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JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
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Unraveling the Protein Targets of Vancomycin in Living *S. aureus* and *E. faecalis* Cells

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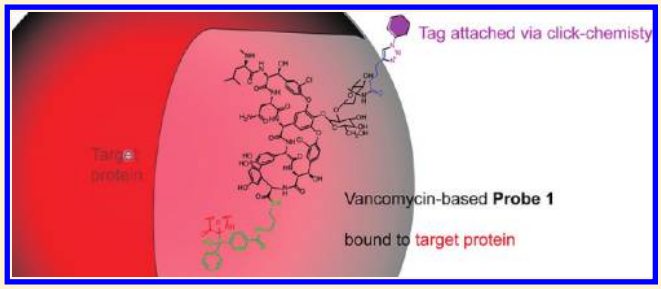
S Supporting Information

ABSTRACT: Vancomycin is a potent glycopeptide antibiotic that has evolved to specifically bind to the D-Ala-D-Ala dipeptide termini of nascent peptidoglycans. Although this mode of action is well established, several studies indicate that vancomycin and analogues exploit noncanonical target sites. In order to address all vancomycin targets in clinically relevant *Staphylococcus aureus* and *Enterococcus faecalis* strains we developed a series of small-molecule photoaffinity probes based on vancomycin. Proteomic profiling revealed the specific labeling of two previously unknown vancomycin targets that are likely to contribute to its antibiotic activity. The specific inhibition of the major staphylococcal autolysin Atl confirms previous observations that vancomycin alters *S. aureus* cell morphology by interaction with the autolytic machinery. Moreover, in *E. faecalis* the vancomycin photoprobe specifically binds to an ABC transporter protein, which likely impedes the uptake of essential nutrients such as sugars and peptides. The labeling of these two prominent membrane targets in living cells reveals a thus far unexplored mode of vancomycin binding and inhibition that could allow a rational design of variants with improved activity.



INTRODUCTION

Vancomycin is a potent glycopeptide antibiotic that specifically binds to the D-Ala-D-Ala dipeptide termini of nascent peptidoglycan. The molecule forms a series of hydrogen bonds with the peptide backbone that block its further processing by penicillin binding proteins (PBPs) and thus the final steps of peptidoglycan and cell wall biosynthesis.[1−3] Although vancomycin was kept as an antibiotic of last resort, resistances to this unique mechanism of action have been observed which currently limit its application as a drug to treat serious methicillin-resistant Gram-positive infections. One prominent form of resistance, observed in enterococcal strains, redesigns cell wall biosynthesis to incorporate D-Ala-D-Lac (lactate) depsipeptide termini which significantly reduces vancomycin binding due to a lack of a crucial hydrogen bond.[1] Multiresistant *Staphylococcus aureus* (MRSA, e.g. Mu50 strain) have also been reported to display reduced susceptibilities to vancomycin.[4,5] One important feature of these cells is a thickened cell wall that is rich in muropeptide components which retain intact D-Ala-D-Ala binding sites and therefore sequester substantial amounts of vancomycin distant to the cell wall biosynthesis site.[4−9] The binding to muropeptides further leads to an inhibition of the *S. aureus* autolytic system by blocking the access of staphylococcal hydrolases to the cell wall substrate that is relevant for cell wall turnover, cell division, and cell separation.[5,7,10] Vancomycin-treated *S. aureus* cells therefore display a lack of daughter cell separation, leading to multicellular clusters. In addition, several other modes of vancomycin or vancomycin analogue action have been reported, indicating that the complex structure of this compound class is suitable to interact with other targets than just D-Ala-D-Ala dipeptides. Studies by Kahne et al. revealed the transglycosylase domain of PBP1b as a specific binding site for a chlorobiphenyl-subsituted vancomycin derivative that leads to inhibition of transglycosylation and therefore exerts a potent antibiotic effect even against vancomycin-resistant enterococcal strains (VRE).[11] Affinity chromatography further verified the direct interaction of immobilized vancomycin derivatives with this enzyme and other enzymes in cell lysates.[11,12] Moreover, recent investigations with biotinylated vancomycin photoprobes indicate that vancomycin binds to VanSsc kinase and induces the expression of resistance genes in *Enterococcus faecalis*.[13] Although this study was carried out in *Escherichia coli* lysates and not in VRE, the labeling of the whole proteome indicated the specific interaction with several other proteins that remained unexplored.

Although useful for the evaluation of compound selectivity, profiling of cell lysates can cause artificial labeling effects due to induced protein degradation and corresponding changes in enzyme activities as well as the unrestricted access of molecules to cytosolic proteins.[14−16] We here introduce a novel generation of vancomycin photoprobes that are capable of labeling protein

Received: May 1, 2011
Published: July 07, 2011

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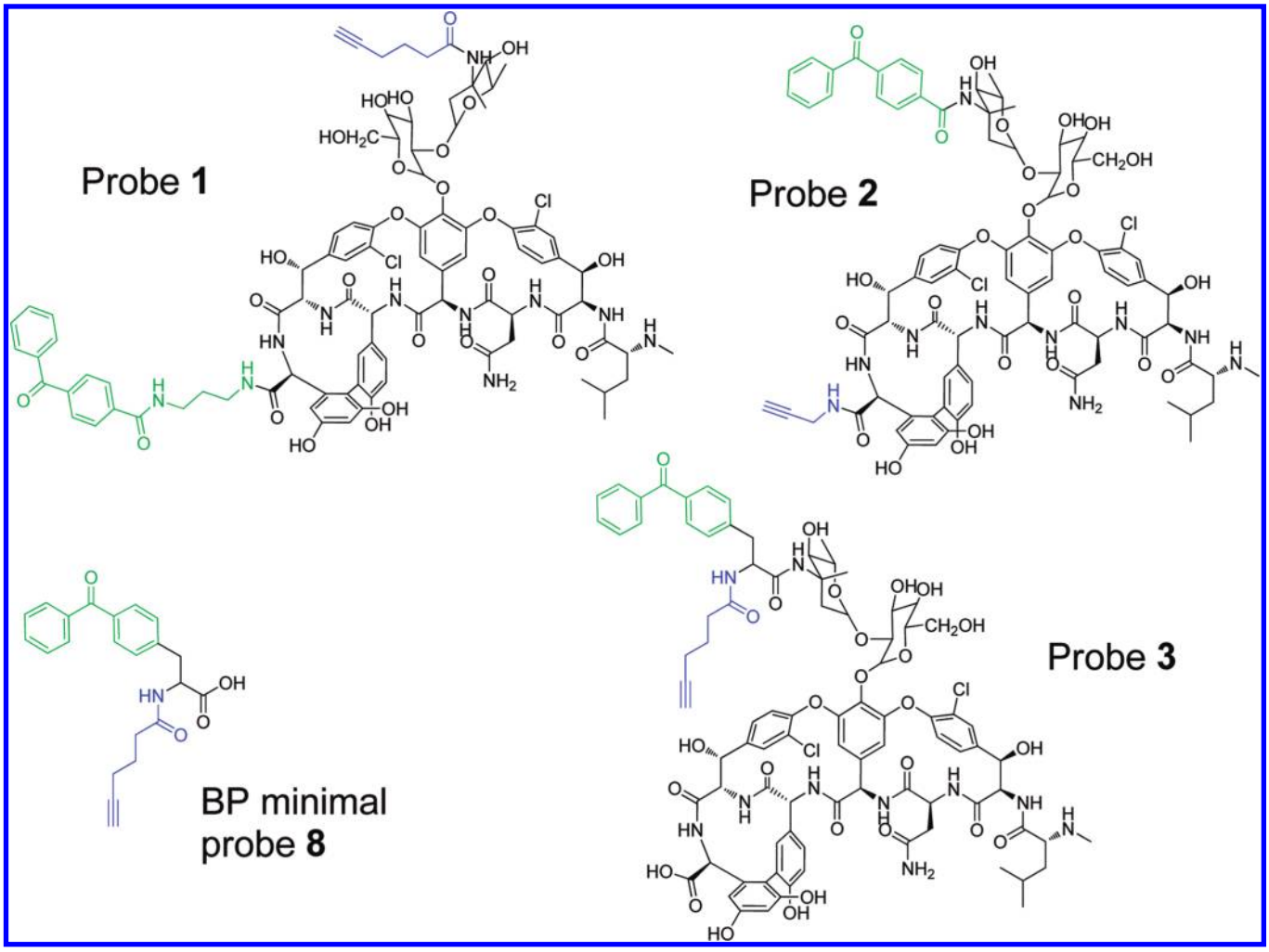


Figure 1. Structures of vancomycin-derived probes **1**−**3** and a benzophenone minimal probe (**BP**) carrying the photo-cross-linker (green) and the alkyne handle (blue) on different positions.

targets in living bacterial cells that are crucial for vancomycin activity.

The *in situ* profiling of *S. aureus* (SA), methicillin-resistant *S. aureus* (MRSA), vancomycin susceptible *E. faecalis* (VSE), and vancomycin resistant *E. faecalis* (VRE) reveals the specific labeling of two previously unknown vancomycin targets that are likely to contribute to its antibacterial activity. In *S. aureus*, the inhibition of the major staphylococcal autolysin Atl confirms previous observations that vancomycin alters *S. aureus* cell morphology.[5,6,10] We here demonstrate that this is not solely done by blocking the access of autolysin to their sequestered peptidoglycan substrate but rather by a direct inhibition of the Atl activity. Moreover, in VSE and VRE vancomycin specifically binds to an ABC transporter protein, which likely impedes the uptake of essential nutrients such as sugars and peptides. The labeling of these two prominent membrane targets in living cells reveals a, thus far, unexplored mode of vancomycin binding and inhibition that could allow a rational design of variants with improved activity.

RESULTS AND DISCUSSION

The design of vancomycin-based affinity probes has to fulfill several prerequisites: First, we required a synthetic strategy that introduces modifications at easy entry points such as the C-terminal carboxylic acid as well as the free amine of the vancosamine sugar. Second, the corresponding probes should be detached from any bulky marker that would affect its interaction with targets during live cell labeling.[17−19] We therefore designed and synthesized three different probe scaffolds that contain a benzophenone photo-cross-linker and a small alkyne handle (Figure 1). The alkyne serves as a benign tag for the modification with marker azides after live cell labeling via the Huisgen/Sharpless/Meldal click chemistry (CC) reaction (Figure 2).[14,20−22] The benzophenone is necessary to establish a covalent bond between the probe scaffold and the target enzyme which is required for visualization, enrichment, and isolation of the labeled proteins. Activation of hexynoic acid via an NHS ester allowed a specific amide bond formation at the free amine of the vancosamine sugar moiety (Scheme 1).[23−28] Subsequently, the free C-terminal carboxylic acid was modified with a benzophenone amide under standard peptide coupling conditions (probe **1**). We utilized closely related synthetic procedures to introduce another probe (probe **2**) with the opposite decoration (benzophenone at vancosamine sugar and alkyne at C-terminal carboxylic acid) as well as one probe (probe **3**) in which both functionalities are attached to the vancosamine sugar (Figure 1).

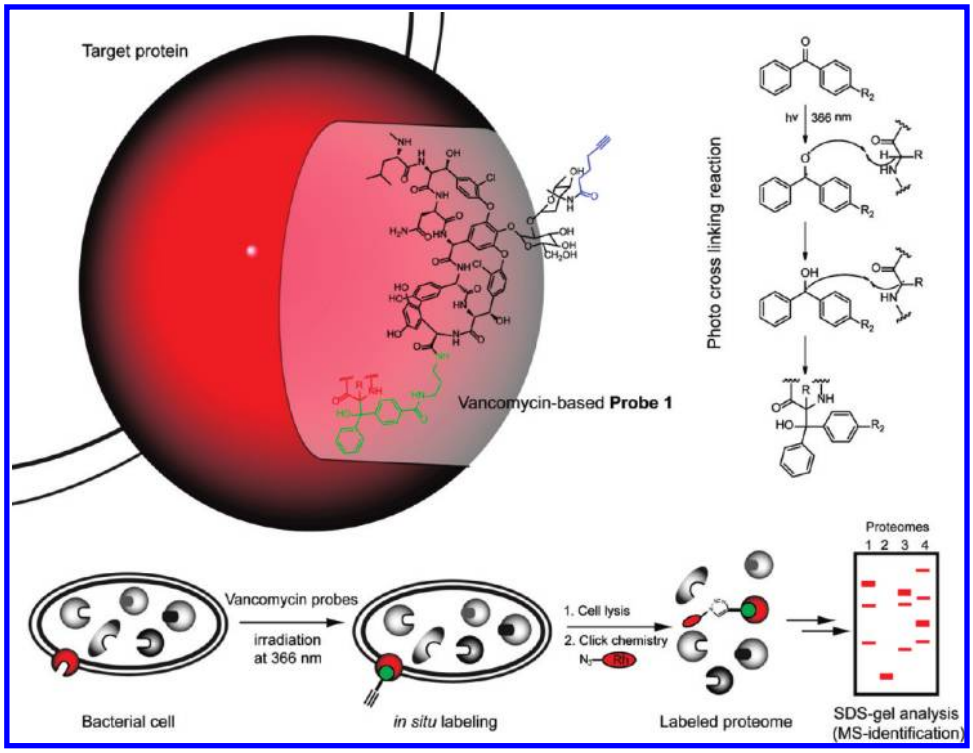
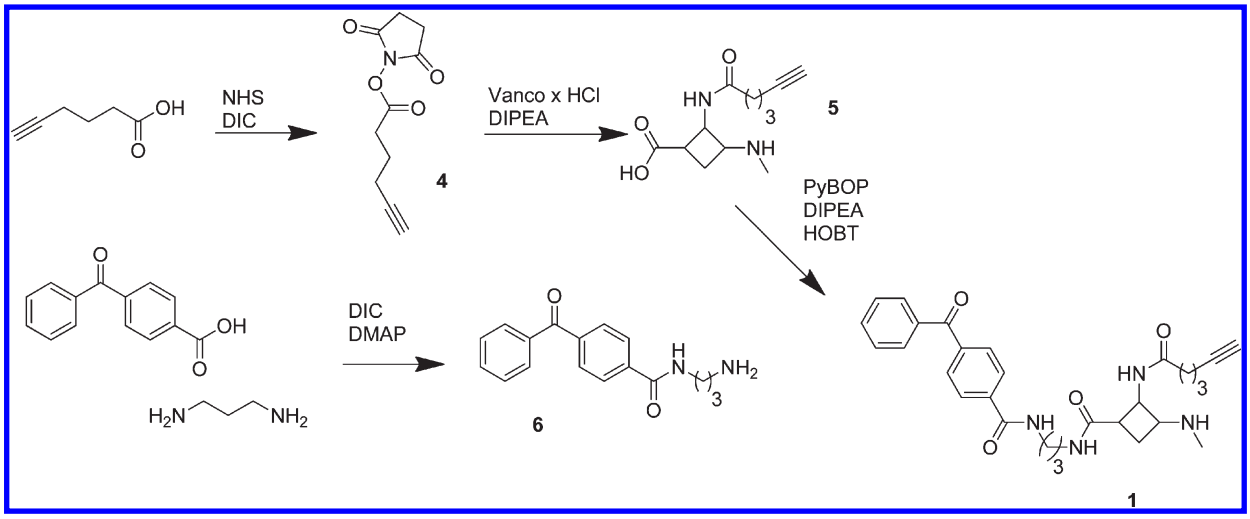


Figure 2. Vancomycin-derived probes are covalently attached to the amino acid backbone of their target proteins via a benzophenone moiety as a photo-activatable cross-linker. The alkyne group of the probes allows the identification of labeled enzymes by the introduction of a fluorescent tag via click chemistry after cell lysis and SDS-gel electrophoresis.

Scheme 1. Synthetic Strategy Leading to Probe 1[a]



[a] The vancomycin glycopeptide core is depicted as a square in **5** and probe **1**. Its peptide backbone runs as a horizontal diagonal (free carboxy terminus left, methylated amino terminus right). The disaccharide is located on the top edge.

To test if and how the structural modifications influence the antibiotic potency of all vancomycin probes we determined their individual minimal inhibitory concentrations (MIC) against *S. aureus*, MRSA, VSE, and VRE (Table 1). Unmodified vancomycin and all three probes displayed potent activities against *S. aureus*, MRSA, and VSE. As expected, a slight drop in potency was observed for VRE. Interestingly, the MIC of probe **3** in VSE drops below 0.6 μM, making it at least 4-fold more potent than vancomycin itself. These intriguing results indicate that the new probes exhibit all suitable traits for successful proteomic tools to unravel vancomycin protein targets.[23]

To evaluate labeling properties of the vancomycin probes we first tested conditions with bacterial lysates and finally optimized probe concentration and time of irradiation with the pathogenic strains VRE and MRSA *in situ* (Figure S1 in Supporting Information [SI], Figure 3 A−C). The cells were treated with various concentrations of probes and irradiated at 366 nm for different durations. Subsequently, bacteria were lysed, rhodamine

Table 1. MIC Values (μM) for Vancomycin Hydrochloride and the Probes 1−3

strain	vancomycin × HCl	probe **1**	probe **2**	probe **3**
S. aureus Mu50	1.3	2.5	2.5	1.3
S. aureus NCTC 8325	1.3	1.3	2.5	2.5
E. faecalis OG1RF (VSE)	2.5	1.3	2.5	<0.6
E. faecalis V583 (VRE)	5	2.5	5	2.5

azide was attached to the probe scaffold under CC conditions, and labeled proteins were visualized via fluorescent SDS gel analysis. A proteome incubation with 1 μM of probe and an irradiation time of 1 h turned out to be optimal in order to achieve saturated target labeling (Figure 3 C). *In situ* labeling of staphylococcal proteomes revealed that all three probes labeled a protein in the membrane with an apparent molecular mass of about 65 kDa (Figure 3B). Labeling of enteroccocal cells with all probes under *in situ* conditions revealed labeling of a single protein band with a molecular weight of about 40 kDa (Figure 3A). As there were no observed differences between *S. aureus* and MRSA as well as VSE and VRE, the labeled proteins likely do not represent resistance-associated targets but rather general interaction partners of vancomycin. All three probes exhibited the same protein target preferences, which indicates that protein labeling is driven solely by the scaffold and not by the attached modifications. We therefore conducted all subsequent studies with probe **1** as a representative example.

To unravel the identity of these two proteins we performed a quantitative proteome analysis by attaching a trifunctional rhodamine−biotin−azide tag under CC conditions for the visualization, enrichment, and identification of proteins by SDS-gel electrophoresis and mass spectrometry.[29] Analysis of the peptide fragments by the SEQUEST algorithm revealed strong hits for the bifunctional autolysin (ATL) in case of MRSA and a peptide ABC transporter (pABC) in case of VSE (Table S1, SI). The MS results were independently confirmed by the recombinant overexpression of both target proteins in *E. coli* and subsequent labeling with probe **1** (Figure 3D). Atl was cloned and expressed in full length (ATLfl) as well as in a shorter version with its amidase domain (ATLam), which was sufficient for successful probe interaction. Labeling occurred only with the native, folded proteins and not with heat-treated unfolded proteins, emphasizing a specific interaction with the probes (Figure 3D). Moreover, these proteins were not labeled by a benzophenone-alkyne control peptide (Figure 1) which shows that target binding is not mediated by the photo-cross-linker but solely by the vancomycin scaffold (Figure S2, SI).[30] Unmodified vancomycin competed for ATLfl, ATLam, and pABC labeling with probe **1**, which further emphasizes a direct and selective interaction of the unmodified natural product with these proteins (Figure 3E, Figure S3, SI). Interestingly, these interactions are likely to contribute to vancomycin antibiotic activity since both targets are important for bacteria to maintain their cell wall (ATL) and import nutrition (pABC).

In order to maintain cell integrity, viability, division, and growth, peptidogylcan must be continuously synthesized and degraded.[31] Peptidoglycan hydrolases are crucial for the degradation process and Atl represents the predominant hydrolase in staphylococci. Atl is a 147 kDa bifunctional enzyme with a 62 kDa *N*-acetylmuramyl-L-alanine amidase (am) and a 51 kDa endo-β-*N*-acetylglucosaminidase domain.[32] After translation, the Atl pro-peptide is cleaved into several processing intermediates that contain the two active domains together with different repeat units.[33,34] Since vancomycin probe incubation with SA and MRSA revealed a strong labeling of an Atl fragment of about 63 kDa it is most likely that vancomycin selectively interacts solely with the amidase unit of Atl. This was confirmed by protein overexpression where all three probes labeled not only the full length (ATLfl) but also the recombinant amidase functional domain (ATLam) (Figure S2, SI).

Previous phenotypic characterization of a *S. aureus* Atl deletion mutant by Götz et al. demonstrated that mutant cells grow in large cell clusters which suggests that Atl is responsible for the splitting of the septum during cell division to allow the separation of daughter cells.[31] This mutation was not lethal since a related Aaa amidase partially recovered the loss of Atl activity. A double mutation of both enzymes, however, significantly impaired bacterial growth.[35,36] In a different study by Tomasz et al. a similar ΔAtl phenotype of cell clustering was observed when sub-MIC concentrations of vancomycin were added to the culture medium of *S. aureus* which strongly suggests an inhibition of the autolytic process.[5,10] The authors suggest that this inhibtion is mediated by binding of vancomycin to muropeptides that sterically blocks access of all cell wall hydrolytic enzymes to the peptidoglycan substrate. Our results strongly emphasize that vancomycin also directly binds to the amidase unit of Atl and inhibits its enzymatic activity. This is further supported by an Atl activity assay in which recombinant ATLam was incubated with the natural peptidoglycan substrate. Atl degrades peptidoglycan, which can be photometrically followed at 600 nm. The dependence of the reaction rate on substrate concentration followed Michaelis−Menten kinetics with a K_M of 0.75 mM and a k_{cat} of 710 s^{-1}. Enzyme inhibition occurred in a concentration-dependent manner (Figure 4). Concentrations ranging from 75 nM to 300 μM of vancomycin show a slight to strong inhibition with an IC_{50} value of 95 μM and a K_I of 30 μM.

Taken together, our results show that vancomycin and corresponding probes directly target the Atl amidase domain in the presence and absence of peptidoglycan and also inhibit its activity. It is therefore most likely that this direct interaction further enhances the observed cell wall clustering phenotype that is caused by steric clashes with the autolytic machinery. This dual strategy of Atl amidase inhibition and limiting the access of all hydrolytic enzymes to their peptidoglycan substrates ensures a fine-tuned regulation of autolysis that may allow a basal level of cell wall remodeling which is probably essential for bacterial survival. One additional function of the autolytic machinery is to trigger antibiotic induced cell degradation. Downregulation of autolysis provides *S. aureus* a unique advantage to survive low-level vancomycin concentrations in its microenvironment.[10] In addition, inhibition of the autolytic machinery increases the thickness of cell walls that further sequester vancomycin molecules and also limits the access of antibiotics to reach their cell wall biosynthesis targets. In fact, these changes in cell morphology and autolytic activity are observed among many strains of vacomycin-resistant *S. aureus*.[4,8,9]

While Atl was the most prominent target in staphylococci, *E. faecalis* does not encode an amidase containing Atl, which accounts for the lack of its detection by our affinity probes. In contrast, probe labeling in living VRE and VSE revealed a peptide ABC transporter as the major proteome target. Peptide ABC transporters have a diverse array of functions in bacteria.[37,38] Some transporters are specialized for the import of essential nutrients such as sugars and peptides, while others carry out the

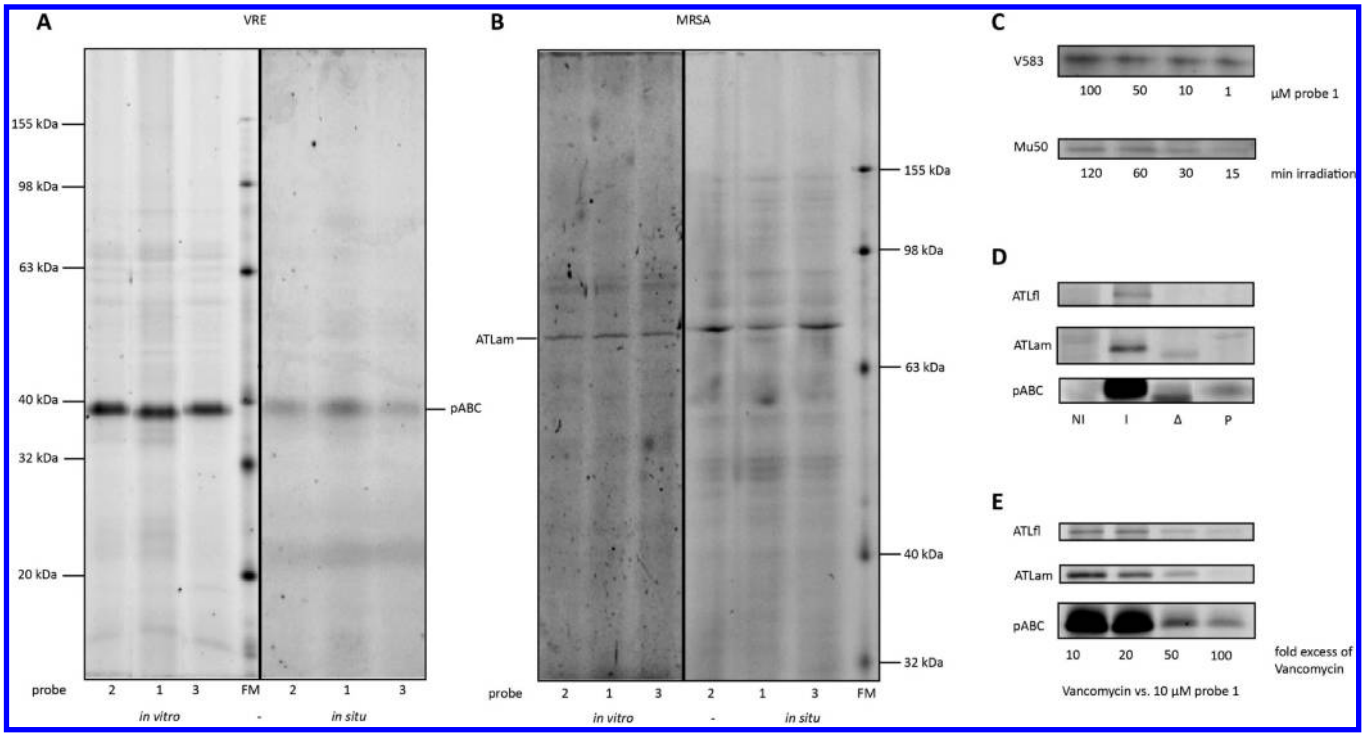


Figure 3. Labeling of bacterial pathogens by vancomycin-based probes **1**, **2** and **3**. Membrane proteins of VRE (A) and MRSA (B) after *in vitro* (left) and *in situ* (right) labeling. (C) Time- and concentration-dependent labeling in VRE and MRSA. (D) Recombinantly expressed ATLfl, ATLam, and pABC. NI = before induction, I = induced, Δ = heat control, P = protein in the proteome. (E) Competitive, concentration-dependent displacement of probe **1** by unmodified vancomycin in labeling experiments with overexpressed target proteins.

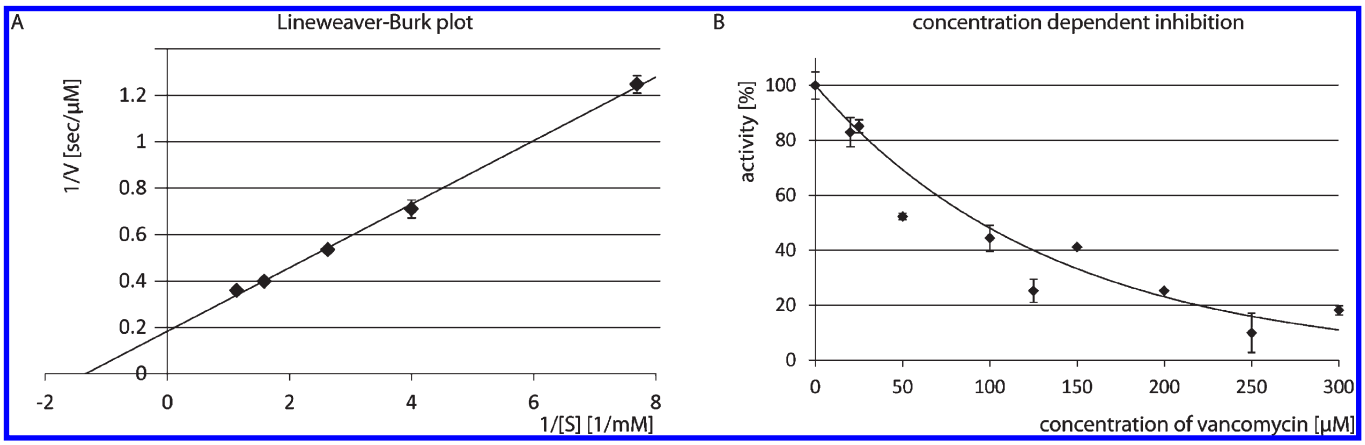


Figure 4. Interaction of vancomycin with ATLam via a peptidoglycan turnover assay. (A) Lineweaver−Burk plot for the degradation of peptidoglycan by ATLam to determine K_M and k_{cat} (B) Concentration-dependent inhibition of ATLam by vancomycin.

export of toxins or, in the case of virulent or drug-resistant bacteria, antibiotics.[39] *E. faecalis* encodes a large number of sugar-uptake systems such as ABC transporters that support bacteria to ferment nonabsorbed sugars in the gastrointestinal tract.[40] The ABC transporter identified here represents a thus far uncharacterized protein that is most likely not involved in antibiotic resistance as it is present in both VRE and VSE strains.[41] This is supported by gene expression data that show that there is no increased transcription upon incubation of bacteria with vancomycin (see Table S2 ([SI]).

■ CONCLUSION

In conclusion, we unraveled the protein targets of vancomycin in living bacterial cells by a chemical proteomic strategy that utilizes affinity-based photo-cross-linking probes. Three functional vancomycin probes were designed, and two unique structural modifications even exceeded the antibiotic potency of the unmodified natural product. It is well established that the binding of vancomycin to the D-Ala-D-Ala motif of nascent peptidoglycan and the corresponding inhibition of cell wall biosynthesis comprise the primary mode of action, which leads to autolysin-triggered cell rupture and death. However, the direct inhibition of the Atl amidase domain represents a mode of action that causes massive defects in cell morphology and enhances the tolerance of *S. aureus* to low concentrations of vancomycin. This provides *S. aureus* with an efficient strategy to evade low vancomycin concentrations without acquiring resistance on the

genetic level. The binding of vancomycin to Atl provides an intriguing perspective to design improved vancomycin inhibitors that exhibit lower affinity for this target and therefore increase antibacterial susceptibility in *S. aureus* and MRSA.

■ EXPERIMENTAL SECTION

General. All chemicals were of reagent grade or better and used without further purification. Chemicals and solvents were purchased from various commercial sources. 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 distilled before use. In all reactions, temperatures were measured externally. All NMR spectra of probe **1** and its precursor **5** were recorded with devices of the Bavarian NMR Center (for ^{1}H and ^{13}C spectra see Schemes S1−S3 in SI). Other ^{1}H, ^{13}C, and two-dimensional (2D) NMR spectra were measured with Bruker Avance 250, Avance 360, or Avance 500 (in cases of **7** and probes **2** and **3** equipped with a CryoProbe) with B-ACS-60/120 auto-samplers and referenced to the residual proton or carbon signal of the deuterated solvent, respectively. ESI mass spectra were recorded with Thermo LTQ Orbitrap XL or LTQ FT Ultra equipped with Dionex UltiMate 3000 Nano or Rapid Separation LC Systems. 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.

Synthetic Strategy to Vancomycin-Based ABPP-Probes. The synthesis was based on commercially available vancomycin hydrochloride. Standard peptide-coupling methods were used to modify the glycopeptide antibiotic as previously described.[23] Full characterization by NMR and HR-MS/MS was carried out for the probes.

Synthesis of 2,5-Dioxopyrrolidin-1-yl hex-5-ynoate 4.[24,25] *N*-Hydroxysuccinimide NHS (1.08 g, 9.35 mmol, 1.05 equiv) and *N,N′*-diisopropylcarbodiimide (DIC) (1450 μL, 9.35 mmol, 1.05 equiv) were added to a solution of 5-hexynoic acid (1.00 g, 984 μL, 8.92 mmol, 1 equiv) in 40 mL of CH_2Cl_2. The reaction was stirred for 16 h at rt. This mixture was washed with 30 mL of H_2O three times, and the combined inorganic phases were re-extracted with 30 mL of DCM. The combined organic phases were washed with sat. NaCl and dried over Na_2SO_4, and the solvent was removed *in vacuo*. The crude product was purified by column chromatography (SiO_2, ethyl acetate/isoHexan = 2:3, R_f = 0.47) yielding 1.19 g of a yellow oil (64%).

^{1}H NMR (360 MHz, $CDCl_3$, δ/ppm): 2.86 (s, 4H, 2 × NCOC**H$_2$**), 2.79 (t, J = 7.4 Hz, 2H, COC**H$_2$**), 2.37 (td, J = 6.9, 2.6 Hz, 2H, CqC**H$_2$**), 2.03 (t, J = 2.6 Hz, 1H, CqC**H**), 1.98 (m, 2H CH_2C**H$_2$**CH_2).

^{13}C NMR (91 MHz, $CDCl_3$, δ/ppm): 169.0 (2 NC═O), 168.2 (1 OC═O), 82.4 (1 Cq), 69.8 (1 CH), 29.7 (1 CH_2), 25.6 (2 CH_2), 23.3 (1 CH_2), 17.6 (1 CH_2).

HR-ESI-MS, positive mode (m/z): 232.0591 [M + Na]$^+$ (calc: 232.0580).

Synthesis of *N′*-Hex-5-ynamide Vancomycin 5.[27] Vancomycin hydrochloride (300 mg, 202 μmol, 1 equiv) was dissolved in 2 mL of DMF. *N,N′*-Diisopropylethylamine (DIPEA) (176 μL, 1.0 mmol, 5 equiv) and a solution of **4** (134 mg, 58 μL, 343 μmol, 1.7 equiv) in 1 mL of DMF were added. The reaction was stirred 24 h at rt and was subsequently poured into 30 mL of ice cold Et_2O. The resulting precipitate was centrifuged at 10000*g* at 4 °C for 15 min. The colorless supernatant was decanted and the pellet air-dried for 30 min. The residue was purified by HPLC, yielding 135 mg of a colorless solid (43%).

HPLC analysis, mobile phase (HPLC grade): A = water with 0.1% (v/v) TFA, B = acetonitrile with 0.1% (v/v) TFA. Gradient: T_0: B = 0%; T_{70}: B = 70%. Retention time: 18.0 min.

^{1}H NMR (600 MHz, d_6-DMSO, δ/ppm): 9.45 (s, 1H), 9.18 (s, 1H), 9.08 (s, 1H), 8.70 (d, J = 18.9 Hz, 1H), 8.57 (s, 1H), 7.89−7.79 (m, 1H), 7.68 (s, 1H), 7.52 (dd, J = 14.6, 8.4 Hz, 1H), 7.47 (s, 1H), 7.44 (d, J = 8.4 Hz, 1H), 7.32 (d, J = 8.4 Hz, 1H), 7.29 (d, J = 8.4 Hz, 1H), 7.17−7.13 (m, 1H), 7.05 (s, 1H), 6.99 (s, 1H), 6.77 (dd, J = 8.4, 2.0 Hz, 1H), 6.71 (d, J = 8.5 Hz, 2H), 6.68 (s, 1H), 6.58 (s, 1H), 6.40 (d, J = 6.6 Hz, 1H), 6.25 (d, J = 2.2 Hz, 1H), 5.98 (d, J = 6.5 Hz, 1H), 5.93 (s, 1H), 5.75 (d, J = 7.9 Hz, 1H), 5.67 (s, 1H), 5.60 (s, 1H), 5.35 (d, J = 5.0 Hz, 1H), 5.31 (dd, J = 6.7, 3.6 Hz, 1H), 5.28 (d, J = 7.7 Hz, 1H), 5.17 (s, 1H), 5.10 (d, J = 6.6 Hz, 1H), 5.08 (d, J = 5.1 Hz, 1H), 5.04 (d, J = 5.1 Hz, 1H), 4.92 (s, 1H), 4.69−4.63 (m, 1H), 4.43 (s, 1H), 4.42 (s, 1H), 4.32 (s, 1H), 4.18 (s, 1H), 4.05 (t, J = 5.4 Hz, 1H), 3.69 (d, J = 5.2 Hz, 1H), 3.53 (d, J = 8.1 Hz, 1H), 3.49 (d, J = 6.0 Hz, 1H), 3.44 (d, J = 6.3 Hz, 1H), 3.30−3.22 (m, 3H), 3.22 (s, 1H), 2.77 (t, J = 2.6 Hz, 2H), 2.63−2.60 (m, 1H), 2.28−2.21 (m, 5H), 2.16−2.06 (m, 4H), 1.97 (d, J = 13.0 Hz, 1H), 1.79 (d, J = 12.7 Hz, 1H), 1.70−1.51 (m, 2H), 1.40 (s, 3H), 1.10 (d, J = 6.3 Hz, 3H), 0.91 (d, J = 5.1 Hz, 3H), 0.86 (d, J = 5.6 Hz, 3H).

^{13}C NMR (151 MHz, d_6-DMSO, δ/ppm): 174.1, 172.6, 171.2, 170.8, 170.4, 169.5, 169.1, 167.8, 166.8, 157.2, 156.5, 155.1, 152.3, 151.3, 149.9, 148.2, 142.5, 139.8, 136.1, 135.7, 134.6, 131.9, 128.6, 127.3, 127.2, 126.2, 125.5, 124.3, 123.4, 121.6, 118.0, 116.2, 107.2, 105.7, 104.6, 102.3, 101.3, 96.7, 84.3, 78.1, 77.0, 76.7, 71.6, 71.3, 70.7, 70.1, 69.7, 63.1, 61.8, 61.2, 61.0, 58.4, 56.7, 54.9, 53.9, 53.7, 50.9, 41.0, 37.1, 33.2, 32.5, 27.8, 24.0, 23.8, 22.7, 22.4, 22.2, 17.7, 16.9.

HR-ESI-MS, positive mode (m/z): 1542.4782 [M + H]$^+$ (calc: 1542.4793).

Synthesis of *N*-(3-Aminopropyl)-4-benzoylbenzamide 6.[26,28] 4-Benzoylbenzoic acid (500 mg, 2.2 mmol, 1 equiv) was dissolved with DMAP (5 mg, cat.) in 5 mL of DMF and stirred at 22 °C for 10 min with DIC (379 μL, 2.4 mmol, 1.1 equiv) for preactivation. 1,3-Diaminopropane (184 μL, 2.2 mmol, 1 equiv) was added, and the reaction was stirred 2 h at 22 °C.

Thirty milliliters of 0.1% (v/v) TFA in H_2O was added, and the aq phase was extracted twice with 30 mL of EtOAc. The combined organic phases were extracted with 50 mL of 0.1% (v/v) TFA in H_2O. The aq phases were pooled, and the solvent was removed *in vacuo*; 447 mg of a yellow foam was obtained (72%) and used without further purification.

^{1}H NMR (360 MHz, d_6-DMSO, δ/ppm): 8.01 (d, J = 8.3 Hz, 2H, H_{arom}), 7.79 (d, J = 8.4 Hz, 2H, H_{arom}), 7.66 (dd, J = 6.3, 2.3 Hz, 2H, 2 × H_{arom}), 7.44 (dd, J = 6.6, 3.6 Hz, 1H, 1 × H_{arom}), 7.12 (dd, J = 6.3, 3.0 Hz, 2H, 2 × H_{arom}), 3.44−3.01 (m, 2H, 2 × C═ONHC**H$_2$**), 2.88 (t, J = 11.8 Hz, 2H, 2 × C**H$_2$**NH_2), 1.88−1.73 (m, 2H, 2 × CH_2C**H$_2$**CH_2).

^{13}C NMR (91 MHz, d_6-DMSO, δ/ppm): 196.7 (Ph**C**═OPh), 162.3 (**C**═ONH), 142.9 (**C**qC═O), 129.7 (2 × C_{arom}), 129.6 (C_{arom}), 128.6 (2 × C_{arom}), 127.3 (C_{arom}), 122.5 (2 × C_{arom}), 118.1 (2 × C_{arom}), 111.2 (C_{arom}), 36.8 (NH**C**H_2), 35.8 (**C**H_2NH_2), 23.3 (CH_2**C**H_2CH_2).

HR-ESI-MS, positive mode (m/z): 283.1448 [M + Na]$^+$ (calc: 283.1441).

***N′*-Hex-5-ynamide C-(3-Aminopropyl)-4-benzoylbenzamide Vancomycin Probe 1.**[23] Vancomycin derivative **5** (20 mg, 13 μmol, 1 equiv) was dissolved together with **6** (7.3 mg, 26 μmol, 2 equiv) in 600 μL of DMF. DIPEA (11.4 μL, 65 μmol, 5 equiv), PyBOP (7.4 mg, 14 μmol, 1.1 equiv), and HOBT (13% H_2O, 2.1 mg, 14 μmol, 1.1 equiv) were added in sequence, and the reaction mixture was shaken for 16 h at 22 °C in the dark. The reaction was crashed into 6 mL of ice-cold Et_2O. The precipitate that formed was centrifuged at 10000*g* and 4 °C for 15 min, and the clear supernatant was removed. The pellet was air-dried for 30 min, and the crude product was purified via HPLC, yielding 1.7 mg of a colorless solid (7%).

HPLC analysis, mobile phase (HPLC grade): A = water with 0.1% (v/v) TFA, B = acetonitrile with 0.1% (v/v) TFA. Gradient: T_0: B = 0%; T_{70}: B = 70%. Retention time: 33.0 min.

^{1}H NMR (500 MHz, d_6-DMSO, δ/ppm): 9.33 (s, 1H), 9.05 (s, 1H), 8.79 (s, 1H), 8.67 (s, 1H), 8.52 (s, 1H), 8.01 (d, J = 8.4 Hz, 2H), 7.86 (s, 1H),

7.81 (d, J = 8.4 Hz, 2H), 7.76 (d, J = 7.1 Hz, 2H), 7.71 (s, 1H), 7.60 (s, 1H), 7.58 (s, 2H), 7.57 (s, 1H), 7.45 (s, 1H), 7.43 (s, 1H), 7.31 (d, J = 8.3 Hz, 1H), 7.22 (s, 1H), 7.17 (s, 1H), 6.98 (s, 2H), 6.76 (d, J = 8.4 Hz, 1H), 6.71 (s, 1H), 6.69 (s, 1H), 6.56 (s, 1H), 6.36 (d, J = 1.8 Hz, 1H), 6.27 (d, J = 2.0 Hz, 1H), 5.93 (s, 1H), 5.79 (s, 1H), 5.75 (s, 1H), 5.69 (s, 1H), 5.62 (s, 1H), 5.32 (s, 1H), 5.31 (s, 1H), 5.31 (s, 1H), 5.30 (s, 1H), 5.18 (s, 1H), 5.17 (s, 1H), 5.16 (s, 1H), 4.93 (s, 1H), 4.66 (d, J = 6.7 Hz, 1H), 4.46 (s, 1H), 4.45 (s, 1H), 4.37 (d, J = 5.4 Hz, 1H), 4.24 (d, J = 10.4 Hz, 1H), 4.05 (s, 1H), 3.68 (d, J = 10.1 Hz, 1H), 3.55 (d, J = 8.8 Hz, 1H), 3.52 (d, J = 10.9 Hz, 1H), 3.40 (covered by H_2O, 3H), 3.30−3.23 (m, 3H), 3.20 (s, 1H), 3.03−2.99 (m, 2H), 2.75 (t, J = 2.6 Hz, 1H), 2.65 (s, 1H), 2.52 (s, 1H), 2.27−2.13 (m, 5H), 2.14−2.04 (m, 8H), 1.98 (d, J = 13.0 Hz, 1H), 1.79 (d, J = 9.8 Hz, 1H), 1.65 (s, 1H), 1.60−1.55 (m, 1H), 1.44−1.39 (m, 1H), 1.23 (s, 1H), 1.02 (d, J = 6.3 Hz, 3H), 0.91 (d, J = 6.1 Hz, 3H), 0.86 (d, J = 6.1 Hz, 3H).

^{13}C NMR (126 MHz, d_6-DMSO, δ/ppm): 195.43, 170.80, 170.35, 170.20, 169.30, 169.22, 169.14, 168.40, 167.70, 166.07, 165.84, 157.07, 156.32, 155.10, 152.53, 151.12, 150.23, 148.27, 142.29, 140.34, 139.18, 137.73, 136.67, 135.61, 133.04, 129.73, 129.68, 129.56, 128.69, 127.43, 127.37, 127.28, 127.22, 127.02, 126.44, 126.25, 125.39, 125.36, 124.35, 123.34, 121.84, 118.34, 118.17, 116.25, 115.96, 107.64, 106.28, 104.83, 102.09, 101.13, 97.55, 84.26, 77.76, 77.13, 76.74, 71.89, 71.39, 70.28, 70.04, 69.66, 63.25, 62.04, 61.30, 61.15, 58.85, 57.87, 54.92, 53.92, 53.71, 51.09, 40.10, 36.79, 36.33, 34.99, 34.52, 31.25, 29.29, 25.95, 24.34, 23.68, 23.07, 22.73, 22.36, 17.41, 17.34.

HR-ESI-MS, positive mode (m/z): 1806.6060 $[M + H]^+$ (calc: 1806.6056).

***N′*-Benzoylbenzamide Vancomycin 7.**[23] To a solution of vancomycin hydrochloride (100 mg, 68 μmol, 1.0 equiv) in 600 μL of DMF was added DIPEA (33 μL, 201 μmol, 3.0 equiv). 4-Benzoylbenzoic acid (16 mg, 71 μmol, 1.05 equiv) was preactivated with PyBOP (39 mg, 74 μmol, 1.1 equiv), HOBT (13% H_2O, 11 mg, 74 μmol, 1.1 equiv), and DIPEA (11 μL, 68 μmol, 1 equiv) in 200 μL of DMF. After 10 min the two solutions were combined and shaken for 2 h at 22 °C. The reaction mixture was subsequently poured into 20 mL of ice-cold Et_2O. The resulting precipitate was treated as described before in the case of probe **1**. The residue was purified by HPLC, yielding 62 mg of a colorless solid (56%).

HPLC analysis, mobile phase (HPLC grade): A = water with 0.1% (v/v) TFA, B = acetonitrile with 0.1% (v/v) TFA. Gradient: T_0: B = 0%; T_{70}: B = 70%. Retention time: 21.6 min.

^{1}H NMR (500 MHz, d_6-DMSO, δ/ppm): 9.47 (s, 1H), 9.19 (s, 1H), 9.09 (s, 1H), 8.70 (s, 1H), 8.57 (s, 1H), 7.95 (d, J = 5.9 Hz, 2H), 7.86 (s, 1H), 7.82 (d, J = 8.5 Hz, 2H), 7.76 (d, J = 8.1 Hz, 2H), 7.67 (s, 1H), 7.59 (s, 1H), 7.58 (s, 2H), 7.56 (s, 1H), 7.46 (s, 1H), 7.45 (s, 1H), 7.35 (s, 1H), 7.29 (d, J = 4.1 Hz, 1H), 7.20 (s, 1H), 7.11 (s, 2H), 6.77 (s, 1H), 6.73 (s, 1H), 6.72 (s, 1H), 6.70 (s, 1H), 6.40 (d, J = 7.8 Hz, 1H), 6.25 (d, J = 4.5 Hz, 1H), 5.97 (br s, 1H), 5.77 (s, 1H), 5.76 (s, 1H), 5.62 (s, 1H), 5.52 (s, 1H), 5.34 (s, 1H), 5.32 (s, 1H), 5.31 (s, 1H), 5.24 (s, 1H), 5.23 (s, 1H), 5.20 (s, 1H), 5.11 (s, 1H), 4.94 (s, 1H), 4.79−4.74 (m, 1H), 4.67 (d, J = 5.3 Hz, 1H), 4.46 (s, 1H), 4.43 (d, J = 5.4 Hz, 1H), 4.20 (s, 1H), 3.95 (s, 1H), 3.70 (d, J = 10.3 Hz, 1H), 3.58 (s, 1H), 3.53 (s, 1H), 3.36 (s, 1H), 3.30−3.17 (m, 3H), 3.14 (s, 1H), 2.81 (s, 1H), 2.67−2.62 (m, 2H), 2.28−2.12 (m, 5H), 2.00−1.86 (m, 6H), 1.78−1.52 (m, 5H), 1.09−1.06 (m, 1H), 0.96 (d, J = 6.4 Hz, 3H), 0.91 (d, J = 5.9 Hz, 3H), 0.86 (d, J = 6.0 Hz, 3H).

^{13}C NMR (126 MHz, d_6-DMSO, δ/ppm): 195.50, 172.62, 170.46, 169.19, 169.16, 167.89, 167.80, 167.58, 166.18, 165.05, 158.26, 157.24, 156.59, 155.13, 152.62, 151.22, 148.31, 142.49, 139.28, 138.85, 137.98, 136.77, 135.70, 133.09, 129.90, 129.75, 129.48, 128.74, 127.49, 127.43, 127.41, 127.35, 127.30, 126.97, 126.50, 126.28, 125.63, 124.41, 123.45, 121.63, 118.11, 118.02, 116.24, 115.80, 113.44, 105.71, 104.85, 102.36, 101.29, 97.62, 78.30, 77.13, 76.77, 71.68, 71.44, 70.32, 70.12, 63.27, 63.17, 61.85, 61.27, 59.60, 58.92, 54.90, 53.99, 53.79, 51.12, 40.12, 35.51, 32.77, 31.28, 24.76, 23.74, 22.81, 22.35, 17.47.

ESI-MS, positive mode (m/z): 1658.4966 $[M + H]^+$ (calc: 1658.4972).

***N′*-Benzoylbenzamide C Propargylamide Vancomycin Probe 2.**[23] To a solution of vancomycin derivative **7** (50 mg, 30 μmol, 1.05 equiv) in 300 μL of DMF 3-amino-1-propyne (1.9 μL, 29 μmol, 1.0 equiv) were added HOBT (13% H_2O, 5 mg, 32 μmol, 1.1 equiv), PyBOP (17 mg, 32 μmol, 1.1 equiv), and DIPEA (15 μL, 90 μmol, 3.2 equiv), and the reaction was stirred 2 h at 22 °C. The reaction mixture was subsequently poured into ice-cold Et_2O and treated as described before for probe **1**.

The residue was purified by HPLC, yielding 38 mg of a colorless solid (75%).

HPLC analysis, mobile phase (HPLC grade): A = water with 0.1% (v/v) TFA, B = acetonitrile with 0.1% (v/v) TFA. Gradient: T_0: B = 0%; T_{70}: B = 70%. Retention time: 22.2 min.

^{1}H NMR (500 MHz, d_6-DMSO, δ/ppm): 9.34 (s, 1H), 9.00 (s, 2H), 8.69 (s, 1H), 8.64 (s, 1H), 8.48 (s, 1H), 8.39 (s, 2H), 7.87 (d, J = 8.0 Hz, 2H), 7.82 (d, J = 7.8 Hz, 2H), 7.77 (d, J = 7.7 Hz, 2H), 7.74 (d, J = 7.4 Hz, 2H), 7.65 (s, 1H), 7.58 (s, 1H), 7.56 (s, 1H), 7.46 (s, 1H), 7.34 (s, 1H), 7.29 (s, 1H), 7.20 (s, 1H), 7.12 (d, J = 8.2 Hz, 1H), 7.08 (d, J = 8.0 Hz, 1H), 6.75 (s, 1H), 6.71 (s, 1H), 6.69 (s, 1H), 6.66 (s, 1H), 6.38 (d, J = 7.4 Hz, 1H), 6.22 (s, 1H), 5.96 (s, 1H), 5.84 (s, 1H), 5.76 (s, 1H), 5.63 (s, 1H), 5.52 (s, 1H), 5.34 (s, 1H), 5.32 (s, 1H), 5.30 (s, 1H), 5.25 (s, 1H), 5.24 (s, 1H), 5.19 (s, 1H), 4.94 (s, 1H), 4.77 (d, J = 6.2 Hz, 1H), 4.55 (s, 1H), 4.45 (s, 1H), 4.40 (s, 1H), 4.22 (s, 1H), 3.96 (s, 1H), 3.91−3.84 (m, 1H), 3.69 (s, 1H), 3.28 (s, 3H), 3.13 (s, 1H), 2.81 (s, 1H), 2.64 (s, 1H), 2.36 (s, 1H), 2.26−2.11 (m, 6H), 2.01−1.93 (m, 1H), 1.80−1.64 (m, 2H), 1.58 (s, 1H), 1.28 (s, 1H), 1.07 (s, 1H), 0.96 (d, J = 6.3 Hz, 3H), 0.91 (d, J = 5.7 Hz, 3H), 0.86 (d, J = 5.9 Hz, 3H).

^{13}C NMR (126 MHz, d_6-DMSO, δ/ppm): 195.46, 170.92, 170.10, 169.13, 169.10, 169.05, 168.19, 167.72, 166.13, 165.00, 158.15, 157.15, 155.11, 152.55, 151.18, 150.19, 148.23, 142.38, 139.24, 138.83, 137.47, 136.72, 136.34, 133.06, 129.87, 129.72, 129.45, 128.71, 127.47, 127.44, 127.27, 127.16, 126.93, 126.42, 126.18, 125.46, 125.41, 124.42, 123.43, 121.82, 118.09, 117.92, 116.33, 115.72, 109.58, 106.46, 104.79, 102.15, 101.26, 96.81, 81.25, 77.21, 77.13, 76.81, 73.00, 71.36, 70.85, 70.28, 69.97, 63.15, 62.02, 61.30, 61.24, 58.96, 57.48, 55.02, 53.87, 53.77, 51.12, 40.19, 35.47, 32.74, 31.23, 28.16, 24.79, 22.82, 22.35, 17.48, 16.81.

ESI-MS, positive mode (m/z): 1696.5234 $[M + H]^+$ (calc: 1696.5225).

3-(4-Benzoylphenyl)-2-(hex-5-ynamido)propanoic Acid 8: BP Minimal Probe.[30] 2-Amino-3-(4-benzoylphenyl)propanoic acid (300 mg, 1.1 mmol, 1.0 equiv) was dissolved in 4 mL of CH_2Cl_2 with DIPEA (474 μL, 2.8 mmol, 2.5 equiv). **1** (242 μL, 1.5 mmol, 1.3 equiv) was added and the reaction was stirred 1.5 h at 22 °C. The solvent was removed *in vacuo* and the crude product purified via HPLC yielding 342 mg of a yellow foam (85%).

HPLC analysis, mobile phase (HPLC grade): A = water with 0.1% (v/v) TFA, B = acetonitrile with 0.1% (v/v) TFA. Gradient: T_0: B = 0%; T_1: B = 60%; T_7: B = 80%. Retention time: 3.5 min.

^{1}H NMR (500 MHz, $CDCl_3$, δ/ppm): 10.00 (s, 1 H, CO_2**H**), 7.75 (d, J = 7.1 Hz, 2 H, $H_{arom.}$), 7.71 (d, J = 8.2 Hz, 2 H, H_{arom}), 7.58 (t, J = 7.4 Hz, 1 H, H_{arom}), 7.46 (t, J = 7.7 Hz, 2 H, H_{arom}), 7.29 (d, J = 8.1 Hz, 2 H, H_{arom}), 6.61 (d, J = 7.6 Hz, 1 H, N**H**), 4.94 (ps. q, J = 6.1 Hz, 1 H, C**H**−CO_2H), 3.35 (dd, J = 14.1, 5.7, 1 H, C_{arom}−C$\mathbf{H_a}$$H_b$), 3.19 (dd, 1 H, J = 14.0, 6.4 Hz, C_{arom}−$CH_a$$\mathbf{H_b}$), 2.36 (t, J = 7.3, 2 H, NHC=OC$\mathbf{H_2}$), 2.25−2.15 (m, 2 H, HC≡CC$\mathbf{H_2}$), 1.95 (t, J = 2.6 Hz, 1 H, **H**C≡C), 1.81 (ps. quint, J = 6.8 Hz, 2 H, HC≡C−CH_2C$\mathbf{H_2}$).

^{13}C NMR (90 MHz, $CDCl_3$, δ/ppm): 196.6, 173.8, 172.9, 141.0, 137.4, 136.4, 132.6, 130.4, 130.1, 130.0, 129.8, 129.4, 128.5, 128.3, 128.3, 83.2, 69.5, 53.1, 37.4, 34.7, 24.0, 17.7.

ESI-MS, positive mode (m/z): 364.1544 $[M + H]^+$ (calc: 364.1549).

***N′*-3-(4-benzoylphenyl)-2-(hex-5-ynamido)propanoic amide vancomycin Probe 3.**[23] To a solution of Vancomycin hydrochloride (100 mg, 68 μmol, 1.0 equivz) in 700 μL of DMF, DIPEA (33 μL, 201 μmol, 3.0 equiv) was added. PyBOP (39 mg, 74 μmol, 1.1 equiv) and HOBT (13% H_2O, 11 mg, 74 μmol, 1.1 equiv) were dissolved in DMF. DIPEA (11 μL, 68 μmol, 1 equiv) and a solution of 3-(4-benzoylphenyl)-2-(hex-5-ynamido)propanoic acid **8** (210 mg/mL in DCM, 120 μL, 71 μmol, 1.05 equiv) were added. After 10 min of preactivating the carboxylic acid, the two solutions were combined and shaken for 2 h at 22 °C. Before HPLC purification, the reaction mixture was treated as described before for probe **1**. 76 mg of a colorless solid (63%) were obtained.

^{1}H NMR (500 MHz, d_6-DMSO, δ/ppm): 9.48 (s, 1H), 9.20 (s, 1H), 9.00 (s, 1H), 8.68 (s, 1H), 8.53 (s, 1H), 8.15 (s, 2H), 7.85 (d, *J* = 10.0 Hz, 1H), 7.71 (d, *J* = 7.7 Hz, 2H), 7.66 (d, *J* = 7.0 Hz, 2H), 7.62 (d, *J* = 7.9 Hz, 1H), 7.57 (s, 1H), 7.56 (s, 2H), 7.54 (s, 1H), 7.45 (s, 1H), 7.38 (s, 1H), 7.33 (s, 1H), 7.25 (s, 1H), 7.17 (s, 1H), 7.08−6.99 (m, 2H), 6.77 (d, *J* = 8.3 Hz, 1H), 6.71 (d, *J* = 8.7 Hz, 1H), 6.61 (s, 1H), 6.49 (s, 1H), 6.40 (s, 1H), 6.25 (s, 1H), 5.98 (s, 1H), 5.76 (s, 1H), 5.74 (s, 1H), 5.60 (s, 1H), 5.52 (s, 1H), 5.32 (s, 1H), 5.31 (s, 1H), 5.26 (s, 1H), 5.24 (s, 1H), 5.22 (s, 1H), 5.17 (s, 1H), 5.11 (s, 1H), 4.94 (s, 1H), 4.94 (s, 1H), 4.68 (d, *J* = 7.2 Hz, 1H), 4.47 (s, 1H), 4.43 (s, 1H), 4.26 (s, 1H), 4.19 (s, 1H), 3.95 (s, 1H), 3.67 (s, 1H), 3.37 (s, 1H), 3.28−3.25 (m, 5H), 2.86 (s, 1H), 2.77−2.71 (m, 1H), 2.65 (s, 1H), 2.25 (s, 5H), 2.13 (s, 6H), 2.00 (s, 1H), 1.78−1.50 (m, 3H), 1.40 (s, 1H), 1.28 (s, 1H), 1.07 (s, 1H), 0.91 (d, *J* = 5.5 Hz, 3H), 0.86 (d, *J* = 5.7 Hz, 3H), 0.80 (d, *J* = 6.2 Hz, 3H).

^{13}C NMR (126 MHz, d_6-DMSO, δ/ppm): 195.74, 172.66, 171.64, 170.96, 170.24, 170.13, 169.94, 169.24, 167.94, 167.48, 166.20, 157.28, 156.62, 155.17, 152.65, 151.26, 150.27, 148.37, 142.49, 139.56, 139.27, 137.34, 136.16, 135.76, 132.64, 129.84, 129.59, 129.47, 128.62, 127.55, 127.45, 127.31, 127.06, 126.61, 126.49, 126.32, 125.64, 125.52, 124.47, 123.52, 121.65, 118.19, 118.06, 116.33, 115.82, 107.53, 105.75, 104.90, 102.41, 101.40, 97.65, 84.14, 77.67, 77.13, 76.75, 71.73, 71.53, 70.82, 70.41, 70.12, 63.18, 61.91, 61.31, 61.27, 59.67, 58.99, 56.74, 54.01, 53.78, 53.31, 51.11, 40.22, 35.89, 35.04, 34.00, 31.30, 30.02, 24.65, 24.40, 23.78, 22.90, 22.37, 17.37, 17.25.

ESI-MS, positive mode (*m*/*z*): 1795.5807 $[M + H]^+$ (calc: 1795.5812).

Bacterial Strains. *Staphylococcus aureus* strains NCTC 8325 and Mu50/ATCC 700699 (both from Institute Pasteur, France), and *Enterococcus faecalis* strains V583/ATCC 700802 and OF1RF/ATCC 47077 (both form LGC Standards) were maintained in BHB (brain-heart broth) medium at 37 °C.

MIC Measurments. Overnight cultures of the bacteria were diluted in fresh BHB medium to OD_{600} = 0.01 and 100 μL was incubated in Nunclon 96-well plates with round-shaped bottoms with 1 μL of the corresponding DMSO stocks of vancomycin and the probes at varying concentrations. The samples were incubated 12 h at 37 °C and the optical densities obtained for MIC calculation. All experiments were conducted at least in triplicates, and DMSO served as control.

Preparation of Proteomes for *in Vitro* Experiments. The proteomes of the bacterial strains were prepared from 1-L liquid cultures in BHB medium harvested 1 h after transition in the stationary phase by centrifugation at 4000*g*. The bacterial cell pellet was washed with PBS, resuspended in PBS buffer, and lysed by sonication with a Bandelin Sonopuls under ice cooling. Membrane and cytosol were separated by centrifugation at 18000*g* for 45 min.

***In Vitro* Analytical and Preparative 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, a total reaction volume of 50 μL was reached. In the case of competitive displacement experiments vancomycin hydrochloride was added in various excess (1 μL of DMSO stocks) and the proteoms were preincubated with the antibiotic for 15 min at 22 °C. Reactions were initiated by addition of 1 μM of probe and allowed to incubate (additional) 15 min at 22 °C. Then they were irradiated in aliquots of 20 μL in open polystyrene microwell plates for 120 min on ice with 366 nm UV light (Benda UV hand lamp NU-15 W). 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 22 °C 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 $CuSO_4$ (1 μL). The reactions were incubated at 22 °C for 1 h. For analytical gel electrophoresis, 50 μL of 2 × 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.

Preparative experiments were carried out in a final volume of 2 mL of proteome sample. 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 a 5-fold volume of prechilled acetone, samples were stored on ice for 60 min and centrifuged at 16000*g* for 10 min. The supernatant was discarded and the pellet 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. Fifty microliters of 2× SDS loading buffer was 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.[45]

***In situ* Experiments.** For analytical *in situ* studies, bacteria were grown in BHB medium and harvested 1 h after reaching stationary phase by centrifugation. After washing with PBS, the cells were resuspended in 200 μL of PBS. Bacteria were incubated 15 min at 22 °C with 10 μM of probe followed by irradiation at 366 nm on ice for 2 h. Competitive *in situ* experiments with vancomycin were performed as described for the *in vitro* case. Subsequently, the cells were washed twice with PBS and lysed by sonication in 100 μL of PBS. The proteomes were separated into cytosolic and membrane fractions, followed by CC as described above.

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 mm (3 μm) column for analysis by tandem MS followed by high-resolution MS using a coupled Dionex Ultimate 3000 LC-Thermo LTQ Orbitrap XL 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. Minimum P-values and Xcorr values of each run as well as the total number of obtained peptides are reported in Table S1, SI.

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 as template,

prepared by standard protocols. *att*B1 forward primer and *att*B2 reverse primer were designed to yield *att*B-PCR Products needed for Gateway Technology. The sequences of these primers can be found in Table S3, SI.

PCR products were separated and identified on 1% agarose gels containing 0.2 μg/mL ethidium bromide, and gel bands were isolated and extracted with an E.ZNA MicroElute Gel Extraction Kit. Concentrations of DNA were measured with a TECAN Infinite 200 PRO and the corresponding NanoQuant Plate. One hundred femtomoles of purified *att*B-PCR product and 50 fmol of *att*P-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 *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 LB medium supplemented with kanamycin. Cells were harvested, and plasmids were isolated using an E.ZNA Plasmid Mini Kit. The corresponding *att*B-containing expression clone was generated by *in vitro* LR recombination reaction of approximately 50 fmol of the *att*L-containing entry clone and 50 fmol of an *att*R-containing destination vector pDEST007 or Invitrogen Champion pET300 NT-DEST using LR Clonase II enzyme mix in TE buffer.[46] The expression clone was transformed in chemically competent BL21 *E. coli* cells (Novagen) and selected on LB agar plates containing 100 μg mL^{-1} ampicillin. Validity of the clones was confirmed by plasmid sequence analysis. Recombinant clones were grown in ampicillin LB medium, and target gene expression was induced with anhydrotetracycline or IPTG, depending on the expression vector used.

ATLam, that was overexpressed using the pET300 NT-DEST, was purified with Profinity IMAC Resin from BioRad corresponding to the manufacture's manual. The protein was desalted into the assay buffer using a GE Healthcare ÄKTApurifier system equipped with a HiTrap desalting column (5 mL). Purity was checked via SDS-PAGE and Coomasie stain.

Peptidoglycan Assay. To evaluate the effect of vancomycin on the degradation of peptidoglycan via ATL the following assay was performed according to published procedures.[47,48] Peptidoglycan from *Staphylococcus aureus* (Sigma-Aldrich) was suspended in ddH_2O to yield 3.3 mg/mL. The molarities given were calculated with respect to the molecular weight of the murein monomers. The measurement of OD_{600} was carried out with a Tecan Infinite 200 PRO plate reader in 96-well plates with lid and flat well shape at 30 °C.

For inhibitory studies the enzyme was preincubated with vancomycin for 5 min and finally diluted to a concentration of 0.5 μg/mL (7.7 nM) with the substrate suspension of $OD_{600} \approx 0.3$. OD_{600} was measured over 30 min, and the 96-well was shaken constantly. The decrease of OD_{600} within this time was set to 100% for the uninhibited reaction. The other reactions are given in relation to this value (Figure 4). The IC_{50} was determined from the fitted curve shown in Figure 4 as the inhibitor concentration where the protein shows half-maximal activity.

For Michaelis−Menten kinetics, substrate concentrations were varied from 1 mM to 0.125 mM, and the corresponding enzyme turnover was monitored as described above. The enzyme concentration was kept constant at 7.7 nM. K_M was determined via a Lineweaver−Burk plot with the *x*-intercept of the graph representing $-1/K_M$ (Figure 4). K_I was directly calculated from these values.[49]

Expression Analysis. *E. faecalis* V583 was grown overnight with half maximal MIC of vancomycin and without antibiotic selection. Total bacterial RNA was isolated with the Bio-Rad Aurum Total RNA mini kit and transcribed to cDNA immediately by reverse transcriptase and a random hexamer primer set using an iScript Select cDNA synthesis kit (Bio-Rad). For RT PCR, specific primers were designed for pABC to amplify a sequence specific for this gene of interest with Primer-BLAST. RT PCR was conducted in a two-step thermal protocol using a SsoFast EvaGreen Supermix kit (Bio-Rad) with white Low-Profile 96-well unskirted PCR plates in a Bio-Rad CFX96 real-time PCR detection system. The polymerase was activated in an initial denaturation step for 30 s at 95.0 °C. Forty cycles were then run, each consisting of 5 s at 95.0 °C, followed by 10 s annealing and elongation at 61 °C. A melting curve was measured to ensure specific replication of the target sequences only.GAP and LAS genes were used as internal standards for ΔΔCq analysis. Primer sequences can be found in Table S3, SI. Cq and Cq mean values are shown in Table S2, SI.

■ ASSOCIATED CONTENT

Supporting Information. Complete ref 40. Additional figures containing fluorescent gel scans, primer sequences, mass spectrometry, real time PCR data, and 1H and 13C NMR-Spectra of Probes 1, 2 and 3. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENT

We thank Matt Nodwell for scientific advice, Mona Wolff for excellent support, and G. Gemmecker and M. Sattler for help with NMR analysis. J.E. was supported by the FOR1406 and the TUM Graduate School (Faculty Center Chemistry). S.S. was supported by the Deutsche Forschungsgemeinschaft (Emmy Noether), SFB749, FOR1406, an ERC starting grant and the Center for Integrated Protein Science Munich CIPSM.

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