

Showdomycin as a Versatile Chemical Tool for the Detection of Pathogenesis-Associated Enzymes in Bacteria

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Abstract: Showdomycin is a potent nucleoside antibiotic that displays a high structural similarity to uridine and pseudouridine. No detailed target analysis of this very unusual electrophilic natural product has been carried out so far. To unravel its biological function, we synthesized a showdomycin probe that can be appended with a fluorophore or a biotin marker via click chemistry and identified diverse enzymes which were important for either the viability or virulence of pathogenic bacteria. Our results indicate that the antibiotic effect of showdomycin against *Staphylococcus aureus* may be due to the inhibition of various essential enzymes, especially MurA1 and MurA2, which are required for cell wall biosynthesis. Although real-time polymerase chain reaction revealed that the MurA2 gene was expressed equally in four *S. aureus* strains, our probe studies showed that MurA2 was activated in only one multiresistant *S. aureus* strain, and only this strain was resistant to elevated concentrations of the MurA inhibitor fosfomycin, suggesting its potential role as an antibiotic bypass mechanism in the case of MurA1 inhibition. Moreover, we utilized this tool to compare enzyme profiles of different pathogenic strains, which provided unique insights in regulatory differences as well as strain-specific signatures.

Introduction

Nature provides a great source of bioactive molecules that are used in medicinal applications for the treatment of many severe diseases, including bacterial infections. The repertoire of small molecules that are produced by microorganisms and sponges exerts a great diversity of biological activities that in many cases is a result of evolutionary processes to develop customized defense strategies against competitors in the fight for limited nutritional resources. The urgent quest to discover novel and biologically active natural product antibiotics for the treatment of multiresistant bacterial pathogens has led to extensive efforts in the fields of natural product isolation and their corresponding total synthesis. However, in many cases the application as a drug is limited since the precise mechanism of action and the dedicated molecular targets remain unknown. We have previously unraveled the molecular targets of biomimetic β -lactones^{1–3} and β -lactams^{4,5} in bacteria by a chemical proteomic strategy called activity-based protein profiling (ABPP).^{6,7} In these studies, several targets with essential roles in virulence and resistance could be detected. Here we extend this strategy to the natural product showdomycin and explore its full complement of biological targets in diverse pathogenic bacteria. We show that a reporter-tagged showdomycin probe labels and inhibits a great diversity of important bacterial

enzymes. Many of those enzyme families have not been targeted by ABPP probes before, which opens new avenues to study their precise function in various proteomes. We further show that the showdomycin probe is an important tool for the discovery of enzymes that display characteristic signature motifs in closely related strains of pathogenic bacteria that help to discriminate multiresistant from antibiotic-sensitive strains. Finally, we identified molecular targets that are likely to be responsible for the antibiotic effects of showdomycin.

Showdomycin is a potent C-glycosyl nucleoside antibiotic that was isolated from *Streptomyces showdoensis*.⁸ It displays a high structural similarity to uridine and pseudouridine, with the exception that the bases are replaced by an electrophilic maleimide moiety (Figure 1). It was therefore speculated that nature designed this nucleoside analogue as a suicide inhibitor to interfere with the uridine metabolism.⁹ So far, several studies found evidence using cell-free *in vitro* experiments with eukaryotes that showdomycin targets uridine, UDP, and other nucleotide-dependent enzymes like kinases, phosphorylases, and dehydrogenases.^{9–11} However, no detailed target analysis of this very unusual electrophilic natural product has been carried out so far for bacteria.

Results and Discussion

To unravel its biological targets, we first applied a total synthesis of showdomycin adapted from a strategy reported by

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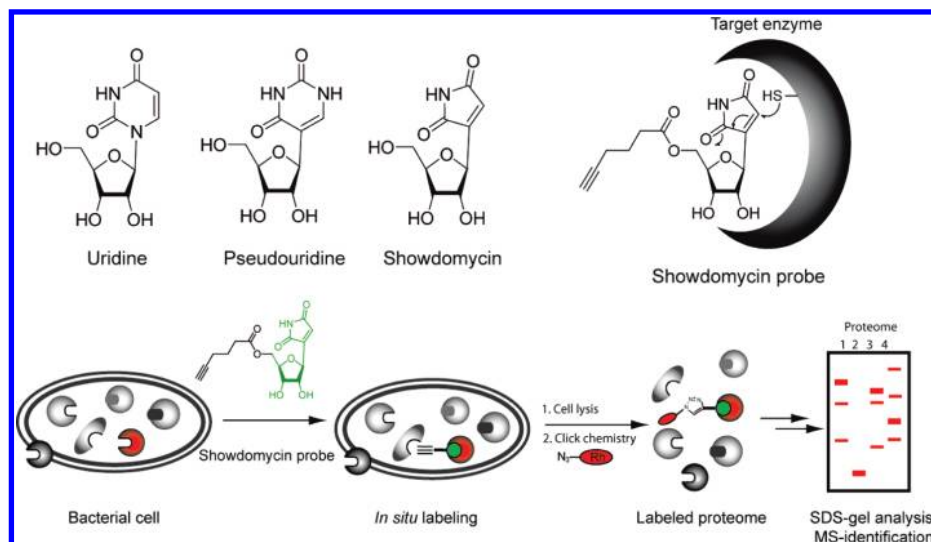


Figure 1. Similarity of showdomycin to uridine and pseudouridine. Sulfhydryl groups in the enzyme's binding site attack the reactive maleimide moiety and covalently bind the probe. The alkyne linker of the probe allows the identification of labeled enzymes by the introduction of a fluorescent tag via click chemistry after cell lysis and SDS gel analysis.

Barrett et al.¹² and attached 5-hexynoic acid via the free primary alcohol group of the ribose moiety (Supporting Information Figure S1) to introduce an alkyne handle for subsequent target analysis. The modification of the alkyne tag via 1,3-dipolar Huisgen cycloaddition (click chemistry, CC)^{13,14} allows us to append a fluorophor or biotin reporter group after proteome labeling for target enzyme visualization via SDS–PAGE or enrichment for target identification via mass spectrometry (MS), respectively. Diastereomeric showdomycin and *epi*-showdomycin probes were separated by preparative HPLC, and the naturally occurring showdomycin isomer was used for all subsequent studies.

To test whether the showdomycin probe still displayed antibacterial activity, we compared minimal inhibitory concentration (MIC) values of *S. aureus* NCTC 8325 that was treated with various concentrations of the two compounds in culture medium. In fact, both molecules gave identical MIC values (500 μ M for showdomycin and 500 μ M for the corresponding probe), indicating that the small alkyne tag modification does not hinder the activity of the natural product. These MIC values are in the order of magnitude determined previously by the agar-streak dilution method for another *S. aureus* strain with a MIC around 220 μ M.⁸ Maleimide is a strong electrophile that reacts preferentially with cysteine residues of proteins, also including those which are not of elevated nucleophilicity and are not part of the active-site pocket. We therefore hypothesized that the maleimide moiety, the reactive group of showdomycin, is fine-tuned by a sterically restricted environment provided by the ribose moiety to ensure specific target interactions, as previously suggested.¹⁵ To evaluate the reactivity of showdomycin, we incubated *S. aureus* proteome lysates with an N-labeled fluorescent maleimide, unhindered at the site of attack, and the showdomycin probe to directly compare the labeling pattern

(Supporting Information Figure S2A). Interestingly, while the maleimide probe labeled a great deal of almost all proteins in the lysate, the showdomycin probe revealed target selectivity for only a limited number of proteins. A corresponding control by which the proteome was heat-inactivated at 95 °C for 5 min prior to probe addition further demonstrated that the labeling of the majority of targets occurred in an activity-based manner, as these proteins do not get labeled when they are unfolded (Supporting Information Figure S2B). MS experiments revealed that in aqueous solution showdomycin reacts only with thiols but not with amines, indicating that cysteine residues are indeed the preferred point of attachment (Supporting Information).

These initial experiments provided evidence that the showdomycin probe is in most cases a selective chemical tool to label distinct enzymes in complex proteomes. We next tested whether the compound is cell permeable and could label enzymes in living pathogens such as *S. aureus*, multiresistant *S. aureus* (MRSA), *P. aeruginosa*, and *L. monocytogenes* strains under *in situ* conditions. After cell lysis, an azide-rhodamine tag was appended to the labeled proteins of the cytosolic proteome fraction via CC prior to fluorescent SDS–PAGE analysis (Figure 2A). Gel bands of labeled proteins were detected by fluorescence scanning and identified by MS. Interestingly, several distinct labeling events were observed, indicating that showdomycin is capable of entering the cell. Some of the targeted proteins were of low abundance, as shown by direct comparison of the relative intensities observed with Coomassie staining vs fluorescence scanning (Figure 2A). The optimal concentration for target labeling was determined by variation of probe concentrations in several bacterial species (Figure 2, Supporting Information Figure S4). It turned out that 50 μ M was the optimal concentration for the full saturation of the majority of targets. Selected enzymes displayed a very sensitive labeling with the showdomycin probe down to 1.3 μ M (Figure 2B). Preincubation of proteomes with 1 mM to 100 μ M unmodified showdomycin and subsequent addition of 10 μ M probe resulted in a total loss of fluorescent signals, indicating that showdomycin and the corresponding probe compete for the same labeling sites (Supporting Information Figure S3). Additionally, various incubation times revealed that the probe very rapidly entered

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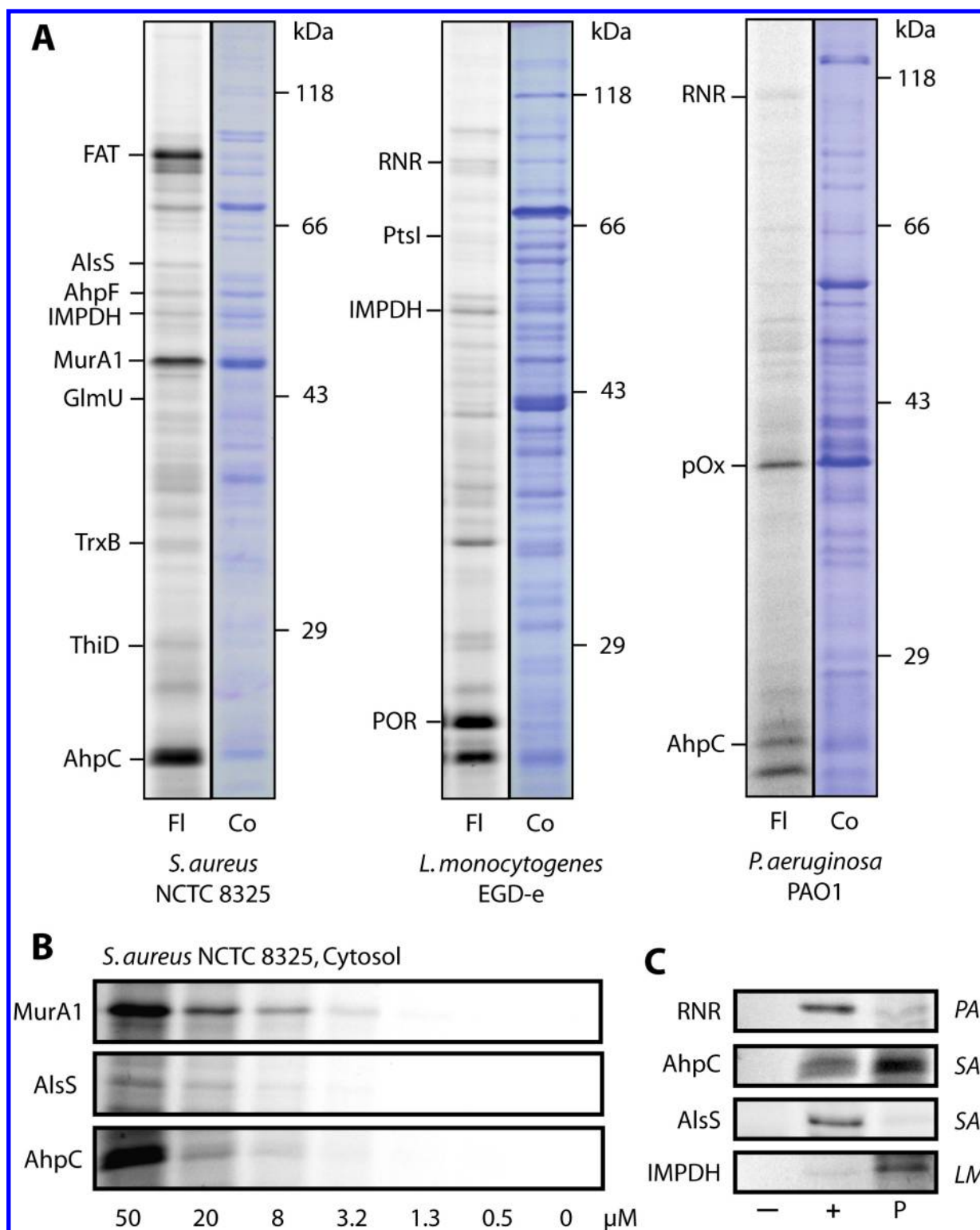


Figure 2. Labeling of the proteomes of bacterial pathogens by the showdomycin probe. (A) Comparison of the fluorescent gels (FI) and Coomassie abundance staining (Co) by *in situ* labeling of enzymes in three different bacterial strains. Enzyme identities are assigned to the corresponding gel band (for a list of abbreviations, please refer to Table 1). (B) The probe exhibits remarkable sensitivity for selected targets, such as MurA1 that is labeled down to 1.3 μM. (C) Examples of recombinantly expressed enzymes (–, before induction; +, after induction; P, native proteome).

the cell and achieved full saturation of targets within 7.5 min (Supporting Information Figure S4).

Taking all bacterial pathogens together, we were able to observe the labeling of about 13 different enzymes by MS (Table 1). MS results were confirmed by recombinant expression of all major hits and subsequent labeling by the corresponding

probe (Figure 2C). The identified enzymes belong to two major classes comprising oxidoreductases and transferases, and interestingly several of them recognize substrates that contain nucleosides, e.g., uridine (Table 1). Many of the corresponding enzyme families require a nucleophilic cysteine residue in their active site for catalysis, which is likely to attack the electrophilic

Table 1. Enzymes of Different Functions and Enzyme Classes (EC) That Were Identified in Pathogenic and Non-pathogenic Strains

function	enzyme	class	organisms ^a
oxidative-stress resistance	alkyl hydroperoxide reductase subunit C (AhpC)	EC 1.11.1	<i>S. aureus</i> NCTC 8325, Mu50, <i>P. putida</i> , <i>P. aeruginosa</i>
	alkyl hydroperoxide reductase subunit F (AhpF)	EC 1.6.4	<i>S. aureus</i> NCTC 8325, Mu50
	thioredoxine reductase (TrxB)	EC 1.8.1	<i>S. aureus</i> NCTC 8325, Mu50
virulence-associated	phosphoenolpyruvate-protein phosphotransferase (PtiI)	EC 2.7.3	<i>S. aureus</i> Mu50, <i>L. monocytogenes</i> EGD-e
	acetolactate synthase (AlsS)	EC 2.2.1	<i>S. aureus</i> NCTC 8325
nucleotide biosynthesis	inosine-5'-monophosphate dehydrogenase (IMPDH)	EC 1.1.1	<i>S. aureus</i> NCTC 8325, Mu50, <i>L. monocytogenes</i> EGD-e
	ribonucleotide-diphosphate reductase subunit alpha (RNR)	EC 1.17.4	<i>L. monocytogenes</i> EGD-e, <i>P. aeruginosa</i>
primary metabolism	phosphomethyl-pyrimidine kinase (ThiD)	EC 2.7.4	<i>S. aureus</i> NCTC 8325
	formate acetyltransferase (FAT)	EC 2.3.1	<i>S. aureus</i> NCTC 8325, Mu50
	cysteine desulfurase (CDS)	EC 2.8.1	<i>P. aeruginosa</i>
resistance/cell-wall biosynthesis	UDP- <i>N</i> -acetylglucosamine 1-carboxyvinyltransferase 1 (MurA1)	EC 2.5.1	<i>S. aureus</i> NCTC 8325, Mu50
	UDP- <i>N</i> -acetylglucosamine 1-carboxyvinyltransferase 2 (MurA2)	EC 2.5.1	<i>S. aureus</i> Mu50
	UDP- <i>N</i> -acetyl-glucosamine pyrophosphorylase, putative (GlmU)	EC 2.7.7	<i>S. aureus</i> NCTC 8325

^a As identified by mass spectrometry; those identified by labeling profile are not given.

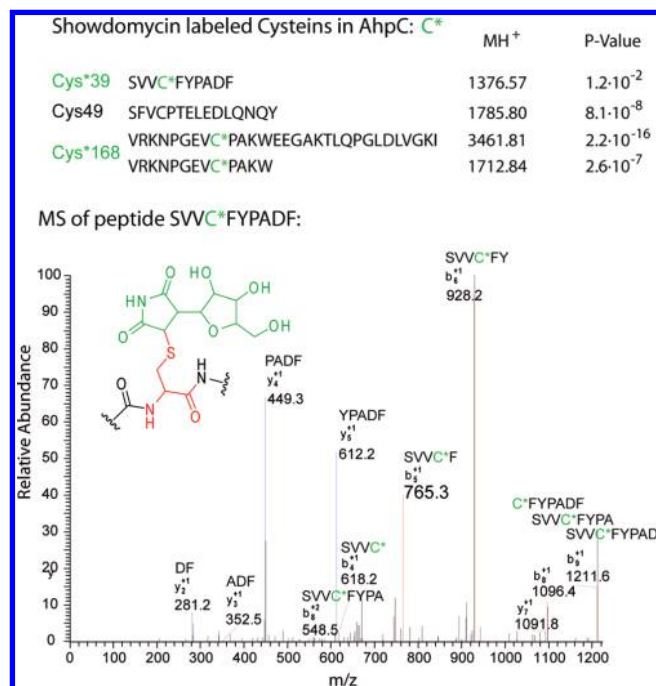


Figure 3. Identification of the binding sites of showdomycin by mass spectrometry (MS). Peptide masses (MH⁺) and *p*-values are given for each peptide containing a cysteine residue. Only Cys39 and Cys168 were found to be modified by showdomycin. A MS fragmentation pattern is given below for the chymotryptic peptide of Cys39.

maleimide of showdomycin. Indeed, a substrate inhibition assay with MurA1 demonstrated that showdomycin mediated inhibition of enzyme activity with an IC₅₀ of 10 μM (Supporting Information Figure S5). Moreover, we analyzed the site of modification in the case of one representative reductase (AhpC, see below) and demonstrated that showdomycin attached selectively to two cysteine residues (Figure 3, Supporting Information Figure S6). The active-site inhibition and great coverage of mechanistically distinct enzyme families that have not been addressed by ABPP before emphasizes the unprecedented utility of showdomycin as a new proteomic tool for the identification of pathogenesis-associated enzyme targets.

Interestingly, a large fraction of the identified enzymes are involved in important cellular functions such as cell wall biosynthesis, oxidative stress resistance, nucleotide biosynthesis, and amino acid biosynthesis. Several targets are of special

medicinal interest (Supporting Information Table 2), including enzymes that are crucial for oxidative stress resistance in *S. aureus*, like alkylhydroperoxide reductase C (AhpC), alkylhydroperoxide reductase F (AhpF), and thioredoxin reductase (TrxB). These enzymes have fundamental functions for colonization and survival of the pathogen in the host.¹⁶ Inosine-5'-monophosphate dehydrogenase (IMPDH) is required for the biosynthesis of GMP and thus for the cells' guanosine phosphate pool.¹⁷ Besides its role for the synthesis of branched amino acids, acetolactate synthase (AlsS) is important for the virulence of *S. aureus*, as genetic knockouts resulted in reduced biofilm formation.¹⁸ The UDP-*N*-acetylglucosamine 1-carboxyvinyltransferase 1 (MurA1) catalyzes the first step in cell wall biosynthesis and is thus an essential bacterial enzyme with major importance as an antibiotic drug target.¹⁹

One of the major challenges in the treatment of bacterial infections is rapid detection of the disease-causing pathogen. In these instances, suitable biomarkers are required that provide a unique readout which is characteristic for the bacterial strain. Our comparison of enzyme-labeling pattern between different resistant and nonresistant strains and between pathogenic and nonpathogenic bacteria (*Listeria*, *Pseudomonas*, and *Staphylococcus*) with the showdomycin probe provided several of these unique signatures (Figure 4 and Supporting Information Figure S7). For instance, the comparative analysis of four different strains of *S. aureus*—the reference strain *S. aureus* NCTC 8325, the highly toxin-producing clinical isolate DSM 19041, and the MRSA strains Mu50 and DSM 18827 (clinical isolate)—revealed that phosphoenolpyruvate-protein phosphotransferase (PtiI) was labeled only in the MRSA strains and the toxin-producing strain, but not in the laboratory strain NCTC 8325 (Figure 4). PtiI was found to be important for the virulence of *S. aureus*, *S. typhimurium*, and *H. influenzae*, as shown by highly attenuated pathogenicity of Δ*ptsI* mutants of these species in a mouse model.²⁰ This may indicate that the more pathogenic strains and

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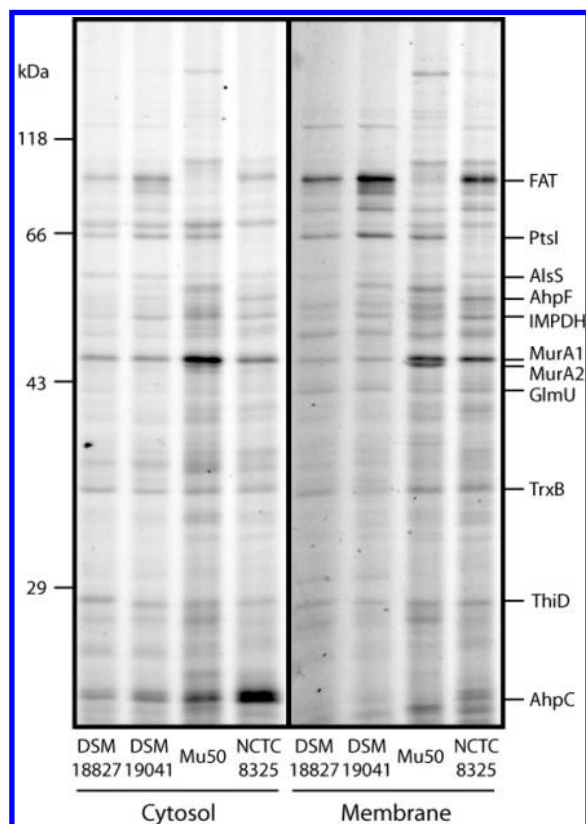


Figure 4. Comparison of the *in situ* labeling profile of four different *S. aureus* strains. The probe concentration was 50 μ M each. Enzyme identities are assigned to the corresponding gel band (for a list of abbreviations, please refer to Table 1).

clinical isolates constitutively exhibit higher PtsI activity, even under culture conditions, compared with the less infective laboratory reference strain. Vice versa, the peroxidase AhpC was found to be highly active in NCTC 8325 but not in the other resistant or toxic strains. Interestingly, bioinformatic gene analysis revealed that even species and strains in which less or no labeling of a certain protein could be detected carried a copy of the gene in their genome that was highly similar to or even identical with the one that was labeled in the reference strain or species (Supporting Information Table 3). This indicates that regulatory processes at expressional or posttranslational levels are the reason for the observed differences in labeling. Furthermore, the comparison of *S. aureus* strains revealed a very distinct labeling of MurA2 which occurred only at the MRSA strain Mu50 and only *in situ* but not *in vitro* (Supporting Information Figure S8). It has been proposed that MurA2 arose from gene duplication of *murA1* in Gram-positive bacteria. The two different copies of MurA are functionally identical and can replace each other when one is inhibited.¹⁹ The two MurA homologues share low sequence identity at the amino acid level. Differences in the active site have made it difficult, so far, to develop MurA-specific antibiotics that effectively inhibit both enzymes.²¹ It was recently shown that mutagenic disruption of either *murA1* or *murA2* did not significantly alter cell growth, while cells could not survive the removal of both genes. It can therefore be speculated that MurA2 represents an essential key as a resistance bypass strategy in the case of MurA1 inhibition,

e.g., by the natural product antibiotic fosfomycin.¹⁹ Interestingly, in our comparative experiment, only the MRSA strain Mu50 maintains two active MurA enzymes, although all *S. aureus* strains share the genetic information for both enzymes. This observation raised the question whether the lack of MurA2 labeling in the nonresistant strains is a consequence of expressional regulation or posttranslational activation. In case of differences in expressional regulation, quantitative real-time polymerase chain reaction (RT PCR) should reveal the amount of mRNA in different strains and allow a direct comparison. We quantified the relative gene expression levels of *murA1* and *murA2* in the four strains by RT PCR. The ratio of *murA1*/*murA2* was found to be 0.93 ± 0.01 for *S. aureus* NCTC 8325, 0.92 ± 0.01 for *S. aureus* Mu50, 0.91 ± 0.01 for *S. aureus* DSM 18827, and 0.90 ± 0.02 for *S. aureus* DSM 19041 (Supporting Information Table 4). Thus, the expressional levels of both *murA1* and *murA2* are comparable in all strains, indicating that the difference in the *in situ* labeling is due to posttranslational regulation of enzyme activity. In fact, regulation of peptidoglycan biosynthesis by MurA inhibition with UDP-MurNAc was already observed previously.²²

Our chemical and genetic functional analysis of the MurA's therefore shows that two constitutively active copies of the MurA enzyme in the MRSA strain Mu50 may contribute to the antibiotic resistance of this strain. Indeed, only Mu50 was resistant against fosfomycin treatment at concentrations up to 500 μ M, while all other strains exhibited IC_{50} values between 1 and 5 μ M of fosfomycin (Supporting Information Figure S9). It should be noted that the *S. aureus* strains were indistinguishable on a proteome level by Coomassie staining, which again illustrates the value of specific enzyme labeling by the activity-based probe (Supporting Information Figure S10). Since showdomycin targets both enzymes, its structure could represent a starting point for customized selective MurA1 and 2 inhibitors.

Conclusion

In conclusion, we were able to establish an ABPP probe based on the reactive nucleoside antibiotic showdomycin and validate it as a tool for labeling and identification of enzymes in pathogenic bacteria. Some of these enzymes indeed had uridine or its derivatives as natural substrates, as the close structural proximity to uridine suggested. Many of the identified enzymes were important for either the viability or virulence of the bacteria. The sum of all essential enzymes targeted by showdomycin, among them especially MurA, which is one of the most intense targets in *S. aureus*, is likely to explain the antibiotic activity of showdomycin and emphasizes the utility of profiling natural products to reveal their molecular targets. Moreover, comparing enzyme activity profiles of different pathogenic strains and between closely related pathogenic and nonpathogenic species provides insights into regulatory differences of these strains as well as unique strain-specific signatures. As demonstrated in this study, the showdomycin probe has a great value as an analytical probe to increase the repertoire of the ABPP toolbox and provides a simple means to study the biological targets of a promising natural product.

Experimental Section

General. All chemicals were of reagent grade or better and used without further purification. Chemicals and solvents were purchased

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from Sigma Aldrich or Acros Organics. For all reactions, only commercially available solvents of purissimum grade, dried over molecular sieves and stored under an 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. If not indicated otherwise, column chromatography was performed on Merck silica gel (Acros Organics, 0.035–0.070 mm, mesh 60 Å). ^1H 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) spectrometer, and ^{13}C NMR spectra were measured with a Varian NMR-System 600 (600 MHz) or a Varian NMR-System 300 (300 MHz) spectrometer, referenced to the residual proton and carbon signal of the deuterated solvent, respectively. For DEI measurements, samples were directly desorbed from platinum wire (20–1600 °C, 120 °C min $^{-1}$) and delivered to a Finnigan MAT 95 mass spectrometer in EI mode (70 eV, 250 °C source). ESI mass spectra were recorded with a Thermo Finnigan LTQ FT spectrometer. HPLC analysis was accomplished with a Waters 2695 separations module, for analytical runs with an X-Bridge BEH130 C18 column (4.6 \times 100 mm) or an X-Bridge BEH130 PREP C18 column (10 \times 150 mm) for preparative applications, and a Waters 2996 PDA detector.

Strategy toward Synthesis of Showdomycin and Its ABPP Probe. The synthesis of showdomycin was adapted from a strategy developed by Barrett et al.²³ Here, the maleimide moiety of showdomycin is introduced via a Wittig reaction of the corresponding triphenylphosphoranylidene succinimide with D-(–)-ribose. Addition of phenylselenenyl chloride triggers ring closure, and subsequent selenoxide elimination restores the functional maleimide, giving showdomycin and 1'-*epi*-showdomycin (Figure S1, Supporting Information). These were coupled via the free primary hydroxyl group of the ribose to DIC/HOBt-activated hex-5-ynoic acid to give the ABPP probe 5'-*O*-hex-5-ynoylshowdomycin and the corresponding 1'-*epi*-diastereoisomer. Separation with HPLC of the last two reactions finally gave the pure products, showdomycin and 5'-*O*-hex-5-ynoylshowdomycin, respectively.

Synthesis of Triphenylphosphoranylidene Succinimide (1). Triphenylphosphine (13.0 g, 50 mmol) was added to maleimide (5.0 g, 51 mmol) in 100 mL of glacial acetic acid and refluxed at 100 °C. After 30 min the reaction was complete, as monitored by TLC (iso-hexane/ethyl acetate 1:1). Diethyl ether was added to the reaction mixture until a pale red precipitate was formed. This was filtered off, washed with diethyl ether, and dried under vacuum to yield 12.89 g (72%) of **1** as an amorphous colorless solid.

^1H NMR (300 MHz, CDCl_3): δ 7.64–7.61 (m, 9 H, Phe), 7.54–7.51 (m, 6 H, Phe), 3.03 (s, 2 H, CH_2).

^{13}C NMR (150 MHz, CDCl_3): δ 177.9 ($J_{\text{C-P}} = 16.8$ Hz, C=O), 171.0 ($J_{\text{C-P}} = 15.1$ Hz, C=O), 133.6 ($J_{\text{C-P}} = 10.5$ Hz, Phe), 133.0 ($J_{\text{C-P}} = 2.2$ Hz, Phe), 129.4 ($J_{\text{C-P}} = 12.6$ Hz, Phe), 125.6 ($J_{\text{C-P}} = 92.3$ Hz, Phe), 38.5 ($J_{\text{C-P}} = 10.2$ Hz, CH_2), 36.9 ($J_{\text{C-P}} = 136.8$ Hz, P=C).

DEI-MS (m/z): 258.0932 [$\text{M} - \text{H}$] $^-$, calcd 358.0997.

Synthesis of (E)-3-(2,3,4,5-Tetrahydroxypentylidene)pyrrolidine-2,5-dione (2). D-(–)-Ribose (2.3 g, 15 mmol) was added to a stirred suspension of **1** (10.8 g, 30 mmol) in 75 mL of dry THF and refluxed at 80 °C under nitrogen. The reaction was complete after 210 h, as monitored by TLC (chloroform/methanol 9:1). After the solvent was evaporated *in vacuo*, the crude product was partitioned between 75 mL of water and 75 mL of dichloromethane. The aqueous phase was collected and concentrated under reduced pressure. Chromatography on a mixture of C_{18} reversed-phase material and silica gel (100:1 w/w) with water as mobile phase yielded a rapidly eluting crude product that was dried *in vacuo* and purified by chromatography on silica gel with ethyl acetate and a gradient of an acetonitrile/methanol (4:1) mixture, reached

from pure ethyl acetate to ethyl acetate/acetonitrile/methanol) 3:1. Evaporation of the solvent yielded 1.20 g (35%) of **2** as a pale red solid.

^1H NMR (200 MHz, D_2O): δ 6.59 (dt, $J = 8.4, 2.3$ Hz, 1 H, C=CH), 4.44 (dd, $J = 8.4, 4.4$ Hz, 1 H, CH-CH=C), 3.69–3.60 (m, 2 H), 3.60–3.52 (m, 2 H), 3.36 (d, $J = 2.4$ Hz, 2 H, $\text{CH}_2-\text{C(O)}$).

^{13}C NMR (100 MHz, D_2O): δ 178.9, 173.1, 134.4, 129.8, 73.5, 71.7, 69.6, 62.7, 33.2.

ESI-MS, positive mode (m/z): 232.0588 [$\text{M} + \text{H}$] $^+$, calcd 232.0816; 249.1052 [$\text{M} + \text{NH}_4$] $^+$, calcd 249.1081. Negative mode (m/z): 230.0678 [$\text{M} - \text{H}$] $^-$, calcd 230.0670; 276.0739 [$\text{M} + \text{FA} - \text{H}$] $^-$, calcd 276.0725; 461.1446 [$2\text{M} - \text{H}$] $^-$, calcd 461.1413.

Synthesis of Showdomycin and 1'-*epi*-Showdomycin (3a,b).

A stirred suspension of **2** (462 mg, 2.0 mmol) in 28 mL of dry acetonitrile was heated to 65 °C, and phenylselenenyl chloride (400 mg, 2.1 mmol) was added. The reaction was stirred for 29 h at 65 °C, allowed to cool to room temperature, and treated with 10 mL of a 10% aqueous H_2O_2 solution. The reaction was monitored by TLC (ethyl acetate/acetonitrile/methanol 15:4:1) and stirred until all selenium-containing material was consumed, as visualized by 1% PdCl_2 in 0.1 M HCl as TLC staining reagent. The solvent was evaporated *in vacuo*, and the products were purified by chromatography on silica gel (ethyl acetate/ethanol 19:1, $R_f = 0.25$). This yielded 230 mg (50%) as a mixture of the diastereoisomers **3a** and **3b**. Purification by preparative HPLC and lyophilization gave the single isomers as white solids. The diastereoisomers were identified using two-dimensional NMR spectra.

Showdomycin (3a). HPLC analysis: mobile phase (HPLC grade) A = water, B = acetonitrile. Gradient: T_0 , B = 0%; T_9 , A = 3%. Retention time = 5.1 min.

^1H NMR (400 MHz, acetone- d_6): δ 6.72 (d, $J = 1.7$ Hz, 1 H, C=CH), 4.71 (dd, $J = 4.8, 1.6$ Hz, 1 H, 1'-H), 4.44 (br, 1 H, OH), 4.20 (dd, $J = 4.9, 4.9$ Hz, 1 H, 2'-H), 4.16 (dd, $J = 5.1, 5.1$ Hz, 1 H, 3'-H), 4.09 (br, 1 H, OH), 3.95 (td, $J = 5.1, 3.3$ Hz, 1 H, 4'-H), 3.89 (app t, 1 H, 5'-OH), 3.81–3.76 (m, 1 H, CH(H)), 3.68–3.62 (m, 1 H, CH(H)).

^{13}C NMR (151 MHz, acetone- d_6): δ 171.3, 170.6, 148.7, 129.1, 84.7, 78.0, 75.4, 71.4, 61.7.

ESI-MS, positive mode (m/z): 229.9854 [$\text{M} + \text{H}$] $^+$, calcd 230.0659; 247.0896 [$\text{M} + \text{NH}_4$] $^+$, calcd 247.0924. Negative mode (m/z): 228.0516 [$\text{M} - \text{H}$] $^-$, calcd 228.0513; 264.0283 [$\text{M} + \text{Cl}$] $^-$, calcd 264.0280; 274.0572 [$\text{M} + \text{FA} - \text{H}$] $^-$, calcd 274.0568; 457.1107 [$2\text{M} - \text{H}$] $^-$, calcd 457.1099.

1'-*epi*-Showdomycin (3b). HPLC analysis: mobile phase (HPLC grade) A = water, B = acetonitrile. Gradient: T_0 , B = 0%; T_9 , A = 3%. Retention time = 4.5 min.

^1H NMR (400 MHz, acetone- d_6): δ 6.48 (d, $J = 2.1$ Hz, 1 H, C=CH), 4.94 (dd, $J = 3.7, 2.1$ Hz, 1 H, 1'-H), 4.40–4.33 (m, 2 H, 2'-H and 3'-H), 4.24 (d, $J = 3.1$ Hz, 1 H, OH), 4.12 (br, 1 H, OH), 3.94 (ddd, $J = 7.3, 4.1, 2.8$ Hz, 1 H, 4'-H), 3.81 (ddd, $J = 11.5, 4.6, 2.9$ Hz, 1 H, CH(H)), 3.73 (app t, 1 H, 5'-OH), 3.63 (ddd, $J = 11.6, 6.5, 4.1$ Hz, 1 H, CH(H)).

^{13}C NMR (151 MHz, acetone- d_6): δ 170.9, 148.7, 128.3, 82.8, 76.7, 72.8, 71.5, 61.8.

ESI-MS, positive mode (m/z): 229.9854 [$\text{M} + \text{H}$] $^+$, calcd 230.0659; 247.0896 [$\text{M} + \text{NH}_4$] $^+$, calcd 247.0924. Negative mode (m/z): 228.0516 [$\text{M} - \text{H}$] $^-$, calcd 228.0513; 264.0283 [$\text{M} + \text{Cl}$] $^-$, calcd 264.0280; 274.0572 [$\text{M} + \text{FA} - \text{H}$] $^-$, calcd 274.0568; 457.1107 [$2\text{M} - \text{H}$] $^-$, calcd 457.1099.

Synthesis of 5'-*O*-Hex-5-ynoylshowdomycin and 5'-*O*-Hex-5-ynoyl-1'-*epi*-showdomycin (4a,b). HOBt (40.5 mg, 0.30 mmol) was dissolved in 800 μL of DMF, and hex-5-ynoic acid (33.1 μL , 0.30 mmol) and 1,3-diisopropylcarbodiimide (46.4 μL , 0.30 mmol) were added. The reaction mixture was stirred for 10 min at room temperature. The mixture of the diastereoisomers **3a** and **3b** (40 mg, 0.17 mmol) was then added in 200 μL of DMF and the reaction stirred for 12 h. The solvent was evaporated *in*

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vacuo and the residue purified via HPLC, giving the showdomycin probe and the 1'-*epi*-showdomycin probe.

5'-O-Hex-5-ynoylshowdomycin (4a). HPLC analysis: mobile phase (HPLC grade) A = water, B = acetonitrile. Gradient: T_0 , B = 0%; T_{40} , A = 40%. Retention time = 20.8 min.

^1H NMR (400 MHz, acetone- d_6): δ 6.60 (d, J = 1.8 Hz, 1 H, C=CH), 4.75 (dd, J = 3.7, 1.8 Hz, 1 H, 1'-H), 4.38–4.34 (dd, J = 11.9, 2.5 Hz, 1 H, 5'-H), 4.27–4.23 (m, 2 H, 2'-H and 5'-H), 4.13–4.08 (m, 2 H, 3'-H and 4'-H), 2.50 (t, J = 7.4 Hz, 2 H, C(O)–CH₂), 2.36 (t, J = 2.7 Hz, 1 H, C=CH), 2.26 (dt, J = 7.1, 2.7 Hz, 2 H, CH₂–C=CH), 1.82 (Ψ -quint, J = 7.4 Hz, 2 H, C(O)–CH₂–CH₂).

ESI-MS, negative mode (m/z): 322.0944 [M – H][–], calcd 322.0932; 358.0730 [M + Cl][–], calcd 358.0699; 645.2015 [2M – H][–], calcd 645.1937.

5'-O-Hex-5-ynoyl-1'-*epi*-showdomycin (4b). HPLC analysis: mobile phase (HPLC grade) A = water, B = acetonitrile. Gradient: T_0 , B = 0%; T_{40} , A = 40%. Retention time = 21.9 min.

^1H NMR (400 MHz, acetone- d_6): δ 6.38 (d, J = 2.11 Hz, 1 H, C=CH), 4.88 (dd, J = 3.6, 2.1 Hz, 1 H, 1'-H), 4.30–4.26 (m, 2 H, 2'-H and 5'-H), 4.19 (dd, J = 8.2, 4.5 Hz, 1 H, 3'-H), 4.08 (dd, J = 11.8, 5.5 Hz, 1 H, 5'-H), 4.01 (ddd, J = 8.2, 5.5, 2.7 Hz, 1 H, 4'-H), 2.39 (t, J = 7.3 Hz, 2 H, C(O)–CH₂), 2.25 (t, J = 2.7 Hz, 1 H, C=CH), 2.16 (dt, J = 7.1, 2.7 Hz, 2 H, CH₂–C=CH), 1.71 (Ψ -quint, J = 7.8 Hz, 2 H, C(O)–CH₂–CH₂).

^{13}C NMR (151 MHz, acetone- d_6): δ 172.2, 170.8, 148.2, 128.4, 83.1, 79.6, 76.7, 73.4, 72.4, 69.6, 63.9, 32.3, 23.7, 17.2.

ESI-MS, positive mode (m/z): 664.2332 [2M + NH₄]⁺, calcd 664.2348. Negative mode (m/z): 322.0942 [M – H][–], calcd 322.0932; 368.1009 [M + FA – H][–], calcd 368.0987; 645.2011 [2M – H][–], calcd 645.1937.

Bacterial Strains. *Staphylococcus aureus* strains NCTC 8325 (Institute Pasteur, France), Mu50/ATCC 700699 (Institute Pasteur, France), DSM 19041 (DSMZ, Germany), and DSM 18827 (DSMZ, Germany) were maintained in brain–heart broth (BHB) medium at 37 °C. DSM 19041 is a highly toxin-producing isolate from a human furuncle, and the MRSA strain DSM 18827 was isolated from tracheal secretion of a clinical case.²⁴ *Listeria monocytogenes* strains EGD-e (Institute Pasteur, France) and F2365 (BCCM/LMG Bacteria Collection, Belgium) as well as the nonpathogenic strain *Listeria welshimeri* SLCC 5334 serovar 6b (DSMZ, Germany) were maintained in BHB medium, while *Pseudomonas aeruginosa* PAO1 (Institute Pasteur, France) and *Pseudomonas putida* KT2440 (ATCC, USA) were maintained in Luria–Bertani (LB) medium. All strains were grown at 37 °C.

MIC Measurements. Overnight cultures of *S. aureus* (NCTC 8325) were diluted in fresh BHB medium to OD₆₀₀ = 0.01, and 100 μL aliquots were incubated in Nunclon round-bottom 96-well plates with 1 μL of the corresponding DMSO stock of showdomycin and the showdomycin probe at varying concentrations. The samples were incubated overnight at 37 °C and the optical densities obtained for MIC calculation. All experiments were conducted at least in triplicate, and DMSO served as control.

Preparation of Proteome for *In Vitro* Experiments. The proteome of the bacterial strains *Staphylococcus aureus* NCTC 8325 was prepared from 1 L liquid cultures in BHB medium, harvested 1 h after transition in the stationary phase by centrifugation at 9000 rpm. The bacterial cell pellet was washed with PBS, resuspended in PBS, and lysed by sonication with a Bandelin Sonopuls instrument under ice cooling. Membrane and cytosol were separated by centrifugation at 9000 rpm for 45 min.

***In Vitro* Labeling Experiments.** Proteome samples were adjusted to a final concentration of 1 mg protein/mL by dilution in PBS prior to probe labeling. Analytical experiments were carried out in 43 μL total volume, such that once CC reagents were added, the total reaction volume was 50 μL . Reactions were initiated by

addition of 50 μM probe and allowed to incubate for 30 min at room temperature. Comparative experiments with maleimide were carried out by incubation of the proteome with 50 μM Atto 550-labeled maleimide (Sigma Aldrich) for 30 min at room temperature. For heat controls the proteome was denatured with 2% SDS (4 μL of 21.5% SDS) at 95 °C for 6 min and cooled to room temperature before the probe was applied. Following incubation, reporter-tagged azide reagents (13 μM rhodamine-azide (1 μL) for analytical or 20 μM rhodamine-biotin-azide for preparative scale) were added, followed by 1 mM TCEP (1 μL) and 100 μM ligand (3 μL). Samples were gently vortexed, and the cycloaddition was initiated by the addition of 1 mM CuSO₄ (1 μL). The reactions were incubated at room temperature for 1 h.^{2,25} For analytical gel electrophoresis, 50 μL of 2 \times SDS loading buffer was added and 50 μL applied on the gel. Fluorescence was recorded in a Fujifilm Las-4000 luminescent image analyzer with a Fujinon VRF43LMD3 lens and a 575DF20 filter.

***In Situ* Experiments.** For analytical and preparative *in situ* studies, bacteria were grown in BHB or LB medium and harvested 1 h after reaching stationary phase by centrifugation. Depending on the cell density at stationary phase, 1 mL of *L. monocytogenes* and *P. aeruginosa* strains and 0.5 mL of *S. aureus* strains were harvested for analytical and 5 and 2.5 mL for preparative studies, respectively. After washing with PBS, the cells were resuspended in 100 and 500 μL of PBS for analytical and preparative experiments. Unless indicated otherwise, bacteria were incubated for 30 min with varying concentrations of probe at room temperature for analytical studies and with a fixed concentration of 100 μM probe for preparative studies. Subsequently, the cells were washed twice with PBS and lysed by sonication in 100 and 500 μL of PBS. The proteomes were separated into cytosolic and membrane fractions, followed by CC as described above. 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 prechilled 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 was washed two times with 200 μL of prechilled methanol and resuspended by sonication. Subsequently, the pellet was dissolved in 1 mL of 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 PBS/0.4% SDS, twice with 1 mL of 6 M urea, and three times with 1 mL of PBS. Aliquots of 50 μL of 2 \times 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.²⁶

Mass Spectrometry and Bioinformatics. 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–ThermoFinnigan LTQ-FT 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 X_{corr} vs charge state filter was set to X_{corr} 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 ≥ 2 , and the peptide probability filter was set to <0.001. These filter values are similar to others previously reported

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for SEQUEST analysis.²⁷ Minimum P values and maximum X_{corr} values of each run as well as the total number of obtained peptides are reported in Table S1 (Supporting Information).

Comparison of labeling with genetic properties and characteristics of the different species and strains was conducted by BLAST search for homologous sequences in the strains. These were used for a more accurate alignment to identify sequence homology via amino acid identities in the sequence by the SIM alignment tool of the Expert Protein Analysis System (ExPASy, Swiss Institute of Bioinformatics). The alignment was computed by the BLOSUM62 comparison matrix, with settings for gap open penalty at 12 and gap extension penalty at 4.

Recombinant Expression. The major hits of MS analysis were recombinantly expressed in *E. coli* as an internal control of the MS results by using Invitrogen Gateway Technology. Target genes were amplified from the corresponding genomes by PCR with an AccuPrime Pfx DNA polymerase kit with 65 ng of genomic DNA, prepared by standard protocols, or alternatively by colony PCR with cells directly from the corresponding cryostocks. *attB1* forward primer and *attB2* reverse primer were designed to yield the following *attB*-PCR products needed for Gateway Technology.

Alkyl hydroperoxide reductase subunit C, *ahpC*, *S. aureus* NCTC 8325: forward primer, 5'-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTA CAT GTC ATT AAT TAA CAA AGA AAT; reverse primer, 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTG TTA GAT TTT ACC TAC TAA ATC.

Acetolactate synthase, *alsS*, *S. aureus* NCTC 8325: forward primer, 5'-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTA CAT GAC TGA T AA AAA GTA CAC T; reverse primer, 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTG TTA GTT AAA TAC GTC AGG TAA.

Inosine monophosphate dehydrogenase, (IMPDH), *L. monocytogenes* EGD-e: forward primer, 5'-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTA CAT GTG GGA AAC AAA ATT TGC A; reverse primer, 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTG TTA AGA AAT ACT ATA ATT TGG CG.

Peroxiredoxin (POR), *L. monocytogenes* EGD-e: forward primer, 5'-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTA CGT GGC AGA ACG TTT AGT A; reverse primer, 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTG TTA AAC AAT TGT TTT TTC ACC G.

Phosphoenolpyruvate—protein phosphotransferase, *ptsI*, *S. aureus* Mu50: forward primer, 5'-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTA CAT GTC TAA ATT AAT TAA AGG TAT T; reverse primer, 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTG TTA TTT TAC GTA GTT GTT AAC T.

Ribonucleotide-diphosphate reductase subunit alpha (RNR), *P. aeruginosa* PAO1: forward primer, 5'-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTA CAT GCA TAC CGA CAC CAC ACG C; reverse primer, 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTG TCA CTG GCA GGC TTC GCA GT.

UDP-*N*-acetylglucosamine 1-carboxyvinyltransferase *murA1*, *S. aureus* NCTC 8325: forward primer, 5'-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTA CAT GGA TAA AAT AGT AAT CAA AGG; reverse primer, 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTG TTA ATC GTT AAT ACG TTC AAT GT.

UDP-*N*-acetylglucosamine 1-carboxyvinyltransferase *murA2*, *S. aureus* Mu50: forward primer, 5'-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTA CAT GGC TCA AGA GGT AAT AAA AAT; reverse primer, 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTG TTA CAT AGT TTC CGT CCA AA.

UDP-*N*-acetylglucosamine pyrophosphorylase *glmU*, *S. aureus* NCTC 8325: forward primer, 5'-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTA CAT GCT AGA TAA AAA TCA ATT AGC; reverse primer, 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTG TTA AAT TAA ACC CAT TCT ACG TA.

PCR products were identified on agarose gels, 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 ND-1000 spectrophotometer. Samples of 100 fmol of purified *attB*-PCR product and 50 fmol of *attP*-containing donor vector pDONR201 in TE buffer 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 $\mu\text{g mL}^{-1}$ kanamycin. Clones of transformed cells were selected and grown in LB medium supplemented with kanamycin. Cells were harvested, and plasmids were isolated using an E.Z.N.A. Plasmid mini kit. The corresponding *attB*-containing expression clone was generated by *in vitro* LR recombination reaction of approximately 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 $\mu\text{g mL}^{-1}$ 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 anhydrotetracycline.

Labeled Proteomes *in Vitro*. Cytosol and membrane fractions of the proteome were labeled separately. Membrane fractions were comparable with the cytosol in terms of intensive and specific labeling pattern, with slight differences in some enzymes. Examples for heat-denatured proteome controls are given in Figure S2 (Supporting Information). The majority of the labeled part of the proteome appears to be heat sensitive and thus can be regarded as the result of specific activity-based binding events. Labeling profiles for both probes, *O'*-hex-5'-ynoylshowdomycin and *O'*-hex-5'-ynoyl-1'-*epi*-showdomycin, are shown in Figure S10 (Supporting Information). Surprisingly, there are no major differences in the labeling profiles of the two isomers, although the showdomycin probe labels more intensively than the 1'-*epi*-showdomycin probe.

MurA1 Inhibition Assay. The inhibition of MurA1 by showdomycin was investigated by an assay system as described previously.^{19,28} Activity was measured as the release of P_i from the MurA1-catalyzed enolpyruvyl transfer reaction with phosphoenolpyruvate (PEP) and UDP-*N*-acetylglucosamine (UDPAG) in a malachite green—molybdate assay. MurA1 from *S. aureus* NCTC 8325 was recombinantly expressed with a Strep-Tag in *E. coli* BL21 cells and purified by affinity chromatography using a StrepTrap HP column. The pure protein was eluted in elution buffer containing 100 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, and 2.5 mM desthiobiotin. Before use, MurA1 was conditioned for at least 10 min with 1 mM UDPAG on ice. In a typical assay, to 5 μL of 500 mM HEPES pH 7.5 were added sequentially 36 μL of H_2O , 2.5 μL of 40 mM UDPAG, 5 μL of MurA1 (17 μg), and 1 μL of showdomycin stock or DMSO. After 10 min incubation at room temperature, 50 μL of 400 μM PEP in 50 mM HEPES pH 7.5 was added to start the reaction. After incubation at room temperature for an appropriate time, 20 μL of the mixture was withdrawn and mixed in a round-bottom 96-well plate with 80 μL of a 3:1 mixture of 0.45% malachite green and 4.2% ammonium heptamolybdate in 4 N HCl. The absorption was read with a TECAN GENios Pro plate reader at 670 nm, and IC_{50} values were calculated from curve fittings by Microcal Origin 6.0 (Figure S5, Supporting Information).

Reactivity of Showdomycin. To study the reactivity of showdomycin toward sulfhydryl and primary amino groups, 17 μL of 10 mM DTT and isopropylamine were incubated with 1 μL of 10 mM showdomycin for 1 h. Next, 80 μL of 25 mM NH_4HCO_3 was added, and after incubation for 12 h, the samples were analyzed

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by ESI-MS. For the DTT treatment, showdomycin was identified with one DTT molecule attached at the maleimide double bond and with a hydrolyzed imide ring ($M = 401.08142$).

ESI-MS, positive mode (m/z): 142.0297 [$M + 2H + Na$] $^{3+}$, calcd 142.0284. Negative mode (m/z): 422.0536 [$M + Na - 2H$] $^{-}$, calcd 422.0561.

Isopropylamine treatment, however, did not produce any adducts, neither by attack of the maleimide double bond nor of the carbonyl groups. Only showdomycin with hydrolyzed ring (due to long exposure to NH_4HCO_3) 29 could be detected ($M = 247.06920$).

ESI-MS, negative mode (m/z): 246.0622 [$M - H$] $^{-}$, calcd 246.0619.

Binding Sites of AhpC. To discover the binding sites of showdomycin in AhpC, purified enzyme was labeled by showdomycin, and after chymotryptic digestion the peptide fragments were analyzed by MS. First, 50 μ g of AhpC (2 pmol) in 17 μ L of PBS was incubated for 30 min at room temperature with 1 μ L of 1 mM showdomycin. The buffer was exchanged to wash away unreacted probe and adjusted to 25 mM NH_4HCO_3 , giving a total volume of 100 μ L. Then 1 mM $CaCl_2$ was added and the digest started with chymotrypsin (sequencing grade, Roche Diagnostics, Germany) for 12 h at room temperature. The sample was brought to 0.1% formic acid and analyzed by LC-MS with MS-MS and high-resolution mass spectra. The spectra of the modified peptides are shown in Figure 3 and Supporting Information Figure S6.

Gene Expression Analysis of MurA Enzymes. *S. aureus* strains NCTC 8325, Mu50, DSM 18827, and DSM 19041 were inoculated from overnight cultures at 1:100 dilution in 20 mL of BHB medium, grown to stationary phase, and kept for an additional 1 h in culture (total of 8 h). Total RNA of the bacteria was isolated using an Aurum Total RNA mini kit (Bio-Rad Laboratories) and transcribed in cDNA by reverse transcriptase and a random primer set using an iScript Select cDNA synthesis kit (Bio-Rad Laboratories). For RT PCR, specific primers were designed for MurA1 and MurA2 to amplify 186 and 210 bp sequences close to the 3' end of the genes, respectively. Because they had identical gene sequences in these regions, the primers were applicable for all strains. Special care was taken in the design to avoid any secondary structure elements in the annealing sequence by using the MFOLD 2.0 (Mobyle@pasteur) package.

MurA1: forward primer, 5'-GCT AAT ATC AAT GTA GAA GGT CGT-3'; reverse primer, 5'-GGC TAT GTT GAC TTA CAC GGT-3'.

MurA2: forward primer, 5'-CAA ATA TTG AAG TTG ACG AAG GCA-3'; reverse primer, 5'-ACT TAA AAG CTT TAG GTG CAG ATA TTT-3'.

RT PCR was conducted in a two-step thermal protocol using a SsoFast EvaGreen Supermix kit (Bio-Rad Laboratories) from an optimized concentration of the cDNA in Thermo-Fast 96 semi-skirted plates (ABgene) using an Eppendorf realplex 4 epgradient S Mastercycler instrument. The Sso7d-fusion polymerase was activated in an initial denaturation step for 30 s at 95.0 $^{\circ}C$. Forty cycles were then run, consisting each of 5 s at 95.0 $^{\circ}C$, followed by 20 s annealing and elongation at 57.6 $^{\circ}C$. A melting curve was measured to ensure specific replication of the target sequences only. For all strains the experiments were run twice in triplicate each, and the MurA1/MurA2 ratios were calculated from C_t values of each strain.

Fosfomycin Growth Inhibition Assay. The resistance of the different *S. aureus* strains against fosfomycin was assessed by studying their growth in BHB medium at various concentrations of the antibiotic. Overnight cultures of *S. aureus* strains NCTC 8325, Mu50, DSM 18827, and DSM 19041 were diluted accordingly to an OD_{600} of 0.01 in fresh BHB medium. In a 96-well plate, 100 μ L of the bacteria in BHB was added to 1 μ L of the corresponding fosfomycin stock dilutions in sterile deionized water. After incubation for 20 h at 37 $^{\circ}C$ under shaking at 250 rpm, the OD_{600} of every well (dilutions in BHB medium) was determined using a spectrophotometer.

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Supporting Information Available: Additional figures containing chemical structures, fluorescent gels, mass spectra, and enzyme inhibition data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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