# A Fluorescent Teixobactin Analogue

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## ABSTRACT:

This report describes the first synthesis and application of a fluorescent teixobactin analogue that exhibits antibiotic activity and binds to the cell walls of Gram-positive bacteria. The teixobactin analogue, Lys(Rhod)$_9$,Arg$_{10}$-teixobactin, has a fluorescent tag at position 9 and an arginine in place of the natural *allo*-enduracididine residue at position 10. The fluorescent teixobactin analogue retains partial antibiotic activity, with minimum inhibitory concentrations of 4–8 μg/mL across a panel of Gram-positive bacteria, as compared to 1–4 μg/mL for the unlabeled Arg$_{10}$-teixobactin analogue. Lys(Rhod)$_9$,Arg$_{10}$-teixobactin is prepared by a regioselective labeling strategy that labels Lys$_9$ with an amine-reactive rhodamine fluorophore during solid-phase peptide synthesis, with the resulting conjugate tolerating subsequent solid-phase peptide synthesis reactions. Treatment of Gram-positive bacteria with Lys(Rhod)$_9$,Arg$_{10}$-teixobactin results in septal and lateral staining, which is consistent with an antibiotic targeting cell wall precursors. Concurrent treatment of Lys(Rhod)$_9$,Arg$_{10}$-teixobactin and BODIPY FL vancomycin results in septal co-localization, providing further evidence that Lys(Rhod)$_9$,Arg$_{10}$-teixobactin binds to cell wall precursors. Controls with either Gram-negative bacteria, or an inactive fluorescent homologue with Gram-positive bacteria, showed little or no staining in fluorescence

micrographic studies. Lys(Rhod)$_9$,Arg$_{10}$-teixobactin can thus serve as a functional probe to study Gram-positive bacteria and their interactions with teixobactin.

## INTRODUCTION

Fluorescent analogues of antibiotics can provide valuable insights into the modes of action of antibiotics and antibiotic resistance and have facilitated the development of new antibiotics.[1,2] Fluorescent antibiotic analogues enable tracking the drug and its interactions not only within bacterial cells but also within mammalian cells,[3,4] human tissue,[5] and live[6-8] and postmortem[9] animals. Penicillin V tagged with BODIPY FL (BOCILLIN-FL) has been used to characterize mechanisms of antibiotic resistance in bacteria by targeting penicillin-binding proteins (PBPs) using profiling assays and flow cytometry.[10-12] Fluorescein-labeled vancomycin has enabled the study of peptidoglycan biosynthesis in *B. subtilis* by fluorescence microscopy and has revealed a possible helical pattern of cylindrical wall synthesis.[13] Vancomycin and ramoplanin functionalized with fluorescent dyes have been used to study the complicated machinery involved in the biosynthesis of peptidoglycan and have revealed information about fundamental growth processes in Gram-positive bacteria.[14] Fluorescent analogues of cephalosporin C have been shown to label a specific subset of PBPs and were used to investigate the localization of PBPs in Gram-positive bacteria.[15] Studies with fluorescent antibiotic analogues are likely to become more important against the growing threat of antibiotic-resistant pathogens because they can illuminate modes of antibiotic action and serve as rapid diagnostics that can detect bacterial infections.

In 2015, the antibiotic teixobactin was reported.[16] Teixobactin is a nonribosomal peptide composed of eleven amino acids and containing a depsipeptide macrocycle. Teixobactin inhibits the biosynthesis of peptidoglycan and teichoic acid, interfering with cell wall formation and resulting in cell lysis.[16,17] Teixobactin binds to the pyrophosphates of lipids II and III and related cell wall precursors, which are thought to be immutable targets, making it difficult or impossible

for bacteria to become resistant. To our knowledge, the cellular localization of teixobactin has not been characterized by fluorescence microscopy.[18]

As part of a program of research aimed at understanding the mechanism of action of teixobactin and developing analogues with improved biological properties, we now introduce a fluorescent analogue of teixobactin and demonstrate that it stains the septa and sidewalls of Gram-positive bacteria. The analogue, $Lys(Rhod)_9,Arg_{10}$-teixobactin, contains the fluorophore sulforhodamine B (Lissamine™ rhodamine B) attached to the ε-amino group of lysine, in place of the native alanine residue at position 9, and arginine in place of the native *allo*-enduracididine residue at position 10 (Figure 1). We incorporated a fluorescent tag at position 9, because structure activity relationship (SAR) studies of the teixobactin pharmacophore established that position 9 tolerates mutations without substantial loss of antibiotic activity.[19,20] We chose sulforhodamine B, because it is suitable for fluorescence microscopy, compatible with solid-phase peptide synthesis (SPPS), easy to incorporate, relatively inexpensive, and commercially available as a single isomer. In aqueous solutions, the sulforhodamine B fluorophore has reported absorption and emission maxima of 564 nm and 583 nm, respectively, with a molar absorptivity coefficient of 71,500 $M^{-1}$ $cm^{-1}$ and a quantum yield of 0.33.[21] Sulforhodamine B is commercially available as the reactive sulfonyl chloride for about $300/g, making it suitable for use as a building block in SPPS. Many other popular fluorescent dyes, including the Alexa Fluor® and BODIPY families, are prohibitively expensive for the reaction scales used in SPPS. Efforts to use fluorescein isothiocyanate (FITC) — another popular and economical fluorophore — were unsuccessful in the synthetic route that we developed, as the label proved incompatible with the cyclization reaction.

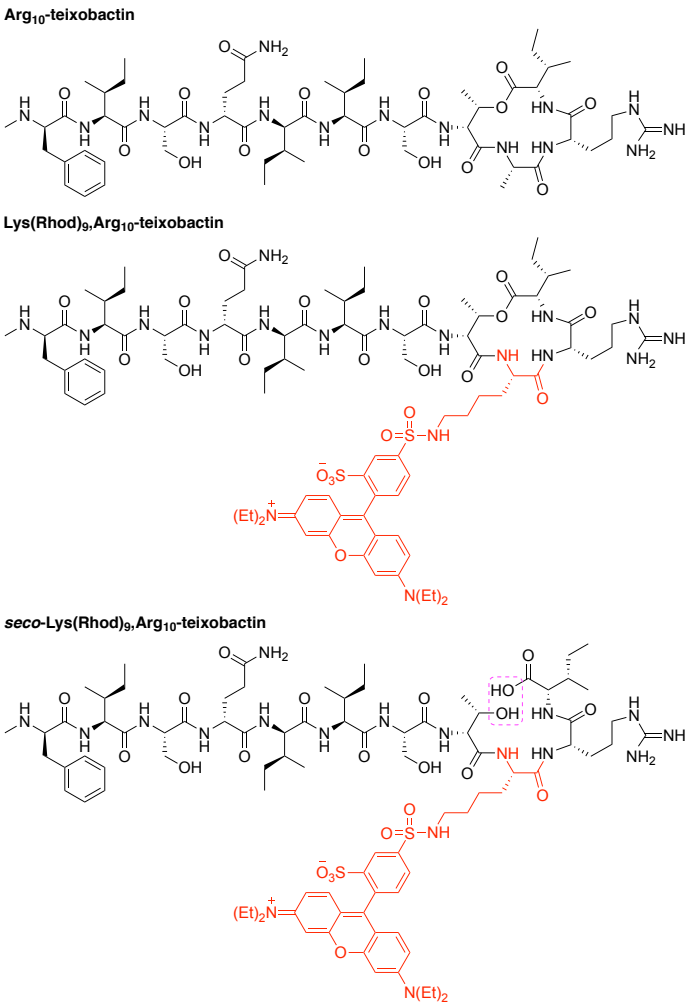


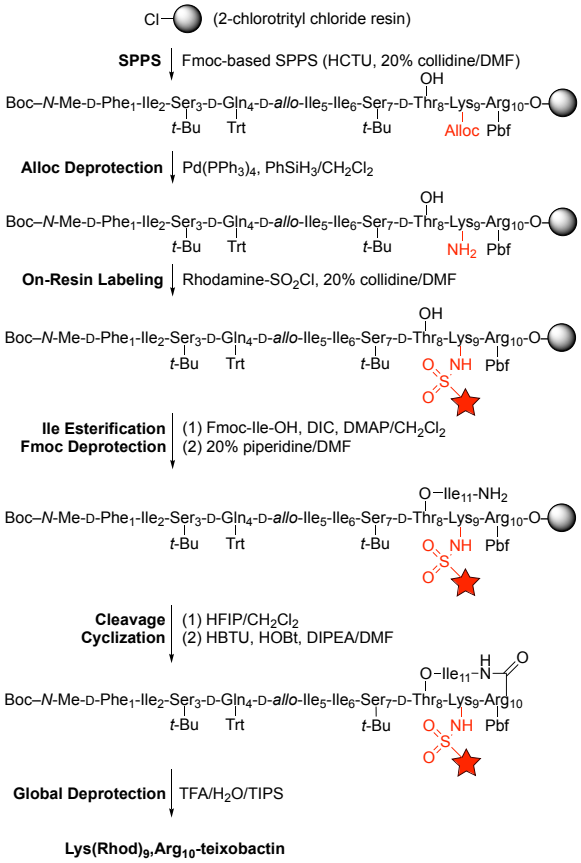

**Figure 1.** Structures of $Arg_{10}$-teixobactin, $Lys(Rhod)_9,Arg_{10}$-teixobactin, and *seco*-$Lys(Rhod)_9,Arg_{10}$-teixobactin.

## RESULTS AND DISCUSSION

We adapted our research group's synthesis of teixobactin analogues[22] to allow incorporation of sulforhodamine B at position 9. We synthesized $Lys(Rhod)_9,Arg_{10}$-teixobactin by Fmoc-based SPPS to give the protected linear peptide, where the side-chain amino group of $Lys_9$ is protected with the allyloxycarbonyl (Alloc) group (Scheme 1). The Alloc protecting group was removed using $Pd(PPh_3)_4$ and $PhSiH_3$, and the side-chain of $Lys_9$ was labeled using sulforhodamine B sulfonyl chloride.[23] The on-resin synthesis allows regioselective labeling, efficient reaction, and removal of excess fluorophore. The rhodamine fluorophore tolerated

subsequent SPPS reactions — $Ile_{11}$ esterification with DIC and DMAP, cleavage with HFIP, cyclization with HBTU and HOBt, and global deprotection with TFA/$H_2O$/TIPS. Synthesis on a 0.16 mmol scale typically yielded 3–11 mg of Lys(Rhod)$_9$,$Arg_{10}$-teixobactin as the trifluoroacetate (TFA) salt after purification by RP-HPLC.[24] We also synthesized the acyclic analogue *seco*-Lys(Rhod)$_9$,$Arg_{10}$-teixobactin as a negative control containing the fluorophore but lacking antibiotic activity (Figure 1).

**Scheme 1. Synthesis of Lys(Rhod)$_9$,$Arg_{10}$-teixobactin**




We evaluated the antimicrobial activity of Lys(Rhod)$_9$,$Arg_{10}$-teixobactin using minimum inhibitory concentration (MIC) assays with five Gram-positive bacteria. We used $Arg_{10}$-teixobactin as a positive control and *E. coli* and the acyclic analogue *seco*-Lys(Rhod)$_9$,$Arg_{10}$-teixobactin as negative controls. Lys(Rhod)$_9$,$Arg_{10}$-teixobactin partially retained antibiotic

activity, with minimum inhibitory concentrations of 4–8 μg/mL against the five Gram-positive bacteria, making it ca. four-fold less active than the parent $Arg_{10}$-teixobactin (Table 1). Comparable diminution of antibiotic activity has been observed in a number of fluorescent analogues of other antibiotics.[2] Like teixobactin and other teixobactin analogues, $Lys(Rhod)_9,Arg_{10}$-teixobactin proved inactive against the Gram-negative bacterium *E. coli*. *seco*-$Lys(Rhod)_9,Arg_{10}$-teixobactin was inactive toward all bacteria tested, reflecting the need for an intact macrolactone ring[22] and establishing that the sulforhodamine B moiety does not impart antibiotic activity.[25]

**Table 1. MIC values of teixobactin analogues in μg/mL**

| | *Bacillus subtilis* ATCC 6051 | *Enterococcus durans* ATCC 6056 | *Streptococcus salivarius* ATCC 13419 | *Staphylococcus aureus* ATCC 29213 | *Staphylococcus epidermidis* ATCC 14990 | *Escherichia coli* ATCC 10798 |
|---|---|---|---|---|---|---|
| $Lys(Rhod)_9,Arg_{10}$-teixobactin | 4 | 8 | 4 | 8 | 8 | >32 |
| $Arg_{10}$-teixobactin | 1 | 4 | 1 | 2 | 2 | >32 |
| *seco*-$Lys(Rhod)_9,Arg_{10}$-teixobactin | >32 | >32 | >32 | >32 | >32 | >32 |

We initially attempted to image *B. subtilis* with a 4 μg/mL $Lys(Rhod)_9,Arg_{10}$-teixobactin solution, which we prepared by diluting a 1 mg/mL DMSO stock solution with sodium phosphate buffer. When we treated *B. subtilis* with this solution, we observed aggregates of the fluorescent teixobactin analogue surrounding the bacteria (Figure 2). These aggregates are visible as a red haze and bright red spots in fluorescence micrographs. The aggregates appear to be a manifestation of the propensity of teixobactin and active teixobactin analogues to form gels and amyloid-like fibrils in water, buffer, and culture media.[20,26] The adhesion of these aggregates to the bacteria may reflect binding of the aggregated teixobactin to the wall teichoic acid (WTA), which in conjunction with peptidoglycan comprises the bacterial cell wall of Gram-positive

bacteria. Our laboratory has previously observed that fibril-like assemblies formed by a teixobactin analogue bind sulfate anions, and we envision that aggregates of teixobactin or Lys(Rhod)$_9$,Arg$_{10}$-teixobactin could bind to the phosphate groups of WTA in a similar fashion.[26]

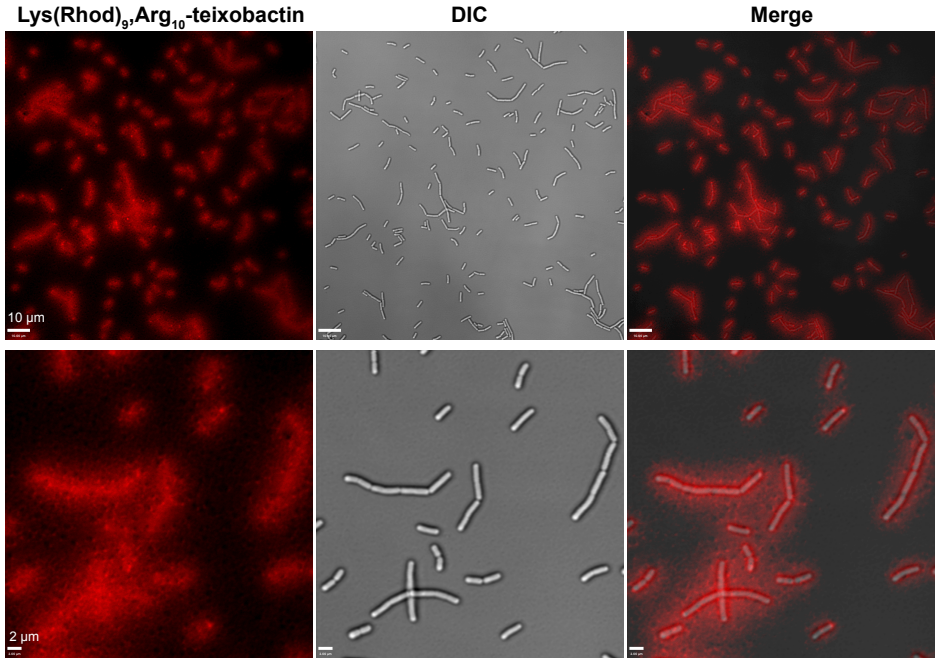



**Figure 2**. Fluorescence and differential interference contrast (DIC) micrographs of *B. subtilis* treated with 4 µg/mL of Lys(Rhod)$_9$,Arg$_{10}$-teixobactin in sodium phosphate buffer containing no polysorbate 80. Fluorescence micrographs were recorded with excitation at 561 nm. Scale bars of the top row images are 10 µm, while the scale bars of the bottom row images are 2 µm.

To eliminate these fluorescent aggregates, we drew upon the original report of teixobactin, in which 0.002% polysorbate 80, a mild nonionic detergent, was added to the test media for MIC assays.[16] When we included 0.002% polysorbate 80 in the diluted solutions of Lys(Rhod)$_9$,Arg$_{10}$-teixobactin, the formation of fluorescent aggregates was reduced. Inclusion of 0.05% polysorbate 80 in the diluted solutions of Lys(Rhod)$_9$,Arg$_{10}$-teixobactin further reduced the formation of aggregates and led to cleaner staining of the bacteria. When we performed MIC assays with either 0.002% or 0.05% polysorbate 80, the antibiotic activity of Lys(Rhod)$_9$,Arg$_{10}$-

teixobactin improved for *B. subtilis* and *S. salivarius*, worsened for *S. aureus* and *E. durans*, and remained the same for *S. epidermidis* (Table 2).

**Table 2. MIC values of teixobactin analogues in μg/mL with 0.002% and 0.05% polysorbate 80**

| | *Bacillus subtilis* ATCC 6051 | *Enterococcus durans* ATCC 6056 | *Streptococcus salivarius* ATCC 13419 | *Staphylococcus aureus* ATCC 29213 | *Staphylococcus epidermidis* ATCC 14990 | *Escherichia coli* ATCC 10798 |
|---|---|---|---|---|---|---|
| Lys(Rhod)$_9$,Arg$_{10}$-teixobactin | 2[a]<br>2[b] | 16[a]<br>16[b] | 1[a]<br>0.25[b] | 16–32[a]<br>32–64[b] | 8[a]<br>8[b] | >32[a]<br>>32[b] |
| Arg$_{10}$-teixobactin | <0.03[a]<br><0.03[b] | 2[a]<br>2[b] | <0.03[a]<br><0.03[b] | 1[a]<br>1[b] | 0.5[a]<br>0.5[b] | >32[a]<br>>32[b] |
| *seco*-Lys(Rhod)$_9$,Arg$_{10}$-teixobactin | >32[a]<br>>32[b] | >32[a]<br>>32[b] | >32[a]<br>>32[b] | >32[a]<br>>32[b] | >32[a]<br>>32[b] | >32[a]<br>>32[b] |

[a]Culture media containing 0.002% polysorbate 80
[b]Culture media containing 0.05% polysorbate 80

When we treated *B. subtilis* with 4 μg/mL Lys(Rhod)$_9$,Arg$_{10}$-teixobactin in the presence of 0.05% polysorbate 80, we observed staining of the bacteria with minimal formation of fluorescent aggregates (Figure 3A).[27] Staining was pronounced at the septa and weaker at the sidewalls. The staining resulted in a banded pattern in chains of *B. subtilis*, with particularly intense bands at the new division sites and weaker bands at the older division sites, which gave rise to an alternating pattern of weaker and stronger bands. The more intense staining of new division sites is consistent with the antibiotic targeting cell wall biosynthesis and cell division,[13,14] thus supporting the model of teixobactin binding to lipid II, lipid III, and related cell wall precursors. To corroborate the staining of Gram-positive bacteria by Lys(Rhod)$_9$,Arg$_{10}$-teixbactin, we treated and imaged *E. durans*, *S. salivarius*, and *S. aureus* (Figure 3B–3D). Treatment of *E. durans*, *S. salivarius*, and *S. aureus* with 4 μg/mL of Lys(Rhod)$_9$,Arg$_{10}$-teixobactin resulted in strong septal staining, with notable lateral staining in *E. durans* and *S. salivarius*.

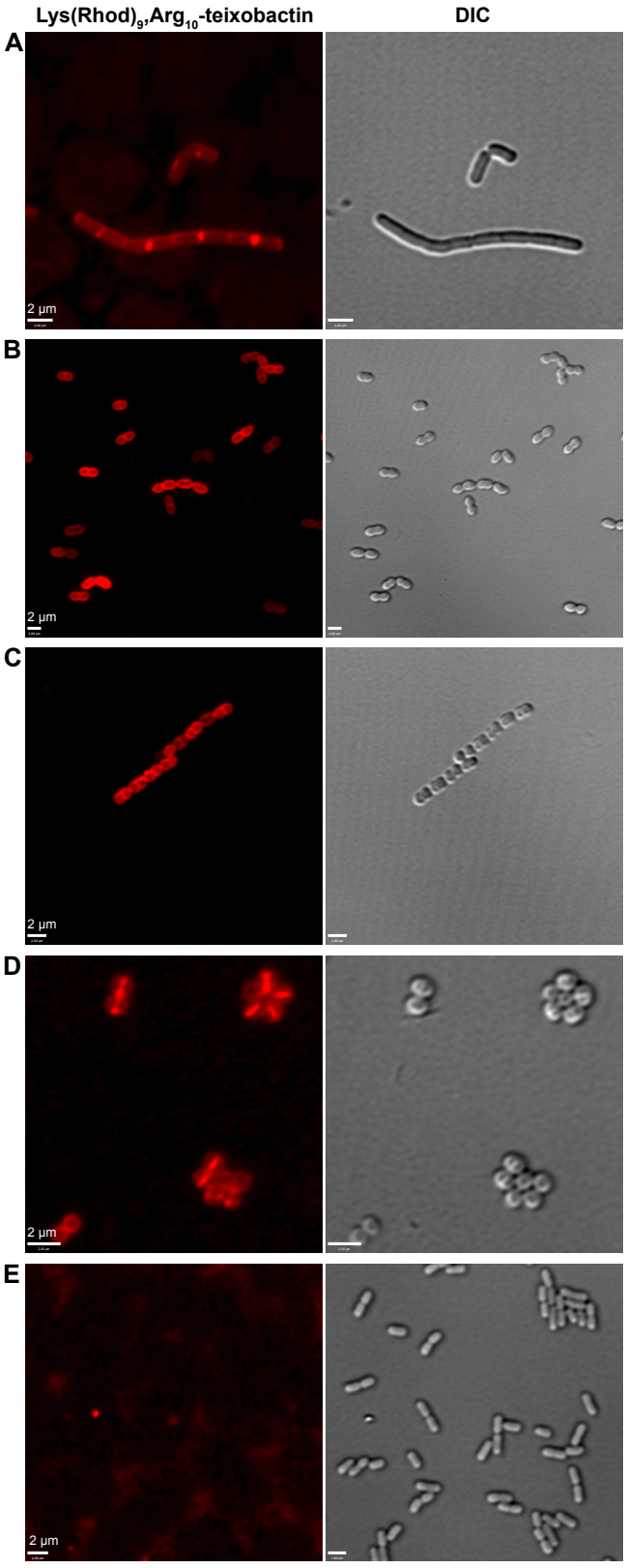



**Figure 3.** Fluorescence and differential interference contrast (DIC) micrographs of (A) *B. subtilis*, (B) *E. durans*, (C) *S. salivarius*, (D) *S. aureus*, and (E) *E. coli* treated with 4 µg/mL of

Lys(Rhod)$_9$,Arg$_{10}$-teixobactin in sodium phosphate buffer containing 0.05% polysorbate 80. Fluorescence micrographs were recorded with excitation at 561 nm. Scale bars are 2 μm.

Walker et al. previously demonstrated that equimolar mixtures of unlabeled vancomycin and BODIPY FL vancomycin resulted in enhanced intensity of septal and sidewall staining in *B. subtilis*.[14] They observed similar effects with equimolar mixtures of labeled and unlabeled ramoplanin. When we treated *B. subtilis* concurrently with 1 μg/mL of Lys(Rhod)$_9$,Arg$_{10}$-teixobactin and 1 μg/mL of Arg$_{10}$-teixobactin, we observed intense staining of both old and new division sites and moderate staining of the sidewalls (Figure 4). The Walker group hypothesized that the improved staining by 1:1 mixtures of the labeled and unlabeled antibiotics reflects improved fluorescence properties of dimeric vancomycin or ramoplanin bearing only a single fluorescent label. We believe a similar effect occurs with the mixture of labeled and unlabeled teixobactin analogues.

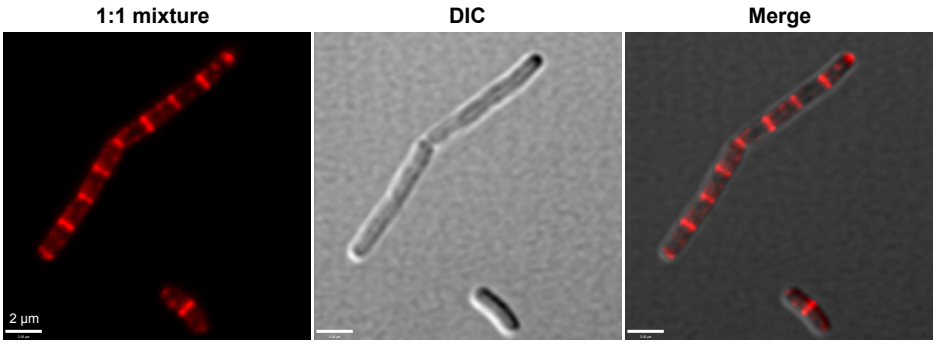



**Figure 4.** Fluorescence and differential interference contrast (DIC) micrographs of *B. subtilis* concurrently treated with 1 μg/mL of Lys(Rhod)$_9$,Arg$_{10}$-teixobactin and 1 μg/mL of Arg$_{10}$-teixobactin in sodium phosphate buffer containing 0.05% polysorbate 80. Fluorescence micrographs were recorded with excitation at 561 nm. Scale bars are 2 μm.

To gain further insight into the staining patterns of Lys(Rhod)$_9$,Arg$_{10}$-teixobactin, we co-stained with BODIPY FL vancomycin, which has previously been used to study peptidoglycan biosynthesis in *B. subtilis*.[14] Although both the teixobactin and vancomycin antibiotics target cell

wall precursors, teixobactin targets the undecaprenyl-pyrophosphate-sugar moieties of peptidoglycan and WTA precursors, while vancomycin targets the D-Ala-D-Ala transpeptidase substrate in peptidoglycan cross-linking.[28] Concurrent treatment of *B. subtilis*, *E. durans*, *S. salivarius*, and *S. aureus* with 4 μg/mL of Lys(Rhod)$_9$,Arg$_{10}$-teixobactin and 4 μg/mL of BODIPY FL vancomycin resulted staining by both fluorescent antibiotics, with prominent staining of the septa and weaker staining of the side walls, and with Lys(Rhod)$_9$,Arg$_{10}$-teixobactin favoring new division sites (Figure 5). The septal co-localization of Lys(Rhod)$_9$,Arg$_{10}$-teixobactin and BODIPY FL vancomycin provides further evidence that Lys(Rhod)$_9$,Arg$_{10}$-teixobactin binds to cell wall precursors. Some differences were observed between the staining by the two antibiotics, which may reflect the differing molecular targets of teixobactin and vancomycin.

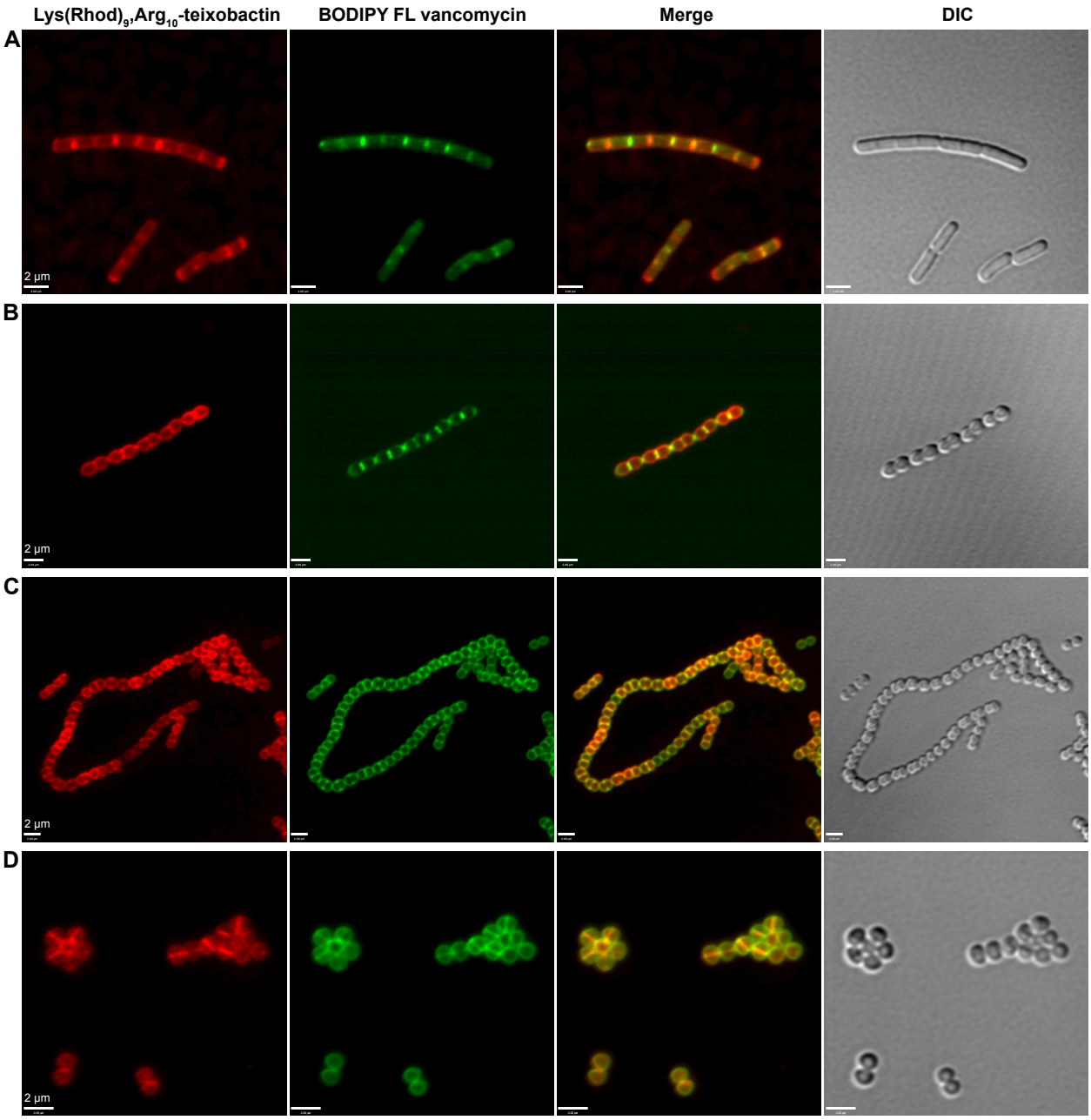



**Figure 5.** Fluorescence and differential interference contrast (DIC) micrographs of (A) *B. subtilis*, (B) *E. durans*, (C) *S. salivarius*, (D) *S. aureus* concurrently treated with 4 µg/mL of Lys(Rhod)$_9$,Arg$_{10}$-teixobactin and 4 µg/mL of BODIPY FL vancomycin in sodium phosphate buffer containing 0.05% polysorbate 80. Fluorescence micrographs were recorded with excitation at 561 nm (left column panel) and 488 nm (right column panel). Scale bars are 2 µm.

We performed three negative controls to establish the specificity of Lys(Rhod)$_9$,Arg$_{10}$-teixobactin toward Gram-positive bacteria: (1) treating *E. coli* (Gram-negative) with

Lys(Rhod)$_9$,Arg$_{10}$-teixobactin, (2) treating *B. subtilis, E. durans*, *S. salivarius*, and *S. aureus* (Gram-positive) with the inactive *seco*-Lys(Rhod)$_9$,Arg$_{10}$-teixobactin analogue, and (3) treating *B. subtilis* with sulforhodamine B *N*-butylsulfonamide. Treatment of *E. coli* with 4 μg/mL of Lys(Rhod)$_9$,Arg$_{10}$-teixobactin resulted in little to no staining (Figure 3E). A few (<2%) of the *E. coli* cells showed brighter staining, at levels comparable to the Gram-positive bacteria. The lack of significant staining in *E. coli* indicates that Lys(Rhod)$_9$,Arg$_{10}$-teixobactin, like teixobactin itself, is specific toward Gram-positive bacteria. Treatment of *B. subtilis*, *E. durans*, and *S. salivarius* and *S. aureus* with 4 μg/mL of *seco*-Lys(Rhod)$_9$,Arg$_{10}$-teixobactin resulted in only very weak staining of cells, at levels only slightly above background (Figure 6). The lack of significant staining from the *seco*-Lys(Rhod)$_9$,Arg$_{10}$-teixobactin analogue suggests that the intact teixobactin pharmacophore is necessary for staining Gram-positive bacteria. Treatment of *B. subtilis* with 4 μg/mL of sulforhodamine B *N*-butylsulfonamide resulted in no staining (Figure S7), further demonstrating that the staining patterns observed in Figures 2–5 result from specific interactions between Lys(Rhod)$_9$,Arg$_{10}$-teixobactin and bacteria, and not from sulforhodamine B itself.

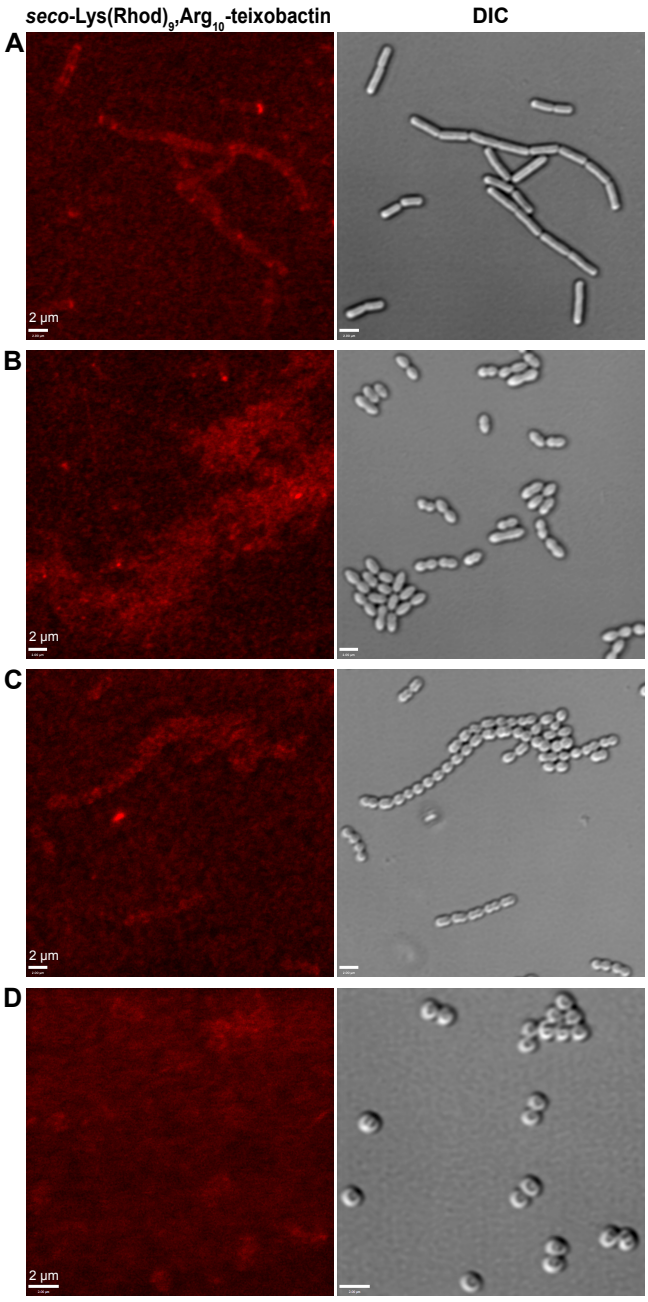



**Figure 6.** Fluorescence and differential interference contrast (DIC) micrographs of (A) *B. subtilis*, (B) *E. durans*, (C) *S. salivarius*, and (D) *S. aureus* treated with 4 µg/mL of *seco*-Lys(Rhod)$_9$,Arg$_{10}$-teixobactin in sodium phosphate buffer containing 0.05% polysorbate 80. Fluorescence micrographs were recorded with excitation at 561 nm. Scale bars are 2 µm.

At the suggestion of a reviewer, we carried out a competition experiment in which we first stained *B. subtilis* with Lys(Rhod)$_9$,Arg$_{10}$-teixobactin and then treated with Arg$_{10}$-

teixobactin, to determine if $Arg_{10}$-teixobactin could displace Lys(Rhod)$_9$,$Arg_{10}$-teixobactin. We also carried out a second experiment in which we first treated *B. subtilis* with $Arg_{10}$-teixobactin and then stained with Lys(Rhod)$_9$,$Arg_{10}$-teixobactin, to see if $Arg_{10}$-teixobactin could block staining by Lys(Rhod)$_9$,$Arg_{10}$-teixobactin (Figure S8). As controls we carried out two complementary experiments in which we first stained *B. subtilis* with BODIPY FL vancomycin and then treated with vancomycin, or first blocked with vancomycin and then stained with BODIPY FL vancomycin (Figure S9). In *all* of the experiments staining was observed. The bacteria that were treated with Lys(Rhod)$_9$,$Arg_{10}$-teixobactin and then with $Arg_{10}$-teixobactin exhibited fluorescent staining; the bacteria that were treated with $Arg_{10}$-teixobactin and then with Lys(Rhod)$_9$,$Arg_{10}$-teixobactin also exhibited fluorescent staining. The bacteria that were treated with BODIPY FL vancomycin and then with vancomycin exhibited fluorescent staining; the bacteria that were treated with vancomycin and then with BODIPY FL vancomycin also exhibited fluorescent staining. These experiments, coupled with the observation that co-treatment with mixtures of fluorescent and non-fluorescent antibiotics result in more pronounced staining, suggest that complete displacement or blocking do not occur and demonstrate that staining with fluorescent vancomycin or teixobactin analogues can survive competition experiments.

## CONCLUSION

Lys(Rhod)$_9$,$Arg_{10}$-teixobactin exhibits antibiotic activity and stains Gram-positive bacteria, allowing its visualization by fluorescence microscopy. The sulforhodamine B fluorophore tolerates reaction conditions used in peptide synthesis, such as coupling agents, piperidine, and trifluoroacetic acid, and is suitable for confocal fluorescence microscopy. MIC studies show that Lys(Rhod)$_9$,$Arg_{10}$-teixobactin exhibits moderate antibiotic activity, with MIC

values of 4–8 μg/mL. This fluorescent teixobactin analogue labels the septa and sidewalls of Gram-positive bacteria, supporting a model in which teixobactin binds to lipids II and III and related cell wall precursors. We anticipate that Lys(Rhod)$_9$,Arg$_{10}$-teixobactin will be useful in studying the mechanism of action of teixobactin, the biosynthesis of peptidoglycan and teichoic acids, and undecaprenyl phosphate metabolism and recycling.[28]

## MATERIALS AND METHODS

**General Methods.** Amino acids, coupling agents, 2-chlorotrityl chloride resin, DIC, palladium-tetrakis(triphenylphosphine), and triisopropylsilane were purchased from Chem-Impex. Phenylsilane was purchased from TCI. DMF (amine-free), DIPEA, 2,4,6-collidine, piperidine, and paraformaldehyde were purchased from Alfa-Aesar. Sulforhodamine B sulfonyl chloride, DMAP, and polysorbate 80 were purchased from Acros Organics. HPLC-grade acetonitrile, dichloromethane, and LB agar were purchased from Fisher Scientific. TFA and hexafluoroisopropanol were purchased from Oakwood Chemical. Difco Mueller Hinton broth was obtained from Becton, Dickinson and Company. Brain Heart Infusion broth was obtained from Teknova. Molecular biology grade agarose and BODIPY FL vancomycin were obtained from Thermo Scientific. Sterile DMSO was obtained from Tocris.

Reagent-grade solvents, chemicals, amino acids, and resin were used as received, with the exception of dichloromethane, which was dried through an alumina column under argon, and dimethylformamide, which was dried through an alumina column and an amine scavenger resin column under argon. Solid-phase peptide synthesis was carried out manually in a solid phase reaction vessel. Analytical reverse-phase HPLC was performed on an Agilent 1200 instrument equipped with an Aeris PEPTIDE 2.6u XB-C18 column (Phenomonex). Preparative reverse-

phase HPLC was performed on a Rainin Dynamax instrument equipped with a ZORBAX SB-C18 column (Agilent). UV detection (214 nm) was used for analytical and preparative HPLC. HPLC grade acetonitrile and 18 MΩ deionized water, each containing 0.1% trifluoroacetic acid, were used for analytical and preparative reverse-phase HPLC. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry was performed on an AB SCIEX TOF/TOF 5800 system and 2,5-dihydroxybenzoic acid was used as the sample matrix. All peptides were prepared and used as the trifluoroacetate salts and were assumed to have one trifluoroacetate ion per ammonium group present in each peptide. Excitation and emission spectra of Lys(Rhod)$_9$,Arg$_{10}$-teixobactin and *seco*-Lys(Rhod)$_9$,Arg$_{10}$-teixobactin were recorded on a Cary Eclipse fluorescence spectrometer. $^1$H NMR spectra of Lys(Rhod)$_9$,Arg$_{10}$-teixobactin were collected on a Bruker Avance 600 MHz NMR equipped with a BBFO cryoprobe.

**Synthesis of Lys(Rhod)$_9$,Arg$_{10}$-teixobactin.**[22] *Resin loading*. 2-Chlorotrityl chloride resin (300 mg, 1.46 mmol/g) was added to a 10-mL Bio-Rad Poly-Prep chromatography column. The resin was suspended in dry $CH_2Cl_2$ (10 mL) and allowed to swell for 30 min. The resin was loaded with a solution of Fmoc-Arg(Pbf)-OH (180 mg, 0.28 mmol, 0.78 equiv) and 2,4,6-collidine (300 μL) in dry $CH_2Cl_2$ (8 mL). The suspension was agitated for 4−12 h until a resin loading of at least 50% was achieved. The solution was drained, and the resin was washed with dry $CH_2Cl_2$ (3x). A mixture of $CH_2Cl_2$/MeOH/DIPEA (17:2:1, 5 mL) was added to the resin and agitated for 1 h to cap any unreacted 2-chlorotrityl chloride sites. The solution was drained, and the resin was washed with dry $CH_2Cl_2$ (3x). The resin was then dried with a flow of nitrogen.

*Quantifying resin loading*. A small portion of loaded resin was removed from the column and dried under vacuum. 1.0 mg of the dried resin was weighed out and transferred to a scintillation vial containing 3 mL of 20% piperidine/DMF and a small magnetic stirring bar. The

reaction mixture was allowed to stir for 10 min and the absorbance at 290 nm was measured. The resin loading was determined to be 0.16 mmol (0.54 mmol/g, 57% loading) using the following formula:

$$\%\ \text{loading} = \frac{A_{290\ \text{nm}} \times V \times 10^3}{6089 \times m_{\text{resin}} \times l} \times 100\%$$

where:

$A_{290}$ nm = Absorbance measured at 290 nm

ε = Molar absorptivity of piperidine adduct (6,089 L $mol^{-1}$ $cm^{-1}$)

$m_{resin}$ = Mass of resin in mg (1.0 mg)

V = Volume of piperidine in DMF in mL (3.0 mL)

*l* = Cell pathlength in cm (1.0 cm)

*Linear peptide synthesis*. The loaded resin was suspended in dry DMF and transferred to a solid-phase peptide synthesis reaction vessel for manual peptide synthesis using Fmoc-Lys(Alloc)-OH, Fmoc-D-Thr-OH (free alcohol OH), Fmoc-Ser(*t*-Bu)-OH, Fmoc-Ile-OH, Fmoc-D-*allo*-Ile-OH, Fmoc-D-Gln(Trt)-OH, Fmoc-Ser(*t*-Bu)-OH, Fmoc-Ile-OH, and Boc-*N*-methyl-D-Phe-OH. The linear peptide was synthesized through the following cycles: *i*. Fmoc deprotection with 20% (*v/v*) piperidine in dry DMF (5 mL) for 10 min (2x), *ii*. resin washing with dry DMF (3x), *iii*. coupling of amino acid (0.64 mmol, 4 equiv) with HCTU (267 mg, 0.64 mmol, 4 equiv) in 20% (*v/v*) 2,4,6-collidine in dry DMF (5 mL) for 20 min, and *iv*. resin washing with dry DMF (3x). After the linear synthesis was completed, the resin was then washed with dry $CH_2Cl_2$ in the solid-phase peptide synthesis reaction vessel (3x).

*On-resin Alloc deprotection*.[29] $PhSiH_3$ (478 μL, 3.87 mmol, 24 equiv) and $Pd(PPh_3)_4$ (19 mg, 0.02 mmol, 0.1 equiv) were dissolved in 4 mL dry $CH_2Cl_2$. The solution was added to the resin in the solid-phase peptide synthesis reaction vessel with agitation by bubbling of nitrogen

gas. After 10 min, the solution was drained, and the resin was washed with dry $CH_2Cl_2$ (8x). The deprotection reaction was repeated once more using the same procedure, and the solution was drained.

*On-resin labeling with sulforhodamine B sulfonyl chloride*. The resin was washed with dry DMF (3x) in the solid-phase peptide synthesis reaction vessel. Sulforhodamine B sulfonyl chloride (188 mg, 0.32 mmol, 2 equiv) was dissolved in 20% 2,4,6-collidine in dry DMF (5 mL). The solution was transferred to the reaction vessel and allowed to react with the resin for 1 h while bubbling under nitrogen gas. The solution was drained and the resin was washed with dry DMF (20x) until residual dye was removed.

NOTE: These procedures were performed in an unlit fume hood and the reaction vessel was protected from excess exposure to light by draping with black felt when not manipulating the resin. During subsequent steps, similar efforts were made to protect the labeled peptide from light.

*Esterification*.[24] The resin was transferred to a 10-mL Bio-Rad Poly-Prep chromatography column and washed with dry $CH_2Cl_2$ (3x). In a test tube, Fmoc-Ile-OH (570 mg, 1.6 mmol, 10 equiv) and diisopropylcarbodiimide (250 μL, 1.6 mmol, 10 equiv) were dissolved in dry $CH_2Cl_2$ (5 mL). The resulting solution was filtered through 0.20-μm nylon filter, and then 4-dimethylaminopyridine (19.7 mg, 0.16 mmol, 1 equiv) was added to the filtrate. The resulting solution was transferred to the resin and was gently agitated for 1 h. The solution was drained and the resin was washed with dry $CH_2Cl_2$ (3x) and DMF (3x).

*Fmoc deprotection and peptide cleavage*. The Fmoc protecting group on $Ile_{11}$ was removed by treatment with 20% piperidine in dry DMF (5 mL) for 10 min (2x). The solution was drained and the resin was washed with dry DMF (3x) and then with dry $CH_2Cl_2$ (3x). To cleave

the peptide, the resin was treated with 20% hexafluoroisopropanol (HFIP) in dry $CH_2Cl_2$ (6 mL) with agitation for 1 h. The filtrate was collected in a round-bottom flask. The HFIP treatment was repeated, and the filtrate was added to the round-bottom flask. The resin was washed with an additional aliquot of 20% HFIP (6 mL) and then washed with dry $CH_2Cl_2$ (3x). The combined filtrates and methylene chloride washes were concentrated under reduced pressure to afford a red oil. The oil was placed under vacuum (≤ 0.1 mmHg) to remove any residual solvents.

*Cyclization*. The oil was dissolved in 125 mL of dry DMF. HBTU (367 mg, 0.97 mmol, 6 equiv) and HOBt (131 mg, 0.97 mmol, 6 equiv) were added to the solution. The reaction mixture was stirred under nitrogen for 30 min. DIPEA (170 μL, 0.97 mmol, 6 equiv) was added over ca. 10 s to the solution, and the reaction mixture was stirred for 12 h. The mixture was concentrated under reduced pressure to afford the cyclized peptide as a pink solid. The solid was placed under vacuum (≤ 0.1 mmHg) to remove any residual solvents.

*Global deprotection, ether precipitation, and purification.* The crude protected peptide was dissolved in a mixture of trifluoroacetic acid (TFA)/triisopropylsilane/$H_2O$ (90:5:5, 10 mL), and the solution was stirred under nitrogen for 1 h. The deprotection mixture was then evenly aliquoted between two 40-mL portions of ice-cold diethyl ether in 50-mL conical tubes. The 50-mL conical tubes were centrifuged (400 x G) for 15 min to precipitate the crude peptide. The diethyl ether supernatant was discarded and the precipitated pellets were dried under nitrogen. The pellets were dissolved in 40% (*v/v*) $CH_3CN$ in water (8 mL) and centrifuged at 3300 rpm (1380 x G) for 5 min, and the solution was filtered through 0.20-μm nylon filter. The peptide was purified by reverse-phase HPLC with $H_2O/CH_3CN$ (gradient elution of 20–60% $CH_3CN$ with 0.1% TFA over 120 min), with the C18 column being heated to 50 °C in a Sterlite plastic bin water bath equipped with a Kitchen Gizmo Sous Vide immersion circulator.[30] Fractions analyzed

by analytical HPLC and MALDI mass spectrometry. The pure fractions were combined and lyophilized to give 5 mg (1.7% yield based on resin loading) of Lys(Rhod)$_9$,Arg$_{10}$-teixobactin trifluoroacetate (TFA) salt as red powder.

**Synthesis of *seco*-Lys(Rhod)$_9$,Arg$_{10}$-teixobactin.**[22] The *seco*-Lys(Rhod)$_9$,Arg$_{10}$-teixobactin analogue was prepared using similar procedures to those described above. The analogue was prepared as a linear peptide by conventional Fmoc-based solid-phase peptide synthesis, starting with Fmoc-Ile-OH on 2-chlorotrityl resin. Purification afforded 8.9 mg (3.0% yield based on resin loading) of *seco*-Lys(Rhod)$_9$,Arg$_{10}$-teixobactin trifluoroacetate (TFA) salt as red powder.

**Preparation of DMSO Stock Solutions.** A 1 mg/mL DMSO stock solution of Lys(Rhod)$_9$,Arg$_{10}$-teixobactin was prepared gravimetrically by dissolving 1.0 mg of the lyophilized peptide in 1 mL of sterile DMSO in an autoclaved Eppendorf tube. BODIPY FL vancomycin (100 μg, ThermoFisher) was dissolved in 100 μL of sterile DMSO to create a 1 mg/mL stock solution. The 1 mg/mL DMSO stock solutions were wrapped in black felt and stored in a −20 °C freezer for subsequent experiments.

NOTE: Solutions of Lys(Rhod)$_9$,Arg$_{10}$-teixobactin were protected from excessive exposure to light in MIC assays and other experiments by use of an unlit biosafety cabinet, black felt, and minimizing exposure to room lights.

**MIC Assays**.[22] *Bacillus subtilis* (ATCC 6051), *Staphylococcus epidermidis* (ATCC 14990), *Staphylococcus aureus* (ATCC 29213), and *Escherichia coli* (ATCC 10798) were cultured from glycerol stocks in Mueller-Hinton broth overnight in a shaking incubator at 37 °C. *Enterococcus durans* (ATCC 6056) and *Streptococcus salivarius* (ATCC 13419) were cultured from glycerol stocks in brain heart infusion broth overnight in a shaking incubator at 37 °C. An

aliquot of the 1 mg/mL antibiotic stock solution was diluted with culture media to make a 64 μg/mL solution. A 200-μL aliquot of the 64 μg/mL solution was transferred to a 96-well plate. Two-fold serial dilutions were made with media across a 96-well plate to achieve a final volume of 100 μL in each well. These solutions had the following concentrations: 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, and 0.0625 μg/mL. The overnight cultures of each bacterium were diluted with either Mueller-Hinton broth (*Bacillus subtilis*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, and *Escherichia coli*) or brain heart infusion broth (*Enterococcus durans* and *Streptococcus salivarius*) to an $OD_{600}$ of 0.075 as measured for 200 μL in a 96-well plate. The diluted mixture was further diluted to 1 x $10^6$ CFU/mL with the appropriate media (dilution of 50x for *B. subtilis*, 60x for *E. durans*, 50x for *S. salivarius*, 10x for *S. aureus*, 10x for *S. epidermidis*, and 50x for *E. coli*).[22] A 100-μL aliquot of the 1 x $10^6$ CFU/mL bacterial solution was added to each well in 96-well plates, resulting in final bacteria concentrations of 5 x $10^5$ CFU/mL in each well. As 100 μL of bacteria were added to each well, the teixobactin analogues were also diluted to the following concentrations: 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625, and 0.03125 μg/mL. The plate was covered with a lid and incubated at 37 °C for 16 h. The optical density measurements were recorded at 700 nm instead of 600 nm due to sulforhodamine absorbance and were measured using a 96-well UV/Vis plate reader (MultiSkan GO, Thermo Scientific). The MIC values were taken as the lowest concentration that had no bacteria growth. Each MIC assay was run in triplicate in three independent runs to ensure reproducibility. MIC assays were performed in test media containing with and without polysorbate 80 (0.002% or 0.05%).

**Fluorescence Microscopy Studies.** *Preparation of sodium phosphate buffers*. A 10x sodium phosphate buffer was prepared by dissolving 14.4 g of $Na_2HPO_4$ (0.100 moles) and 2.4 g

of $KH_2PO_4$ (0.018 moles) in 1 L of 18 MΩ deionized water. The solution was stirred and gently heated on a hot plate until the buffer salts were completely dissolved. The pH of the 10x sodium phosphate buffer was adjusted to 7.4 using either 6 M HCl or 6 M NaOH and was subsequently sterile filtered. To create a 1x sodium phosphate buffer (12 mM), the 10x sodium phosphate buffer was diluted 10-fold using 18 MΩ deionized water and, if necessary, the pH was adjusted using either 6 M HCl or 6 M NaOH. If needed, the desired amount of polysorbate 80 was added to the 12 mM sodium phosphate buffer, with gentle heating and stirring on a hot plate until the polysorbate 80 dissolved. All buffers were sterile filtered.

*Culturing bacteria for imaging.* Bacteria were allowed to grow overnight (ca. 16 h) in the appropriate broth (Mueller-Hinton broth or brain heart infusion broth) in a shaking incubator at 37 °C. The following morning, the cultures were diluted 1:100 in the appropriate broth and were allowed to grow exponentially in a shaking incubator at 37 °C. Once an $OD_{600}$ of ca. 0.3 was achieved, 500 µL of bacteria was transferred to a sterile Eppendorf tube and the bacteria were centrifuged at 4000 rpm (1300 x G) for 5 min.

*Preparation of 2% agarose beds for imaging bacteria*. A 2% stock solution of agarose was prepared by adding 1 g of agarose into 50 mL of sodium phosphate buffer, autoclaving, and allowing the solution to cool until it completely solidified. While the bacteria were growing in the shaking incubator, fresh 2% agarose beds were prepared to immobilize bacteria for fluorescence microscopy studies as follow: On a laboratory bench equipped with an alcohol burner [to help maintain sterility], microscope slides were gently warmed on a hot plate. While the slides were gently warming, the solidified 2% agarose solution was heated in a microwave oven until it became a homogenous liquid. Once the microscope slides were warm to the touch, a 75-µL aliquot of the molten 2% agarose solution was applied to each microscope slide, and a No.

1.5 coverslip was immediately applied gently to the drop of agarose. The assembly was allowed to set for at least 45 minutes before use.

*Preparation of Lys(Rhod)$_9$,Arg$_{10}$-teixobactin and BODIPY FL vancomycin solutions for fluorescence microscopy studies*. While the bacteria were being centrifuged, a 4 μg/mL solution of Lys(Rhod)$_9$,Arg$_{10}$-teixobactin was freshly prepared and then used immediately to stain the bacteria. The 4 μg/mL solution was prepared by diluting 2.4 μL of the 1 mg/mL DMSO stock solution with 597.6 μL of sterile sodium phosphate buffer containing 0.05% polysorbate 80. A solution containing 1 μg/mL of Lys(Rhod)$_9$,Arg$_{10}$-teixobactin and 1 μg/mL of Arg$_{10}$-teixobactin was prepared by combining 2 μL of each 1 mg/mL DMSO stock solution and then diluting with 1.996 mL of sterile sodium phosphate buffer containing 0.05% polysorbate 80. A solution containing 4 μg/mL of Lys(Rhod)$_9$,Arg$_{10}$-teixobactin and 4 μg/mL of BODIPY FL vancomycin was prepared by combining 2.4 μL of each 1 mg/mL DMSO stock solution, followed by dilution with 595.2 μL of sterile sodium phosphate buffer containing 0.05% polysorbate 80. The fluorescent antibiotic solutions were subsequently vortexed for 30 seconds and then used immediately to stain the bacteria, in order to avoid aggregation of the Lys(Rhod)$_9$,Arg$_{10}$-teixobactin probe. [The stained bacteria should also be imaged immediately, as formation of Lys(Rhod)$_9$,Arg$_{10}$-teixobactin aggregates on the bacteria was observed when the stained bacteria were incubated on the microscope slides.]

*Staining bacteria for fluorescence microscopy studies*. After centrifuging the bacteria (see above), the supernatant was removed, the pellet was resuspended in 500 μL of 4 μg/mL of the probe solution, and the bacteria were incubated in a shaking incubator at 37 °C for 10 min. The bacteria were centrifuged at 4000 rpm (1300 x G) for 5 min, and the supernatant was removed. The pellet was resuspended in 500 μL of sterile sodium phosphate buffer containing

0.05% polysorbate 80, the suspension was centrifuged at 4000 rpm (1300 x G) for 5 min, and the supernatant was removed. This washing process was repeated two additional times. After the last wash, the cells were resuspended in 200−500 μL of sterile sodium phosphate buffer containing 0.05% polysorbate 80. [The volume of phosphate buffer was selected based on the size of the pellet remaining after the washing steps.] On a sterile bench, the coverslip of each agarose bed was removed, and a 5-μL aliquot of the stained bacteria was applied to the coverslip. The coverslip was then sandwiched on top of the agarose bed.

*S. aureus staining and fixation*. Methicillin sensitive *S. aureus* (ATCC 29213) was fixed with formalin prior to imaging, because it is a BSL-2 pathogen. *S. aureus* was cultured, diluted, stained, and washed using the same protocols as described above. After the final wash, the stained *S. aureus* cells were centrifuged and the supernatant was removed. The pellet was resuspended in 50 μL of sodium phosphate buffer containing 0.05% polysorbate 80. 500 μL of 4% formalin in sodium phosphate buffer[31] was added, and the suspension was gently mixed by pipetting. The Eppendorf tube containing the cells was wrapped in black felt and was agitated for 20 min at room temperature on an orbital shaker. The cells isolated by centrifugation at 4000 rpm (1300 x G) for 5 min and then resuspended in 500 μL sodium phosphate buffer containing 0.05% polysorbate 80. The stained, fixed *S. aureus* cells were then immobilized onto agarose pads using the same procedure described above.

*Imaging the bacteria.* The stained bacteria were immediately imaged on a Zeiss LSM 780 confocal fluorescence microscope. Images were collected with a 63x oil immersion objective lens, with additional optical zoom used as needed to provide detailed images. Fluorescence micrographs of bacteria treated with Lys(Rhod)$_9$,Arg$_{10}$-teixobactin or *seco*-Lys(Rhod)$_9$,Arg$_{10}$-teixobactin were recorded with excitation at 561 nm and emission between 568–639 nm.

Fluorescence micrographs of bacteria treated with BODIPY FL vancomycin were recorded with excitation at 488 nm and emission between 490–544 nm. For both the Lys(Rhod)$_9$,Arg$_{10}$-teixobactin and BODIPY FL vancomycin channels, the pinhole size was set to 53–64 μm and the gain was set to 650–700 AU. The image brightness of the Lys(Rhod)$_9$,Arg$_{10}$-teixobactin and BODIPY FL vancomycin channels were adjusted linearly using Volocity 6.3 (Quorum Technologies), and a medium filter in the Volocity software was used to reduce noise in all channels.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:

Additional fluorescence micrographic images; HPLC traces, mass spectra, and excitation and emission spectra of Lys(Rhod)$_9$,Arg$_{10}$-teixobactin and *seco*-Lys(Rhod)$_9$,Arg$_{10}$-teixobactin; HPLC trace and mass spectrum of Arg$_{10}$-teixobactin.

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### Notes

The authors declare no competing financial interest.

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23) The on-resin synthesis of the Lys(Rhod)$_9$ residue from Lys(Alloc) and sulforhodamine B sulfonyl chloride allows use of off-the-shelf building blocks. It should also be possible to directly incorporate Fmoc-Lys(Rhod)-OH as a building block in the solid phase peptide synthesis. Doing so requires the synthesis of Fmoc-Lys(Rhod)-OH, which is not commercially available.

24) Our laboratory has previously observed that the Ile esterification using DIC and DMAP gives a ca. 2:1 epimeric mixture of Ile and D-*allo*-Ile at position 11.[22] HPLC purification of the final peptide product allows removal of the Lys(Rhod)$_9$,Arg$_{10}$,D-*allo*-Ile$_{11}$-teixobactin epimer and affords pure Lys(Rhod)$_9$,Arg$_{10}$-teixobactin (Figure S13).

25) To test the effect of Lys(Rhod)$_9$,Arg$_{10}$-teixobactin staining on the viability of *B. subtilis*, we carried out the following time-lapse microscopy experiment. We treated *B. subtilis* with either sodium phosphate buffer containing 0.05% polysorbate 80 (Figure S10), 4 μg/mL Lys(Rhod)$_9$,Arg$_{10}$-teixobactin (1 x MIC, Figure S11), or 8 μg/mL Lys(Rhod)$_9$,Arg$_{10}$-teixobactin (2 x MIC, Figure S12) and performed time-lapse imaging at 37 °C. After 90 minutes, the bacteria treated with 8 μg/mL Lys(Rhod)$_9$,Arg$_{10}$-teixobactin, showed no evidence of growth, while the bacteria treated with either the vehicle control or 4 μg/mL Lys(Rhod)$_9$,Arg$_{10}$-teixobactin grew.

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