

# Facile Removal of Leader Peptides from Lanthipeptides by Incorporation of a Hydroxy Acid

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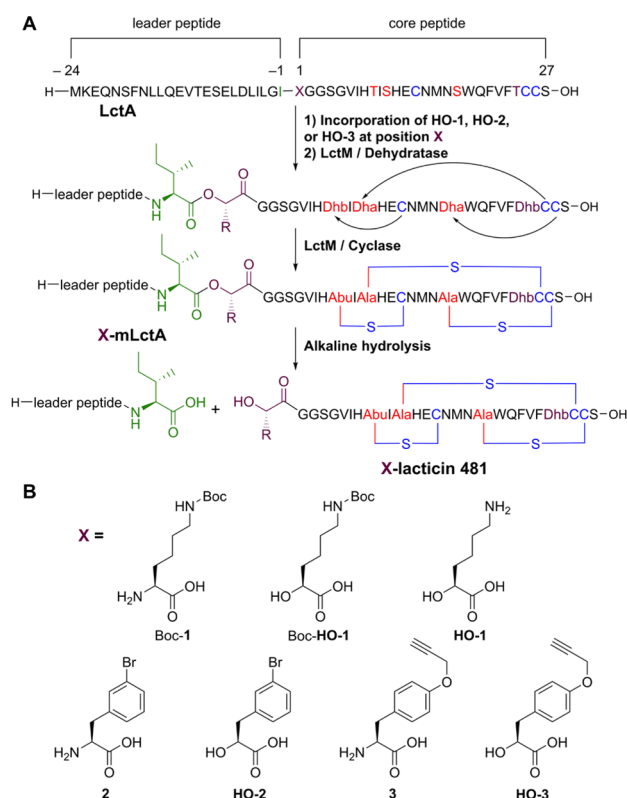
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**S** Supporting Information

**ABSTRACT:** The biosynthesis of ribosomally synthesized and post-translationally modified peptide (RiPP) natural products typically involves a precursor peptide which contains a leader peptide that is important for the modification process, and that is removed in the final step by a protease. Genome mining efforts for new RiPPs are often hampered by the lack of a general method to remove the leader peptides. We describe here the incorporation of hydroxy acids into the precursor peptides in *E. coli* which results in connection of the leader peptide via an ester linkage that is readily cleaved by simple hydrolysis. We demonstrate the method for two lantibiotics, lactacin 481 and nukacin ISK-1.

Recent genome sequencing efforts have revealed that ribosomally synthesized and post-translationally modified peptides (RiPPs) comprise a much larger class of natural products than previously anticipated.<sup>1</sup> The relatively short RiPP biosynthetic pathways lend themselves well to heterologous expression and genome mining,<sup>2–6</sup> and the gene-encoded precursor peptides make them attractive for bioengineering efforts.<sup>7–15</sup> Based on the currently sequenced bacterial genomes, the lanthionine-containing peptides (lantipeptides) are the largest class of RiPPs. They are characterized by intramolecular thioether cross-links called lanthionine (Lan) and methyllanthionine (MeLan). Lantipeptides are biosynthesized from a linear peptide, generically called LanA (e.g., LctA for lactacin 481, NukA for nukacin ISK-1). They consist of a C-terminal core region that is processed into the mature lanthipeptide and an N-terminal leader region important for recognition by the post-translational modification enzymes (Figure 1A). For class II lanthipeptides, LanM enzymes dehydrate specific Ser and Thr residues to dehydroalanine (Dha) and dehydrobutyrine (Dhb), respectively. The enzyme then promotes Michael-type additions of Cys residues to the dehydro amino acids (e.g., Figure 1A). The complex ring topology provides the lanthipeptides with restricted conformations that bestow antimicrobial properties on a large subset of family members called lantibiotics.<sup>16</sup> Several lantibiotics, including derivatives of nukacin ISK-1 and lactacin 481,<sup>17,18</sup> have been produced in *Escherichia coli* by coexpression of the precursor peptides with LanM.<sup>19–21</sup> However, the removal of the leader peptide often presents a major hurdle. In the producing organisms, a protease removes the leader peptide by

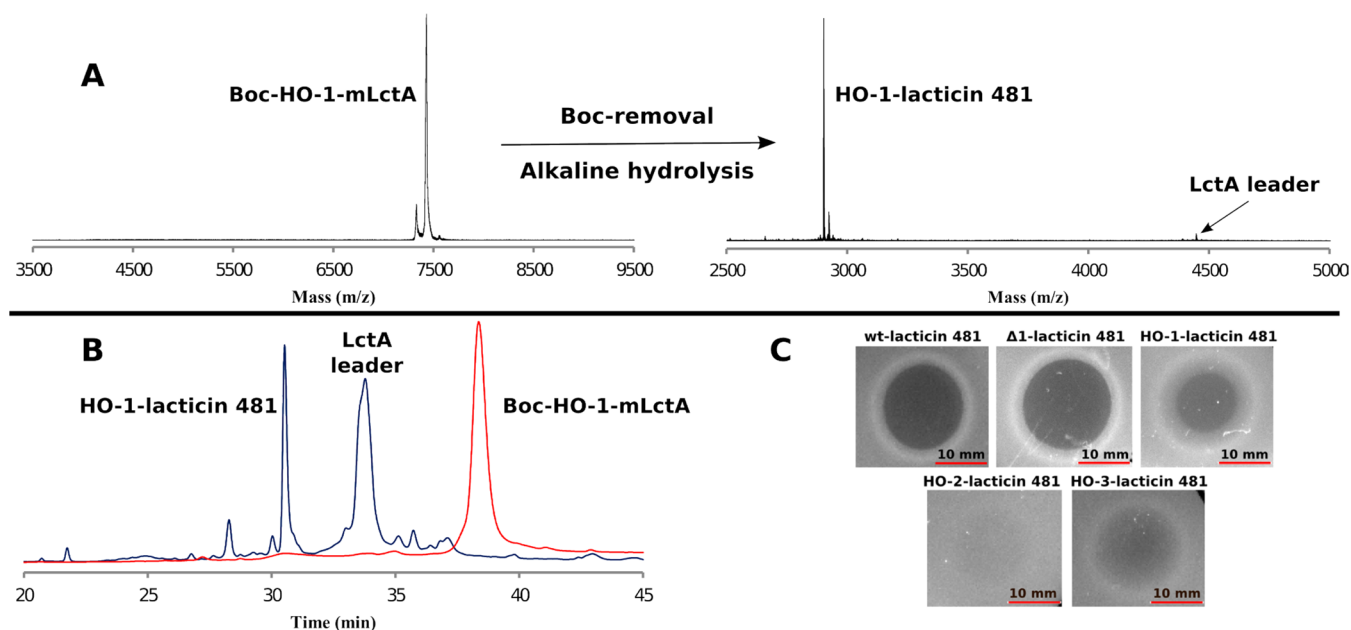


**Figure 1.** (A) Biosynthesis of lactacin 481 analogues by incorporating Boc-HO-1, -HO-2, or HO-3 at position 1 of the core peptide (X) using the amber stop codon suppression method. Wild-type lactacin 481 contains a lysine at position 1. Also, position -1 (green) was mutated from Ala to Ile as described in the text. (B) Structures of non-proteinogenic amino and hydroxy acids introduced into lactacin 481 or nukacin ISK-1 at residue 1 (X).

proteolysis after residue -1, the last amino acid of the leader peptide (Figure 1A). Unfortunately, the protease domain of class II lanthipeptides are membrane associated as part of an ATP-binding cassette transporter (LanT), and the soluble protease domains have low in vitro activity.<sup>21,22</sup> The use of commercial proteases (e.g., trypsin, LysC, or GluC) to remove the leader peptide, by mutating the -1 position on LanA to Arg, Lys, or Glu, is a common leader peptide removal strategy,

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**Figure 2.** (A) MALDI-TOF MS spectra depicting fully modified LctA containing Boc-HO-1 at position 1 (Boc-HO-1-mLctA), and the Boc-removal from and alkaline hydrolysis of Boc-HO-1-mLctA, yielding HO-1-lactacin 481 and LctA leader peptide. The leader peptide ionizes poorly in the mass spectrum, but is seen clearly by HPLC. Boc-HO-1-mLctA ( $[M + H]^+$ ; calcd = 7432 Da; obsd = 7428 Da). HO-1-lactacin 481 ( $[M + H]^+$ ; calcd = 2901.3 Da; obsd = 2901.3 Da; monoisotopic mass spectrum). (B) Analytical RP-HPLC trace of HO-1-mLctA before (red) and after (blue) Boc removal and alkaline hydrolysis. (C) Antimicrobial assays of lactacin 481 and analogues against *L. lactis* HP. A total of 10  $\mu$ L of 12.5  $\mu$ M compound solution was added to each spot. Plates were incubated at 30  $^{\circ}$ C for 18 h.

but it is only possible if the core peptide does not contain the proteolytic cleavage site.<sup>19,23–27</sup> Indeed, in previous studies that produced lactacin 481 in *E. coli*, removal of the LctA leader peptide was achieved with endoproteinase LysC, which cleaved after residue 1, producing analogues lacking the N-terminal lysine residue.<sup>28</sup> A longer protease recognition site such as Factor Xa or TEV protease could overcome cleavage in the core peptide, but introduction of longer sites often interferes with post-translational processing, or the proteolysis step is prevented because of the introduction of non-proteinogenic structures at the P1' site (amino acid C-terminal to the cleavage site).<sup>24,29,30</sup> A photolabile linker has been employed to produce lactacin 481 that alleviates any sequence dependence, but the linker was introduced by solid-phase peptide synthesis and is not amenable to heterologous lanthipeptide biosynthesis.<sup>31</sup> In this work we developed a chemical leader peptide removal strategy that can be used with bacterially expressed lanthipeptides and, in principle, also other RiPPs.

Previous studies have demonstrated that the ribosomal peptide synthesis machinery of *E. coli* can incorporate hydroxy acids.<sup>32,33</sup> The resulting ester bonds can then be site-specifically cleaved by alkaline hydrolysis.<sup>34,35</sup> Therefore, we investigated whether a hydroxy acid could be incorporated in the first position of a lanthipeptide without interfering with the post-translational modifications. We demonstrate herein that this methodology is successful for the *in vivo* production and subsequent leader peptide removal of analogues of lactacin 481 and nukacin ISK-1.

We chose lactacin 481 because of the difficulty of removing its leader peptide without removing the N-terminal Lys. Recently, the pyrrolysyl-tRNA synthetase (PylRS)-tRNA<sub>CUA</sub><sup>Pyl</sup> (PylT) pair,<sup>36</sup> which naturally incorporates pyrrolysine (Pyl) in response to the amber stop codon (UAG), was shown to incorporate the hydroxy acid of Boc<sup>L</sup>-lysine (Boc-1, Figure

1B).<sup>37</sup> To ensure that the lactacin synthetase LctM would fully modify the LctA precursor peptide analogue, we first attempted incorporation of Boc-1 into LctA using PylRS. Duet plasmids were designed to contain *pylRS*, *pylT*, *his<sub>6</sub>-lctA(A-11/K1X)*, and *lctM* for coexpression in *E. coli* (Supporting Information). The codon for the first amino acid of the LctA core region was replaced with the amber stop codon *tag* such that non-canonical amino or hydroxy acids (X, Figure 1) could be incorporated. In addition, the last residue of the leader peptide was mutated from Ala to Ile (A-11) because previous studies have shown that cleavage of ester bonds in a peptide backbone is slow when the ester is preceded by a large hydrophobic residue.<sup>35</sup>

N-Terminally His<sub>6</sub>-tagged LctA(A-11/K1X), LctM, PylRS, and PylT were expressed in media supplemented with Boc-1. After purification of the peptide by immobilized metal affinity chromatography (IMAC), analysis by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) demonstrated that LctA was fully modified (mLctA) with Boc-1 incorporated (Boc-1-mLctA; Figure S1). When the coexpression was performed in the absence of Boc-1, the full-length LctA analogue was not detected. Incorporation of hydroxy acid Boc-HO-1 (Figure 1B) was attempted next. His<sub>6</sub>-LctA(A-11/K1X) was coexpressed with LctM, PylRS, and PylT in the presence of Boc-HO-1, purified by IMAC and reversed-phase high-performance liquid chromatography (RP-HPLC), and analyzed by MALDI-TOF MS. Indeed, the hydroxy acid was incorporated into LctA, which was fully modified by LctM (Boc-HO-1-mLctA; Figure 2A).

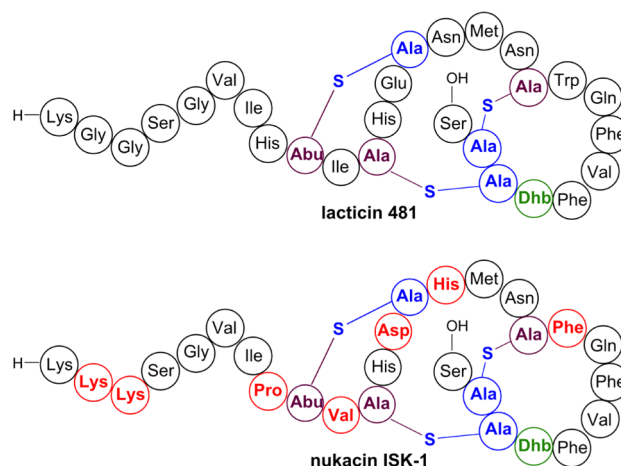
To extend the ester backbone insertion to amino acids other than Lys, we next investigated a mutant form of PylRS (mut-PylRS) that can load analogues of the amino acids Phe and Tyr onto tRNA<sub>CUA</sub><sup>Pyl</sup>, including 3-bromo-L-phenylalanine (H-Phe(3-Br)-OH; 2) and O-propargyl-L-tyrosine (H-Tyr(propargyl)-

OH; 3).<sup>38–40</sup> Indeed, compounds **2** and **3** were successfully incorporated in position 1 of LctA, and the resulting mutants were fully modified by LctM in *E. coli* to produce 2-mLctA and 3-mLctA (Figure S1). Unlike the observations with Boc-1, a very small peak corresponding to L-Phe incorporation at the amber stop codon was also observed by MALDI-TOF MS (Figure S1), consistent with weak recognition of L-Phe by mut-PylRS.<sup>38</sup> However, incorporation of L-Phe was very minor compared to incorporation of **2** or **3**, suggesting the non-proteinogenic amino acid outcompetes L-Phe in vivo. Introduction of the corresponding hydroxy acids was then investigated by coexpression of His<sub>6</sub>-LctA(A-11/K1X) with LctM, mut-PylRS, and PylT in media containing HO-Phe(3-Br)-OH (**HO-2**) or HO-Tyr(propargyl)-OH (**HO-3**; Figure 1B). IMAC and RP-HPLC purification followed by MALDI-TOF MS analysis demonstrated that the major products were fully modified LctA analogues containing **HO-2** and **HO-3** (**HO-2**-mLctA and **HO-3**-mLctA; Figures S2 and S3).

To test the hydrolytic cleavage of the leader peptides, the Boc group of Boc-**HO-1**-mLctA was first removed by incubating the modified peptide in 5% TFA at 50 °C. The pH was then increased to 10.5 to facilitate hydrolysis. Analysis by MALDI-TOF MS and RP-HPLC showed consumption of the full-length LctA analogue and production of the leader peptide and the anticipated lactacin analogue with a hydroxyl group at its N-terminus (**HO-1**-lactacin 481) (Figures 2A,B and S2). The bioactivity of **HO-1**-lactacin 481 was compared to wild-type (wt) lactacin 481, isolated from *Lactococcus lactis* subsp. *lactis* 481, and Δ1-lactacin 481 heterologously produced in *E. coli* and proteolyzed with LysC. Both agar diffusion antimicrobial assays (Figure 2C) and liquid media growth assays (Figure S4) with *L. lactis* HP cells showed that **HO-1**-lactacin 481 (IC<sub>50</sub> = 200 ± 70 nM) exhibited decreased activity compared to wt lactacin 481 (IC<sub>50</sub> = 100 ± 25 nM) and Δ1-lactacin 481 (IC<sub>50</sub> = 90 ± 20 nM). Collectively, these data show that the N-terminal Lys is not as important as previously suggested based on studies on a mutant that lacked the N-terminal Lys but also had two other mutations compared to wt lactacin 481.<sup>41</sup>

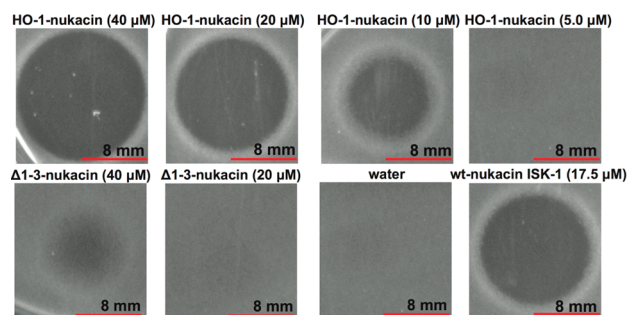
Alkaline hydrolysis of **HO-2**-mLctA and **HO-3**-mLctA successfully produced lactacin 481 analogues with the desired N-terminal mutations (**HO-2**-lactacin 481 and **HO-3**-lactacin 481) (Figure S2). The antimicrobial activities of **HO-2**- and **HO-3**-lactacin 481 were compared with that of **HO-1**-lactacin 481 by agar diffusion assays (Figure 2C) and liquid media growth assays (Figure S4) with *L. lactis* HP cells. **HO-1**-lactacin 481 was >10-fold more active than **HO-2**-lactacin 481 (IC<sub>50</sub> = 3.50 ± 0.15 μM) and more than 3-fold more active than **HO-3**-lactacin 481 (IC<sub>50</sub> = 700 ± 280 nM).

We next applied the methodology to nukacin ISK-1 (Figure 3) because, like lactacin 481, it has an N-terminal lysine. Moreover, in all previous attempts to biosynthesize nukacin ISK-1 in *E. coli*, the removal of the leader peptide was achieved with endoproteinase LysC, which removed the first three residues of the core peptide yielding Δ1-3-nukacin ISK-1 and decreasing the bioactivity of the compound 32-fold.<sup>17,42</sup> A coexpression vector was constructed encoding NukM and His<sub>6</sub>-NukA. After expression in *E. coli*, His<sub>6</sub>-mNukA was purified by IMAC and RP-HPLC. Removal of the leader peptide with LysC resulted in Δ1-3-nukacin ISK-1 as shown by MALDI-TOF MS (Figure S5). The incorporation of hydroxy acid HO-1 into His<sub>6</sub>-NukA(A-11/K1X) was then attempted. Coexpression of the NukA mutant with NukM, PylRS, and PylT in the



**Figure 3.** Structures of the lanthipeptides lactacin 481 and nukacin ISK-1. Residues in nukacin ISK-1 that differ from lactacin 481 are highlighted in red.

presence of Boc-**HO-1** resulted in cyclized NukA containing the hydroxy acid (Boc-**HO-1**-mNukA; Figure S6). The Boc-group of Boc-**HO-1**-mNukA was removed with TFA followed by alkaline hydrolysis yielding nukacin ISK-1 with an N-terminal hydroxyl group (**HO-1**-nukacin ISK-1; Figure S6). The nukacin ISK-1 analogues were then tested by agar diffusion antimicrobial assays (Figure 4) and liquid media growth assays



**Figure 4.** Zones of growth inhibition caused by nukacin ISK-1 and analogues against *L. lactis* HP. A total of 10 μL of solution at the concentrations listed was applied to each spot.

(Figure S4) against *L. lactis* HP and compared to authentic nukacin ISK-1 isolated from the producer strain *Staphylococcus warneri* JCM 11004. The wt compound (IC<sub>50</sub> = 400 ± 80 nM) was more active than **HO-1**-nukacin ISK-1 (IC<sub>50</sub> = 560 ± 100 nM), indicating that the N-terminal amine of nukacin ISK-1 contributes to the bioactivity. Importantly, **HO-1**-nukacin ISK-1 displayed much stronger bioactivity than Δ1-3-nukacin ISK-1 (IC<sub>50</sub> = 5900 ± 2500 nM), which was only weakly bioactive, in agreement with a previous study.<sup>42</sup> Thus, unlike the observations with lactacin 481, the use of an ester linkage between the leader and core peptides resulted in a considerably more active product than use of endoproteinase LysC, illustrating the utility of this approach for lantibiotics with N-termini important for bioactivity. Another important potential application of this method is in synthetic biology efforts, as RiPPs are well suited for generation of non-natural cyclic compounds/scaffolds that can be screened/selected for various purposes.<sup>12,13</sup> In this arena, there is no obvious inherent advantage or disadvantage whether the peptide contains an N-terminal amino or hydroxyl group at the start of the selection

process. However, the ability to readily remove the leader peptide in a general manner without the need to screen various proteases is highly enabling.

In conclusion, we produced analogues of lactacin 481 and nukacin ISK-1 by hydrolytic removal of the leader peptide. Unlike methods using commercial proteases, the ester bond hydrolysis reaction is not sequence-dependent and is completely site-selective. Furthermore, the method can be readily multiplexed. This general methodology may therefore find use for lanthipeptides and other classes of RiPPs, and many applications can be envisioned in synthetic biology when an N-terminal amino group is not important.

## ■ ASSOCIATED CONTENT

### ■ Supporting Information

Experimental procedures and supporting figures. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b04681.

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### Notes

The authors declare no competing financial interest.

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