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β-Lactam Probes As Selective Chemical-Proteomic Tools for the Identification and Functional Characterization of Resistance Associated Enzymes in MRSA

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Abstract: With the development of antibiotic resistant bacterial strains, infectious diseases have become again a life threatening problem. One of the reasons for this dilemma is the limited number and breadth of current therapeutic targets for which several resistance strategies have evolved over time. To identify resistance associated targets and to understand their function, activity, and regulation, we utilized a novel strategy based on small synthetic β-lactam molecules that were applied in activity based protein profiling experiments (ABPP) to comparatively profile *in situ* enzyme activities in antibiotic sensitive and resistant *S. aureus* strains (MRSA). Several enzyme activities which are unique to the MRSA strain including known resistant associated targets, involved in cell wall biosynthesis and antibiotic sensing, could be identified. In addition, we also identified uncharacterized enzymes which turned out to be capable of hydrolyzing β-lactam antibiotics. This technology could therefore represent a valuable tool to monitor the activity and function of other yet unexplored resistance associated enzymes in pathogenic bacteria and help to discover new drug targets for customized therapeutic interventions.

Introduction

Infectious diseases caused by methicillin resistant *S. aureus* strains (MRSA) have become largely untreatable by currently available antibiotics with major consequences for public health.[1] With the recent advent of community acquired MRSA, the bacteria have made a dangerous spread from hospitals to the public. Since the majority of current antibiotics targets only the classical repertoire of cellular functions such as DNA replication and protein and cell wall biosynthesis, it is an urgent goal to identify novel, customized targets in pathogenic and resistant bacteria for functional characterization and subsequent drug development. We have recently introduced small synthetic β-lactam probes for activity based protein profiling experiments[2] (ABPP, pioneered by Cravatt et al.)[3] to specifically label the active site of several enzyme classes in nonpathogenic bacterial proteomes. A typical probe of this selection contains a short alkyne handle which is attached to the β-lactam core scaffold as a benign tag for visualization and enrichment of labeled proteins. The modification of the alkyne tag via the 1,3-dipolar Huisgen cycloaddition (click chemistry, CC) allows the introduction of the bulky reporter group (e.g., rhodamine) after enzyme binding and cell preparation (Figure 1).[4] Labeled enzymes can be run on SDS gels, visualized by fluorescent scanning, and subsequently identified by mass spectrometry. In previous experiments, we showed that these probes were cell permeable and labeled not only the classical set of cell wall biosynthesis associated penicillin binding proteins (PBPs) but also several other enzymes with roles for instance in resistance and in fatty acid biosynthesis. Several probes with a monolactam core that are unrelated to classical antibiotics exhibited stable labeling and inhibition of several β-lactamases, an enzyme class that contributes to bacterial resistance by hydrolyzing the β-lactam core of many antibiotics. These initial experiments suggested that the artificial lactam probes are stable enough to resist β-lactamase hydrolysis and might therefore be suitable tools to detect and monitor the activities of other resistance associated proteins in multiresistant pathogenic bacteria. Pathogenic multiresistant strains such as MRSA exhibit several pathways for β-lactam antibiotic resistance including signal sensing and transducing systems. One major reason for β-lactam resistance of MRSA is the expression of PBP2′. PBP2′ is not inhibited by classical β-lactam antibiotics and is therefore able to perform its physiological role of cross-linking the cell wall in the face of challenge by the antibiotic.[5] The expression of this enzyme is regulated by the sensor/signal transducer protein MecR1 which experiences acylation by β-lactams on the cell surface and transduces the signal into the cytoplasm.

Here we show that our set of antibiotic and artificial β-lactam probes were effective in the comparative *in situ* profiling of bacterial proteomes derived from antibiotic sensitive (SA) and resistant *S. aureus* strains (MRSA). In fact, several antibiotic probes were able to label the extracellular MecR1 sensor domain and artificial β-lactam probes labeled PBP2′ which nicely demonstrates the proof of concept that these probes can be used

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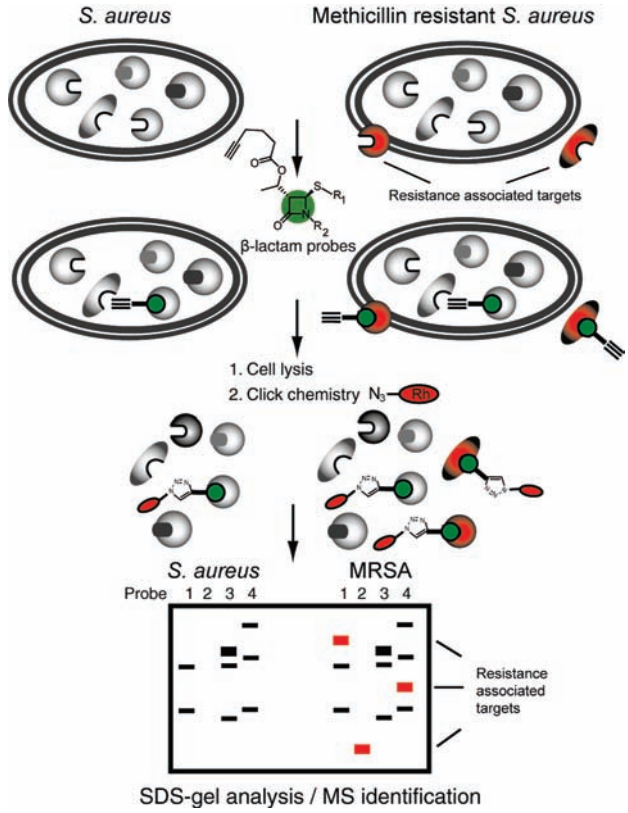

Figure 1. Comparative profiling of antibiotic sensitive and resistant *S. aureus* strains with β-lactam probes. Intact bacterial cells are separately incubated by selected β-lactam probes *in situ*. After cell lysis, the labeled enzymes are separated and visualized by fluorescent SDS-PAGE.

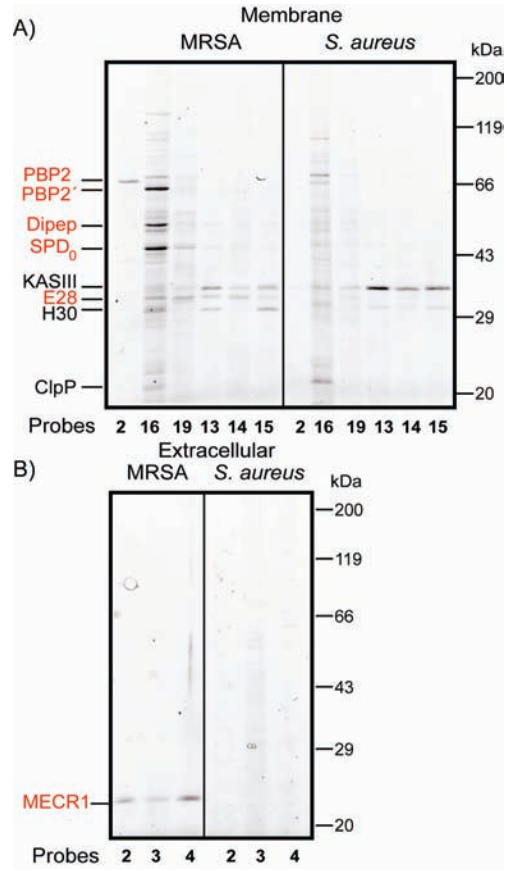

Figure 2. Comparative *in situ* profiling of *S. aureus* and MRSA strains with selected β-lactam probes. (A) Fluorescent gels of *S. aureus* and MRSA membrane fractions after treatment with 250 μM AmpN (**2**), 300 μM SPHx (**13**), SBzHx (**14**), and OctHx (**15**), and 100 μM of the more reactive NCO (**16**) and PNCO (**19**) probes. Enzyme identities are assigned to the corresponding gel band (for a list of abbreviations, please refer to Table 1). Names highlighted in red refer to enzymes that are unique to MRSA. (B) Fluorescent gel of *S. aureus* and MRSA secreted proteomes with 100 μM AmpN (**2**), Azt (**3**), and CephN (**4**).

to monitor the activity and function of known resistance associated enzymes. Furthermore, several enzyme activities of unknown function could be identified that were only active in the MRSA strain. In-depth functional analysis revealed that two uncharacterized MRSA enzymes were effective in the hydrolysis of β-lactam antibiotics of the penicillin family. Interestingly, one of these enzymes is a metalloenzyme that most likely belongs to the metallo-β-lactamase family, a novel, rising resistance associated enzyme class that is yet less explored and lacks chemical tools to monitor activity and function. Therefore, these targets may prove valuable for therapeutic intervention or as diagnostic markers in MRSA detection and disease classification for customized therapies.

Results and Discussion

Comparative Profiling of Enzyme Activities in SA and MRSA. Based on our previous experiments, we selected a subset of the best β-lactam probes for comparative *in situ* profiling experiments in *S. aureus* and MRSA. To cover a broad diversity of enzyme targets we selected three antibiotic probes (**2**−**4**, with scaffolds derived from ampicilin, cephalosporin, and aztreonam) with high affinities for PBPs and seven synthetic probes (**13**−**19**) that label PBP unrelated targets with nucleophilic active site Cys or Ser residues (Supporting Information Figure S1). *S. aureus* and MRSA strains were grown to a stationary phase and subsequently incubated *in situ* with the probes for 2 h (Experimental Section). Depending on individual reactivities, the probe concentrations for these experiments varied from 250 μM for **2**, 300 μM for **13**, **14**, and **15**, to 100 μM for **16** and **19**. After incubation, the cells were harvested, lysed, and subsequently reacted with a rhodamine azide tag under click chemistry[6−8] conditions for visualization of bound enzyme targets. In addition, we collected and labeled the extracellular proteomes of the two strains which are likely to contain relevant resistance associated enzyme activities.

Comparison of the membrane fractions of *S. aureus* and MRSA showed that individual probes displayed highly distinct reactivity profiles (Figure 2A). Among those, several enzyme activities of comparable intensity could be observed in both proteomes. Subsequent identification by LC−MS analysis (Experimental Section) revealed the identity of these targets (Table 1). Similar to our previous β-lactam profiling experiments in antibiotic sensitive strains, the probes picked up, e.g., an uncharacterized hydrolase (Hy30), a metabolic enzyme (KASIII), and a virulence regulator (ClpP). However, the striking feature of this approach is the detection of differences in activity pattern between *S. aureus* and MRSA. In fact, five

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Table 1. List of All Enzymes Labeled by β-Lactam Probes in SA and MRSA

Enzyme	Observed in SA	Observed in MRSA	Function
Dipeptidase PepV (Dipep)	No	Yes	Unknown
hypothetical protein SAV2581 (Hy30)	Yes (weak)	Yes	Unknown
Serine proteinase Do (SPD_0)	No	Yes	Unknown
Carboxyesterase precursor-like protein (E28)	No	Yes	Unknown
Penicillin binding protein 2 (PBP2)	No	Yes	Cell wall cross-link
Penicillin binding protein 2′ (PBP2′)	No	Yes	Cell wall cross-link in MRSA
Chain A, Mecr1 Unbound Extracellular Antibiotic-Sensor Domain (MECR1)	No	Yes	Antibiotic sensing in MRSA
3-oxoacyl-(acyl carrier protein) synthase III (KASIII)	Yes	Yes	Fatty acid biosynthesis
ATP-dependent Clp protease proteolytic subunit (ClpP)	Yes	Yes	Virulence regulation

enzyme activities were unique to MRSA which suggested a potential role of these enzymes in the resistance development. Mass spectrometric analysis revealed their identities as PBP2, PBP2′, a serine protease (SPD_0), an esterase (E28), and a dipeptidase (Dipep). By contrast, no enzyme activities that appeared only in antibiotic sensitive *S. aureus* were observed. MS results (Supporting Information Table S1) were confirmed by recombinant expression of all major hits and subsequent labeling by the corresponding probes (Supporting Information Figure S2). Labeling experiments with corresponding heat denatured samples revealed that all enzyme targets required their native fold for specific probe binding (Supporting Information Figure S2).

Identification of Known Resistance Associated Enzymes. PBP2′ is the major cause for the global resistance of MRSA strains to nearly all β-lactam antibiotics.[9,10] The enzyme takes over the total cell wall biosynthesis capacity in MRSA replacing the action of all other PBPs that get inactivated by β-lactams. Unlike these original PBPs, PBP2′ displays a very low binding affinity to nearly all β-lactams. Only recently, some novel β-lactam based compounds have been reported for the successful inhibition of PBP2′.[11] Interestingly, one of these compounds exerts a free hydroxyl group in the C1′ position of the β-lactam core structure which is comparable to the **NCO** (**16**) probe that labeled PBP2′ in our experiments. To test whether this free hydroxyl group is essential for PBP2′ labeling, we synthesized a novel derivative of this probe that displayed a TBDMS protecting group at the C1′ position (**PNCO**, **19**). Proteomic profiling by this compound revealed indeed no labeling signal for PBP2′ but also for all other targets that have been observed with the parental **NCO** (**16**) probe, suggesting an important role of the free hydroxyl group for PBP2′ binding.

The expression of PBP2′ in resistant strains is induced by the action of the MECR1 antibiotic sensing domain.[12] This resistance regulator senses the presence of β-lactams in the extracellular environment by covalent binding and induces the expression of PBP2′ by an intracellular cascade. Comparative profiling of the extracellular proteomes of *S. aureus* and MRSA revealed an intense fluorescent protein band that was labeled by the antibiotic probes **2**, **3**, and **4** only in MRSA (Figure 2B). Subsequent mass spectrometric analysis identified this enzyme as the MECR1 antibiotic binding domain which is known to sense β-lactams by active site acylation.[13,14] This binding domain is part of a bigger intramembrane complex that likely got shedded during bacterial growth and sensing, explaining its occurrence in the extracellular proteome fraction.

Identification of Uncharacterized Enzymes in MRSA. In addition to this pair of resistance associated enzymes with known functions, we also identified three uncharacterized enzymes that were only active in MRSA and have not been linked to resistance before. Two of these enzymes belong to the serine hydrolase enzyme family that covalently bind β-lactam probes by nucleophilic ring-opening reactions. Interestingly, one dipeptidase (Dipep) exhibited strong sequence homologies with the metalloprotease (MP) enzyme family that utilize active site zinc atoms for catalysis. To test whether this enzyme is indeed a MP, several experiments with the recombinant dipeptidase in *E. coli* lysates were performed. First, we utilized well established hydroxamate based MP probes that chelate the active site zinc atom and attach irreversibly via a photoactivatable benzophenone cross-linker.[4,15] Since the substrate preference of the dipeptidase was unknown, we used probes with different side chains and observed intense labeling in the case of a positively charged lysine residue, moderate labeling with a hydrophobic leucine, and no labeling with a negatively charged aspartate (Figure 3, Supporting Information Figure S1). These results indicate that the enzyme is indeed a MP that prefers positively charged amino acids for binding. In addition, separate preincubation of the dipeptidase with 1 mM GM6001, a broad spectrum MP inhibitor, and 1 mM PMSF, a broadband serine hydrolase inhibitor, and subsequent labeling with 10 μM of the **NCO** (**16**) β-lactam probe revealed competition for the same active site in the case of the GM6001 MP inhibitor but not for PMSF which further supports the MP identity (Figure 3).

Although the MRSA dipeptidase is an uncharacterized enzyme of unknown function, it displays high homology to metalloproteases (e.g., dipeptidase V) that contain two metal centers. Interestingly, prominent members of this family are

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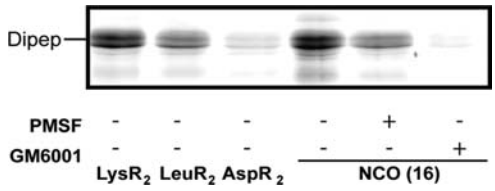

Figure 3. Characterization of a MRSA dipeptidase. Lanes 1−3: Labeling of recombinantly expressed MRSA dipeptidase in *E. coli* lysate (double band refers to enzyme with and without strep tag) with 1 μM of three established hydroxamate based metalloprotease UV-cross-linking probes $LysR_2$, $LeuR_2$, and $AspR_2$.[4] Lane 4: Labeling with the NCO (**16**) β-lactam probe. Lane 5: Competitive labeling with 100 μM NCO (**16**) and an excess of 1 mM PMSF (phenylmethanesulphonylfluoride, serine protease inhibitor). Lane 6: Competitive labeling with NCO (**16**) and an excess of 1 mM GM6001 (hydroxamate based metalloprotease inhibitor).

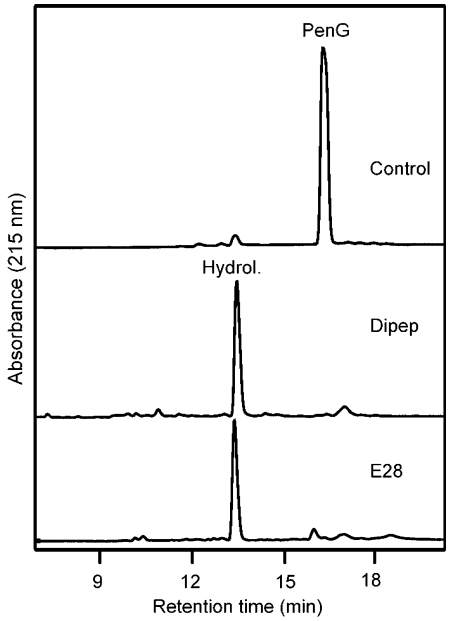

Figure 4. HPLC assay for functional studies of uncharacterized enzymes. Incubation of 0.5 mM Penicillin G (PenG) with Dipep (10 μM) or E28 (10 μM) for 1 h at 25 °C leads to a total conversion to the hydrolysis (Hydrol.) product.

metallo-β-lactamases which are crucial for bacterial resistance.[16] These enzymes are not affected by conventional β-lactamase inhibitors, and their increasing number as well as the lack of tools to monitor their function and activity poses a major problem in the development of novel antibiotic therapies. To investigate if the uncharacterized MRSA dipeptidase displays β-lactamase activity, we incubated the enzyme with several β-lactam antibiotics of different families including penicillin G, aztreonam, and cefotaxim. Indeed, HPLC assays revealed that the enzyme was effective in hydrolyzing penicillin G (Figure 4) with a Michaelis constant (K_M) of 285.89 μM and a turnover number (k_{cat}) of 0.47 min^{-1} (Supporting Information Figure S3). A control experiment with MMP7, a human matrix metalloprotease, did not reveal any hydrolytic activity with penicillin G demonstrating that the hydrolysis is enzyme dependent (Supporting Information Figure S4). In an additional control experiment, we incubated a commercially available β-lactamase form of *Bacillus cereus* with Penicillin G that revealed the same hydrolytic product peak on HPLC. In all cases the identity of the hydrolytic product was confirmed by high resolution MS analysis (calculated mass for $C_{16}H_{21}N_2O_5S^+$: 353.1166, observed mass: 353.1154). These results suggest that the MRSA dipeptidase can be classified as a metallo-β-lactamase that could be involved in MRSA resistance. Interestingly, an uncharacterized serine esterase (E28) from MRSA revealed also significant hydrolysis of penicillin G (Figure 4) with a Michaelis constant (K_M) of 125.91 μM and a turnover number (k_{cat}) of 1.01 min^{-1} (Supporting Information Figure S3). Conversely, both enzymes did not hydrolyze cefotaxim and aztreonam indicating a preference for the penicillin scaffold. This notion is supported by the observed hydrolytic activity of both enzymes against the related antibiotic ampicillin (Supporting Information Figure S5). While the K_M values for both purified enzymes are in agreement with literature published results for serine- and metallo- β-lactamases, the turnover numbers (k_{cat}) are in the lower range of most reported results.[16,17] This could be explained by not yet fully optimized assay conditions that mimic the native situation, or it may result from a more specialized role of these enzymes in resistance to β-lactams *in vivo* that were not monitored in this assay. Future studies will be conducted to elucidate their precise function by chemical and genetic knockouts.

Conclusion

MRSA strains have evolved several resistance mechanisms to β-lactam antibiotics. One prominent pathway is carried out by the concerted action of a pair of enzymes, MecR1 and PBP2′. MecR1 senses the presence of antibiotics in the extracellular milieu and communicates the arrival of β-lactam antibiotics inside the cell. Here, MRSA can adapt to this situation rapidly by the expression of PBP2′ that takes over all of the cell wall cross-linking capacity since it is not inhibited by classical β-lactam antibiotics. Our set of conventional and artificial β-lactam probes was efficient for monitoring the activity of these two important enzymes which are the major cause of MRSA resistance. While MecR1 was labeled solely by probes that resemble dedicated classical antibiotics, PBP2′ was only picked up by an artificial β-lactam probe (**NCO16**) which emphasizes the utility of using diverse sets of compounds (conventional and artificial β-lactams). Contrary to other probes used in this study, the amide-activated compound **NCO** (**16**) is probably of suitable reactivity to acylate the active site of PBP2′ overcoming the limited reactivity of this enzyme toward β-lactams. In addition to this important pair of resistance associated proteins, we identified several other enzymes of unknown functions. Subsequent analysis of two hydrolytic enzymes that were only active in MRSA led to their classification as β-lactamases with a preference for the hydrolysis of penicillins. Interestingly, one of these enzymes belongs most likely to the rising and yet largely unexplored class of metallo-β-lactamases, which are insensitive to current β-lactamase inhibitors and pose a major problem to antibiotic therapies. These results suggest that our strategy may be a valuable tool to monitor the activity and function of other yet unexplored resistance associated enzymes in pathogenic bacteria and to discover dedicated new drug targets for customized therapeutic interventions.

Experimental Section

General. All chemicals were of reagent grade or better and used without further purification. Chemicals and solvents were purchased from Sigma Aldrich or Acros Organics. For all reactions, only

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commercially available solvents of ultrapure grade, dried over molecular sieve, 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. All experiments were carried out under nitrogen. Column chromatography was performed on Merck silica gel (Acros Organics 0.035−0.070 mm, mesh 60 Å). ^{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 ^{13}C NMR spectra were measured with a Varian NMR-System 600 (600 MHz) and a Varian NMR-System 300 (300 MHz) and referenced to the residual proton and carbon signal of the deuterated solvent, respectively. ESI spectra were recorded with a Thermo Finnigan LTQ FT. Proteomic experiments were performed on a Thermo Orbitrap XL coupled with a Dionex Ultimate 3000 nano-HPLC. Regular HPLC analysis was accomplished with a Waters 2695 separations module, a X-Bridge BEH130 C18 column (4.6 mm × 100 mm) and a Waters 2996 PDA detector. Mobile phase (HPLC grade): A = water, 0.1% (v/v) TFA; B = acetonitril, 0.1% (v/v) TFA.

Synthesis of [3*R*(1′*R*,4*R*)]-3-[1-(*tert*-Butyl-dimethyl-silanyloxy)ethyl]-4-(2-diethylamino-ethyl-sulfanyl)-1-hex-5-ynoyl-azetidin-2-one (19, PNCO). [3*R*(1′*R*,4*R*)]-3-[1-(*tert*-Butyl-dimethyl-silanyloxy)ethyl]-4-(2-diethylaminoethylsulfanyl)azetidin-2-one[2] (23.5 mg, 0.065 mmol, 1.0 equiv) was dissolved in 0.8 mL of DCM followed by the addition of NEt_3 (27.2 μL, 19.7 mg, 0.20 mmol, 3.0 equiv), DMAP (1.8 mg, 0.015 mmol, 0.2 equiv), and Hex-5-ynoyl chloride (28.0 mg, 0.21 mmol, 3.3 equiv) at 12 °C in a nitrogen atmosphere. After stirring for 50 min the reaction mixture was concentrated under reduced pressure and extracted with EtOAc. The EtOAc solution was washed with H_2O, dried over $MgSO_4$, and evaporated under reduced pressure. The residue was purified by HPLC to afford **19** (6.3 mg, 21%) as a yellowish oil. ^{1}H NMR (600 MHz, $CDCl_3$) δ 5.21 (d, J = 3.3 Hz, 1 H, N−C*H*S), 4.23 (dquart, J = 7.2, 2.6 Hz, 1 H, C*H*−O−Si), 3.13 (psquint, J = 13.0, 6.6, 6.6 Hz, 1 H, S−C*H*H−CH_2), 2.94 (pstr, J = 2.7 Hz, 1 H, *O*=C−C*H*), 2.86 (psquint, J = 13.1, 6.7, 6.7 Hz, 1 H, S−CH*H*−CH_2), 2.70 (m, 2 H, N−CO−CH_2), 2.64−2,68 (m, 2 H, S−CH_2−CH_2), 2.45−2.55 (m, 4 H, N−(CH_2−CH_3)$_2$), 2.12−2.22 (m, 2H, CH_2−C≡CH), 1.89 (tr, J = 2.6 Hz, 1 H, C≡C*H*), 1.76−1.84 (m, 2 H, CH_2−CH_2−C≡CH), 1.17 (d, J = 6.6 Hz, 3 H, CH_3−CHOSi), 0.95 (tr, 6 H, J = 6.9 Hz, N−(CH_2−CH_3)$_2$), 0.73 (s, 9 H, Si−*t*Bu), −0.06 (s, 3 H, Si−CH_3), −0.02 (s, 3 H, Si−CH_3). ^{13}C NMR (151 MHz, $CDCl_3$) δ 169.8, 165.4, 83.0, 69.0, 64.2, 63.9, 56.1, 52.6, 46.4, 35.1, 30.4, 25.3, 22.4, 21.6, 17.6, 17.5, 11.4, −4.5, −5.5. HRMS calcd for $C_{23}H_{43}N_2O_3SSi^+$ ($M+H^+$) 455.27582, found 455.2762.

Preparation of Proteomes. Bacterial strains *Staphylococcus aureus* NCTC 8325 (Institute Pasteur, France) and Mu50/ATCC 700699 (Institute Pasteur, France) were grown to a stationary phase, harvested by centrifugation at 8.000 rpm 1 h after entrance in the stationary phase (1 mL for analytical and 5 mL for preparative studies), washed once with PBS, and resuspended in 100 and 500 μL of PBS for analytical and preparative studies, respectively. Unless indicated otherwise, the cell suspensions were incubated for 2 h with varying concentrations of probes at rt. Probes were applied from DMSO stocks whereby DMSO never exceeded 2% in the final solution. After incubation, the cells were washed thoroughly three times with PBS to remove excess probe from the supernatant. Subsequently the cells were lysed in 100 μL (500 μL for preparative) of PBS by sonication with a Bandelin Sonopuls (3 × 20 s), pulsed at 70% max. power. Preparation of secreted proteomes: The supernatant was sterile filtered, and the probes were added to 500 μL (5 mL in preparative experiments) of the corresponding extracellular filtrate. After 1 h of incubation at room temperature, 500 μL (5 mL for preparative scale) of ice cooled EtOH were added. Samples were allowed to stand overnight at 4 °C and were then centrifuged for 30 min at 13.000 rpm. The supernatant was discarded, and the remaining precipitate was resuspended in 19 μL of PBS.

Labeling of Proteomes. For analytical experiments 44 μL of proteome were used to append a reporter tag via click chemistry (CC), such that once CC reagents were added, the total reaction volume was 50 μL. In preparative purposes the total volume of 500 μL was consumed per experiment. Reporter-tagged azide reagents (13 μM rhodamine-azide for analytical or 20 μM rhodamine-biotin-azide for preparative scale) were added followed by 1 mM TCEP and 100 μM ligand. Samples were gently vortexed, and the cycloaddition was initiated by the addition of 1 mM $CuSO_4$. The reactions were incubated at room temperature for 1 h. For analytical gel electrophoresis, 50 μL of 2 × SDS loading buffer were added and 50 μL were applied onto 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 prechilled acetone. Samples were stored on ice for 20 min and centrifuged at 13000 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 0.2% 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/0.2% SDS, twice with 1 mL of 6 M urea, and three times with 1 mL PBS. 50 μL of 2 × SDS loading buffer were added, and the proteins were released for preparative SDS-PAGE by 6 min incubation at 95 °C. Gel bands were isolated, washed, and tryptically digested as described previously.[4]

Metalloprotease Labeling. Labeling of recombinantly expressed dipeptidase (Dipep) in *E. coli* BL21 lysates was carried out as described previously.[4] Competitive experiments with an excess of PMSF (2 mM) or GM6001 (2 mM) were allowed to preincubate for 15 min at room temperature followed by the addition of 100 μM NCO (**16**) probe. After incubation for 1 h at room temperature, CC was performed.

HPLC Assays and Kinetics. Reactions were carried out in PBS buffer, pH 7.3, in a total volume of 50 μL. The penicillin G concentration was 500 μM for standard reactions and varied for kinetic investigations. Reactions were initiated by the addition of enzyme to give final concentrations of 5 μM for Dipep and 3 μM for E28. Reactions were quenched at various time points by addition of 50 μL of cold acetone, and the products were analyzed by HPLC. The identity of the hydrolytic product was confirmed by ESI-MS. Concentrations of penicillin G were calculated using experimentally determined absorptions at 215 nm and assumed to be the same for the hydrolyzed product. Kinetic characterization of the hydrolysis reaction was carried out by determining duplicate initial rates at 56 substrate concentrations using two time points at each concentration within the linear region of the enzymes determined by time courses.

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-Thermo 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 ≥2, and the peptide probability filter was set to <0.001. These filter values are similar to others previously reported for SEQUEST

analysis.[18] Maximum P-values and Xcorr values of each run as well as the total number of obtained peptides are reported in Table S1.

Recombinant Expression. The major hits of MS analysis were recombinantly expressed in *E. coli* as an internal control of the MS results by using the 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 according to standard protocols. *att*B1 forward primer and *att*B2 reverse primer were designed to yield *att*B-PCR products needed for Gateway Technology. Primer: E28. Forward primer: 5′-ggg gac aag ttt gta caa aaa agc agg ctt tat gca gat aaa att acc aaa a. Reverse primer: 5′-ggg gac cac ttt gta caa gaa agc tgg gtg tta ttc tga cca gtc taa t. Hy30, Forward primer: 5′-ggg gac aag ttt gta caa aaa agc agg ctt tat gga aac ttt aga att ac. Reverse primer: 5′-ggg gac cac ttt gta caa gaa agc tgg gtg tta acc cca cat att taa t. PBP2, Forward primer: 5′-ggg gac aag ttt gta caa aaa agc agg ctt tat gac gga aaa caa agg a. Reverse Primer: 5′-ggg gac cac ttt gta caa gaa agc tgg gtg tta gtt gaa tat acc tgt taa t. Dipep, Forward Primer: 5′-ggg gac aag ttt gta caa aaa agc agg ctt ttt cat tat gcg tgg agg aat aa. Reverse Primer: 5′-ggg gac cac ttt gta caa gaa agc tgg gtg tta ttc ctc cac gca taa tga a. SPD_0, Forward Primer: 5′-ggg gac aag ttt gta caa aaa agc agg ctt tat gtc aga ttt taa tca tac ag. Reverse Primer: 5′-ggg gac cac ttt gta caa gaa agc tgg gtg tta tct aaa gaa atc tct atc g. PCR products were identified on agarose gels, and gel bands were isolated and extracted with an E.ZNA MicroElute Gel Extraction Kit. Concentrations of DNA were measured by a NanoDrop Spectrophotometer ND-1000. 100 fmol of purified *att*B-PCR product and 50 fmol of *att*P-containing donor vector pDONR201 in TE buffer were used for an *in vitro* BP recombination reaction with BP Clonase II enzyme mix to yield the appropriate *att*L-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^{-1} kanamycin. Clones of transformed cells were selected and grown in kanamycin LB medium. Cells were harvested, and plasmids were isolated using an E.ZNA Plasmid Mini Kit. The corresponding *att*B-containing expression clone was generated by an *in vitro* LR recombination reaction of ~50 fmol of the *att*L-containing entry clone and 50 fmol of the *att*R-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^{-1} carbenicillin. The 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.

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Supporting Information Available: Additional figures containing probe structures, heat controls, labeling of recombinant enzymes, Lineweaver−Burk plots, HPLC traces, NMR spectra, and mass spectrometry data. This material is available free of charge via the Internet at http://pubs.acs.org.

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