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A General Method for Fluorescent Labeling of the N-Termini of Lanthipeptides and Its Application to Visualize their Cellular Localization

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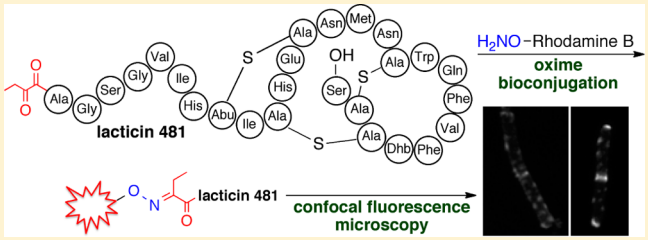
Supporting Information

ABSTRACT: Labeling of natural products with biophysical probes has greatly contributed to investigations of their modes of action and has provided tools for visualization of their targets. A general challenge is the availability of a suitable functional group for chemoselective modification. We demonstrate here that an N-terminal ketone is readily introduced into various lanthipeptides by the generation of a cryptic N-terminal dehydro amino acid by the cognate biosynthetic enzymes. Spontaneous hydrolysis of the N-terminal enamines results in α-ketoamides that site-specifically react with an aminooxy-derivatized alkyne or fluorophore. The methodology was successfully applied to prochlorosins 1.7 and 2.8, as well as the lantibiotics lacticin 481, haloduracin α, and haloduracin β. The fluorescently modified lantibiotics were added to bacteria, and their cellular localization was visualized by confocal fluorescence microscopy. Lacticin 481 and haloduracin α localized predominantly at sites of new and old cell division as well as in punctate patterns along the long axis of rod-shaped bacilli, similar to the localization of lipid II. On the other hand, haloduracin β was localized nonspecifically in the absence of haloduracin α, but formed specific patterns when coadministered with haloduracin α. Using two-color labeling, colocalization of both components of the two-component lantibiotic haloduracin was demonstrated. These data with living cells supports a model in which the α component recognizes lipid II and then recruits the β-component.



INTRODUCTION

Lanthipeptides are a rapidly expanding subclass of ribosomally synthesized and post-translationally modified peptides (RiPPs)[1] that are characterized by thioether cross-links.[2] Many lanthipeptides have antimicrobial activities against Gram-positive bacteria, including multidrug resistant strains;[3] such lanthipeptides are termed lantibiotics. Currently, the mode(s) of action of only a small subset of lantibiotics has been deduced, including the best studied family member nisin. Fluorescent microscopy with fluorescently labeled nisin has greatly contributed to understanding its mechanism of action.[4] Site-specific labeling of nisin was possible because it contains only a single carboxylate (the C-terminus). However, for most lantibiotics/lanthipeptides, no such chemoselective handle for convenient modification is available because they contain multiple reactive groups, and hence to date, biophysical labeling studies of lanthipeptides have been limited. For instance, labeling of the lanthipeptides duramycin and nukacin ISK-1 with amine-reactive probes resulted in labeling of at least two positions.[5,6] Similarly, most lanthipeptides contain multiple dehydroamino acids, and therefore, the use of Michael-type additions to introduce biophysical probes[7] would also result in multiple sites of labeling. In recent years, complete chemical synthesis of lantibiotics on a solid support has been reported[8−10] and could potentially be a viable route to lanthipeptides carrying biophysical probes, but such an application has not been reported to date and would not be a source of renewable materials. Therefore, development of a general means to label lantibiotics without affecting their bioactivities would aid the investigation of this large and promising class of compounds.[11,12]

Lanthipeptides are biosynthesized from a ribosomally generated linear peptide that is generically called LanA (e.g., NisA for nisin, NukA for nukacin, etc). The LanA peptides consist of a leader peptide that is important for recognition by the post-translational modification enzymes and a core peptide that is modified into the mature lanthipeptide. Ser and Thr residues in the core peptides are first dehydrated to generate dehydroalanine (Dha) and dehydrobutyrine (Dhb) residues, respectively, and the thiols of Cys residues subsequently add to these dehydro amino acids to form thioether structures called lanthionine and methyllanthionine, respectively (Figure 1A).[2] For class II lanthipeptides, both the dehydration and cyclization processes are catalyzed by a single synthetase that is generically called LanM, as exemplified in Figure 1B for prochlorosin 1.7 (Pcn1.7), which is generated by the lanthionine synthetase ProcM. In a final step, a protease removes the leader peptide.

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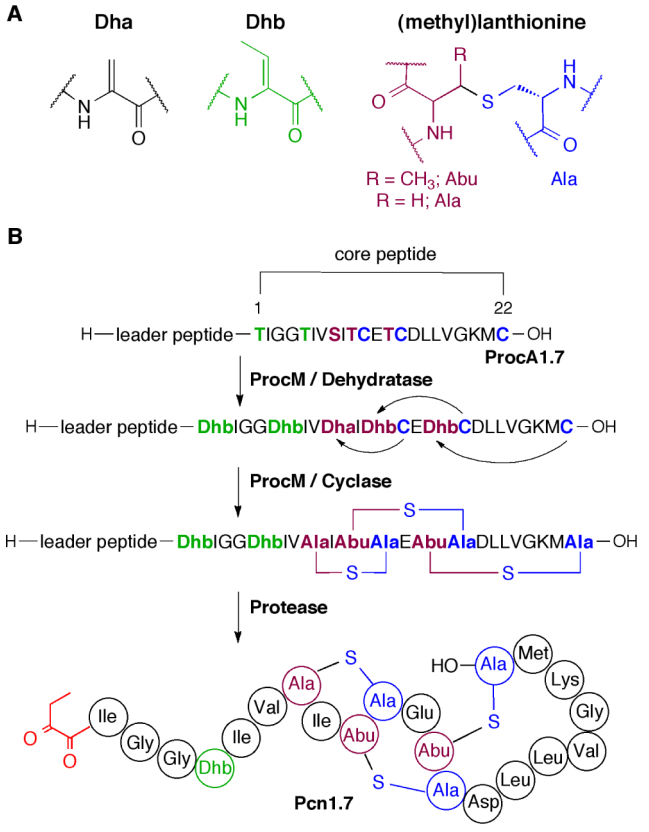

Figure 1. (A) Structures of dehydroalanine (Dha), dehydrobutyrine (Dhb), lanthionine (R = H), and methyllanthionine (R = CH_3). Structure in purple (R = CH_3) is 2-aminobutyric acid (Abu). (B) Biosynthesis of Pcn1.7 with its naturally occurring N-terminal 2-oxobutyryl group (red). For the sequence of the leader peptide, see Figure 3.

One approach that might allow site-specific labeling of either the N- or C-termini of lanthipeptides involves removal of any Lys, or Asp and Glu residues. Indeed, because of their ribosomal origin, site directed mutagenesis has been applied extensively to lanthipeptides and generally (but not always) does not affect the post-translational modification process.[13−16] Unfortunately, Lys and Asp/Glu residues have been shown to be very important for the activities of many lantibiotics,[17−25] rendering this approach ineffective for most applications.

Many site-specific peptide- and protein-labeling techniques have emerged in recent years that use either chemical or enzymatic methods to introduce chemical handles for derivatization.[26] For most enzymatic approaches, a peptide tag is incorporated into a protein or peptide of interest at the gene level.[27−31] Through site-specific enzymatic modification of the tag, a reactive chemical functionality is introduced that may be used for a 'bioorthogonal' conjugation with a reporter molecule carrying a suitable complementary functionality. The termini of peptides and proteins have been particularly attractive for the incorporation of chemical probes because it generally minimizes undesired effects of the probe on function.[32−36] Many of these labeling strategies have incorporated a ketone or aldehyde into a peptide or protein of interest followed by bioconjugation with an aminooxy-, hydrazine-, or alkoxyamine-containing biophysical probe.[27,37−39] These reactions are chemoselective with respect to most peptides and proteins because the twenty canonical amino acids do not contain reactive carbonyl groups. However, introduction of a peptide tag at the N- or C-terminus of a lanthipeptide is still a considerable perturbation in structure because lanthipeptides are typically only ∼20−25 amino acids in length. Interestingly, an N-terminal ketone (2-oxobutyryl or pyruvyl) is a naturally occurring post-translational modification in some lanthipeptides, including Pep5,[40] plantaracin-W β,[41] and Pcn1.7 (Figure 1B).[42] The N-terminal ketone arises from a dehydrated Ser/Thr at position 1 of the core peptide. After proteolytic removal of the leader peptide, the N-terminal dehydro amino acid hydrolyzes to an α-ketoamide (Figure 2A). We hypothesized that such a ketone handle could be used to site-specifically label this subset of lanthipeptides with minimal perturbation, and that a similar approach might be used for the installation of a 2-oxobutyryl group onto the N-termini of lanthipeptides that naturally lack this functionality. We demonstrate here that this methodology is successful for prochlorosins 1.7 and 2.8, lacticin 481, haloduracin α, and haloduracin β (for structures, see Figures 1B and 2B). The resulting ketones of the latter three compounds were conjugated to a fluorescent probe, and the distribution of the fluorescent lantibiotic analogues in bacteria was visualized by confocal fluorescence microscopy.

RESULTS

Installation of a Ketone on the N-Terminus of Pcn1.7.

The proposed methodology requires solutions to two hurdles. The first is to successfully install a dehydroamino acid at the first amino acid of the core peptide, and the second is to site-specifically remove the leader peptide through proteolysis after residue −1 (amino acids in the leader peptide are indicated with negative residue numbers counting backward from the natural protease cleavage site). We first investigated the site-specific labeling of a lanthipeptide with a naturally occurring N-terminal 2-oxobutyryl group for which the first hurdle is already solved in nature. We chose Pcn1.7 (Figure 1B) because it has been produced by coexpression of the precursor peptide ProcA1.7 with the lanthionine synthetase ProcM in *Escherichia coli*,[43] and because biophysical labeling of prochlorosins may help in elucidating their biological functions, which at present are unknown.[42] The second hurdle, site-specific removal of the leader peptide in vitro, is challenging for Pcn1.7. The cognate proteases that remove the leader peptides in lanthipeptide-producing bacteria are often membrane bound,[44] their soluble domains have low in vitro activity,[45,46] and for the prochlorosins, the protease(s) have not been identified altogether. Hence, although a few successful examples have been reported,[47] the cognate proteases are not a general solution for in vitro removal of leader peptides. To address these limitations, various other methods for in vitro removal of the leader peptides have been reported,[48] including incorporation of Lys or Glu at the −1 position for subsequent treatment with endoproteinases LysC or GluC, respectively.[49−52] Whereas these approaches have worked in select cases, use of GluC and LysC typically does not work if the core peptide contains a Lys or Glu, unless that residue is "deactivated" for proteolysis by adjacent post-translationally modified amino acids.

For Pcn1.7, leader peptide removal by inserting a Lys at position −1 and treatment of the ProcM-modified ProcA1.7 mutant with LysC was unsuccessful in previous work as cleavage at a Lys in the core peptide was observed.[43] An alternative approach was therefore used in this study, involving mutation of the last residue of the ProcA1.7 leader peptide from Gly to Glu.[42] The gene encoding His_6-ProcA1.7(G−1E)

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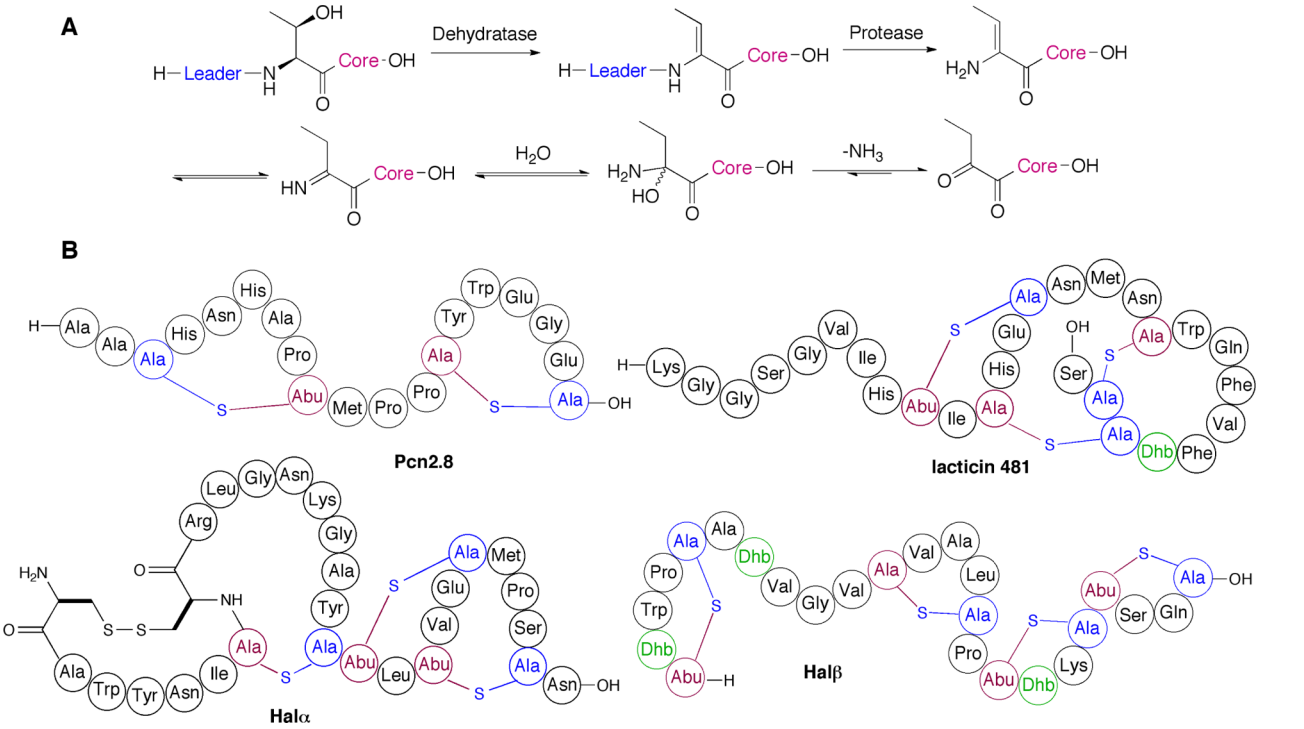

Figure 2. (A) Proposed installation of an N-terminal 2-oxobutyryl group. Dehydration of a Thr at the N-terminus of the core peptide followed by proteolysis yields the core peptide with an N-terminal enamine. In aqueous conditions the enamine spontaneously converts to a 2-oxobutyryl group. (B) Structures of the lanthipeptides Pcn2.8, lacticin 481, Hal*α*, and Hal*β*.

was inserted into multiple cloning site 1 (MCS1) of a pRSFDuet plasmid in which the gene encoding the modification enzyme ProcM was inserted in MCS2. Coexpression in *E. coli* resulted in the desired 5-fold dehydrated His_6-ProcA1.7(G−1E). After GluC cleavage, the major ion observed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) corresponded to a peptide that had been proteolyzed after Glu−6 rather than after Glu−1, resulting in Pcn1.7 with a five amino acid overhang (see Figure 3 for peptide sequences). This reluctance of a protease to cleave next to a post-translationally modified amino acid (Dhb1 for Pcn1.7) and therefore prefer alternative cleavage sites in the leader peptide is a common observation[43,52,53] that hampers the generality of the proposed methodology. Similar difficulties were also observed with use of a larger recognition sequence such as the IleGluGlyArg motif that is recognized by Factor Xa.[52]

ProcA1.7$_{(-68)-(-20)}$QANSQKNLSDAELEGVALLTIGGTIVSITCETCDLLVGKMC

ProcA2.8$_{(-64)-(-20)}$LNSHRQNLSDDELEGVAGKTACHNHAPSMPPSYWEGEC

LctA$_{(-24)-(-15)}$QEVTESELDLILGAKAAALLKTAGSGVIHTISHECNMNSWQFVFTCCS

HalA1$_{(-41)-(-20)}$GDIFQELEDQDILAGVNGETCAWYNISCRLGNKGAYCTLTVECMPSCN

HalA2$_{(-41)-(-20)}$EVNEKELSSLAGSGDVHAETATTWPCATVGVSVALCPTTKCTSQC

Figure 3. Peptide sequences that yield lanthipeptides with an N-terminal 2-oxobutyryl group. Native core peptides are underlined. Red, inserted Thr; blue, protease cleavage site; green, aminopeptidase cleavage sequence; brown, other amino acid insertions or mutations (see text). The Thr at the N-terminus of the core peptide in ProcA1.7 is naturally occurring. The leader peptide segments that are not shown in the figure are: ProcA1.7$_{(-68)}$MSEEQLKAFIAKVQADTSLQEQLKVEGADVVAIAKASGFAITTEDLKAH$_{(-20)}$, ProcA2.8$_{(-64)}$MSEEQLKAFLTKVQADTSLQEQLKIEGADVVAIAKAAGFSITTED$_{(-20)}$, LctA$_{(-24)}$MKEQNSFNLL$_{(-15)}$, HalA1$_{(-41)}$MTNLLKEWKMPLERTHNNSNPA$_{(-20)}$, and HalA2$_{(-41)}$MVNSKDLRNPEFRKAQGLQFVD$_{(-20)}$.

To overcome these obstacles, we envisioned a two-stage leader peptide removal strategy in which most of the leader peptide is initially removed by trypsin, GluC, or LysC, and an aminopeptidase is used subsequently to remove the remaining amino acids of the leader peptide. Aminopeptidase activity was expected to self-terminate after reaching the first dehydrated amino acid (Dha1 or Dhb1) assuming that the conversion of the N-terminal enamine to an *α*-ketoamide (Figure 2A) occurs faster than aminopeptidase-catalyzed hydrolysis. The last two amino acids of the leader peptide of many lanthipeptides consist of the "double-glycine" motif,[54] which poses a problem because commercial Leu-selective aminopeptidase from *Aeromonas proteolytica* is not very effective at removing this sequence.[46] Thus, a new mutant, His_6-ProcA1.7(G−2L/G−1L), was generated (Figure 3). Coexpression of this double mutant with ProcM resulted in 5-fold dehydrated ProcA demonstrating that the two Leu residues did not interfere with post-translational processing. The peptide was purified by immobilized metal affinity chromatography (IMAC) and HPLC resulting in a typical yield of about 8 mg/L of purified product. The peptide was then treated with GluC first (cleaves after Glu−6) and then aminopeptidase (removes GVALL, Figure 3). The product was analyzed by MALDI-TOF MS and was found to be identical to the anticipated mass of Pcn1.7 with an N-terminal ketone (Figure S1A).

To determine if the N-terminal ketone on Pcn1.7 could be used as a site-specific handle for labeling with small molecules, HPLC-purified Pcn1.7 was reacted with the synthetic aminooxy-alkyne (***1***) (Figure 4). Analysis by MALDI-TOF MS of the product indicated that the alkyne-functionalized Pcn1.7 (alk-Pcn1.7) was indeed generated (Figure S2A). Addition of aniline

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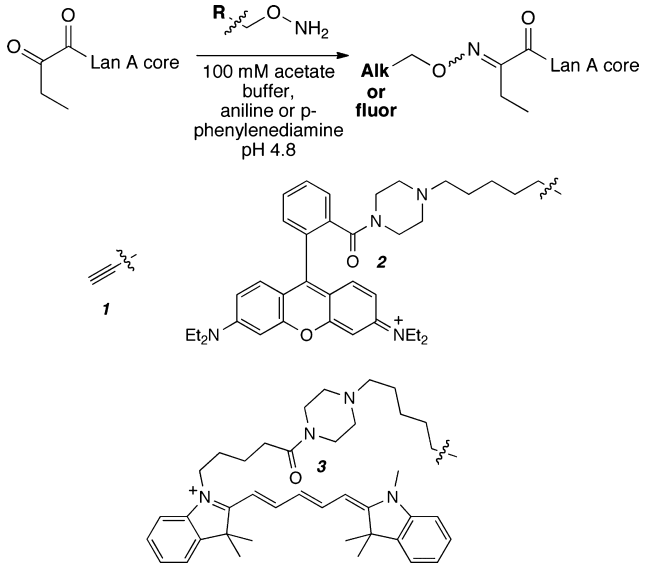

Figure 4. Aniline- or *p*-phenylenediamine-catalyzed oxime bioconjugation between keto-LanA core and an aminooxy moiety functionalized with either an alkyne (alk, ***1***) or the fluorophores (fluor) rhodamine B (***2***) or Cy5 (***3***).

(100 mM) to the buffered solution of peptide and ***1*** dramatically increased the product yield.[55] When the bioconjugation was attempted with GluC-cleaved ProcA1.7-(G−1E), an analogue lacking the N-terminal ketone, no reaction occurred demonstrating that oxime formation was specific for the N-terminus of Pcn1.7 (Figure S3).

Installation of a Non-Native N-Terminal 2-Oxobutyryl Group in Lanthipeptides. Unlike Pcn1.7, most lanthipeptides do not contain an N-terminal 2-oxobutyryl group. Therefore, to make the labeling strategy general for other lanthipeptides, a ketone needs to be appended to their N-termini. Harnessing the well-established substrate tolerance of the lanthipeptide synthetases,[14] we hypothesized that installation of a Ser or Thr between the leader and the core peptides or replacing the first residue of the core peptide with a Ser or Thr might result in dehydration by the cognate dehydratase. Proteolytic removal of the leader peptide would then afford the modified core peptide with an N-terminal ketone. We chose to install a Thr rather than Ser because an analysis of almost 40 lanthipeptides found that Ser escaped dehydration more than twice as often as Thr.[56]

Installation of a Ketone on the N-Terminus of Prochlorosin 2.8. We tested the methodology with prochlorosin 2.8 (Pcn2.8) (Figure 2B). Because the core peptide of Pcn2.8 does not contain a Lys, a G−1K mutation was introduced in addition to replacement of Ala1 with Thr (Figure 3). The His_6-ProcA2.8(G−1K/A1T) mutant peptide was coexpressed with the modification enzyme ProcM in *E. coli* cells. After purification by IMAC and cleavage of the leader peptide from the modified core peptide with trypsin, analysis by MALDI-TOF MS demonstrated a major ion corresponding to the core peptide with three dehydrations (Figure S1B), compared to two dehydrations in wild-type Pcn2.8 (Figure 2B). The mass of the product was 1 Da larger than the calculated peptide mass containing an N-terminal enamine, demonstrating the formation of a 2-oxobutyryl group on the N-terminus of Pcn2.8 (keto-Pcn2.8) (Figure S1B). Keto-Pcn2.8 was obtained in a typical yield of 3 mg/L after HPLC purification, and was site-specifically labeled with ***1*** in an aniline-catalyzed conjugation (Figure S2B). As with Pcn1.7, the alkyne added only once to keto-Pcn2.8.

Installation of a Ketone on the N-Terminus of Haloduracin *α* and Haloduracin *β*. The experiments with the two prochlorosins established the feasibility of the site-specific labeling approach, but because their bioactivities are unknown, we could not assess whether N-terminal labeling interfered with activity. The two-peptide systems are an interesting subclass of lantibiotics, which achieve maximum antimicrobial activity from the synergistic activity of two disparate peptides that are produced by the same organism. One example is haloduracin (Figure 2B), consisting of Hal*α* produced from the HalA1 precursor peptide by the lanthionine synthetase HalM1, and Hal*β*, generated from the HalA2 precursor peptide by the synthetase HalM2.[57,58] The keto-Hal*α* and keto-Hal*β* analogues were obtained using the previously described methodology. Briefly, for Hal*α*, a gene encoding His_6-HalA1(A−1E-C1 ins T) (Figure 3) was generated by site-directed mutagenesis in which the last residue of the leader peptide (Ala−1) was mutated to Glu for leader peptide removal and a Thr was inserted between this Glu and the first residue of the core peptide (Cys1). The gene was inserted into MCS1 of a pRSFDuet plasmid and the gene encoding HalM1 was inserted in MCS2. Because of the relatively low yield of haloduracin production in *E. coli* in a previous study,[49] a second copy of the *halA1* mutant was inserted into a pET15b plasmid in an attempt to increase the peptide yield after coexpression. To obtain keto-Hal*β*, a gene encoding His_6-HalA2(Q−1E-T1 ins TA) (Figure 3) was generated by site-directed mutagenesis. In this mutant peptide, the last residue of the leader peptide of HalA2 (Gln−1) was mutated to Glu and a ThrAla sequence was inserted between the Glu and the first residue of the core peptide (Thr1). The additional Ala was added to separate the cleavage site from the N-terminal methyllanthionine ring as only partial proteolytic leader peptide removal was observed in a mutant lacking this additional Ala residue. The *halA2* mutant gene was inserted into MCS1 of a pRSFDuet plasmid containing the *halM2* gene in MCS2. Coexpression of both the HalA1 and HalA2 mutant peptides with their cognate lanthionine synthetases, and purification by IMAC and HPLC resulted in fully modified peptides in yields of about 8 mg/L for the HalA1 analogue and 10 mg/L for the HalA2 analogue. Subsequent cleavage of their leader peptides with endoprotease GluC yielded Hal*α* and Hal*β* analogues containing a 2-oxobutyryl moiety (keto-Hal*α*, keto-Hal*β*) (Figure S1C,D). The keto analogues were each reacted with ***1*** in aniline-catalyzed conjugations to yield alkyne-labeled alk-Hal*α* and alk-Hal*β* (Figure S2C,D).

Installation of a Ketone on the N-Terminus of Lacticin 481. Previous work determined that Ser4 is not modified in wild type (wt) lacticin 481 (Figure 2B) because it is both too close to the leader peptide and flanked by two Gly residues.[59] This Ser was only dehydrated by the synthetase LctM when three Ala residues were inserted between the leader and the core peptides and Gly3 and Gly5 were mutated to Ile and His, respectively.[59] We therefore anticipated that a Thr inserted directly between the leader and the core peptides in lacticin 481 would not be dehydrated based on its close proximity to the leader peptide and its flanking amino acids. Taking these previous studies as a guideline, a gene encoding the peptide His_6-LctA(K1-G2A ins AAALLKT) (Figure 3) was prepared by PCR with appropriate primers (see Supporting Information).

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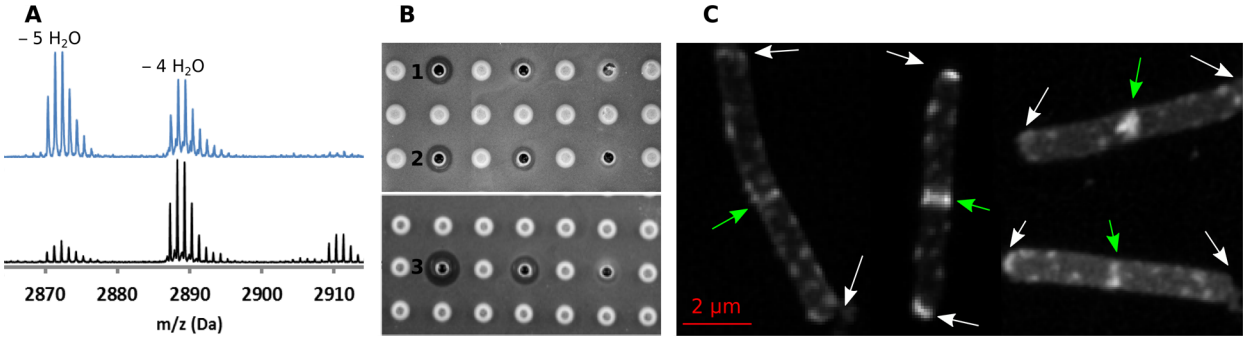

Figure 5. Biosynthesis, bioactivity, and cellular distribution of lacticin 481 analogues. (A) MALDI-TOF MS of LysC cleaved His_6-LctA(K1-G2A ins AAALLKT) overexpressed in *E. coli* with 3 copies (blue) and 1 copy (black) of the *lctM* gene. (B) Zones of growth inhibition displayed by wt lacticin 481 (row 1), alk-lacticin 481 (row 2) and rho-lacticin 481 (row 3) against *B. subtilis* 168. Concentrations from left to right: 50, 25, and 12.5 μM; 20 μL were added to each well. (C) Staining of *B. subtilis* 168 with rho-lacticin 481 (1 μM) to analyze its cellular distribution. New and old cellular division sites are represented by green and white arrows, respectively.

In this peptide, the sequence AAALLKT was inserted between residues 1 and 2 of the core peptide to place the inserted Thr at a longer distance from the end of the leader peptide. Furthermore, the second residue of the core peptide (Gly) was mutated to Ala to increase the efficiency of dehydration of the inserted Thr residue. Finally, a Lys was placed before the inserted Thr such that the leader peptide could be removed with LysC or trypsin. This mutant LctA peptide and its cognate synthetase LctM were coexpressed from a pRSFDuet plasmid in *E. coli* cells.[60] After LysC cleavage of modified His_6-LctA(K1-G2A ins AAALLKT), MALDI-TOF MS analysis of the resulting peptide showed that a majority of the product contained only four dehydrations, the number of dehydrations in wt lacticin 481 (Figure 5A, black). Only a small portion of the peptide was successfully dehydrated five times. Further analysis of this peptide with five dehydrations by MALDI-TOF MS showed that an N-terminal 2-oxobutyryl had been introduced onto lacticin 481 (keto-lacticin 481).

We hypothesized that an increase in the concentration of LctM in the coexpression system would lead to an enhancement in dehydration efficiency of the inserted Thr. We increased the concentration of LctM by adding copies of *lctM* to pCDFDuet and pACYCDuet vectors. Coexpression of the *his_6-lctA(K1-G2A ins AAALLKT)* construct with 3 copies of *lctM* afforded the post-transalationally modified LctA in a typical yield of 3 mg/L, and with the 5-fold dehydrated peptide as the major product (Figure 5A, blue). Treatment of the 5-fold dehydrated product with LysC followed by aniline-catalyzed conjugation with ***1*** resulted in the desired alk-lacticin 481 (Figure S2E). We note that this analogue lacks the N-terminal Lys that is found in wt lacticin 481. However, previous studies have shown that the Δ1K analogue is only about three times less active than the wt compound.[21]

Bioactivities of Alkyne-Labeled Lacticin 481, Haloduracin *α*, and Haloduracin *β*. alk-Lacticin 481, alk-Hal*α*, and alk-Hal*β* were used for agar well-diffusion antimicrobial assays against *Bacillus subtilis* 168. The zones of growth inhibition compared with those obtained with the wt standard showed that alk-lacticin 481 retained its activity (Figure 5B). Because haloduracin is a two-component lantibiotic, both components, alk-Hal*α* and alk-Hal*β*, were administered individually and in tandem to *B. subtilis* 168. Like wt haloduracin, alk-Hal*α* and alk-Hal*β* alone displayed little bioactivity. However, the alk-Hal*α* and -*β* peptides exhibited potent antibacterial activity when combined (Figure S4).

Fluorescent Labeling of Lacticin 481, Haloduracin *α*, and Haloduracin *β*. Aminooxy-rhodamine (***2***) and aminooxy-Cy5 (***3***) (Figure 4) were synthesized using published procedures with minor changes (see Supporting Information).[61−63] Very recently, *p*-phenylenediamine was reported to be an improved catalyst over aniline for oxime bioconjugations, in part because of its increased water solubility.[64] Therefore, keto-lacticin 481 and keto-Hal*α* were labeled with ***2*** via *p*-phenylenediamine-catalyzed oxime bioconjugations, providing rho-lacticin 481 and rho-Hal*α* in modestly improved yields compared to aniline as catalyst. Similarly, keto-Hal*β* was ligated with ***2*** or ***3*** yielding rho- or Cy5-Hal*β*. The purity of each peptide was assessed by analytical RP-HPLC (Figure S5) and analysis by MALDI-TOF MS showed that the aminooxy-fluorophore added only once (Figure S6). Reaction of Δ1K lacticin 481, which lacked the N-terminal ketone, with ***2*** did not yield an addition product, indicating the fluorophore was incorporated at the N-terminus of the lantibiotic analogue (Figure S7).

The bioactivities of the fluorescently labeled peptides were tested by agar diffusion and liquid media growth assays and compared to their respective wt peptides. Rho-lacticin 481 was active at micromolar concentrations against *B. subtilis* (Figure 5B) and *Lactococcus lactis* HP but with a minimum inhibitory concentration (MIC) that was increased 6-fold compared to wt lacticin 481 (Table S1). Rho-Hal*α* and rho/Cy5-Hal*β* displayed antimicrobial activity against *B. subtilis* 168 when added together with their unlabeled partner (Hal*β* and Hal*α*, respectively), but exhibited only low level bioactivity when administered alone, as is also observed for the wt peptides (Figure S4). The MICs of rho-Hal*α* when coincubated with wt Hal*β* and of rho- or Cy5-Hal*β* when coincubated with wt Hal*α* were increased about 8- to 15-fold compared to the wt peptides (Table S1). Although not surprisingly their bioactivities were decreased, a similar mode of action of the fluorescently labeled peptides compared to the wt lantibiotics is suggested by additional experiments discussed later.

Lacticin 481 Binds to Lipid II on Cell Surfaces. Lacticin 481 was recently shown to inhibit the transglycosylation step of peptidoglycan biosynthesis, most likely by binding to the cell wall precursor lipid II.[24] This hypothesis is also supported by a recent study that showed using ITC that nukacin ISK-1, a close structural analogue of lacticin 481, binds to lipid II.[65] Therefore, analysis of the interaction of rho-lacticin 481 with

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sensitive bacteria by confocal fluorescence microscopy was anticipated to show preferential staining of lipid II.

Previous studies of the peptidoglycan architecture in *B. subtilis* using fluorescently labeled vancomycin and ramoplanin, two lipid II-binding antibiotics, demonstrated intense staining at the midcell, corresponding to new division sites, and at the poles, corresponding to old division sites.[66,67] In addition, staining of the bacterial sidewalls was observed as peripheral dots connected by weak transverse bands, suggesting a helical structure. More recently, two research groups used the cellular biosynthetic machinery to incorporate alkyne, azide, and fluorescently functionalized D-alanine analogues into the lipid II of several bacteria, including *B. subtilis*.[68,69] The alkyne and azide analogues were then visualized in living cells with azide- or alkyne-linked fluorophores via click chemistry. Analysis of these bacteria by fluorescence microscopy also showed a punctate distribution of the lipid II analogues with intense bands at the midcell of the bacteria. Thus, these metabolic labeling studies provide an independent and complementary determination of lipid II localization. In the current work, rho-lacticin 481 was incubated with *B. subtilis* 168 at sublethal concentrations and the staining pattern was examined by confocal fluorescence microscopy. In most bacteria, the fluorescence intensity was greatest at midcell (Figure 5C, green arrows). We also observed peripheral dots with weak transverse bands along the longitudinal axis of the rod-shaped bacilli (Figure 5). These results are very similar to the patterns seen with the lipid II-binding antibiotics and fluorescent lipid II analogues in *B. subtilis*, strongly suggesting that lacticin 481 binds lipid II in living cells.

Lacticin 481-E13A Does Not Localize in a Specific Pattern on Cell Surfaces. A conserved Glu/Asp is found in several class II lantibiotics and is integral to their bioactivities.[17,19,20,23,24] In lacticin 481, this residue is Glu13 and a Glu13Ala mutant abolished antimicrobial activity.[24] Thus, a fluorescent analogue of this mutant was used to confirm that the observed localization of rho-lacticin 481 is mediated by the peptide and not the fluorophore. To this end, an LctA mutant (His_6-LctA-K1-G2A ins AAALLKT/E13A) was coexpressed with LctM in *E. coli*, purified by IMAC, and the leader peptide removed with LysC. The resulting modified core peptide was purified by RP-HPLC yielding keto-lacticin 481-E13A (Figure S1E). The peptide was labeled with **2** to provide rho-lacticin 481-E13A (Figure S6E), incubated with *B. subtilis* 168, and the staining pattern was examined by confocal fluorescence microscopy. Very little staining was observed (Figure S8A), even at concentrations 20 times larger than that used to stain *B. subtilis* 168 with rho-lacticin 481, demonstrating that Glu13 is essential for the localization of lacticin 481 and that the rhodamine in rho-lacticin 481 is not responsible for the observed localization pattern.

Haloduracin β Colocalizes with Haloduracin α. The two components of haloduracin act synergistically to achieve their bioactivity. Halα binds to lipid II with high affinity to inhibit peptidoglycan biosynthesis.[23] Halβ either does not bind to lipid II or has a much reduced affinity, but it is required for optimal bioactivity. These and other observations[70−72] have led to a model that Halα binds lipid II and that this complex is recognized by Halβ, but experimental evidence of the binding interactions of Halβ are lacking.

To probe Halα localization on bacterial cells, we added rho-Halα at sublethal concentrations to *B. subtilis* 168 cells and analyzed the cells by confocal fluorescence microscopy. Similar to the results seen with rho-lacticin 481, the most intense fluorescent signal was at midcell (Figure 6A). In addition, a staining pattern of peripheral dots with weak transverse bands was seen on the bacterial sidewall, consistent with binding of

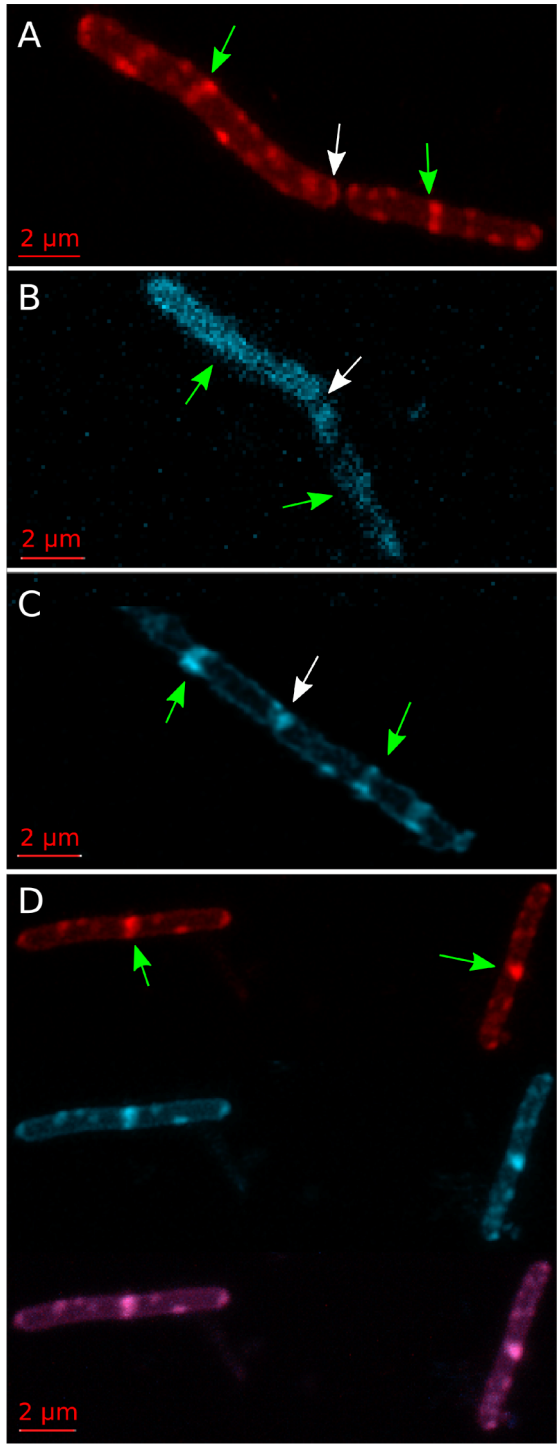

Figure 6. Cellular distribution of haloduracin analogues. (A) Staining of *B. subtilis* 168 with 0.25 μM rho-Halα (red). (B) Staining of *B. subtilis* 168 with 4 μM Cy5-Halβ (blue). (C) Staining of *B. subtilis* 168 with 1 μM each of wt Halα + Cy5-Halβ (blue). (D) Staining of *B. subtilis* 168 with 1 μM each of rho-Halα (red) + Cy5-Halβ (blue). Top, rhodamine channel (red); center, Cy5 channel (blue); bottom, merge of rhodamine and Cy5 (purple). New and old cellular division sites are represented by green and white arrows, respectively.

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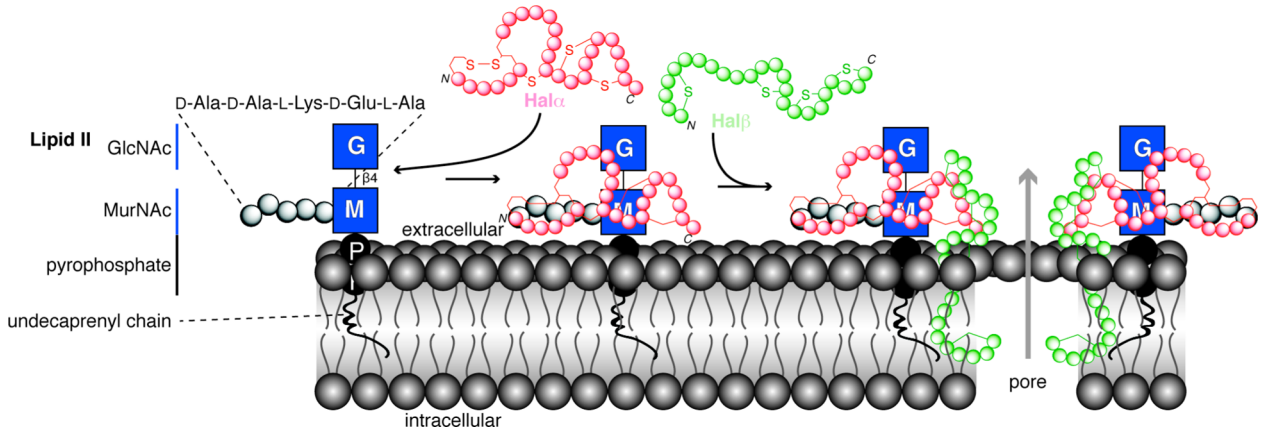

Figure 7. Proposed mode of action of the two-component lantibiotic haloduracin. First, Hal*α* associates with the cell wall precursor lipid II. Then, Hal*β* binds to the Hal*α*-lipid II complex causing pore formation. GlcNAc, *N*-acetylglucosamine (blue squares labeled G); MurNAc, *N*-acetylmuramic acid (blue squares labeled M). The molecular details of the binding interaction of Hal*α* and lipid II are not known.

Hal*α* to lipid II. However, when the experiment was repeated in the presence of 50 equiv of wt Hal*α*, no fluorescent staining was observed, suggesting that the unlabeled peptide competes for the same binding site on the cell surface (Figure S8B). When rho-Hal*α* was preincubated with 50 equiv of wt Hal*β* (25 *μ*M), rho-Hal*α* still localized at the midcell and poles (Figure S8C), but the transverse bands were less frequently observed. These observations suggest that Hal*β* does not compete with Hal*α* for its binding target. We next administered Cy5-Hal*β* to *B. subtilis* cells. Consistent with the model, no specific fluorescent pattern was observed (Figure 6B). Some nonspecific labeling was seen, likely because of a hydrophobic interaction between the lantibiotic and bacterial membrane. This interaction is expected to increase upon addition of the hydrophobic Cy5 functionality. Interestingly, when Cy5-Hal*β* and unlabeled Hal*α* were added in concert, the Hal*β* analogue did localize in a specific pattern (Figure 6C). Preferential staining was observed at midcell and poles. Furthermore, coincubation of rho-Hal*α* and Cy5-Hal*β* with *B. subtilis* cells showed colocalization of Hal*α* and Hal*β* (Figure 6D). To rule out a role for the cyanine group of Cy5-Hal*β*, the microscopy experiments were also conducted with rho-Hal*β*. As anticipated, rho-Hal*β* localized in a site-specific pattern on *B. subtilis* cells only in the presence of Hal*α* (Figure S9). The localization of rho-Hal*β* in the presence of wt Hal*α* was not altered in the presence of 50 equiv of Hal*β* (25 *μ*M); higher concentrations could not be used because of toxicity to the cells, which is already starting to be visible at 25 *μ*M Hal*β*.

DISCUSSION

This study demonstrates the successful use of the biosynthetic machinery of five different lanthipeptides to introduce a ketone group at their N-termini, and illustrates the utility of the method for site-specific labeling of this class of compounds. This strategy has a distinct advantage over other lanthipeptide-specific labeling techniques such as introduction of non-proteinogenic amino acids[49,73] because of its generality, scalability, and relatively high yields. Several structurally unique N-termini were successfully modified including lanthipeptides containing an N-terminal disulfide bond (Hal*α*) and an N-terminal lanthionine cross-link (Hal*β*). To produce keto-Pcn2.8 and keto-Hal*α*, the only perturbation compared to our previously developed lanthipeptide coexpression system was the insertion of a Thr directly after the proteolytic cleavage site. In these peptides the modification enzyme successfully dehydrated the inserted Thr, and proteolytic removal of the leader peptide yielded the lanthipeptide with an N-terminal ketone. In the biosynthesis of Pcn1.7 and keto-Hal*β*, the inserted Thr was successfully dehydrated but the cleavage of the leader peptide from the core peptide occurred at an undesired location, giving the modified core peptide with an N-terminal amino acid overhang originating from the leader peptide. This problem was overcome in Hal*β* by inserting an Ala directly after the N-terminal Thr, which distanced the proteolytic cleavage site from the lanthionine ring at the N-terminus of Hal*β* (Figure 2). The protease, GluC, could then cleave the leader peptide at the desired site, presumably because the D-stereocenter of the lanthionine ring no longer interfered with protease binding. Conversely, in ProcA1.7 the two amino acids immediately preceding the inserted Thr were mutated to Leu and the leader peptide was removed in a two-step procedure of treatment with GluC and aminopeptidase. The biosynthesis of keto-lacticin 481 proved to be the most challenging and required distancing the inserted Thr from the leader peptide and introduction of two extra copies of the *lctM* gene into the coexpression system.

Using the fluorescent labeling strategy, we were able to visualize the distribution of lacticin 481 and haloduracin on *B. subtilis* 168 cells. The most intense labeling with rho-lacticin 481 was at the new cell division sites at the bacterial midcell, which we believe represents the relatively large amounts of lipid II present during cell division. In most cases, rho-lacticin 481 also stained the sidewall of *B. subtilis* as peripheral dots, which in some images could be seen to be linked by transverse bands. In contrast, incubation of *B. subtilis* with the Glu13Ala mutant of lacticin 481 did not result in a specific pattern, consistent with the previous proposals that this conserved residue is critical to mediate interactions with lipid II in a variety of lantibiotics.[17,19,20,23,24] The results with the fluorescently labeled lacticin 481 mutant also strongly suggest that the observed specific localization pattern is a consequence of the activity of lacticin 481 and not the fluorophore.

We also gained a deeper understanding of the mode of action of the two-component lantibiotic haloduracin using the fluorescently labeled analogues. Incubation of sublethal concentrations of rho-Hal*α* with *B. subtilis* cells showed a staining pattern similar to that seen with rho-lacticin 481, consistent with binding to lipid II. This conclusion is also supported by the observation that unlabeled wt Hal*α* abolished

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the specific labeling pattern of rho-Halα. Rho- and Cy5-Halβ did not exhibit a specific localization pattern unless wt Halα was also present. In the presence of Halα, the fluorescently labeled Halβ analogues localized at the poles and midcell, suggesting colocalization. Localization in transverse bands was also observed but less frequently for unknown reasons. Use of rho-Halα and Cy5-Halβ also showed colocalization at the midcell and poles. Fluorescently labeled Halβ in the presence of Halα could not be displaced from these sites by unlabeled Halβ at the maximum nontoxic concentrations achievable, possibly because of the higher affinity of the fluorescently labeled analogues for the membrane. Collectively, these results support the previously proposed model of synergistic activity, in which Halα binds to lipid II, followed by the binding of Halβ to the Halα-lipid II complex (Figure 7), although the resolution of confocal microscopy is such that colocalization in these experiments does not necessarily mean complex formation. Further experiments will be needed to confirm or refute a direct molecular interaction between Halβ and the Halα-lipid II complex.

■ CONCLUSION

In summary, we report a general labeling strategy for lanthipeptides, which may be used for studies on family members for which the mode(s) of action and targets are currently unknown, such as the prochlorosins and the Pep5/epilancin class of lantibiotics. Potential applications also include use of ^{99m}Tc-labeled lanthipeptides for imaging purposes[5] and introduction of photoaffinity probes.

■ EXPERIMENTAL SECTION

Heterologous Production. The lanthipeptides were expressed using *E. coli* BL21 cells transformed with a single pRSFDuet plasmid containing the *lanA* and *lanM* genes (see SI for plasmid construction). For Halα, two plasmids were used to increase the yield of production (pRSFDuet(*halA1(G−1E-C1 ins T)/halM1* and pET15b(*halA1)G−1E-C1 ins T*)) and for lacticin 481 three plasmids were used (pRSFDuet(*lctA(K1-G2A ins AAALLKT)/lctM*, pACYCDuet(*lctM*), and pCDFDuet(*lctM*)).

Overexpression and Purification of His$_6$-Tagged Modified Peptide Mutants. An overnight culture of *E. coli* BL21 cells was added to an overexpression culture flask containing either terrific broth (TB) or LB broth (1:100; volume overnight culture: volume overexpression culture) and appropriate antibiotics depending on the plasmids used (1:1000 v:v; kanamycin (50 mg/mL), chloramphenicol (25 mg/mL), spectinomycin (25 mg/mL), ampicillin (100 mg/mL)). The culture was then incubated in a 37 °C shaker (220 rpm) until the optical density reached 0.6 (LB) or 1.0 (TB). For HalA1 and HalA2, overexpression was immediately induced with isopropyl β-D-1-thiogalactopyranoside (IPTG, 0.25 mM final concentration) and the flask was incubated overnight in a shaker at 37 °C and 220 rpm. Conversely, for ProcA1.7, ProcA2.8, and LctA, the culture was first placed on ice (15 min). Overexpression was then induced by the addition of IPTG and the flask was incubated overnight in a shaker at 18 °C and 220 rpm. The following morning the cells were harvested by centrifugation (11,867 × *g*, 4 °C, 20 min), the supernatant was discarded and the cells were lysed using a cell homogenizer (Avestin Emulsiflex-C3; 5,000 PSI) in LanA start buffer (1 mL per 100 mL culture; 20 mM NaH_2PO_4, 500 mM NaCl, 0.5 mM imidazole, 20% glycerol, pH 7.5). The soluble and insoluble layers were then separated by centrifugation (22,789 × *g*, 4 °C, 20 min) and the soluble layer was saved for purification. The insoluble layer was suspended in LanA lysis buffer (1 mL per 100 mL culture; 6 M guanidine HCl, 20 mM NaH_2PO_4, pH 7.5, 500 mM NaCl, 0.5 mM imidazole) and the cells were lysed by sonication. The soluble and insoluble layers were again separated by centrifugation (22,789 × *g*, 4 °C, 20 min) and the modified His$_6$-LanA peptide was purified, separately, from the filtered soluble layers (0.45 μm) using a Ni HisTrap HP column (5 mL, GE Healthcare). The column was washed with LanA wash buffer (4 M guanidine-HCl, 20 mM NaH_2PO_4, pH 7.5, 500 mM NaCl, 30 mM imidazole) to remove nonspecific binding proteins. The His$_6$-LanA peptide was eluted with LanA elute buffer (3 × 5 mL, 4 M guanidine HCl, 20 mM NaH_2PO_4, 100 mM NaCl, 500 mM imidazole, pH 7.5). Finally, the peptide was further purified by preparative reversed phase high-performance liquid chromatography (RP-HPLC) (described in the SI) and lyophilized.

Proteolytic Cleavage of LanA Mutants. To lyophilized peptide was added HEPES buffer (1 mL per 5 mg of peptide, pH 7.5, 50 mM final concentration) and the necessary protease (5 μL/L overexpression culture; stock solutions used: LysC = 3 U/100 μL, GluC = 2 mg/mL, trypsin = 40 μM). To the HalA1 mutant reaction was also added TCEP (1 mM final concentration). The reaction was incubated overnight at room temperature (GluC, trypsin) or at 37 °C (LysC). The following morning the progress of the reaction was analyzed by MALDI-TOF MS and additional enzyme was added in 5 μL aliquots if the reaction was not complete. Once complete, the products were purified by preparative RP-HPLC (described in SI).

Aminopeptidase Cleavage of GluC-Cleaved ProcA1.7(G−2L/G−1L). To a reaction vessel was added HPLC-purified GluC-cleaved ProcA1.7(G−2L/G−1L), HEPES buffer (1 mL per 5 mg of peptide, pH 7.5, 100 mM final concentration), and aminopeptidase (A8200, 6 μL/1 mg peptide, 100 U/mL stock solution). The reaction was incubated at 37 °C for 24 h. The reaction product was used in a bioconjugation reaction with ***1*** without further purification.

Representative Bioconjugation of keto-LanA and *1*. To a reaction vessel was added keto-LanA (1.45 μmol) and ***1*** (14.5 μmol, 1.54 mg, 10 equiv). Next, a solution of H_2O (2.04 mL), acetate buffer (75 μL, 4 M, pH 4.75, final concentration 100 mM), and aniline (885 μL, 339 mM, final concentration 100 mM) was added. This solution was deoxygenated with N_2 prior to addition to prevent large amounts of oxidized product. The reaction was stirred for 4 h at room temperature. The crude material was then purified by preparative RP-HPLC.

Representative Bioconjugation of keto-LanA and *2* or *3*. To keto-LanA (0.48 μmol) in a 15 mL conical vial was added ***2*** or ***3*** (24.2 μmol, 50 equiv) dissolved in a degassed solution of acetate buffer (100 mM, 2 mL). To this solution was added *p*-phenylenediamine (86.4 mg, 400 mM final concentration) and the reaction was vortexed to dissolve the catalyst. The reaction was incubated for 3 h and diluted with 60% MeCN, 0.1% TFA (2 mL). The crude material was then purified by preparative RP-HPLC, except rho-Halα, which was purified by several rounds of analytical RP-HPLC to separate the fluorescently labeled peptide from a rhodamine impurity. Excess ***2*** or ***3*** was recovered during HPLC purification and used in subsequent reactions. For yields of each individual bioconjugation, see the Supporting Information.

Confocal Fluorescence Microscopy with *B. subtilis*. A 150 μL aliquot from an overnight culture of *B. subtilis* 168 was used to inoculate LB (5 mL) and the culture was grown to an OD of 0.5. This culture was transferred into 2.0 mL Eppendorf tubes (1.5 mL) and centrifuged at 1,431 × *g* for 2 min in a microfuge. Into the tubes with cells were added cold solutions of fluorescently labeled lantibiotic at the desired concentration in 1X Dulbecco's Phosphate-Buffered Saline (D-PBS) (1 mL, Fisher Scientific). The reactions were kept on ice for 30 min. At this time the reactions were centrifuged at 4 °C (1 min, 1,431 × *g*). The supernatant was carefully removed and the cells were carefully suspended in 1 mL of 1X D-PBS. The solution was again centrifuged, the supernatant was removed, and cells were carefully suspended in 1 mL of 1X D-PBS. Finally, the suspension was centrifuged and D-PBS was removed to give a suspension of around 200 μL. Aliquots of the suspension (10 μL) and liquified low gelling temperature agarose (10 μL, 1.5%) were added to a microscopy plate and the localization of the lantibiotic was analyzed by confocal fluorescence microscopy (for details, see SI).

Minimum Inhibitory Concentration Determination. 96-well and 48-well microtiter plates (Corning Costar) were utilized for

anaerobic and aerobic cultures, respectively. For 96-well plates, each well contained 90 μL of an overnight culture containing *L. lactis* HP (approximately 1×10^8 CFU mL^{-1}) and a 10× stock of peptide (10 μL) of the desired concentration. The plates were incubated at 30 °C overnight with no agitation. For 48-well plates, each well contained 180 μL of an overnight culture containing *B. subtilis* 168 (approximately 1×10^8 CFU mL^{-1}) and a 10× stock of peptide (20 μL) of the desired concentration. The plates were incubated at 30 °C overnight with moderate shaking. In addition, each 96- and 48-well plate contained several blank (growth media with no bacteria) and control (SDW in place of peptide) wells. The optical density at 630 nm (OD_{630}) was recorded at hourly intervals from 0 to 6 h and a final recording at 22−24 h. For experiments with Cy5-Hal*β*, the optical density was read at 570 nm (OD_{570}) to minimize the undesired excitation of Cy5. The MIC was determined as the lowest concentration at which no cell growth was observed after 22−24 h.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures for plasmid construction, purification and characterization of LanA analogues, as well as supporting figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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