focused on the putative amino acid residue that might be involved in binding. One recent study by Cravatt and co-workers emphasized the presence of nucleophilic cysteines in some AARS[33] and since **2** exhibits an electrophilic Michael acceptor system it was likely that a cysteine residue of STS is involved in the nucleophilic attack. Thiols are known to covalently interact with α,β-unsaturated ketones[34,35] and in order to validate this hypothesis for STS we pre-incubated the STS expression lysate with various excess of iodoacetamide, a chemical alkylation reagent for thiols. Interestingly, an only 5-fold excess of iodoacetamide over **2** showed a strong reduction of band intensities and indeed emphasized the preference of **2** towards cysteine residues in STS (Fig. 5B).

STS contains five cysteines, however none of those has been reported to be essential for catalysis. As probe **2** could potentially react with all of the five residues (or even with more than one) we individually substituted all five cysteine residues with alanine and purified the mutant enzymes. For identification of the probe binding site, equal amounts of each purified STS mutant (5 μM final) were reacted with 10 μM of **2** and subsequently compared to the wild type enzyme *via* fluorescent gel analysis. Except minor changes in the fluorescence intensities all five mutants were still labeled with probe **2** suggesting that indeed more than one cysteine is involved in binding (Fig. 7).

In a next step we applied intact protein MS analysis to determine the stoichiometry of STS and bound probe **2**. After the incubation of 200 μM of **2** with 20 μM of STS the protein was loaded on the MS and the resulting spectra showed the attachment of up to two molecules compared to the unmodified protein control (Fig. 8). In addition to the mutant labeling studies, this result suggests the presence of two reactive cysteines within STS. However, all our attempts to determine the site of alkylation by MS/MS sequencing failed due to a low ionization of cysteine containing peptides.

As all our results suggested that cysteine residues were the site of probe attachment we addressed the question if a cysteine modification mechanism could explain the inhibition of STS. We thus individually investigated the catalytic activities of the five C to A mutants in comparison with the wild type enzyme. Interestingly, all five mutant enzymes displayed no catalytic activity emphasizing that a perturbation of one of the cysteines is already sufficient to directly interfere with the amino acylation of tRNAs by STS (Fig. S8, ESI†). Further structural studies by X-ray crystallography are necessary to elucidate the nature of the cysteine network and its direct or indirect role in STS catalysis.

Conclusion

Chalcones represent privileged structures that are present in many natural products. Their potent bioactivities against tumor cells as well as pathogenic bacteria make them interesting

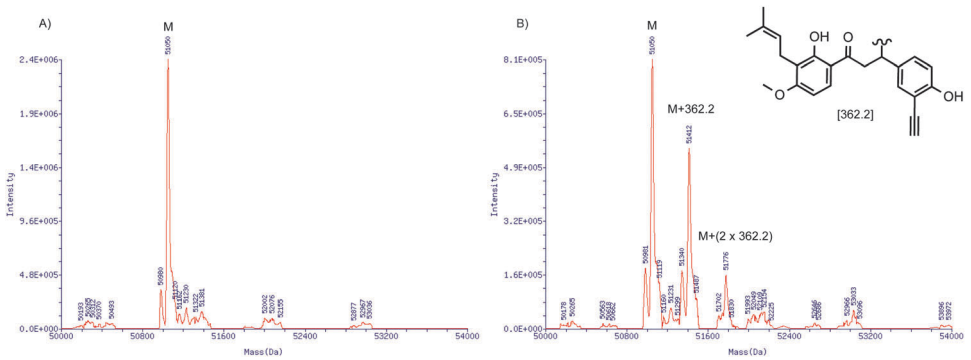


Fig. 8 Full length MS experiment for recombinant STS before (A) and after (B) incubation with probe **2**.

candidates for pharmacological studies. We here utilized a chemical-proteomic strategy in order to characterize 4-HD in terms of its antibacterial activity as well as possible modes of action. As 4-HD is heat sensitive we utilized a chemical cleavable linker that is not only essential for 4-HD enrichment and cleavage but also useful for the implementation as a standard proteomic tool that facilitates the analysis of other labile molecules of natural or synthetic origin. Our studies suggest that 4-HD directly inhibits STS activity by the alkylation of essential cysteine residues within the enzyme and thus may induce cell death by the attenuation of bacterial protein biosynthesis. Although STS represents a prominent hit for the 4-HD derived probe **2** we cannot exclude that the compound binds reversibly or irreversibly to other cellular targets as well. However, as tRNA synthetases represent validated antibacterial targets we believe that the chalcone core motif will be useful for future optimization studies and be a suitable chemical tool *e.g.* for co-crystallization to determine the mode of inhibition.

Experimental section

General methods

Reagents and solvents were of reagent grade and used without further purification as obtained from commercial sources. Reactions sensitive to air and moisture were carried out under an inert atmosphere. Column chromatography was performed on Merck silica gel (Geduran Si 60, 0.040–0.063 mm). HPLC analysis was performed on a Waters 2695 separation module, a Waters PAD 2996 and a Waters XBridge C18 column (4.6 × 100 mm). For preparative scale HPLC separation, a Waters 2545 quaternary gradient module in combination with a Waters PAD 2998 and a Waters XBridge C18 (30 × 150 mm) or a YMC Triart C18 (10 × 250 mm) column was used. Mobile phase: water, 0.1% (v/v) TFA and acetonitrile, 0.1% (v/v) TFA unless otherwise noted. ^{1}H NMR and ^{13}C NMR spectra were recorded at rt on Bruker Avance 360, Avance 500 and Avance III 500 spectrometers and were calibrated to the residual proton and carbon signal of the deuterated solvent. Mass spectra were obtained on a Thermo Scientific DFS High Resolution GC/MS (EI) and a LTQ FT Ultra (ESI†).

S. aureus strain NCTC 8325 was cultured in BHB-medium consisting of 17.5 g L^{-1} brain heart infusion, 2.5 g L^{-1} Na_2HPO_4, 2.0 g L^{-1} glucose, 10.0 g L^{-1} peptone, 5.0 g L^{-1} NaCl, final pH 7.5. *E. coli* was cultured in LB-medium: 10.0 g L^{-1} peptone, 5 g L^{-1} NaCl, 5 g L^{-1} yeast extract, final pH 7.5. All strains were grown at 37 °C unless otherwise noted. For selective growth, media with the following final antibiotic concentrations were used: ampicillin 100 μg mL^{-1}, kanamycin 25 μg mL^{-1}, chloramphenicol 10 μg mL^{-1}. Primers for mutagenesis and PCR were purchased from Eurofins MWG Operon. For all biochemistry applications of **1**, DMSO stock solutions were prepared to keep the final DMSO concentrations $\leq$2% unless reported otherwise. PCR reactions were performed using the Phusion® High-Fidelity PCR kit (NEB) with a C1000 Thermal Cycler (BioRad) following the standard manufacturers protocols. DNA-bands on agarose were purified with an Omega gel extraction kit and plasmids were isolated from overnight cultures using the Omega plasmid mini kit. DNA and protein concentrations were measured using a TECAN Infinite M200 PRO. Plasmid sequencing was done by GATC Biotech AG. Gene expression was induced with 0.1 mM anhydrotetracycline. For protein purification an ÄKTApurifier (P900, UPC900, Frac950) was used.

3-Bromo-4-(methoxymethoxy)benzaldehyde (4)

Compound **4** was prepared according to the method of Chi *et al*. The spectral data matched with those reported in the literature.[20]

1-(4-Methoxy-2-((3-methylbut-2-en-1-yl)oxy)phenyl)ethanone (7)

Compound **7** was prepared according to the method of Matsui *et al*. The spectral data matched with those reported in the literature.[19]

4-(Methoxymethoxy)-3-((trimethylsilyl)ethynyl)benzaldehyde (5)

To a solution of **4** (3.0 g, 12 mmol) in piperidine (100 mL) was added PPh_3 (42 mg, 0.3 mmol), CuI (23 mg, 0.1 mmol) and trimethylsilylacetylene (7 mL, 48.8 mmol) under an inert atmosphere. The solution was degassed and $PdCl_2$ (42 mg, 0.3 mmol) was added. The mixture was refluxed for 24 h, concentrated to dryness and purified by column chromatography to yield **5** (1.9 g, 7.2 mmol, 59%) as a colourless solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.30 (s, 9*H*), 3.56 (s, 3*H*), 5.35 (s, 2*H*), 7.23 (d, *J* = 8.6 Hz, 1*H*), 7.81 (dd, *J* = 2.0, 8.6 Hz, 1*H*), 7.99 (d, *J* = 2.0 Hz, 1*H*), 9.89 (s, 1*H*). ^{13}C NMR (125 MHz, $CDCl_3$) δ −0.1, 56.6, 94.7, 99.5, 100.1, 114.4, 114.6, 130.3, 131.3, 136.1, 162.6, 190.3. HRMS (EI): $[M]^+$ $C_{14}H_{18}O_3{}^{28}Si$ calcd 262.1020, found 262.1013. R_f (20% EtOAc–hexanes) = 0.6.

(*E*)-3-(3-Ethynyl-4-(methoxymethoxy)phenyl)-1-(4-methoxy-2-((3-methylbut-2-en-1-yl)oxy)phenyl)prop-2-en-1-one (8)

A solution of **5** (1.9 g, 7.3 mmol), **7** (2.0 g, 8.7 mmol) and 3 M NaOH (24 mL) in EtOH (50 mL) was stirred for 16 h at rt. The solution was adjusted to pH = 2 with 1 M HCl and extracted with EtOAc. The solvent was removed under reduced pressure and the crude product was purified by HPLC to yield **8** (1.1 g, 2.7 mmol, 37%) as a yellow solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.78 (s, 3*H*), 1.82 (s, 3*H*), 3.33 (s, 1*H*), 3.55 (s, 3*H*), 3.89 (s, 3*H*), 4.61 (d, *J* = 6.8 Hz, 2*H*), 5.32 (s, 2*H*), 5.57 (t, *J* = 6.7 Hz, 1*H*), 6.52 (d, *J* = 2.1 Hz, 1*H*), 6.59 (dd, *J* = 2.2, 8.7 Hz, 1*H*), 7.16 (d, *J* = 8.7 Hz, 1*H*), 7.51 (dd, *J* = 2.1, 8.7 Hz, 1*H*), 7.61 (d, *J* = 3.1 Hz, 2*H*), 7.73 (d, *J* = 2.1 Hz, 1*H*), 7.86 (d, *J* = 8.7 Hz, 1*H*). ^{13}C NMR (125 MHz, $CDCl_3$) δ 18.3, 25.9, 55.6, 56.4, 65.5, 79.5, 81.5, 94.7, 99.6, 105.5, 112.9, 114.8, 118.9, 122.1, 126.7, 129.4, 130.7, 133.2, 133.4, 139.1, 139.9, 159.4, 160.1, 164.3, 189.8. HRMS (ESI): $[M + H]^+$ $C_{25}H_{27}O_5$ calcd 407.1853, found 407.1852.

(*E*)-3-(3-Ethynyl-4-(methoxymethoxy)phenyl)-1-(2-hydroxy-4-methoxy-3-(3-methylbut-2-en-1-yl)phenyl)prop-2-en-1-one (9)

A suspension of **8** (1.4 g, 3.4 mmol) and montmorillonite K10 (1.4 g) in CH_2Cl_2 was stirred for 1.5 h at 0 °C. Solids were removed by filtration. The solvent was removed under reduced

pressure and the crude product was purified by HPLC to yield **9** (430 mg, 1.1 mmol, 31%) as a yellow solid. ^{1}H NMR (500 MHz, $CDCl_3$) 1.71 (s, 3*H*), 1.83 (s, 3*H*), 3.36 (s, 1*H*), 3.42 (d, *J* = 7.0 Hz, 2*H*), 3.56 (s, 3*H*), 3.94 (s, 3*H*), 5.26 (t, *J* = 7.1 Hz, 1*H*), 5.34 (s, 2*H*), 6.53 (d, *J* = 9.0 Hz, 1*H*), 7.22 (d, *J* = 8.7 Hz, 1*H*), 7.52 (d, *J* = 15.4 Hz, 1*H*), 7.59 (dd, *J* = 2.1, 8.7 Hz, 1*H*), 7.78 7.85 (m, 3*H*). ^{13}C NMR (125 MHz, $CDCl_3$) δ 17.9, 21.7, 25.9, 55.8, 56.5, 79.2, 81.8, 94.7, 102.1, 113.0, 114.6, 114.9, 117.6, 119.6, 122.0, 128.6, 129.2, 131.0, 132.0, 133.8, 142.6, 159.9, 163.0, 163.3, 192.0. HRMS (ESI): $[M + H]^+$ $C_{25}H_{27}O_5$ calcd 407.1853, found 407.1853.

(*E*)-3-(3-Ethynyl-4-hydroxyphenyl)-1-(2-hydroxy-4-methoxy-3-(3-methylbut-2-en-1-yl)phenyl)prop-2-en-1-one (2)

To a solution of **9** (224 mg, 0.6 mmol) in MeOH was added *p*-TsOH (105 mg, 0.6 mmol) and the mixture was stirred for 3 d at 30 °C. The solvent was removed under reduced pressure and the crude product was purified by HPLC to yield **2** (122 mg, 0.3 mmol, 62%) as a yellow solid. ^{1}H NMR (500 MHz, $CDCl_3$) 1.71 (s, 3*H*), 1.83 (s, 3*H*), 3.41 (d, *J* = 7.0 Hz, 2*H*), 3.56 (s, 1*H*), 3.94 (s, 3*H*), 5.25 (d, *J* = 7.1 Hz, 1*H*), 6.53 (d, *J* = 9.0 Hz, 1*H*), 7.04 (d, *J* = 8.6 Hz, 1*H*), 7.50 (d, *J* = 15.4 Hz, 1*H*), 7.62 (dd, *J* = 2.1, 8.6 Hz, 1*H*), 7.72 (d, *J* = 2.1 Hz, 1*H*), 7.77, 7.83 (m, 2*H*). ^{13}C NMR (125 MHz, $CDCl_3$) δ 17.9, 21.7, 25.9, 55.8, 77.4, 85.3, 102.1, 109.2, 114.6, 115.6, 117.6, 119.1, 122.0, 127.6, 129.2, 131.4, 132.0, 132.4, 142.8, 159.1, 163.0, 163.3, 192.0. HRMS (ESI): $[M + H]^+$ $C_{23}H_{24}O_4$ calcd 363.1591, found 363.1580.

4-Hydroxyderricin (1)

Compound **1** was prepared according to the method of Matsui *et al*. The spectral data matched with those reported in the literature.[19]

(*E*)-4-((5-(2-((((9*H*-Fluoren-9-yl)methoxy)carbonyl)amino)ethyl)-2-hydroxyphenyl)diazenyl)benzoic acid (11)

The diazobenzene building block **11** was synthesized following literature procedures.[26]

Biotin–diazo–N_3 linker 12

The synthesis of click-cleavable linker **12** was performed on a Rink resin. Fmoc–Lys(azide)–OH, Fmoc–PEG–OH and Fmoc–Lys(Boc)–OH were coupled *via* DIC–HOBt (3 eq. with respect to resin loading). The Fmoc group of Fmoc–Lys(Boc) was removed from the resin by treatment with 20% piperidine in DMF (20 min). The diazobenzene cleavable building block **11** (1.5 eq.), HBTU and DIEA were reacted with the resin overnight. After Fmoc–deprotection biotin (1.5 eq.) was coupled using DIC–HOBt overnight, the resin-bound building block was cleaved by incubation with a solution of TFA : TIS : H_2O (95% : 2.5% : 2.5%) for 2 hours. The peptide was precipitated in cold diethyl ether and purified by HPLC yielding an orange solid (10.5 mg, yield 5.6%). ESI-MS: $[M + H]^+$ calculated for $C_{43}H_{63}N_{13}O_9S$ 938.4592, found 938.4686.

Rh–biotin–diazo–N_3 linker 13

Building block **12** (10 mg, 0.011 mmol) was reacted overnight in DMSO (200 μL) with 5-(and 6-)carboxytetramethylrhodamine, succinimidyl ester (4.68 mg, 0.009 mmol) and DIEA (7.6 μL, 0.044 mol). The solution was purified by HPLC to give a red compound (10 mg; yield 83%). ESI-MS: $[M + H]^+$ calculated for $C_{68}H_{83}N_{15}O_{13}S$ 1350.6015, found 1350.6502.

Minimum inhibitory concentration assay

MICs were measured in 96 well plates. 99 μL of a diluted overnight culture in fresh medium (final OD_{600} = 0.01) were supplemented with 1 μL of DMSO probe stocks with various concentrations. Plates were incubated at 37 °C with shaking at 220–250 rpm for 14–16 h. All experiments were conducted in triplicate in at least three independent experiments. Reported MICs represent the lowest concentrations of probe where no bacterial growth was visible.

In vitro labeling

S. aureus NCTC 8325 was grown in BHB medium as described above. One hour after reaching the stationary phase they were harvested by centrifugation, washed and lysed under ice cooling using a Bandelin Sonopuls HD 2070 (3 × 20 s, 80% power) in PBS buffer. Insoluble components were separated by centrifugation (30 min, 9000 rpm) and discarded. The protein concentration of the soluble fraction was measured *via* Bradford assay and adjusted to 1 mg mL^{-1} by dilution in PBS. For heat controls the proteome was denatured with 2% SDS at 98 °C for 10 min. Competitive labeling experiments were performed with proteome that was pre-incubated (20 min) with iodoacetamide or **1**. Then probe **2** was added to the final concentrations reported and mixtures were incubated at rt. After 1 h click reagents were added to the final concentrations of 200 μM rhodamine–azide (Rh–N_3), 1.2 mM tris(2-carboxyethyl)phosphine (TCEP), 96 μM tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA) ligand and 1 mM $CuSO_4$ with a final volume of 50 μL. After 1 h at rt 2× BME loading buffer (63 mM Tris–HCl, 10% glycerin, 0.0025% bromophenolblue, 2% SDS, 5% β-mercaptoethanol, pH = 8.3) was added. Samples were separated by SDS-PAGE and analyzed by fluorescence recorded using a Fujifilm LAS-4000 luminescent image analyzer (Fujinon VRF43LMD3 lens, 575DF20 filter). Total protein expression levels were compared subsequently by Coomassie staining.

Enrichment strategies

Preparative labeling experiments were based on the described analytical protocol with the following amounts: protein concentration 6 mg mL^{-1}, 200 μM of **1**, 10 μM of linker (Rh–biotin–N_3 or Rh–biotin–diazo–N_3) with a 1 mL total reaction volume. Previous to click chemistry proteins were precipitated with an equal volume of pre-cooled acetone, washed with methanol and resuspended in PBS to remove excess of probe. Click reagents were added and after 1 h at rt proteins were again precipitated with acetone. The pellet was washed three times with methanol, resuspended in 0.2% SDS–PBS and incubated with avidin–agarose beads (Sigma-Aldrich, 50 μL) with gentle mixing. This enrichment protocol was also performed with a control lacking **1** to compare the results of the biotin–avidin-enrichment with the background of unspecific protein binding on avidin–agarose beads.

After 1 h beads were washed three times with 0.2% SDS–PBS, twice with 6 M urea and again three times with 0.2% SDS–PBS. For thermal elution beads were incubated for 6 min with 50 μL BME buffer at 95 °C. For chemical elution beads were 3× incubated for 15 min with 20 μL of elution buffer (100 mM ammonium hydrogen carbonate, 25 mM sodium hydrosulfite).[25] Released proteins were applied to a preparative SDS-PAGE and fluorescent bands were isolated, washed and digested with trypsin as described previously.[18]

Mass spectrometry and bioinformatics[36]

ESI-MS spectra were recorded using a Thermo LTQ Orbitrap XL coupled to a Dionex UltiMate 3000 RSLC nano. The peptides were loaded on a Dionex Acclaim® PepMap 100, 75 μm × 2 cm, C18 (3 μm) and subsequently eluted and separated by a Dionex Acclaim® PepMap RLSC 75 μm × 15 cm, C18 (2 μm). Mass spectrometry data were searched against the corresponding databases *via* the software Proteome Discoverer 1.3 (Thermo Scientific) using the SEQUEST algorithm. The search was limited to only tryptic peptides, two missed cleavage sites, precursor mass tolerance of 10 ppm and fragment mass tolerance of 0.8 Da. Filters were set to further refine the search results. The X_{corr} *vs.* charge state filter was set to X_{corr} values of 1.5, 2.0, 2.25 and 2.5 for charge states +1, +2, +3 and +4, respectively. The number of different peptides had to be ≥ 2 and the peptide confidence filter was set to at least medium. These filter values are similar to others previously reported for SEQUEST analysis. X_{corr} values (Score) of each run, the peptide spectrum matches (PSM) as well as the total number of obtained peptides and unique peptides are reported in Table S1 (ESI†).

Overexpression and purification of Strep-tagged *S. aureus* NCTC 8325 wild type enzymes in *E. coli* BL21 (DE3)

Recombinant expression in *E. coli* was performed using the Invitrogen Gateway protocols. Genes were amplified from genomic DNA with Gateway primers given in Table S2 (ESI†). The purified *attB*-PCR products were cloned with the Gateway BP Clonase II Mix into the entry-vector pDONOR201 (Invitrogen) for amplification in XL1-Blue Competent Cells (Agilent Technologies) using kanamycin selection. The purified and sequenced plasmid was then used in the LR reaction (Gateway LR Clonase II Enzyme Mix) for recombination with the destination vector pDest007. After transformation into chemically competent BL21 (DE3) cells (NEB) recombinant Strep-tag clones were grown in LB_{amp} medium at 37 °C until $OD_{600} \sim 0.6$. Target gene expression was induced with anhydrotetracycline at 18 °C overnight. Purification of the soluble lysate fraction was carried out using Strep-Tactin Superflow Plus columns (Qiagen) and concentrated using Amicon® Ultra-4 Centrifugal Filter Units NMWL 10 000 (Millipore). Desalted stock solutions were stored in 10 mM Tris buffer (pH 7.5) at −80 °C.

Site-directed mutagenesis

Cysteine to alanine mutations were introduced by fusion PCR using primers given in Tables S2 and S3 (ESI†). Single fragments were amplified from plasmid DNA, purified by agarose gel electrophoresis and subsequently used in the fusion reaction. Cloning and overexpression were performed as described above. The mutations were verified by sequence analysis.

Aminoacylation assay and IC_{50} determination

Inhibition of the seryl-tRNA-synthetase was measured by formation and separation of [α-^{32}P]-AMP from [α-^{32}P]-ATP by thin layer chromatography.[37] DMSO solutions of **1** (1 μL) were placed in reaction tubes and diluted with 16 μL assay buffer (100 mM HEPES (pH 7.2), 10 mM $MgCl_2$, 30 mM KCl). One tube with 1 μL DMSO was used as the positive control for 100% enzyme activity determination. 1 μL of purified enzyme was added (1 μL assay buffer as no enzyme control for background subtraction) and the enzyme–inhibitor mixtures were pre-incubated for 20 min at rt. The reaction was started by addition of a 7 μL reaction mixture resulting in final concentrations of 5 μM ATP, 50 nM [α-^{32}P]-ATP (Hartmann Analytic) 1 mM serine, 4 mg mL^{-1} *E. coli* total tRNA (Roche) and 320 nM enzyme.[32] After incubation at 37 °C for 1 h the reaction was stopped by spotting 1 μL volume per reaction on polyethylenimine–cellulose plates (Merck Bioscience). The plates were developed with a mobile phase of 0.5 M LiCl in 2 N formic acid. Spot intensities were determined using a Typhoon 9200 Variable Mode Imager using Image Quant for Quantification (GE Healthcare). All measurements were performed in triplicate in at least 4 independent trials. AMP spot identities were validated using a commercial [α-^{32}P]-AMP (Hartmann Analytic). Inhibition values were corrected for background hydrolysis (no enzyme reaction), normalized and plotted against the concentrations of **2**.

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