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A Biochemical Lanthanide-Encoding Approach Enables Quantitative Monitoring of the Bacterial Response to Vancomycin Treatment

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ABSTRACT: A pathogenic bacterium has its own mechanisms for not only pathogenic attack but also exogenous invasion defense, in which the bacterial cell wall is the front line of attack and defense. We developed a biochemical lanthanide-encoding approach to quantify the uncanonical D-amino acid (D-X) that was edited in a small proportion into the terminal acyl-D-Ala-D-X of nascent peptidoglycan UDP-MurNAc-pentapeptides in the bacterial cell wall. This approach overcomes the difficulties regarding quantification and accuracy issues encountered by the popular optical imaging and traditional high-performance liquid chromatography-based methods. Newly synthesized *azide*-D-Leu and *ketone*-D-Met were used together with *alkynyl*-D-Ala for their metabolic assembly and then bioorthogonally encoded by the correspondingly fabricated *DBCO*-DOTA-Gd, H_2NO-DOTA-Eu, and *azide*-DOTA-Sm tags. This approach allows direct quantification of the D-X in situ in the cell wall using ^{158}Gd, ^{153}Eu, and ^{154}Sm species-unspecific isotope dilution inductively coupled plasma mass spectrometry, avoiding any tedious and complex "cell-broken" pretreatment procedures that might induce racemization of the D-X. The obtained site-specific and accurate in situ information about the D-X enables quantitative monitoring of the bacterial response when *Staphylococcus aureus* meets vancomycin, showing that the amounts of *azide*-D-Leu and *ketone*-D-Met assembled are more important after determining the structure- and composition-dependent bacterial antibiotic resistance mechanisms. In addition, we found that the combined use of vancomycin and D-Ala restores the efficacy of vancomycin and might be a wise and simple way to combat vancomycin intermediate-resistant *S. aureus*.

Antibiotics are some of the greatest discoveries in medical history.[1] Most antibiotic classes used today were identified in the antibiotic golden age from the 1940s to the 1960s followed by semisynthetic and fully synthetic antibiotics to the currently genomics- and proteomics-driven post-antibiotic discovery era.[2] However, we are now suffering from a serious antibiotic resistance crisis because the appearance of unprecedented multidrug resistance exceeds the supply of newly approved antibiotics for efficaciously clinical usage. Encoded by corresponding conserved and mutated genes, the putative mechanisms of intrinsic and acquired bacterial antibiotic resistance include (1) the prevention of access of the drug to targets, (2) changes in the structure and protection of antibiotic targets, and (3) direct modification or inactivation of antibiotics. The unique bacterial cell envelope is the first hurdle to prevent antibiotics from entering the bacterial cells and thus limits their consequent efficacy.[3,4] Generally, the bacterial cell envelope is composed of a peptidoglycan (PG) layer-supported negatively charged lipopolysaccharide (LPS) outer membrane that prevents the penetration of large and hydrophobic compounds and an inner hydrophobic phospholipid cytoplasmic membrane that restricts the penetration of hydrophilic compounds. This unique structure is an almost impenetrable barrier from a chemophysics perspective. Although some antibiotics could pass through the barrier with the aids of chimeric peptides, chaperone proteins, or β-barrel porins anchored on the PG layer, some of them might be extruded eventually by the trans-envelope multidrug resistance pumps, leading to the failure or deprecation of most developed antibiotics. However, the classic β-lactam and glycopeptide antibiotics discovered more than 70 years ago are two exceptions and still in use today. This is clearly because their main targets (penicillin binding proteins that are in charge of assembling PG and the penultimate acyl-D-Ala-D-Ala of PG pentapeptides[5]) are located in the periplasm; therefore, they do not need to cross the barrier. For example, vancomycin, a typical glycopeptide antibiotic that contains two basic and four acidic groups with an isoelectric point of 8.30 and an average charge of +0.67 as penta- and tetraprotonated species that are predominant in the physiological pH,[6] can easily move to the LPS outer membrane and reach its acyl-D-Ala-D-Ala target in the bacterial cell wall, remaining one of the choices for the treatment of methicillin-resistant *Staphylococcus aureus*.[7] Nevertheless, bacterial vancomycin resistance still happened.[8] The known change of the vancomycin's target from acyl-D-Ala-D-Ala to acyl-D-Ala-D-lactate (Lac) is responsible for the resistance, resulting in the 1000-fold lower binding affinity of vancomycin due to the loss of one crucial hydrogen bond (H-bond) of the five H-bonds in the binding domain.[9,10] This alteration of the terminal D-Ala to D-Lac conferred by the mutated Van gene operons has been generally believed to

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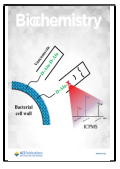

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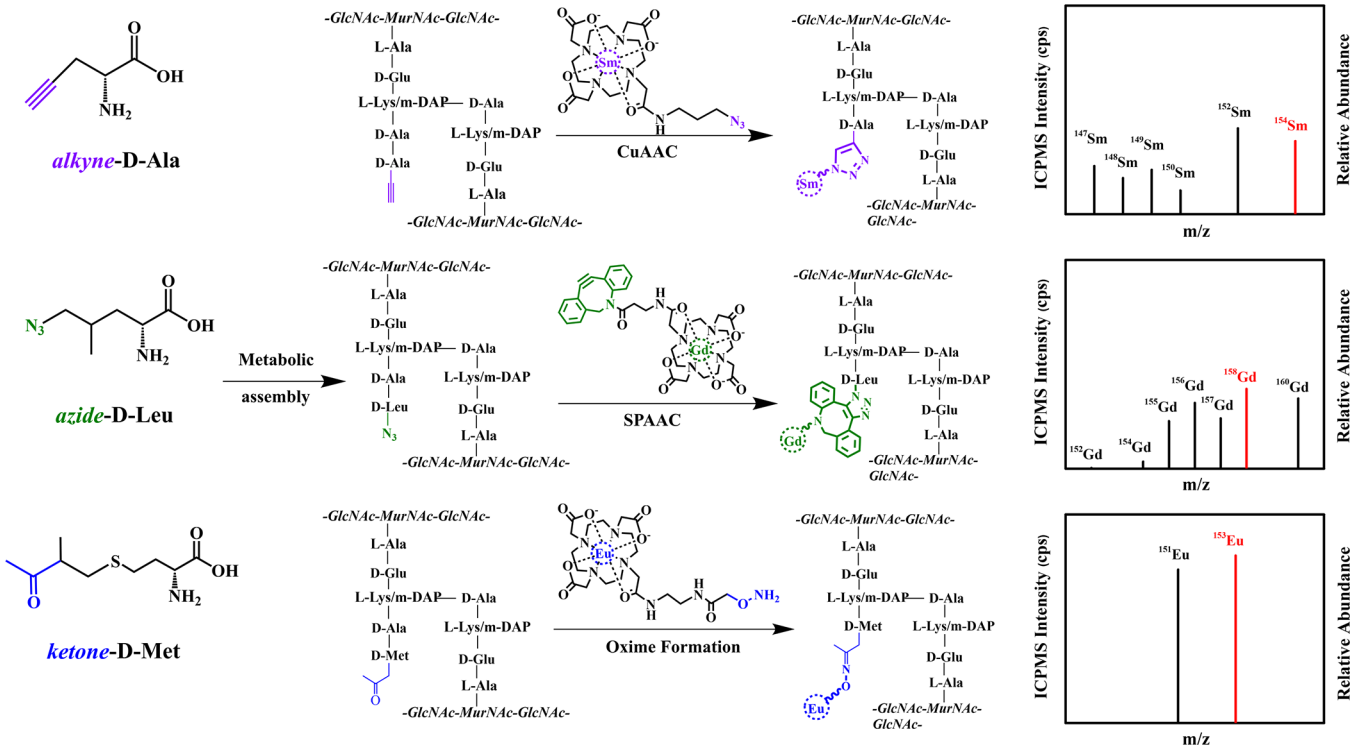
Scheme 1. Metabolic Assembly of *alkyne*-D-Ala, *azide*-D-Leu, and *ketone*-D-Met into the Terminal Acyl-D-Ala-D-X of Bacterial PG Pentapeptides and Site-Specific Ln Encoding by Corresponding *DBCO*-DOTA-Gd, H_2NO-DOTA-Eu, and *azide*-DOTA-Sm Tags via the Bioorthogonal Cu(I)-Catalyzed Azide−Alkyne Cycloaddition Click Reaction, Strain-Promoted Azide−Alkyne Cycloaddition, and Oxime Formation Reactions for Subsequent In Situ Quantification Using ^{154}Sm-, ^{158}Gd-, and ^{153}Eu-SUID-ICPMS.



account for vancomycin-resistant *S. aureus* [VRSA; minimum inhibitory concentration (MIC) ≥ 16 μg/mL], while the prevalence of vancomycin intermediate-resistant *S. aureus* (VISA; MIC = 4−8 μg/mL) and heterogeneous VISA (*h*VISA; MIC ≤ 2 μg/mL) is much more frequently encountered than VRSA.[8] VISA was suggested to be caused by editing a small proportion of another D-amino acid (D-X) instead of D-Ala into the terminal acyl-D-Ala-D-X of newly biosynthesized UDP-MurNAc-pentapeptides,[11,12] triggering apparently insignificant losses in the vancomycin binding affinity strength compared with that of acyl-D-Ala-D-Lac. Confirmation of whether VISA resulted from this hypothesis is vital to rationally understand the bacterial response mechanism behind the phenotype manifestation and thus efficaciously clinical management, in which quantitative information about the change in the D-X is the key point.

Metabolic assembly of radioactive D-amino acid surrogates and especially the recently developed fluorescent D-amino acid (FDAA)-based methods,[13−15] which enable spatiotemporal tracking of PG synthesis and modification in real time and understanding the difference between the Gram-positive and -negative bacteria, might be a workable choice for detecting the insignificant but nonnegligible changes in the terminal acyl-D-Ala-D-X. However, we know that the FDAA-based methods have not been used to obtain quantitative information because the intrinsic characteristics of fluorescence show that the readout emission fluorescent intensity depends on the intensity of the excitation laser source and somehow photobleaching with the fluorescence half-life time from a few to dozens of seconds; on the contrary, considering a relatively shorter wavelength and a stronger laser power are usually desired to obtain a higher fluorophore brightness, these might, in turn, bring certain irradiation-induced phototoxicity to bacterial cell viability. With respect to the traditional high-performance liquid chromatography (HPLC)-based techniques with a chiral derivatization optical assay or soft-ionization mass spectrometry such as ESI-MS/MS,[16] they could provide quantitative information regarding the content and configuration of the acyl-D-Ala-D-X but could not afford much desired in situ information because bacterial cells must be subjected to tedious and complex "cell-broken" pretreatment procedures before the determination. More seriously, unavoidable racemization of the target D-X caused during the pretreatments led to some extent of uncertainty in the results obtained. This uncertainty might make it difficult to discriminate the insignificant but nonnegligible change in the D-X. Development of a new approach to determine the not easily measured D-X is definitely important for examining the bacterial response to vancomycin, finding a smart way to combat VISA.

Here, we report a biochemical lanthanide-encoding approach to quantify D-X using species-unspecific isotope dilution inductively coupled plasma mass spectrometry (SUID-ICPMS) (Scheme 1). During this process, *azide*-D-Leu and *ketone*-D-Met were newly synthesized and used together with *alkyne*-D-Ala for their metabolic incorporation into the terminal acyl-D-Ala-D-X of nascent UDP-MurNAc-pentapeptides of *S. aureus*; then they were bioorthogonally encoded in situ by correspondingly fabricated *azide* (N_3)-, *dibenzocyclooctyne* (*DBCO*)-, and *hydroxylamine* (H_2NO)-functionalized 1,4,7,10-tetraazacyclodecane-1,4,7-trisacetic acid (DOTA)-lanthanide (Ln = Sm, Gd, or Eu) tags, converting the determination of D-X to nonradiative Gd, Eu, and Sm. Because

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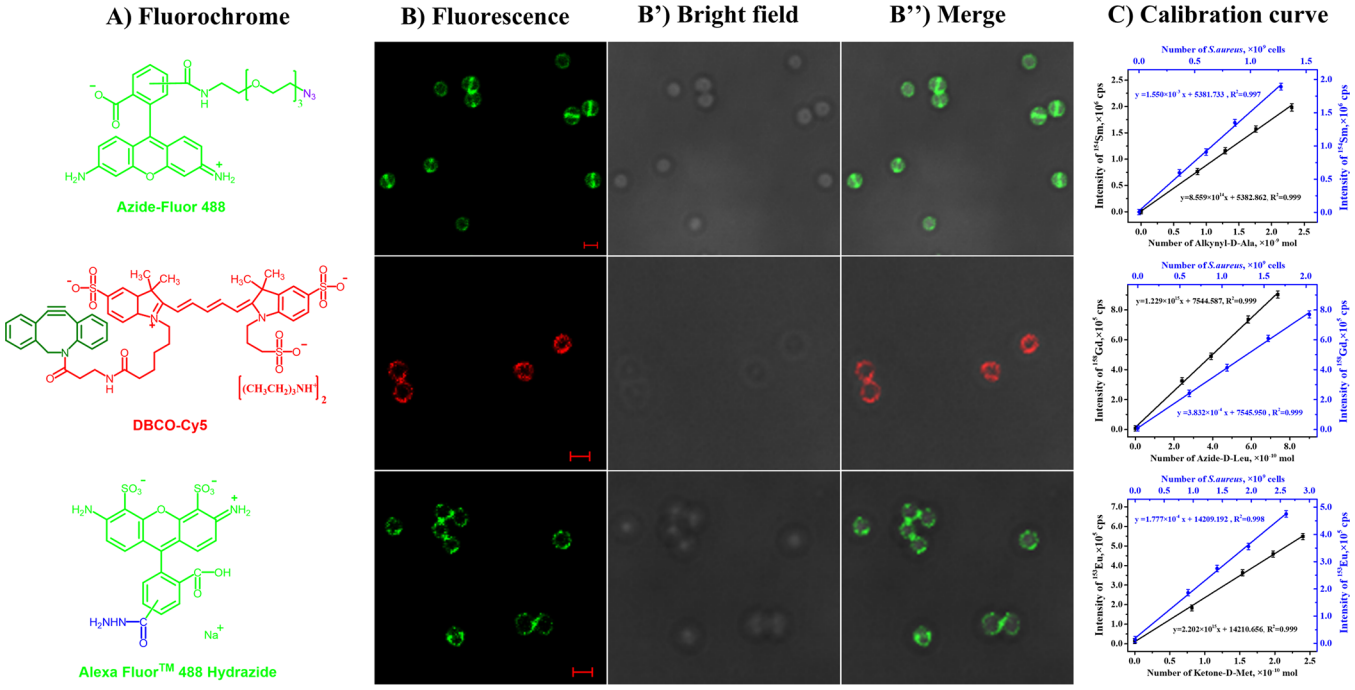

Figure 1. (A) Structure of the fluorochrome used for CLSM visualization. CLSM images of alkynyl-D-Ala assembled in the PG-supported cell wall of *S. aureus* after labeling with *azide*-Fluor 488 (top), *azide*-D-Leu DBCO-Cy5 (middle), and *ketone*-D-Met Alexa Fluor 488 hydrazide (bottom) (B, dark field; B′, bright field; B″, merged). (C) Calibration curves of ICPMS intensities of ^{154}Sm, ^{158}Gd, and ^{153}Eu against the number of N_3-DOTA-Sm-encoded *alkynyl*-D-Ala, *DBCO*-DOTA-Gd-encoded *azide*-D-Leu, and H_2NO-DOTA-Eu-encoded *ketone*-D-Met as well as the corresponding bacterial cell number.

of the feature of a very hard ionization ICP source, these site-specific Ln-encoded and in situ-preserved D-Ala, D-Leu, and D-Met can be directly quantified via the determination of Sm, Gd, and Eu using ^{154}Sm-, ^{158}Gd-, and ^{153}Eu-SUID-ICPMS, respectively, just after washing out the excess free Ln tags without any possible racemization-inducing "cell-broken" pretreatment procedures and HPLC separation, allowing us to obtain accurate information about the D-X and thus rationally rethink the response of *S. aureus* to the administration of vancomycin.

We first synthesized *azide*-D-Leu and *ketone*-D-Met (*alkynyl*-D-Ala was used as received) and the corresponding N_3-DOTA-Sm, *DBCO*-DOTA-Gd, and H_2NO-DOTA-Eu tags (Scheme 1 and Figures S1−S28; see details in the Supporting Information). The cytotoxicity experiments indicated that *alkynyl*-D-Ala, *azide*-D-Leu, and *ketone*-D-Met (each at 1 mM) do not cause any adverse effects on *S. aureus* growth (Figure S29 and Table S1), indicating that they have good compatibility and can be used in the following metabolic assembly experiments. We next evaluated the conjugation efficiency between *alkyne*-D-Ala and N_3-DOTA-Sm, *azide*-D-Leu, and *DBCO*-DOTA-Gd, as well as *ketone*-D-Met and H_2NO-DOTA-Eu in vitro using ESI-Q-TOF-MS. As expected, the complete conjugation between *alkyne*-D-Ala and N_3-DOTA-Sm with a simple 1:1 stoichiometry (Figure S30: ESI-Q-TOF-MS at m/z 749.2390) was achieved via the Cu(I)-catalyzed azide−alkyne cycloaddition with a very fast reaction rate,[17] and that between *azide*-D-Leu and *DBCO*-DOTA-Gd (Figure S32; ESI-Q-TOF-MS at m/z 990.3097) through the strain-promoted azide−alkyne cycloaddition[18] with the second-order rate constant of 2.267 M^{-1} s^{-1} (Figure S31). The oxime formation reaction between *ketone*-D-Met and H_2NO-DOTA-Eu was completed with a 1:1 stoichiometry (Figure S34; ESI-Q-TOF-MS at m/z 869.2321) using *m*-phenylenediamine as the catalyst[19] at pH 4.5 with a second-order rate constant of 0.11 M^{-1} s^{-1} (Figure S33). It should be noted that the mandatory use of copper and *m*-phenylenediamine catalysts to promote the reaction rate of conjugations might bring cytotoxicity to bacterial cells that normally grow under physiological conditions. Fortunately, these Ln-encoding procedures aiming to quantify the uncanonical D-X are performed after bacterial cell cultivation and, thus, can be safely used avoiding the adverse impacts to the bacterial cell growth.

Subsequently, *S. aureus* strains were cultured at 37 °C for 10 h in Mueller-Hinton broth culture medium together with *alkynyl*-D-Ala, *azide*-D-Leu, and *ketone*-D-Met (each at 1 mM), respectively, while the respective control experiments were performed under the same conditions but without adding the corresponding uncanonical D-X (see the details in the Supporting Information). Before in situ and site-specific Ln encoding of the *alkynyl*-D-Ala, *azide*-D-Leu, and *ketone*-D-Met assembled at the terminus of PG-pentapeptides in the bacterial PG-supported cell wall, *azide*-Fluor 488, *DBCO*-Cy5, and Alexa Fluor™ 488 hydrazide (Figure 1A) that have the same or very similar reactive groups as those of Ln tags were used to label them under the same experimental conditions of Ln encoding. The clearly observed CLSM images of PG-supported bacterial cell wall implied the success of the D-X metabolic assembly and selective dye labeling (Figure 1B and Figure S35). Ln encoding and ICPMS quantification were then performed (see the details in the Supporting Information and Figure S36). The detection limit (DL, 3σ) and quantification limit (QL, 10σ) of *alkyne*-D-Ala reach 17.2 and 57.3 fmol (corresponding to 1.04 × 10^8 and 3.45 × 10^8 molecules), those of *azide*-D-Leu 15.8 and 52.7 fmol (9.51 × 10^7 and 3.17 × 10^8

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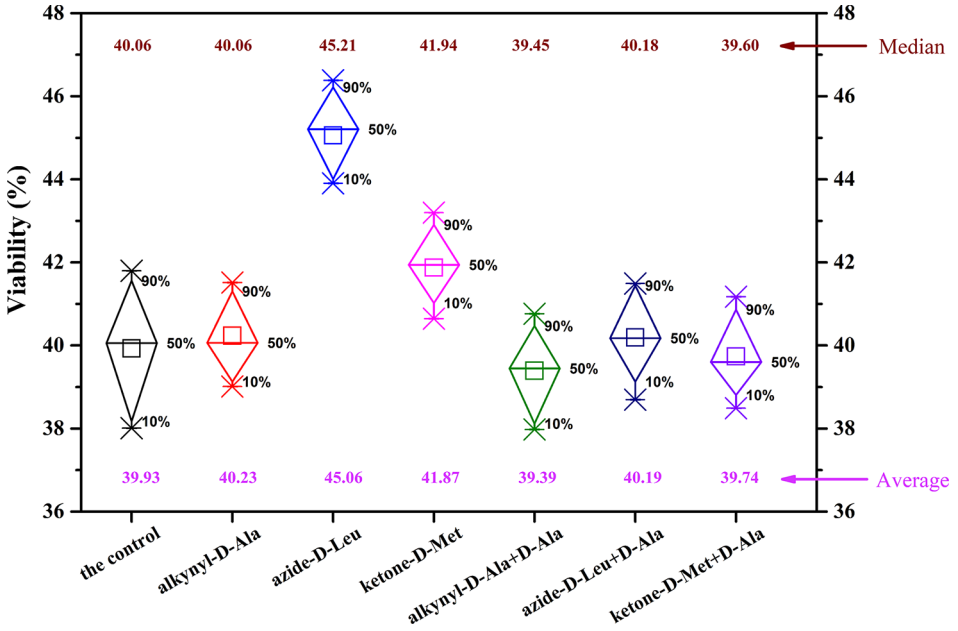

Figure 2. Viability of *S. aureus* under the administration of vancomycin. *S. aureus* strains (1.0×10^9 mL^{-1}) were cultivated for 10 h in Mueller-Hinton broth without (the control) and with *alkynyl*-D-Ala, *azide*-D-Leu, and *ketone*-D-Met, as well as *alkynyl*-D-Ala with D-Ala, *azide*-D-Leu with D-Ala, and *ketone*-D-Met with D-Ala, respectively, at a final concentration of 1 mM each. The viability of *S. aureus* was obtained by directly counting the number of viable *S. aureus* using the Ln-encoding ^{154}Sm-, ^{158}Gd-, and ^{153}Eu-SUID-ICPMS approach (Figure 1C) 1 h after vancomycin had been administrated (5 μg/mL). Each experiment was repeated 21 times.

molecules), and those of *ketone*-D-Met 10.3 and 34.3 fmol (6.20×10^7 and 2.06×10^8 molecules), respectively. These DL and QL were calculated on the basis of the simple 1:1 stoichiometry from 17.2 (DL) and 57.3 (QL) fmol of ^{154}Sm, 15.8 and 52.7 fmol of ^{158}Gd, and 10.3 and 34.3 fmol of ^{153}Eu obtained on ICPMS owing to monitored isotope abundances of 22.74% (^{154}Sm), 24.84% (^{158}Gd), and 52.19% (^{153}Eu). The number of D-X on one bacterial cell can then be quantified using ^{154}Sm-, ^{158}Gd-, and ^{153}Eu-SUID-ICPMS (Figure 1C), indicating that the average number is $(1.09 \pm 0.02) \times 10^6$ *alkynyl*-D-Ala/*S. aureus* (RSD of 3.2%; $n = 7$), $(1.88 \pm 0.04) \times 10^5$ *azide*-D-Leu (3.8%; $n = 7$), and $(4.86 \pm 0.07) \times 10^4$ *ketone*-D-Met (3.5%; $n = 7$), respectively, when using 0.86×10^9, 1.02×10^9, and 1.30×10^9 cells of the corresponding D-X assembled *S. aureus* that were counted by flow cytometry. Moreover, on the basis of the number of *alkynyl*-D-Ala, *azide*-D-Leu, and *ketone*-D-Met per bacterial cell, at least 95 ± 4, 506 ± 23, and 1276 ± 129 ($n = 7$) (DL) and 317 ± 15, 1687 ± 81, and 4253 ± 379 ($n = 7$) (QL) viable *S. aureus*, respectively, can be directly counted via the corresponding constructed calibration curves ($R^2 > 0.995$ and a linear dynamic range of >6 orders of magnitude) of ICPMS intensity of the encoded Ln tag against bacterial cell number (Figure 1C).

Vancomycin (5 μg/mL) was then administrated for an additional 1 h incubation after a 10 h cultivation of *S. aureus* (1.0×10^9 mL^{-1}) in Mueller-Hinton broth without (the control) and with *alkynyl*-D-Ala, *azide*-D-Leu, and *ketone*-D-Met, as well as *alkynyl*-D-Ala with D-Ala, *azide*-D-Leu with D-Ala, and *ketone*-D-Met with D-Ala at 1 mM each, respectively. The viability, which was obtained by directly counting the number of viable *S. aureus* using ^{154}Sm-, ^{158}Gd-, and ^{153}Eu-SUID-ICPMS (Figure 1C), indicated that the mean and average values of 40.06% and 40.23%, respectively, of the *S. aureus* cultured with *alkyne*-D-Ala are almost insignificantly different from 40.06% and 39.93%, respectively, of the control (p = 0.35), while that of *azide*-D-Leu increases by 5.13% with mean and average values of 45.21% and 45.06%, respectively ($p = 9.32 \times 10^{-18}$), and *ketone*-D-Met by 1.94% with values of 41.94% and 41.87%, respectively ($p = 1.46 \times 10^{-10}$) (Figure 2). Such a small promotionally increased viability caused by *azide*-D-Leu [$(1.88 \pm 0.04) \times 10^5$; $n = 21$] and *ketone*-D-Met [$(4.86 \pm 0.07) \times 10^4$; $n = 21$] determined by our Ln-encoding SUID-ICPMS approach confirms the D-X incorporation that competed with the original D-Ala should be responsible for VISA. Although the much smaller size (2.4 Å × 2.8 Å × 6.6 Å = 44.35 Å^3) of D-Ala-D-Lac compared to that (3.2 Å × 3.5 Å × 6.7 Å = 75.04 Å^3) of D-Ala-D-Ala estimated by the PyMOL Molecular Graphics System version 2.0 (https://pymol.org) seemingly favors the interaction with vancomycin, the change of an O atom in D-Ala to a N atom in Lac was believed to be responsible for VRSA because of the known loss of one crucial H-bond,[9,10] while a significant increase in size caused by the isobutane group in D-Leu [(3.4 Å × 6.5 Å × 7.1 Å = 156.91 Å^3) of D-Ala-D-Leu-*azide*] and ethyl(methyl) sulfide in D-Met [(3.5 Å × 6.7 Å × 10.2 Å = 239.19 Å^3) of D-Ala-D-Met-*ketone*] relative to the methyl group in D-Ala weakens the still existing H-bonds with vancomycin, in addition to the decreased polarities of D-Ala-D-Leu-*azide* (logP = −1.88) and D-Ala-D-Met-*ketone* (logP = −2.51) compared with those of D-Ala-D-Ala (logP = −3.26) and D-Ala-D-Ala-*alkyne* (logP = −3.17) that were calculated with MOLINPIRATION (http://www.molinspiration.com/cgi-bin/properties). These remarkable increases in size and decreases in polarity certainly resulted in the reduced affinity of vancomycin for D-Ala-D-Leu and D-Ala-D-Met. Results from autodocking prediction (AutoDock version 4.2, http://autodock.scripps.edu) indicated that the energy of bindingof vancomycin to acetyl-D-Ala-D-Ala is −6.05 kcal/mol, that of D-Ala-D-Ala-*alkyne* −5.48 kcal/mol, that of D-Ala-D-Leu-*azide* −4.60 kcal/mol, and that of D-Ala-D-Met-*ketone* −3.24 kcal/mol as well as that of D-Ala-D-Lac −3.00

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kcal/mol, again suggesting the binding of vancomycin to D-Ala-D-Ala is strongest followed by D-Ala-D-Ala-*alkyne*, D-Ala-D-Leu-*azide*, D-Ala-D-Met-*ketone*, and D-Ala-D-Lac. It is very interesting that, however, the viability of *S. aureus* assembled by *azide*-D-Leu is reversibly significantly higher by ∼3% than that of *ketone*-D-Met under the same vancomycin administration (Figure 2). This observed phenomenon was mainly ascribed to the fact that almost 4-fold higher *azide*-D-Leu than *ketone*-D-Met has been metabolically incorporated into the PG-pentapeptides in the cell wall, besides the relatively lower polarity of D-Ala-D-Leu-*azide* than D-Ala-D-Met-*ketone*. After we had determined the structure-dependent bacterial antibiotic resistance mechanism,[20] the highly sensitive and quantitative information about the *alkyne*-D-Ala, *azide*-D-Leu, and *ketone*-D-Met assembled in the bacterial cell wall obtained using the developed biochemical Ln-encoding ^{154}Sm-, ^{158}Gd-, and ^{153}Eu-SUID-ICPMS approach is extremely crucial for understanding the uncanonical D-X assembly-induced VISA. More interestingly, we found that the bacterial viability became incredibly nonsignificant when D-Ala was added together with alkynyl-D-Ala, azide-D-Leu, and ketone-D-Met to *S. aureus* compared to the control with p values of 0.12, 0.43, and 0.56, respectively. The >1 order of magnitude decreases in the average numbers of $(1.43 \pm 0.03) \times 10^5$ *alkynyl*-D-Ala (RSD = 4.1%; $n = 21$) in the case of D-Ala with *alkynyl*-D-Ala on *S. aureus* cells, $(5.71 \pm 0.08) \times 10^3$ *azide*-D-Leu (3.5%; $n = 21$) of D-Ala with *azide*-D-Leu, and $(2.12 \pm 0.05) \times 10^3$ *ketone*-D-Met (4.0%; $n = 21$) of D-Ala with *ketone*-D-Met should be responsible for the renascence of vancomycin efficacy. It is understandable that D-Ala might compete with the D-X to be installed and recover the terminus D-Ala-D-Ala that vancomycin likes. In addition, these observed phenomena hint that D-Ala addition may improve the bactericidal efficacy of vancomycin.

The distinctive characteristic of bacteria producing and using D-amino acids has been discovered to be a commonly innate feature in the bacterial kingdom, and more importantly, the bacterial biosynthetic machinery accepts a set of structurally diverse D-amino acid substrates and incorporates them into PG fragments.[21−23] Changes in the terminal D-X with larger side chains and lower polarities are sufficient to reduce the large extent of binding of vancomycin via H-bonds, despite the fact they still allow the modified PG to cross-link via catalysis by the transpeptidase enzymes. Such a feature of bacteria themselves synchronizes growth inhibition via PG biosynthesis when the population density becomes saturating in the stationary phase; on the contrary, it may in turn promote bacterial resistance to diverse exogenous threats. The threats awaken the silent bacterial genes or cause gene mutation to edit less D-X, which may be supplied from the bacterial symbiotic environment, other than D-Ala into the netlike PG layer, leading to acquisition of antibiotic resistance. *S. aureus* meets vancomycin is such a classic case.[8,11] D-Leu and D-Met are just two models of D-amino acids, and *S. aureus* is a representative of the bacteria exemplified here to understand the bacterial response to vancomycin treatment. It is conceivable that other D-amino acids with a greater difference in both structure and physicochemical property and other bacterial species might produce more significant antibiotic resistance that can be uncovered with the aid of our biochemical Ln-encoding approach that converts the D-X to more sensitive Ln mass signals that can be quantified using SUID-ICPMS. Once the structure and component-dependent antibiotic resistance mechanisms are known, the amount of uncanonical D-X incorporated into the terminal acyl-D-Ala-D-X does matter. Can we predict carefully from a chemist's perspective, if any, that the combinational use of vancomycin and D-Ala is a wise and simple way to combat VISA? To be sure, doctors' clinical trials are needed in the future. Before the realistic success of genomics- and proteomics-mediated antibiotics discovery, we must combined the use of or repurpose old drugs to treat existing and new bacterial infectious diseases.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.biochem.0c00614.

Materials and instrumentation, synthesis and characterization of *azide*-D-Leu and *ketone*-D-Met as well as the corresponding Ln tags, cytotoxicity experiments, evaluation of conjugation efficiency and kinetics between the uncanonical D-X and lanthanide tags, *S. aureus* growth conditions, in situ fluorescent labeling and CLSM imaging of the D-X, quantification of the D-X assembled in *S. aureus*, and counting of viable *S. aureus* cells (PDF)

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Notes

The authors declare no competing financial interest.

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