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Synthesis and Application of Fluorescent Teixobactin Analogues

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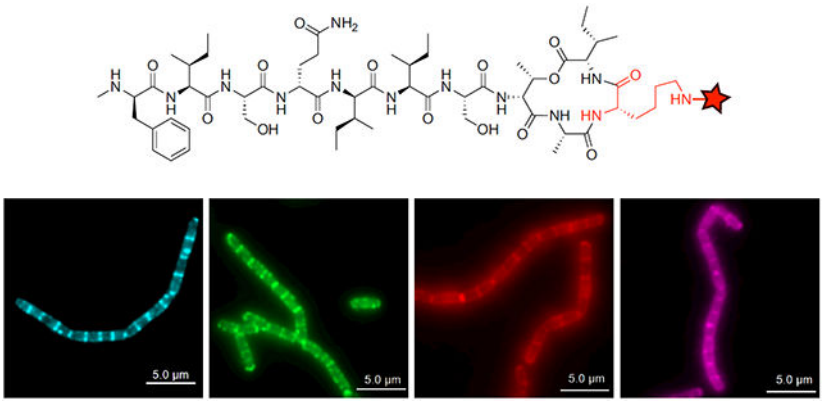
Abstract

Teixobactin is a promising new antibiotic that kills a spectrum of Gram-positive pathogens that are considered to be urgent threats by the CDC and the WHO. Better understanding of the novel mechanism of action of teixobactin may assist in developing new antibiotics and furthering our understanding of antibiotic resistance. This chapter describes the synthesis and application of fluorescent teixobactin analogues in fluorescence microscopy to study the mode of action of teixobactin. The first part of this chapter describes the synthesis and purification of fluorescent teixobactin analogues using two synthetic approaches. The second part of this chapter describes the application of the fluorescent teixobactin analogues to visualize their interactions with molecular targets in *B. subtilis* using fluorescence microscopy. The methods described herein provide synthetic access to chemical probes that may help further the understanding of antibiotic resistance.

Graphical Abstract



Keywords

teixobactin; antibiotics; antibiotic resistance; fluorescent probes; fluorescence microscopy; bacteria; microbiology; cell wall; lipid II; solid-phase peptide synthesis

1. Introduction

A new peptide antibiotic, teixobactin (Figure 1), was reported in 2015 (Ling et al., 2015). The introduction of teixobactin was met with much excitement because it kills

Gram-positive pathogens without developing resistance and has excellent antibacterial activity against resistant pathogens, including ones that are considered to be urgent and serious threats by the CDC and the WHO (Centers for Disease Control and Prevention, 2019; Homma et al., 2016; Ling et al., 2015; World Health Organization, 2020). Teixobactin has broad-spectrum activity against many Gram-positive pathogens, including *Staphylococcus aureus*, MRSA, VRE, *Streptococcus pneumoniae, Mycobacterium tuberculosis, Clostridioides difficile*, and *Bacillus anthracis*, and teixobactin is able to kill these bacteria with excellent potency (MIC values range from 0.005–0.5 μg/mL) (Ling et al., 2015). Another remarkable property of teixobactin is its in-vivo activity, where it protects mice against death from MRSA at 0.2 mg/kg, making it more than an order of magnitude more effective than vancomycin. (Ling et al., 2015) Thus, teixobactin, with its impressive antibacterial properties and ability to evade resistance, has the transformative potential to compensate for the increasing threat of antibiotic resistance.

Here we describe the synthesis of various fluorescent teixobactin analogues (Figure 2) and their application in fluorescence microscopy. We developed two synthetic methods to label teixobactin analogues with fluorophores. In the first method, the teixobactin analogue is labeled on-resin with sulforhodamine B sulfonyl chloride during the solid-phase peptide synthesis of the teixobactin analogue. In the second method, we label the Lys_{10}-teixobactin peptide using a variety of NHS esters. We then summarize how to stain *B. subtilis* with the fluorescent teixobactin analogues and visualize the interactions between *B. subtilis* and the fluorescent antibiotics using fluorescence microscopy.

2. Synthesis of fluorescent teixobactin analogues on-resin using sulforhodamine B sulfonyl chloride

Teixobactin analogues can be synthesized using Fmoc-based solid-phase peptide synthesis (SPPS) (Yang et al., 2016). We modified our research group's synthesis of teixobactin analogues to enable the conjugation of sulforhodamine B sulfonyl chloride to peptides during the solid-phase peptide synthesis (Morris et al., 2020). The resulting fluorescent analogue, $Lys(Rhod)_9,Arg_{10}$-teixobactin, contains sulforhodamine B on the ε-amino group of lysine, in place of $alanin_9$, and arginine in place of *allo*-$enduracididine_{10}$ (Figure 2). Structure activity relationship (SAR) studies of the teixobactin pharmacophore established that position 9 withstands non-native residues without abrogation of antibiotic activity (Abdel Monaim et al., 2016; Chen et al., 2017), so we incorporated the fluorophore at position 9. These SAR studies also established that position 10 tolerates non-native residues (Abdel Monaim et al., 2016; Chen et al., 2017), so we also synthesized $Lys(Rhod)_{10}$-teixobactin as a comparison. We chose sulforhodamine B, because of its suitability for fluorescence microscopy, compatibility with SPPS, ease of incorporation, and availability as a single isomer. In this section, we describe the synthesis of $Lys(Rhod)_9,Arg_{10}$-teixobactin and $Lys(Rhod)_{10}$-teixobactin using SPPS.

Peptide synthesis equipment and reagents:

- Bio-Rad Poly-Prep chromatography column (Bio-rad, cat. #7311550)
- Solid-phase peptide synthesis vessel (Chemglass, cat. #CG-1866)

- Benchmark Tube Rocker (Benchmark Scientific, cat. #M2100)
- Water purification system to generate high purity (18 MΩ) deionized water (Barnstead Nanopure, cat. #50131922)
- 250-mL round-bottom flask
- Rotary evaporator
- Glass scintillation vial
- 0.2-μm syringe filter (Fisher Scientific, cat. #13100100)
- Preparative RP-HPLC instrument
- Analytical RP-HPLC instrument
- Agilent Zorbax 21.2 x 250 mm SB-C18 column (Agilent, cat. #897250-102)
- Phenomenex Aeris PEPTIDE 2.6u XB-C18 column (Phenomenex, cat. #00B-4505-AN)
- Kitchen Gizmo Sous Vide immersion circulator
- Centrifuge for 15-mL centrifuge tubes
- Lyophilizer
- 2-Chlorotrityl chloride resin (Chem Impex, cat. #03498)
- Sulforhodamine B sulfony1 chloride
- DCM
- DMF (Alfa Aesar, contains a packet of amine scavenger) (Alfa Aesar, cat.#A13547)
- 2,4,6-Collidine
- DIPEA
- Fmoc-protected amino acids
- Piperidine
- HCTU
- HBTU
- HATU
- HOBt
- HOAt
- Palladium-tetrakis(triphenylphosphine)
- Phenylsilane
- DIC
- DMAP

- HFIP
- TFA
- TIPS
- Diethyl ether (anhydrous)
- Acetonitrile

2.1 Loading the resin

1. Place 2-chlorotrityl chloride resin (300 mg, 1.46 mmol/g) in a 10-mL Bio-Rad Poly-Prep chromatography column.
2. Add anhydrous DCM (10 mL) to the Poly-Prep column and rock for 30 minutes.
3. Drain the solution from the column using a flow of nitrogen gas and add 0.78 equivalents of Fmoc-Arg(Pbf)-OH (180 mg, 0.28 mmol, 0.78 equiv) dissolved in anhydrous DCM (8 mL) with 2,4,6-collidine (0.3 mL).
4. Rock for 4–12 h.
5. Drain the solution after 4–12 h.

2.2 Capping the resin

1. Cap unreacted resin sites by adding a solution of DCM (4.25 mL), methanol (0.5 mL), DIPEA (0.25 mL) to the resin and rock for 1 h.
2. Drain the capping solution and wash the resin three times with anhydrous DCM (3 x 10 mL) and three times with anhydrous DMF (3 x 10 mL).
3. Prepare a solid-phase peptide synthesis vessel with a line to introduce nitrogen through the frit and a line to pull vacuum to evacuate solvent into a waste collection flask.
4. Add a final portion of DMF (10 mL) to the Poly-Prep column and transfer the resin to the coupling vessel.

2.3 Coupling amino acids

Subject the loaded resin to cycles of peptide coupling. In each cycle, 20% piperidine is first used to remove the Fmoc protecting group from the amine. An amino acid is then activated with coupling agents and added to extend the amino acid chain.

1. Add 20% (*v/v*) piperidine in DMF (5 mL) to the coupling vessel containing the resin and bubble with nitrogen for 5 minutes to remove the Fmoc protecting group from the initial Arg(Pbf) amino acid.
2. After 5 minutes, drain the solution from the coupling vessel.
3. Repeat step 1 once and drain the second round of deprotection before washing the resin.

4. Wash the resin three times with DMF (3 x 10 mL), ensuring the inner walls of the vessel are well rinsed.
5. Weigh out 4.0 equivalents of the next desired Fmoc-protected amino acid (residue 9) and 4.0 equivalents of HCTU coupling agent and dissolve in 20% (*v/v*) 2,4,6-collidine in DMF (5 mL).
6. Add the solution to the coupling vessel and bubble with nitrogen for at least 20 minutes.
7. Drain the solution from the coupling vessel and wash the resin three times with DMF (3 x 10 mL), ensuring the inner walls of the vessel are well rinsed.
8. Repeat steps 1–7 until all linear amino acids have been coupled (residues 9-1).

2.4 On-resin Alloc deprotection

1. Mix 24 equiv of $PhSiH_3$ (0.48 mL, 3.9 mmol, 24 equiv) and 0.10 equiv of $Pd(PPh_3)_4$ (19 mg, 0.016 mmol, 0.10 equiv).
2. Dissolve in dry DCM (4 mL).
3. Add the solution to the coupling vessel and bubble with nitrogen for 10 minutes.
4. Drain the solution from the coupling vessel.
5. Wash the resin 4 times with DCM (4 x 10 mL).
6. Repeat steps 1–5

2.5 On-resin labeling with sulforhodamine B sulfonyl chloride

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.

1. Wash the resin three times with DMF (3 x 10 mL).
2. Weigh out 2.0 equiv of sulforhodamine B sulfonyl chloride (188 mg, 0.32 mmol, 2.0 equiv) and dissolve in 20% (*v/v*) 2,4,6-collidine in DMF (5 mL).
3. Add the solution to the coupling vessel and bubble with nitrogen for 1 h.
4. Drain the solution from the coupling vessel and wash the resin 10 times with DMF (10 x 10 mL).

2.6 On-resin esterification of D-Thr

1. Add a final portion of DMF (10 mL) to the vessel and pipet the resin back into the Poly Prep column.
2. Once all of the resin has been transferred, drain the remaining DMF from the Poly Prep column and wash the resin three times with dry DCM (3 x 10 mL).
3. Weigh out 10 equiv of Fmoc-Ile-OH (570 mg, 1.6 mmol, 10 equiv).

4. Dissolve in dry DCM (5 mL) and add 10 equiv of DIC (0.25 mL, 1.6 mmol, 10 equiv).
5. Filter the solution through a 0.20-μm nylon filter.
6. Weigh out 1.0 equiv of DMAP (19.7 mg, 0.16 mmol, 1 equiv) and add to filtrate.
7. Add the resulting solution to the resin and rock for 1 h.
8. Drain the solution and wash the resin three times with dry DCM (3 x 10 mL).

2.7 Fmoc deprotection and cleavage of the peptide from the resin

1. Wash the resin three times with DMF (3 x 10 mL).
2. Remove the Fmoc protecting group from Ile_{11} by adding 20% (*v/v*) piperidine in DMF (5 mL) and rock for 10 minutes.
3. Drain the solution and repeat step 2.
4. Drain the solution and wash the resin 3 times with DMF (3 x 10 mL).
5. Cleave the peptide from the resin by adding HFIP (1.2 mL) in dry DCM (4.8 mL) to the Poly Prep column. Rock for 1 h.
6. After 1 h, filter the suspension and collect the filtrate in a 250 mL round-bottom flask.
7. Add additional solution of HFIP (1.2 mL) in DCM (4.8 mL) to the remaining resin and rock for 30 minutes.
8. Filter the suspension and collect the filtrate in the same 250 mL round-bottom flask.
9. Wash the resin three times with dry DCM (3 x 10 mL) and collect the washes in the same 250 mL round-bottom flask.
10. Concentrate by rotary evaporation until dry.

2.8 Cyclizing the peptide

1. Add dry DMF (125 mL) to the flask, followed by 6.0 equivalents of HOBt (131 mg, 0.97 mmol, 6 equiv) and 6.0 equivalents of HBTU (367 mg, 0.97 mmol, 6 equiv).
2. Stir the reaction mixture for 30 minutes under nitrogen.
3. Add DIPEA (170 μL, 0.97 mmol, 6 equiv) to the solution and stir under nitrogen for 12 h.
4. After 12 h, remove the solvent by rotary evaporation.
5. The peptide can be stored under vacuum or directly subject to global deprotection and purification by RP-HPLC.

2.9 Deprotecting amino acid side chain protecting groups

Note: Global deprotection with trifluoroacetic acid (TFA) will remove trityl (Trt), O-*tert*-butyl (O*t*Bu), *tert*-butyloxycarbonyl (Boc), and pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) protecting groups. It does not remove other protecting groups, such as acetamidomethyl (Acm), benzyloxycarbonyl (Cbz), Alloc, or Dde.

1. To the dried, cyclized peptide, add 9 mL of TFA, 0.5 mL of TIPS, and 0.5 mL of 18 MΩ deionized water.
2. Stir at room temperature under nitrogen for 1 h.
3. After 1 h, you may remove the majority of the solution by rotary evaporation on a system dedicated for acidic solutions. It is not advised to evaporate this solution on a shared rotovap. If a rotovap dedicated to acidic solutions is not available, proceed directly to ether precipitation.

2.10 Precipitating the peptide with ether

1. Fill a flask with 100 mL of ether and chill in an ice bath for 20 minutes.
2. Divide the TFA solution containing the peptide equally into two 50 mL conical tubes.
3. Add 35 mL of cold ether to the conical tubes containing peptide. Swirl gently and incubate in the ice bath for 5 minutes.
4. After 5 minutes, centrifuge the conical tubes at 400 x *g* for 15 minutes. A pellet will form.
5. Decant the ether without disturbing the pellet.
6. Repeat steps 3–5 twice.
7. Dry pellets under nitrogen.
8. Once all of the ether has evaporated, proceed to purification.

2.11 Purifying the peptide by HPLC

1. Dissolve the deprotected peptide pellets in 40% (*v*/*v*) acetonitrile in water (8 mL) and centrifuged at 3300 rpm (1380 x *g*) for 5 minutes.
2. Filter the solution through a 0.20-μm nylon filter into a 15 mL conical tube.
3. First use an analytical HPLC instrument with a C18 column to obtain a chromatogram of your crude peptide before purifying as a guide for purification. [*Note*: The Ile esterification reaction generates a ca. 2:1 mixture with the D-*allo*-Ile_{11} epimer, which needs to be removed during the HPLC purification.]
4. The peptide is now ready to be purified using a preparative HPLC instrument with a C18 column.
5. Equilibrate the preparative HPLC instrument by running 20% acetonitrile with 0.1% TFA and 80% water with 0.1% TFA.

6. Heat the C18 column to 50 °C in a Sterlite plastic bin water bath equipped with a Sous Vide immersion circulator.
7. Load the peptide onto the column.
8. Once the peptide is loaded onto the column, increase the percent of acetonitrile by a gradient of 20-60% over 120 minutes until all peaks elute off of the column and are collected. [*Note:* Pausing the gradient and running isocratically when a peak elutes can assist in the separation of multiple peaks.]
9. Collect the fractions of interest and analyze by analytical HPLC and mass spectrometry.
10. Combine fractions containing the peptide mass with sharp analytical HPLC chromatograms and concentrate to dryness by rotary evaporation. [*Note*: Make sure to exclude fractions corresponding to the epimeric impurity.]
11. Reconstitute the peptide in 18 MΩ deionized water (10 mL) and transfer to a 15 mL conical tube.
12. Freeze the tube containing the peptide and obtain dry peptide via lyophilization.

2.12 Synthesis of Lys(Rhod)$_{10}$-teixobactin

1. Adapt the procedure from Lys(Rhod)$_9$,Arg$_{10}$-teixobactin to synthesize Lys(Rhod)$_{10}$-teixobactin by replacing Fmoc-Arg(Pbf)$_{10}$-OH with Fmoc-Lys(alloc)$_{10}$-OH and Fmoc-Lys(alloc)$_9$-OH with Fmoc-Ala$_9$-OH.

3. Synthesis of fluorescent teixobactin analogues using fluorophore NHS esters

Introduction of fluorophores after the synthesis of teixobactin analogues through NHS ester chemistry provides an attractive alternative to introduction of sulforhodamine B during SPPS, because it allows the creation of teixobactin analogues bearing a variety of popular fluorophores. It is now our preferred method of preparing fluorescent teixobactin analogues. We found that treatment of Lys$_{10}$-teixobactin with stoichiometric amounts of fluorophore NHS esters under basic conditions cleanly yields Lys(fluorophore)$_{10}$-teixobactin (Morris et al.). This NHS ester labeling strategy provides flexibility in fluorophore choice, and the fluorescent teixobactin conjugates are easy to purify using reverse-phase HPLC. In this section, we explain how to synthesize and purify Lys(fluorophore)$_{10}$-teixobactin conjugates.

Peptide synthesis equipment and reagents:

- Bio-Rad Poly-Prep chromatography column (Bio-rad, cat. #7311550)
- Solid-phase peptide synthesis vessel (Chemglass, cat. #CG-1866)
- Benchmark Tube Rocker (Benchmark Scientific, cat. #M2100)
- Water purification system to generate high purity (18 MΩ) deionized water (Barnstead Nanopure, cat. #50131922)

- 250-mL round-bottom flask
- Rotary evaporator
- Glass scintillation vial
- 0.2-μm syringe filter (Fisher Scientific, cat. #13100100)
- Preparative RP-HPLC instrument
- Analytical RP-HPLC instrument
- Agilent Zorbax 21.2 x 250 mm SB-C18 column (Agilent, cat. #897250-102)
- Agilent Zorbax 9.4 x 250 mm SB-C18 semipreparative column (Agilent, cat. # 889885-202)
- Phenomenex Aeris PEPTIDE 2.6u XB-C18 column (Phenomenex, cat. # 00B-4505-AN)
- Kitchen Gizmo Sous Vide immersion circulator
- Centrifuge
- Lyophilizer
- 2-Chlorotrityl chloride resin (Chem Impex, cat. #03498)
- BODIPY FL NHS ester (Lumiprobe, cat. #21420)
- Cy3 NHS ester (Lumiprobe, cat. #21020)
- Cy5 NHS ester (Lumiprobe, cat. #23020)
- DCM
- DMF (Alfa Aesar, contains a packet of amine scavenger) (Alfa Aesar, cat. #A13547)
- 2,4,6-Collidine
- DIPEA
- Fmoc-protected amino acids
- Piperidine
- HCTU
- HBTU
- HATU
- HOBt
- HOAt
- DIC
- DMAP
- HFIP

- TFA
- TIPS
- DMSO
- Diethyl ether (anhydrous)
- Acetonitrile

3.1 Loading the resin

1. Place 2-chlorotrityl chloride resin (300 mg, 1.6 mmol/g) in a 10-mL Bio-Rad Poly-Prep chromatography column.
2. Add anhydrous DCM (10 mL) to the Poly-Prep column and rock for 30 minutes.
3. Drain the solution from the column using a flow of nitrogen gas and add 0.56 equivalents of Fmoc-Lys(Boc)-OH (148 mg, 0.32 mmol, 0.56 equiv) dissolved in anhydrous DCM (8 mL) with 2,4,6-collidine (0.3 mL).
4. Rock for 4–12 h.
5. Drain the solution after 4–12 h.

3.2 Capping the resin

1. Cap unreacted resin sites by adding a solution of DCM (4.25 mL), methanol (0.5 mL), DIPEA (0.25 mL) to the resin and rock for 1 h.
2. Drain the capping solution and wash the resin three times with anhydrous DCM (3 x 10 mL) and three times with anhydrous DMF (3 x 10 mL).
3. Prepare a solid-phase peptide synthesis vessel with a line to introduce nitrogen through the frit and a line to pull vacuum to evacuate solvent into a waste collection flask.
4. Add a final portion of DMF (10 mL) to the Poly-Prep column and transfer the resin to the coupling vessel.

3.3 Coupling amino acids

Subject the loaded resin to cycles of peptide coupling. In each cycle, 20% piperidine is first used to remove the Fmoc protecting group from the amine. An amino acid is then activated with coupling agents and added to extend the amino acid chain.

1. Add 20% (*v/v*) piperidine in DMF (5 mL) to the coupling vessel containing the resin and bubble with nitrogen for 5 minutes to remove the Fmoc protecting group from the initial Lys(Boc) amino acid.
2. After 5 minutes, drain the solution from the coupling vessel.
3. Repeat step 1 once and drain the second round of deprotection before washing the resin.

4. Wash the resin three times with DMF (3 x 10 mL), ensuring the inner walls of the vessel are well rinsed.
5. Weigh out 4.0 equivalents of the next desired Fmoc-protected amino acid (residue 9) and 4.0 equivalents of HCTU coupling agent and dissolve in 20% (*v/v*) 2,4,6-collidine in DMF (5 mL).
6. Add the solution to the coupling vessel and bubble with nitrogen for at least 20 minutes.
7. Drain the solution from the coupling vessel and wash the resin three times with DMF (3 x 10 mL), ensuring the inner walls of the vessel are well rinsed.
8. Repeat steps 1–7 until all linear amino acids have been coupled with the final amino acid being Boc-*N*-Me-D-Phe-OH (residues 9-1).

3.4 On-resin esterification of D-Thr

1. Add a final portion of DMF (10 mL) to the vessel and pipet the resin back into the Poly Prep column.
2. Once all of the resin has been transferred, drain the remaining DMF from the Poly Prep column and wash the resin three times with dry DCM (3 x 10 mL).
3. Weigh out 10 equiv of Fmoc-Ile-OH (640 mg, 1.8 mmol, 10 equiv).
4. Dissolve in dry DCM (5 mL) and add 10 equiv of DIC (0.28 mL, 1.8 mmol, 10 equiv)
5. Filter the solution through a 0.20-μm nylon filter.
6. Weigh out 1.0 equiv of DMAP (22.1 mg, 0.18 mmol, 1 equiv) and add to filtrate.
7. Add the resulting solution to the resin and rock for 1 h.
8. Drain the solution and wash the resin three times with dry DCM (3 x 10 mL).

3.5 Fmoc deprotection and cleavage of the peptide from the resin

1. Wash the resin three times with DMF (3 x 10 mL).
2. Remove the Fmoc protecting group from Ile_{11} by adding 20% (*v/v*) piperidine in DMF (5 mL) and rock for 10 minutes.
3. Drain the solution and repeat step 2 once.
4. Drain the solution and wash the resin 3 times with DMF (3 x 10 mL).
5. Cleave the peptide from the resin by adding HFIP (1.2 mL) in dry DCM (4.8 mL) to the Poly Prep column. Rock for 1 h.
6. After 1 h, filter the suspension and collect the filtrate in a 250 mL round-bottom flask.
7. Add additional solution of HFIP (1.2 mL) in DCM (4.8 mL) to the remaining resin and rock for 30 minutes.

8. Filter the suspension and collect the filtrate in the same 250 mL round-bottom flask.
9. Wash the resin three times with dry DCM (3 x 10 mL) and collect the washes in the same 250 mL round-bottom flask.
10. Concentrate by rotary evaporation until dry.

3.6 Cyclizing the peptide

1. Add dry DMF (125 mL) to the flask, followed by 6.0 equivalents of HOBt (146 mg, 1.08 mmol, 6 equiv) and 6.0 equivalents of HBTU (412 mg, 1.08 mmol, 6 equiv).
2. Stir reaction for 30 minutes under nitrogen.
3. Add DIPEA (189 μL, 1.08 mmol, 6 equiv) to the solution and stir under nitrogen for 12 h.
4. After 12 h, remove the solvent by rotary evaporation.
5. The peptide can be stored under vacuum or directly subject to global deprotection and purification by RP-HPLC.

3.7 Deprotecting amino acid side chain protecting groups

Note: Global deprotection with trifluoroacetic acid (TFA) will remove trityl (Trt), O-tert-butyl (OtBu), tert-butyloxycarbonyl (Boc), and pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) protecting groups. It does not remove other protecting groups, such as acetamidomethyl (Acm), benzyloxycarbonyl (Cbz), Alloc, or Dde.

1. To the dried, cyclized peptide, add 9 mL of TFA, 0.5 mL of TIPS, and 0.5 mL of 18 MΩ deionized water.
2. Stir at room temperature under nitrogen for 1 h.
3. After 1 h, you may remove the majority of the solution by rotary evaporation on a system dedicated for acidic solutions. It is not advised to evaporate this solution on a shared rotovap. If a rotovap dedicated to acidic solutions is not available, proceed directly to ether precipitation.

3.8 Precipitating the peptide with ether

1. Fill a flask with 100 mL of ether and chill in an ice bath for 20 minutes.
2. Divide the TFA solution containing the peptide equally into two 50 mL conical tubes.
3. Add 35 mL of cold ether to the conical tubes containing peptide. Swirl gently and incubate in the ice bath for 5 minutes.
4. After 5 minutes, centrifuge the conical tubes at 400 x *g* for 15 minutes. A pellet will form.
5. Decant the ether without disturbing the pellet.

6. Repeat steps 3–5 twice.
7. Dry the pellets under nitrogen.
8. Once all of the ether has evaporated, proceed to purification.

3.9 Purifying the peptide by HPLC

1. Dissolve the deprotected peptide pellets in 40% (*v/v*) acetonitrile in water (8 mL) and centrifuged at 3300 rpm (1380 x *g*) for 5 minutes.
2. Filter the solution through a 0.20-μm nylon filter into a 15 mL conical tube.
3. First use analytical HPLC instrument with a C18 column to obtain a chromatogram of your crude peptide before purifying as a guide for purification. [*Note*: The Ile esterification reaction generates a ca. 2:1 mixture with the D-*allo*-Ile_{11} epimer, which needs to be removed during the HPLC purification.]
4. The peptide is now ready to be purified using a preparative HPLC instrument with a C18 column.
5. Equilibrate the preparative HPLC instrument by running 20% acetonitrile with 0.1% TFA and 80% water with 0.1% TFA.
6. Load the peptide onto the column.
7. Once the peptide is loaded onto the column, increase the percent of acetonitrile by a gradient of 20–60% over 120 minutes until all peaks elute off of the column and are collected. [*Note:* Pausing the gradient and running isocratically when a peak elutes can assist in the separation of multiple peaks.]
8. Collect the fractions of interest and analyze by analytical HPLC and mass spectrometry.
9. Combine fractions containing the peptide mass with sharp analytical HPLC chromatograms and concentrate to dryness by rotary evaporation. [*Note*: Make sure to exclude fractions corresponding to the epimeric impurity.]
10. Reconstitute the peptide in 18 MΩ deionized water (10 mL) and transfer to a 15 mL conical tube.
11. Freeze the tube containing the peptide and obtain dry peptide via lyophilization.

3.10 Preparation of NHS ester stock solution

1. Prepare a 50 mg/mL stock solution of the NHS ester by dissolving the NHS ester (5 mg) in 100 μL of dry DMSO.
2. Wrap the NHS ester stock solution in black felt and store in a desiccator in a −20 °C freezer for subsequent reactions.

3.11 Labeling of Lys_{10}-teixobactin with fluorophore NHS esters

1. Weigh out 1.0 equiv of Lys_{10}-teixobactin (5.1 mg, 3.5 μmol, 1.0 equiv) into a tared Eppendorf tube.

2. Dissolve the peptide in dry DMF (59.3 μL) and 5.0 equiv of DIPEA (3.1 μL, 17.7 μmol, 5.0 equiv).
3. To the solution, add 1.2 equiv of NHS ester 50 mg/mL stock solution (37.6 μL, 4.2 μmol, 1.2 equiv) and mix with a pipette.
4. Cover the Eppendorf tube with black felt and place on rocker for 10–60 min.
5. Monitor reaction progress with analytical HPLC.

3.12 Purifying the labeled peptide by HPLC

1. Dilute the reaction mixture to 3 mL with 40% (*v/v*) acetonitrile in water
2. The peptide is now ready to be purified using a preparative HPLC instrument with a 9.4 x 250 mm C18 semipreparative column.
3. Equilibrate the preparative HPLC instrument by running 20% acetonitrile with 0.1% TFA and 80% water with 0.1% TFA.
4. Heat the C18 semipreparative column to 50 °C in a Sterlite plastic bin water bath equipped with a Sous Vide immersion circulator.
5. Load the peptide onto the column.
6. Once the peptide is loaded onto the column, increase the percent of acetonitrile by a gradient of 20–60% over 60 minutes until all peaks elute off of the column and are collected. [*Note:* Pausing the gradient and running isocratically when a peak elutes can assist in the separation of multiple peaks.]
7. Collect the fractions of interest and analyze by analytical HPLC and mass spectrometry.
8. Combine fractions containing the peptide mass with sharp analytical HPLC chromatograms and concentrate to dryness by rotary evaporation.
9. Reconstitute the peptide in 18 MΩ deionized water (10 mL) and transfer to. 15 mL conical tube.
10. Freeze the tube containing the peptide and obtain dry peptide via lyophilization.

4. Application of fluorescent teixobactin analogues in fluorescence microscopy

Fluorescent antibiotics are valuable chemical biology tools to study the mechanisms of action of antibiotics and antibiotic resistance (Stone et al., 2018). Fluorescent antibiotics allow visualization of interactions with their molecular targets in bacteria, and provide qualitative and quantitative ways to understand the modes of actions of the antibiotics (Stone et al., 2018). In this section, we describe a simple protocol to stain *B. subtilis* with fluorescent teixobactin analogues and then use fluorescence microscopy to visualize the localization of the fluorescent antibiotics in bacteria (Figure 3). We also report optimized staining concentrations of the fluorescent teixobactin analogues to achieve bright staining of *B. subtilis*: 4 μg/mL for $Lys(Rhod)_9,Arg_{10}$-teixobactin and 1 μg/mL for the

Lys(fluorophore)$_{10}$-teixobactin analogues. This protocol can be adapted for staining bacteria with other fluorescent antibiotics, such as BODIPY FL vancomycin.

Items and equipment for staining and imaging bacteria

- BSL2 biosafety cabinet
- Sterile laboratory bench equipped with alcohol burner (suitable for BSL1 bacteria)
- Frozen glycerol stock of *Bacillus subtilis* (ATCC 6051)
- Shaking incubator for bacteria
- 1 mg/mL DMSO stock solutions of fluorescent teixobactin analogues
- Sterile Mueller Hinton broth (pH 7.4)
- Fluorescence microscope with 60x oil objective and appropriate filter cubes (e.g., Keyence BZ-X810 fluorescence microscope)
- Immersion oil for 60x oil objective
- 12 mM sodium phosphate buffer (pH 7.4) containing 0.05% polysorbate 80
- 12 mM sodium phosphate buffer (pH 7.4)
- Sterile 14-mL round bottom culture tubes
- Sterile pipette tips
- Sterile 1.5-mL Eppendorf tubes
- Microcentrifuge (set to 4000 rpm)
- P20, P200, and P1000 micropipettes
- 2% agarose in 12 mM sodium phosphate buffer (pH 7.4)
- Microwave for laboratory use
- Microscope slides
- No. 1.5 Coverslips

4.1 Preparation of DMSO stock solutions of fluorescent teixobactin analogues

1. Weigh out 1.0 mg of a lyophilized fluorescent teixobactin into a sterile 1.5-mL Eppendorf tube.
2. In a biosafety cabinet, dissolve the peptide in 1.0 mL of sterile DMSO to create a 1.0 mg/mL stock solution. [*Note*: Wrap the DMSO stock solutions in black felt and store in a −20 °C freezer to preserve the stock solutions for future experiments].

4.2 Preparation of the primary culture of *B. subtilis*

1. In a sterile biosafety cabinet, aseptically pipette 2.0 mL of Mueller Hinton broth in a 14-mL round bottom culture tube.
2. Retrieve a frozen glycerol stock of *Bacillus subtilis* (ATCC 6051) from the −80 °C freezer and aseptically place it inside the biosafety cabinet.
3. In the biosafety cabinet, submerge a sterile pipette tip into a frozen glycerol stock of *Bacillus subtilis* (ATCC 6051) and eject the inoculated pipette tip into the round bottom tube.
4. Quickly return the *B. subtilis* glycerol stock to the −80 °C freezer.
5. Place the inoculated round bottom tube in a shaking incubator set to 37 °C and 225 rpm, and allow the culture to incubate overnight (ca. 16 h).

4.3 Preparation of the secondary culture of *B. subtilis*

1. On the same day of imaging, aseptically transfer the overnight culture of *B. subtilis* from the shaking incubator to the biosafety cabinet.
2. In the biosafety cabinet, dilute the overnight *B. subtilis* culture 1:100 in a new round bottom tube by adding 20 μL of primary culture in 1980 μL of fresh Mueller Hinton broth to create a secondary culture of *B. subtilis. [Note*: Be sure to adjust the volume of your secondary culture depending on how many *B. subtilis* samples you plan on imaging. Plan to use 500 μL of secondary culture for each sample. For example, if you plan to image four samples, then a 2-mL secondary culture of *B. subtilis* should be sufficient. It is also suggested to prepare a *B. subtilis* sample that is only treated with sodium phosphate buffer, to use as a vehicle control for autofluorescence during the fluorescence microscopy step.]
3. Transfer the secondary culture into a shaking incubator set to 37 °C and 225 rpm.
4. Allow the secondary culture of *B. subtilis* to grow until an OD_{600} of ca. 0.3 is achieved. While the secondary culture is growing, prepare the 2% agarose pads (see next subsection).

4.4 Preparation of 2% agarose pads for imaging bacteria

1. Add 1.0 g of agarose in 50 mL of 12 mM sodium phosphate buffer (pH 7.4) in a 250-mL Pyrex bottle to create a 2% agarose solution.
2. Sterilize the colloidal agarose solution using an autoclave, and allow the solution to cool and solidify.
3. On the same day of imaging, set up a hot plate on a sterile laboratory bench equipped with an alcohol burner. Sterilize the bench using 70% alcohol and wipe the bench with clean paper towels until dry. Ignite the alcohol burner and place twice as many microscope slides as needed on the hot plate using the lowest setting. [*Note*: It is recommended to make twice as many agarose pads relative to

the number of your samples since some of the agarose pads may break or dry out by the time you add bacteria to them.]

4. While the microscope slides are warming, melt the 2% agarose by microwaving it in 15-second portions, until the agarose is completely dissolved. Aseptically transfer the molten solution of 2% agarose to the sterile laboratory bench space.

5. Once the microscope slides are warm to the touch, pipette 75 μL of the molten 2% agarose onto the microscope slide and quickly cover the agarose with a no. 1.5 coverslip. Repeat this step for each microscope slide.

6. Allow the agarose pads to set for 45 min before use. [*Note*: Fresh agarose pads must be used for imaging experiments. Agarose pads older than 10 h must be discarded, as they will be too dry to be useful for imaging].

4.5 Preparation of diluted probe solutions and staining bacteria for fluorescence microscopy studies

1. Begin this step when the secondary culture of *B. subtilis* has achieved an OD_{600} of ca. 0.3. The diluted probe solutions of fluorescent teixobactin analogues need to be prepared right before staining the bacteria in order to avoid aggregation of the teixobactin analogues.

2. In an unlit biosafety cabinet, aliquot 500 μL of the secondary culture of *B. subtilis* into as many Eppendorf tubes as needed. [Note: Keep the biosafety cabinet light off to avoid excessive light exposure to the fluorescent teixobactin solutions.]

3. Centrifuge the aliquoted *B. subtilis* samples in a microcentrifuge for 5 min at 4000 rpm.

4. While the bacteria are being centrifuged, prepare the diluted solutions of the fluorescent teixobactin analogues using the 1 mg/mL DMSO stock solutions. To make a 4 μg/mL solution of $Lys(Rhod)_9,Arg_{10}$-teixobactin, dilute 4 μL of the 1 mg/mL DMSO stock solution with 996 μL of 12 mM sodium phosphate buffer (pH 7.4) containing 0.05% polysorbate 80. To make a 1 μg/mL solution of $Lys(Rhod)_{10}$-teixobactin, $Lys(BDY\ FL)_{10}$-teixobactin, $Lys(Cy3)_{10}$-teixobactin, or $Lys(Cy5)_{10}$-teixobactin, dilute 1 μL of the 1 mg/mL DMSO stock solution with 999 μL of 12 mM sodium phosphate buffer (pH 7.4). [Note: The $Lys(Rhod)_{10}$-teixobactin, $Lys(BDY\ FL)_{10}$-teixobactin, $Lys(Cy3)_{10}$-teixobactin, or $Lys(Cy5)_{10}$-teixobactin can be diluted in sodium phosphate buffer lacking polysorbate 80. These probes do not aggregate as quickly as $Lys(Rhod)_9,Arg_{10}$-teixobactin, which requires sodium phosphate buffer containing 0.05% polysorbate 80].

5. Vortex the diluted probe solutions for 30 s.

6. Once the bacteria are finished centrifuging, remove the supernatant in each Eppendorf tube in the biosafety cabinet, being careful not to remove the bacterial pellet at the bottom of the tube.

7. In a biosafety cabinet, resuspend each bacterial pellet with 1 mL of the appropriate diluted fluorescent teixobactin solution, and mix the solution by pipetting.
8. Incubate the resuspended *B. subtilis* samples in a shaking incubator set to 37 °C and 225 rpm for 10 min.
9. Microcentrifuge each sample for 5 min at 4000 rpm.
10. In a biosafety cabinet, remove the supernatant of each Eppendorf tube, being careful not to remove the pellet at the bottom of the tube.
11. In a biosafety cabinet, resuspend each bacterial pellet with 1 mL of sodium phosphate buffer. [*Note*: Be sure to use sodium phosphate buffer containing 0.05% polysorbate 80 for the $Lys(Rhod)_9,Arg_{10}$-teixobactin sample.]
12. Repeat steps 9–11 two more times to finish rinsing the bacteria to remove excess probe from the bacteria.
13. After rinsing the bacteria, resuspend the bacteria pellets in 100–500 μL of sodium phosphate buffer. The volume of phosphate buffer should be based on the size of the pellet remaining after the washing steps. [*Note*: Be sure to use sodium phosphate buffer containing 0.05% polysorbate 80 for the $Lys(Rhod)_9,Arg_{10}$-teixobactin sample.]
14. On a sterile bench, carefully remove the coverslip on each microscope slide using a razor blade. [*Safety Note*: Razor blades are sharp.]
15. Pipette 5–15 μL of each stained bacteria sample onto each agarose pad.
16. Cover each agarose pad with its corresponding coverslip.

4.6 Imaging the bacteria

1. Image the stained bacteria using a fluorescence microscope. [Note: The stained bacteria should be immediately imaged in order to prevent aggregation of the fluorescent teixobactin analogues.]
2. Using the brightfield at 60x oil magnification, locate the bacteria in the sample by adjusting the focus.
3. Once the bacteria have been located in the brightfield, switch to the appropriate fluorescence filter to visualize the fluorescent bacteria. [*Note*: Be sure to use a low exposure setting to prevent overexposing the fluorescence signal].
4. Adjust the focus and exposure of the fluorescence in order to generate a clear fluorescence micrograph of the bacteria, and then acquire the image. [*Note*: The fluorescent teixobactin analogues stain the sidewalls and septa of *B. subtilis*.]

5. Conclusion

In this chapter, we have described the synthesis and application of fluorescent teixobactin analogues in fluorescence microscopy. The teixobactin analogues are labeled with

fluorophores using two different synthetic approaches, where the NHS ester method is the preferred approach since it offers flexibility in choice of fluorophore. Using fluorescence microscopy, we determined that the fluorescent teixobactin analogues stain the sidewalls and septa of *B. subtilis*, consistent with an antibiotic targeting peptidoglycan precursors including lipid II. We anticipate that the fluorescent teixobactin analogues described herein will be useful tools to study the mode of operation of teixobactin, peptidoglycan and teichoic acid biosynthesis, and undecaprenyl phosphate metabolism.

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Abbreviations:

DCM	methylene chloride
DIC	*N,N′*-diisopropylcarbodiimide
DMAP	4-dimethylaminopyridine
DMF	*N,N*-dimethylformamide
DMSO	dimethyl sulfoxide
DIPEA	*N,N*-diisopropylethylamine
HCTU	[(6-chlorobenzotriazol-1-yl)oxy-(dimethylamino)methylidene]-dimethylazanium hexafluorophosphate
HBTU	[benzotriazol-1-yloxy(dimethylamino)methylidene]-dimethylazanium hexafluorophosphate
HATU	[dimethylamino(triazolo[4,5-b]pyridin-3-yloxy)methylidene]-dimethylazanium hexafluorophosphate
HOBt	1-hydroxybenzotriazole
HOAt	1-hydroxy-7-azabenzotriazole
HFIP	1,1,1,3,3,3-hexafluoroisopropanol
NHS	*N*-hydroxysuccinimide
TFA	trifluoroacetic acid
TIPS	triisopropylsilane

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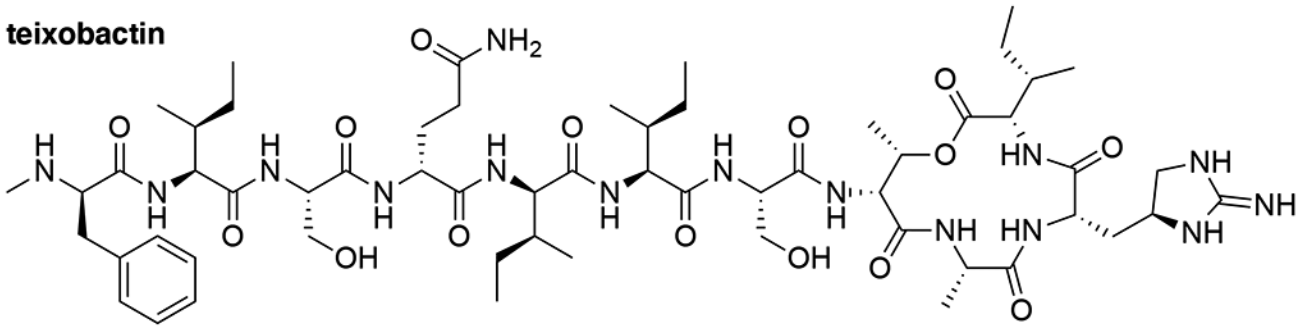


Fig. 1.
Chemical structure of teixobactin.

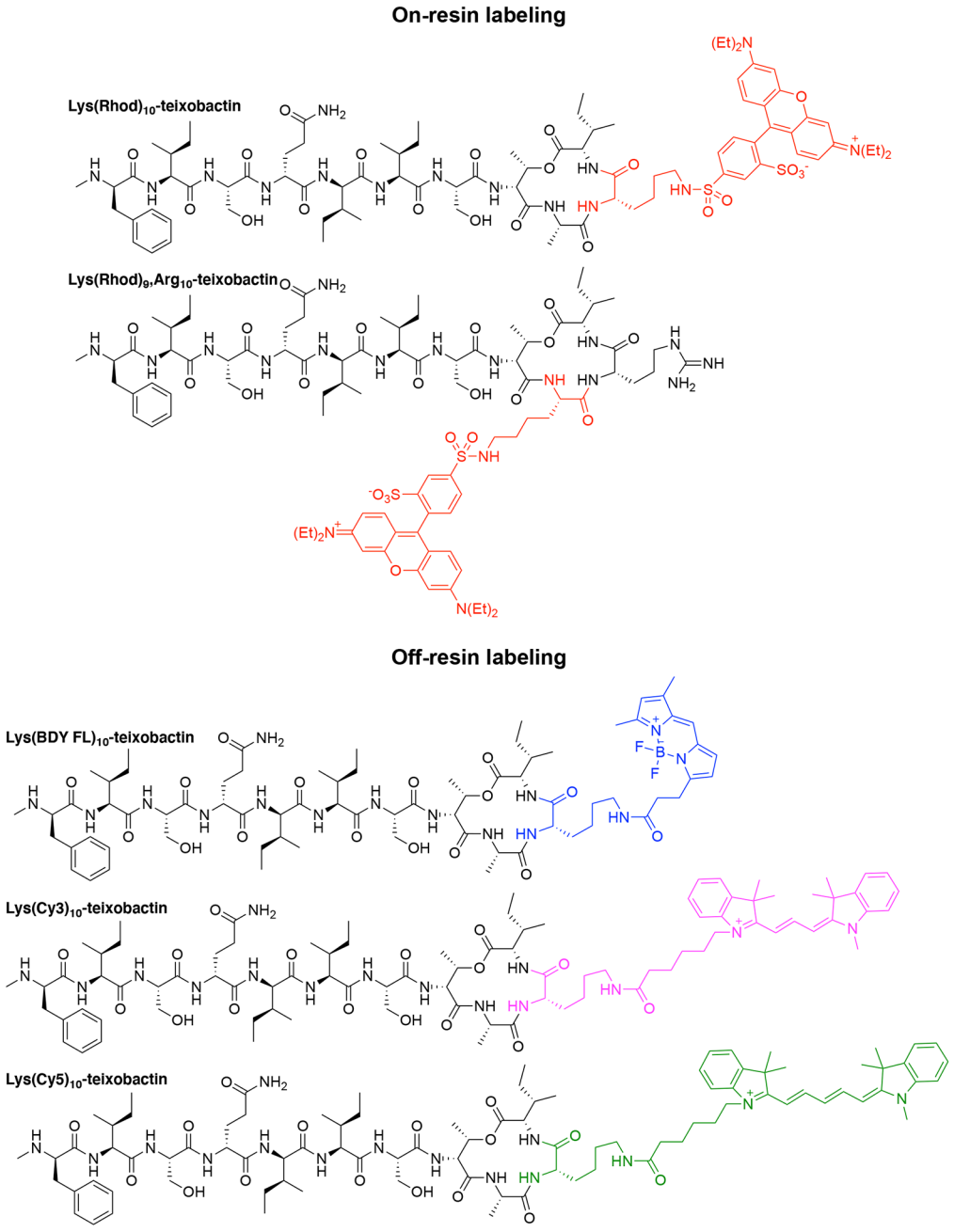


Fig. 2.
Fluorescent teixobactin analogues described in this chapter.

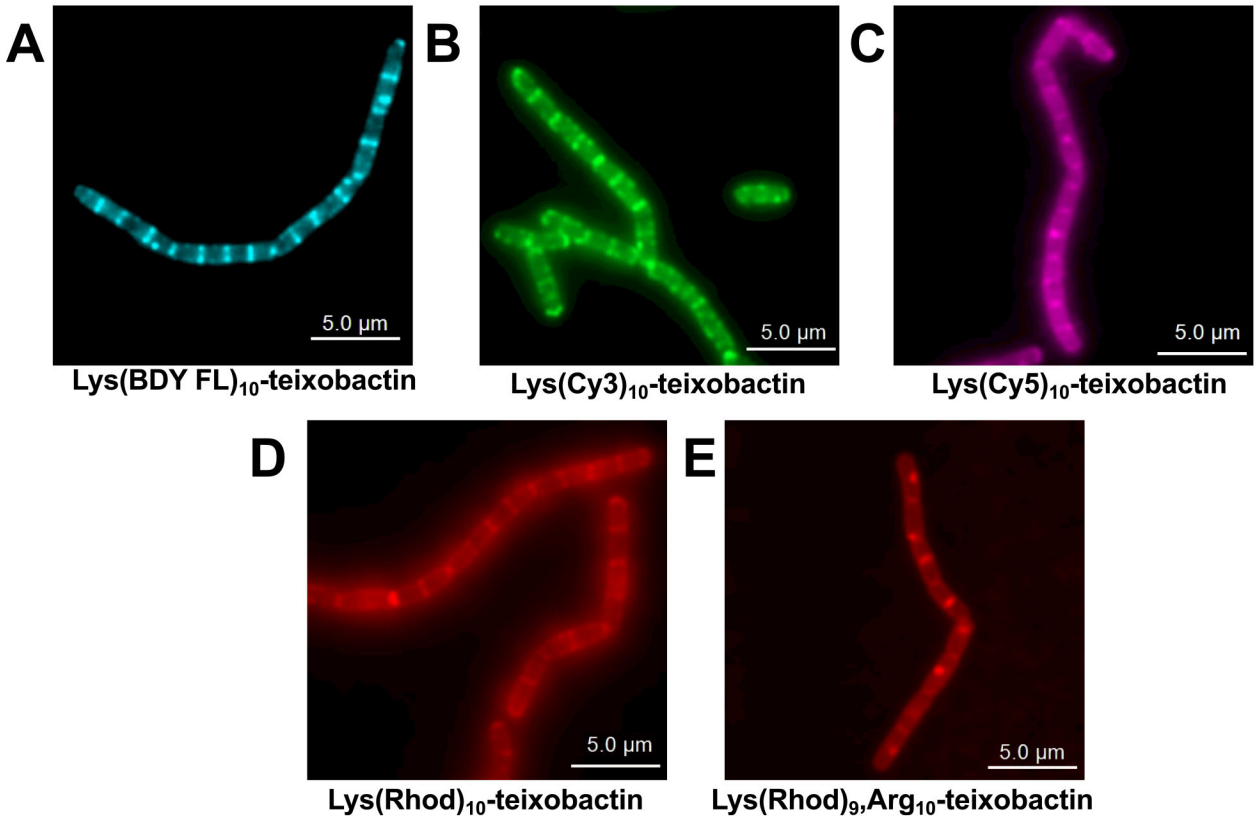
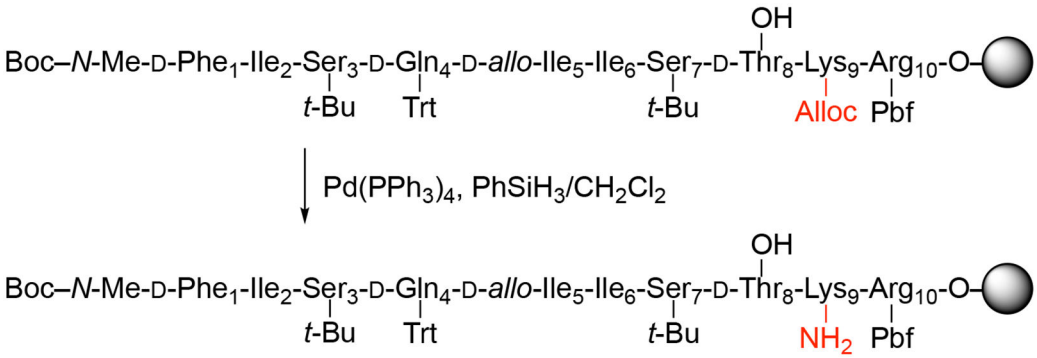


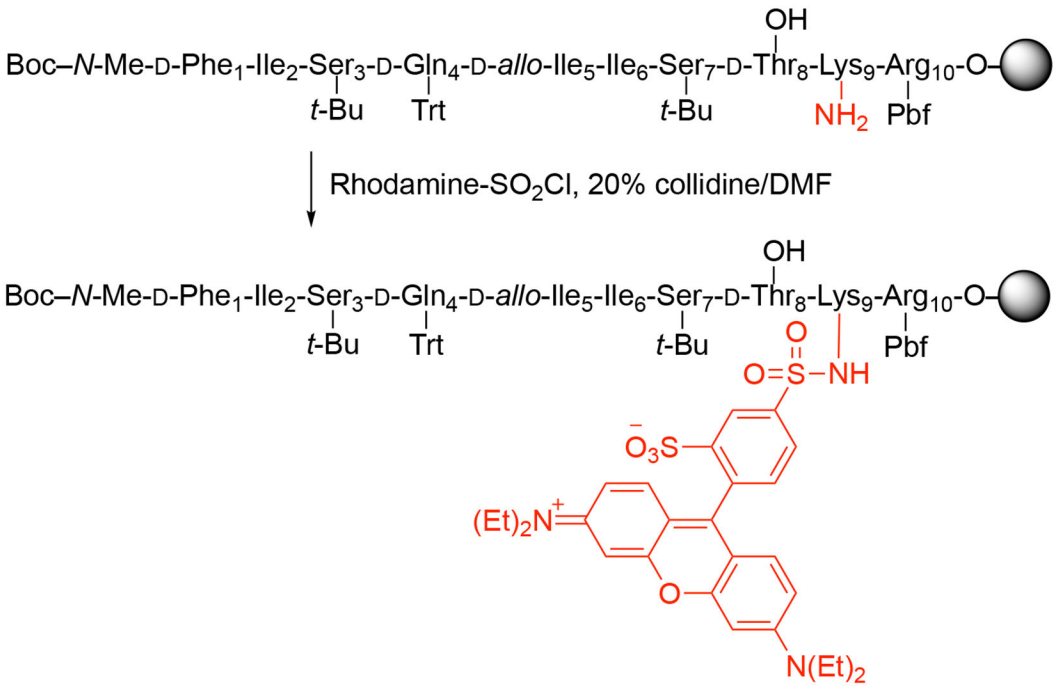
Fig. 3.

Fluorescence micrographs of *B. subtilis* stained with: (A) 1 μg/mL of Lys(BDY FL)$_{10}$-teixobactin, (B) 1 μg/mL of Lys(Cy3)$_{10}$-teixobactin, (C) 1 μg/mL of Lys(Cy5)$_{10}$-teixobactin, (D) 1 μg/mL of Lys(Rhod)$_{10}$-teixobactin, and (E) 4 μg/mL of Lys(Rhod)$_9$,Arg$_{10}$-teixobactin. Scale bars are 5 μm. Fluorescence micrographs were obtained from a Keyence BZ-X810 fluorescence microscope.



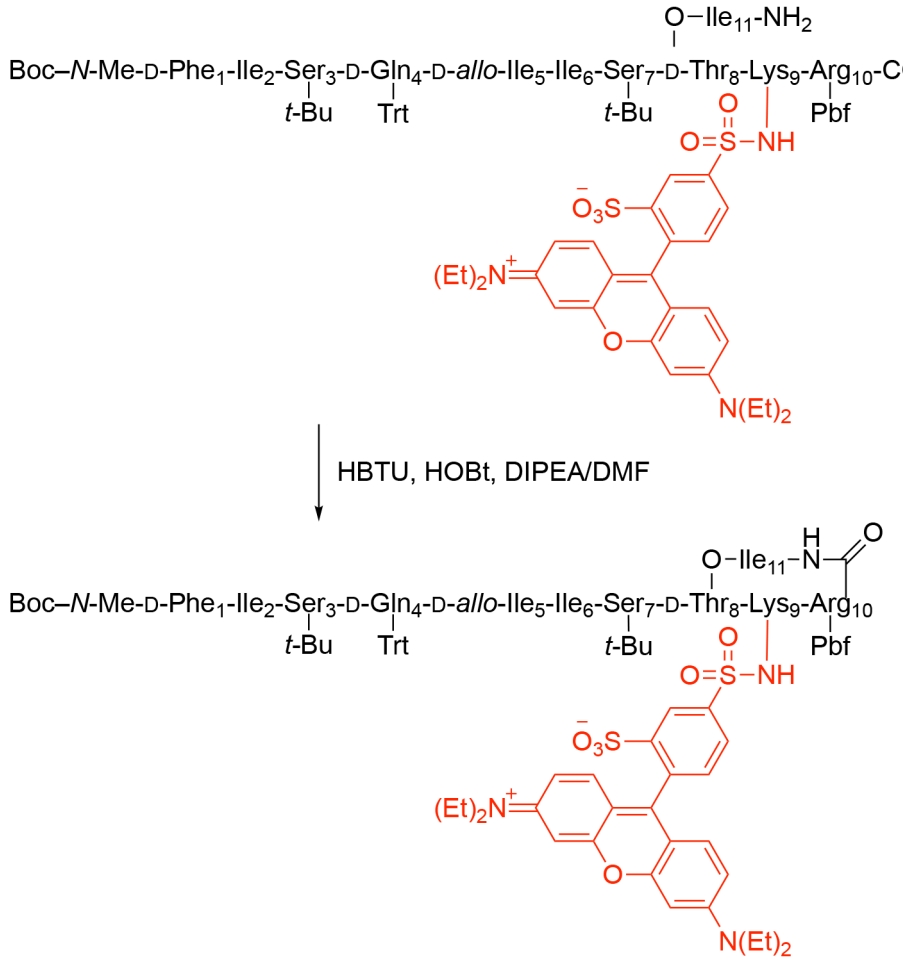
Scheme 1.

Removal of the Alloc protecting group in the synthesis of a sulforhodamine B labeled teixobactin analogue.

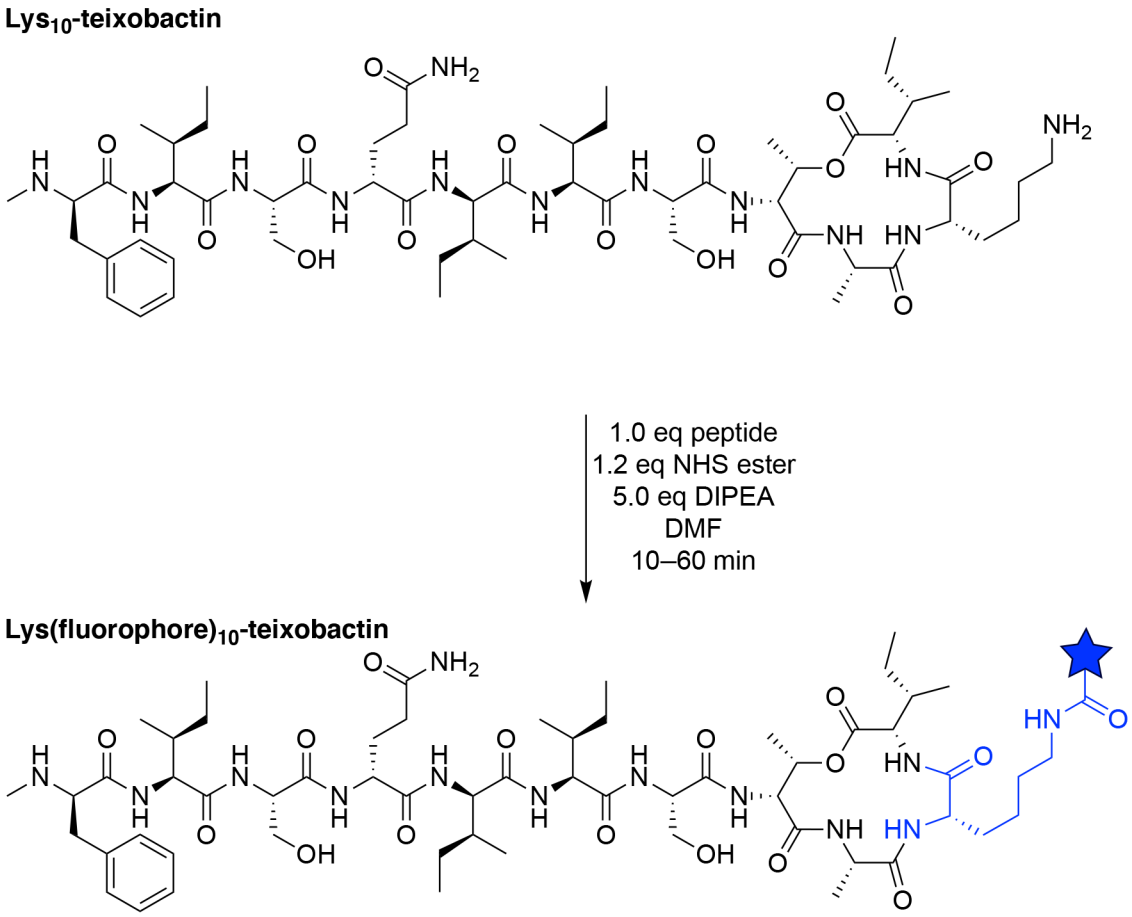


Scheme 2.

On-resin labeling of Lys(Rhod)$_9$,Arg$_{10}$-teixobactin with sulforhodamine B sulfonyl chloride.



Scheme 3.
Cyclization to generate the macrolactone.



Scheme 4.
Labeling of Lys_{10}-teixobactin with a fluorophore NHS ester.