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Research Article

Synthesis and Characterization of Heterodimers and Fluorescent Nisin Species by Incorporation of Methionine Analogues and Subsequent Click Chemistry

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Cite This: *ACS Synth. Biol.* 2020, 9, 2525−2536

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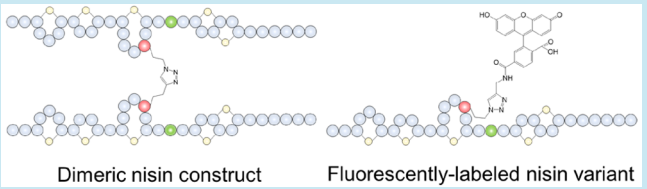
ABSTRACT: Noncanonical amino acids form a highly diverse pool of building blocks that can render unique physicochemical properties to peptides and proteins. Here, four methionine analogues with unsaturated and varying side chain lengths were successfully incorporated at four different positions in nisin in *Lactococcus lactis* through force feeding. This approach allows for residue-specific incorporation of methionine analogues into nisin to expand their structural diversity and alter their activity profiles. Moreover, the insertion of methionine analogues with biorthogonal chemical reactivity, e.g., azidohomoalanine and homopropargylglycine, provides the opportunity for chemical coupling to functional moieties and fluorescent probes as well as for intermolecular coupling of nisin variants. All resulting nisin conjugates retained antimicrobial activity, which substantiates the potential of this method as a tool to further study its localization and mode of action.



KEYWORDS: *nisin, methionine analogues, click chemistry, dimers, fluorescence*

Lantibiotics are antimicrobial peptides harboring unusual post-translationally modified amino acid residues such as dehydroalanine (Dha) and dehydrobutyrine (Dhb), lanthionine (Lan) and methyllanthionine (MeLan), that are introduced by a promiscuous post-translational modification (PTM) machinery.[1,2] The unique biosynthetic pathways and relatively low genetic complexity of biosynthesis make lantibiotics good candidates for synthetic biology and bioengineering, to expand the antimicrobial arsenal.[2] Various synthetic and biosynthetic strategies have been developed to increase the diversity of lantibiotics.[3−5] The uncommon amino acids (Dha, Dhb, Lan, MeLan) in lantibiotics play an important role in their biological activity and structural stability. Other noncanonical amino acids (ncAAs) offer a further highly diverse pool of building blocks that can introduce unique physicochemical properties.[6] By incorporating non-natural functional groups with unique features, we can dramatically expand the chemical and functional space of lantibiotic structures and enable the design of novel lantibiotics with enhanced properties (e.g., stability, specificity, bioavailability, and half-life).[7−9] The use of this approach allows for the *in vivo* production of new lantibiotics with an expanded amino acid repertoire.[8] Among ncAAs, the analogues of methionine are of particular interest, as some of them (e.g., azidohomoalanine and homopropargylglycine) possess unique reactive groups which can serve as chemical handles to conjugate with fluorophores, glycans, PEGs, lipids, peptide moieties, and other antimicrobial moieties through click chemistry.[10]

Copper(I)-catalyzed azide−alkyne cycloaddition (CuAAC), referred to as "click chemistry", was first reported by Sharpless and co-workers in 2001.[11] It is a region-selective copper(I) catalytic cycloaddition reaction between an azide and an alkyne that gives rise to a triazole. Peptide modification using click chemistry has been the subject of several studies for the development of target-specific bacterial probes and for expanding their bioactivity and application.[12−17] Prompted by these recent reports, we used nisin as a model to explore the potential of this approach for lantibiotic engineering. Nisin is the best studied lanthipeptide to date.[18] It is produced by *Lactococcus lactis* and has potent activity against a broad spectrum of Gram-positive bacteria, including many antibiotic-resistant organisms, such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant Enterococci (VRE). Mature nisin, encoded by *nisA* as a linear precursor peptide (57 aa) that consists of a leader peptide (23 aa) and a propeptide to be modified (34 aa), is released after modification and cleavage of the leader.[19] Gratifyingly, the modification machinery of nisin has a broad substrate specificity, which

Received: June 9, 2020
Published: July 28, 2020

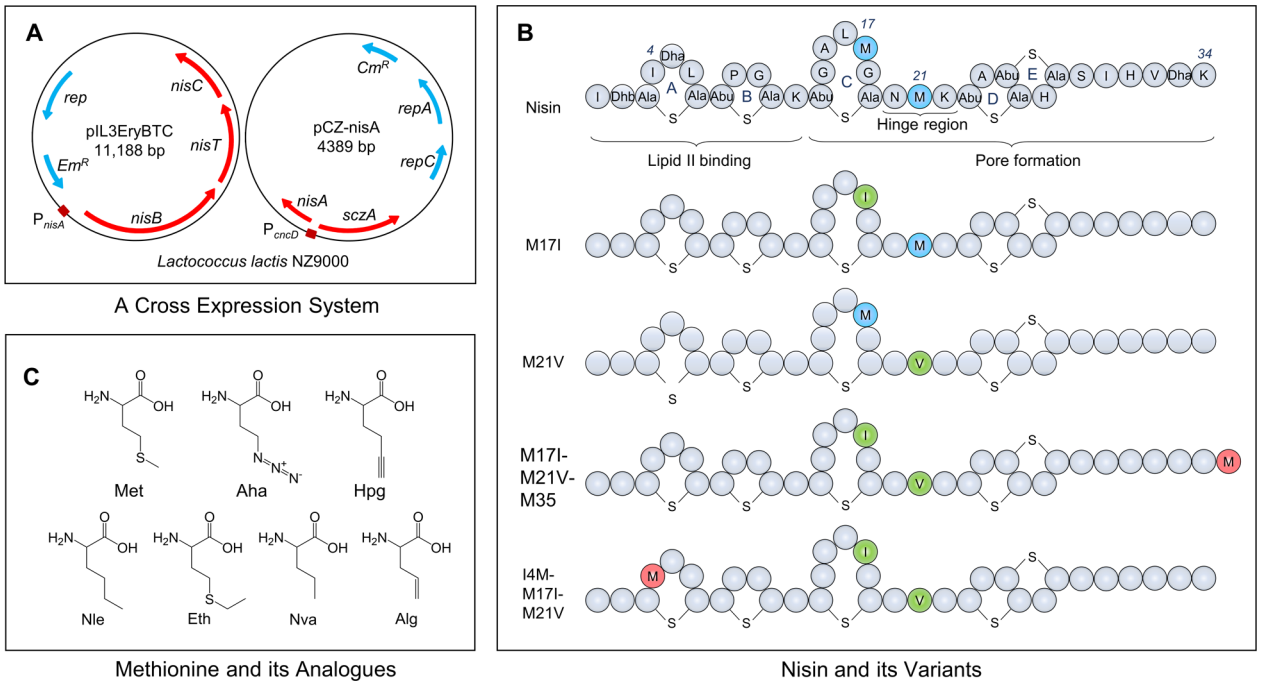


Figure 1. (A) A cross expression system with two plasmids. *SczA*, encoding the repressor of P_{czcD}; P_{czcD}, a zinc inducible promoter; *nisA*, encoding NisA; *repA* and *repC*, encoding plasmid replication proteins; *CmR*, chloramphenicol resistance gene; P_{nisA}, a nisin inducible promoter; *nisB*, encoding NisB; *nisT*, encoding NisT; *nisC*, encoding NisC; *EmR*, erythromycin resistance gene. (B) Peptide sequence of nisin and nisin derivatives. Lipid II binding site (rings AB), pore formation domain (rings CDE), and hinge region (NMK) are indicated; Positions 17, 21, and 35, which served to incorporate methionine analogues of nisin are indicated; Dha, dehydroalanine; Dhb, dehydrobutyrine; A-S-A, lanthionine; Abu-S-A, methyllanthionine; In blue, wildtype Met positions; In green, Met residues replaced by Ile or Val; In red, Met residues at novel positions. (C) Structures of methionine and its analogues. Met, L-methionine; Aha, L-azidohomoalanine; Hpg, L-homopropargylglycine; Nle, L-norleucine; Eth, L-ethionine; Nva, L-norvaline; Alg, L-allyglycine.

allows for the divergence from the original core-peptide to produce variant peptides.

Here, we describe the incorporation of six different methionine analogues with unsaturated, unique chemical handles and varying side chain length, *i.e.*, Aha (azidohomoalanine), Hpg (homopropargylglycine), Nle (norleucine), Eth (ethionine), Nva (norvaline), and Alg (allyglycine), at four different positions in the lantibiotic nisin by using a methionine auxotrophic strain of *Lactococcus lactis*. Previous studies have shown that with mutations at sites I4, M17, and M21, nisin can retain or even have enhanced bioactivity.[20] To broaden the structural diversity and test the effects of single replacements of methionine with respective analogues, four single methionine nisin mutants, *i.e.*, M17I, M21V, M17I-M21V-M35, and I4M-M17I-M21V, were constructed. As methionine is essential for the synthesis of post-translational modification enzymes, a cross-expression system was developed utilizing separate promoters, allowing for the separate induction of expression of target genes and biosynthetic enzymes. The amino acid replacement and incorporation efficiency of ncAAs into nisin derivatives were determined by matrix assisted laser desorption/ionization time-of-flight analyzer (MALDI-TOF) and liquid chromatography−mass spectrometry (LC-MS). Twelve nisin derivatives were purified in large scale by HPLC and their antimicrobial activities were determined. In addition, six Aha- or Hpg-containing nisin derivatives were coupled either mutually or with nisin ABC-azide (A, B, and C denoting the first three lanthionine rings of nisin; Figure 1), Cy5-azide and 6-FAM-alkyne through click chemistry to obtain six dimeric nisin constructs, three nisin hybrids, and six fluorescently labeled nisin variants.

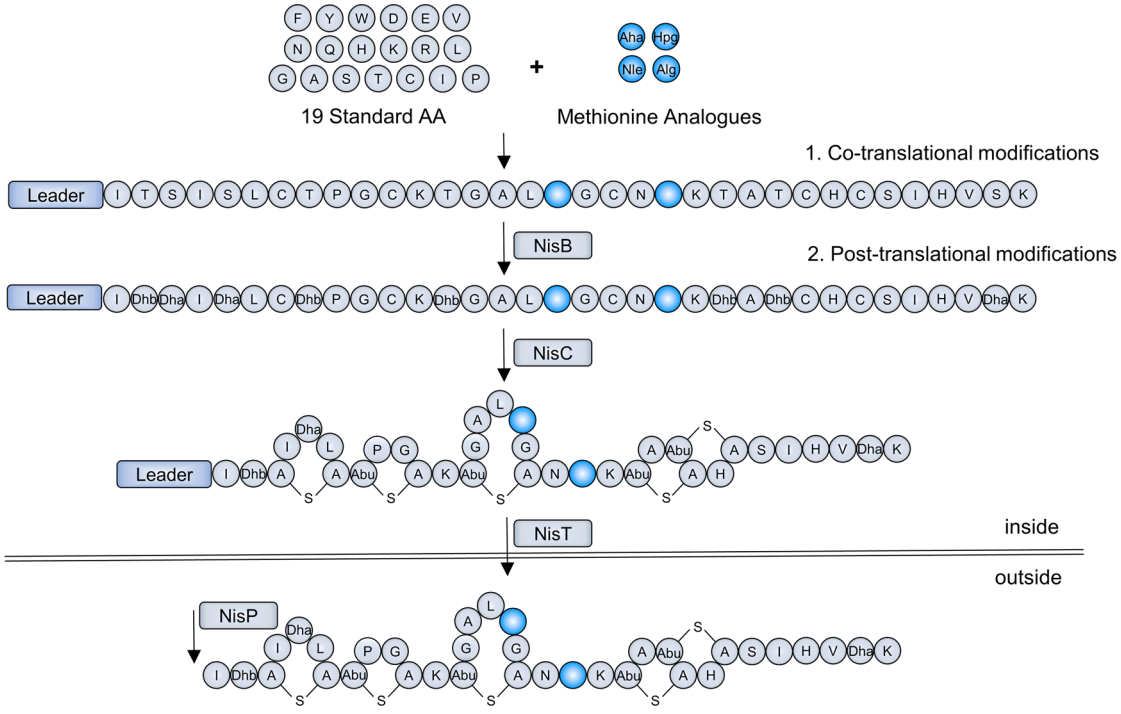
RESULTS AND DISCUSSION

A Cross Expression System to Incorporate Methionine Analogues into Nisin by Use of *L. lactis* as a Host. Two *in vivo* approaches have been developed for incorporating ncAAs into peptides.[8] The first approach is "site-specific incorporation".[21] For this method, the coexpression of orthogonal amber suppressor aminoacyl-tRNA synthetase (AARS/tRNA) pairs is necessary. Specific mutations can be introduced into the peptide sequence by reassigning the amber nonsense stop codon during translation. However, the screening and development of orthogonal AARS/tRNA pairs is time-consuming and the production yield of this method is extremely low. Conversely, "residue-specific incorporation", the second approach, that generally does not suffer from such drawback, is a more promising strategy.[22] This method typically involves replacing natural amino acids with the ncAAs of interest by using auxotrophic strains. It is able to generate broad and efficient structural diversity by directly incorporating ncAAs *via* translation into bioactive peptides.

Various expression hosts have been developed for the incorporation of ncAAs.[7] Until now, the Gram-negative *Escherichia coli* is the only prokaryotic expression host used for the incorporation of methionine analogues.[23−25] Here, the Gram-positive expression host *L. lactis*, a methionine-auxotrophic strain, is used for the incorporation of methionine analogues into the lantibiotic nisin. After ribosomal synthesis of the precursor peptide with 19 standard amino acids and with variable methionine analogues, the unmodified prenisin is processed by its dedicated modification machinery (Scheme 1). First, the serine and threonine residues in the core peptide

https://dx.doi.org/10.1021/acssynbio.0c00308
ACS Synth. Biol. 2020, 9, 2525−2536

Scheme 1. Incorporation of Methionine Analogues into Nisin[a]



[a](1) Cotranslational modifications, insertion of methionine analogues into the precursor peptide. (2) Post-translational modifications, converting the linear precursor peptide into an active polycyclic peptide.

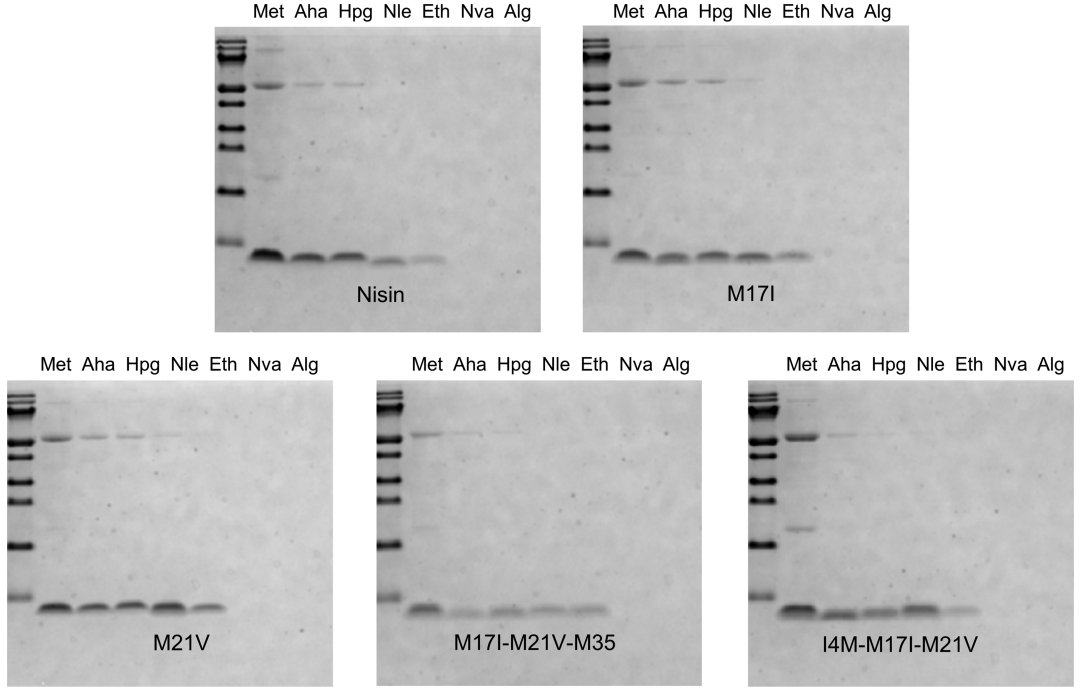


Figure 2. Coomassie-blue stained Tricine-SDS-PAGE gel. Each well contained TCA-precipitated prepeptides from 1 mL supernatant.

are Dha and Dhb by the dehydratase NisB. The dehydrated residues are then coupled to specific cysteine by the cyclase NisC to form lanthionine rings. Subsequently, the modified prenisin is transported out of the cell by the ABC-type transporter NisT, and then the leader is cleaved off by the extracellular protease NisP to liberate the active peptide.

As methionine is essential for the translation of post-translational modification (PTM) enzymes and transporter, a cross expression system, which allows for the expression of prenisin derivatives and PTM enzymes and transporter at different time was used for this study. *L. lactis* NZ9000 was transformed with a plasmid encoding the expression of NisBTC under the control of the P_{nisA} promoter and the other plasmid encoding the expression of prenisin derivatives was controlled by the P_{czcD} promoter. The expression of NisBTC was first conducted by the supplementation of

https://dx.doi.org/10.1021/acssynbio.0c00308
ACS Synth. Biol. 2020, 9, 2525−2536

methionine, after which the medium was replaced by new medium lacking methionine, but containing methionine analogues to express prenisin derivatives (Figure 1A). Although the expression of the modification machinery NisBTC was induced in advance, no effect on the modification efficiency was observed.

Production of Nisin and Its Derivatives. There are two methionine residues in the core peptide of nisin, which are located at sites 17 and 21. Previous studies showed that nisin with the mutation at sites I4, M17, and M21 could retain or even have increased antimicrobial activity.[20] To test the effects of single methionine replacement with analogues in bioactive nisin, four single methionine mutants, *i.e.*, M17I, M21V, M17I-M21V-M35, and I4M-M17I-M21V were constructed (Figure 1B). The choice for Ile or Val as substituents was to retain good antimicrobial activity, since both residues share the hydrophobic character of Met and are sterically not very different, though branched. Six methionine analogues, *i.e.*, Aha, Hpg, Nle, Eth, Nva, and Alg were selected for the incorporation (Figure 1C), and each combination was tested.

The expression level of nisin and its derivatives is shown in Figure 2. The protein quantities in the first five lanes showed that Aha, Hpg, Nle, and Eth can be incorporated into nisin and its derivatives at varying levels. However, incorporation of Nva and Alg was not observed at any moment, and addition of these analogues to a culture lacking methionine led to arrested cell growth. These results strongly indicate that Nva and Alg cannot be incorporated by *L. lactis*. The highest production yield was observed when normal methionine was supplemented. Effectively, a lower production yield was observed in the presence of methionine analogues, in particular with Eth, regardless of the position within the molecule. Methionine analogue incorporation in nisin, M17I, and M21V gave much higher yields of fully modified peptides than M17I-M21V-M35 and I4M-M17I-M21V, which may be due to the intolerance of the modification machinery and/or the transporter to a change of chemical structures at sites I4 and M35. Surprisingly, the production yield of fully modified M21V is even higher than that of WT nisin. To assess the presence of post-translational modifications and incorporation of ncAAs, all samples were further analyzed by HPLC and MALDI-TOF. The resulting spectra showed that the production yield of nisin and its derivatives with Aha and Hpg are much higher than the ones with Nle and Eth. In addition, we found that Nle and Eth had a negative influence on the dehydration rate, as large fractions of 7 times dehydrated peptides were observed.

LC-MS Analysis of Nisin Derivatives. In order to estimate the incorporation efficiency of methionine analogues at different positions, the precipitated precursor peptides were subjected to LC-MS. The LC-MS data showed that the incorporation efficiency of Aha and Hpg into mutants M17I, M21V, and I4M-M17I-M21V was more than 91%, while the incorporation efficiency of Nle and Eth was 88% and 71−73%, respectively. Remarkably, the incorporation efficiency of Aha and Hpg into M17I-M21V-M35 was at least 99.5%, and the peaks of peptides containing methionine were undetectable. However, the incorporation efficiency of Nle and Eth was only 51 and 71%, respectively. In the case of nisin, the incorporation efficiency was 88% for Aha, 87% for Hpg, 77% for Nle, and 56% for Eth. Generally, the incorporation efficiency of ncAAs declined in the order Aha > Hpg > Nle > Eth (Table 1).

Incorporation of Aha and Hpg into nisin, M17I, and M21V did not affect the dehydration efficiency, as peptides with 7 times dehydrated residues were nearly undetectable, suggesting they are excellent methionine surrogates. It may be due to the rate of activation of Aha and Hpg by methionyl-tRNA synthetase (MetRS) during translation, which finally results in the higher yield and efficient modification. However, introducing Nle and Eth resulted in a large fraction of peptides with 7 times dehydration. It may be that the integration speed of Nle and Eth during translation is relatively slow which leads to a lower yield and insufficient modification. The dehydration of M17I-M21V-M35 and I4M-M17I-M21V was dramatically affected by the mutation. Additionally, methionine and ethionine can be oxidized, and peaks corresponding to oxidized products were indeed observed. Furthermore, the first methionine of prenisin is usually cleaved by the enzyme methionine aminopeptidase (MAP). However, a large portion of precursor peptide produced by this system contained the N-terminal Met. The molecular weight of both peaks is shown in Table 2.

Table 1. Incorporation Efficiency of Nisin and Its Derivatives Analyzed by LC-MS[a]

peptide		incorporation efficiency (%)
Nisin	Aha	88
	Hpg	87
	Nle	77
	Eth	56
M17I	Aha	96
	Hpg	92
	Nle	88
	Eth	71
M21V	Aha	99
	Hpg	91
	Nle	88
	Eth	73
M17I-M21V-M35	Aha	>99.5
	Hpg	>99.5
	Nle	51
	Eth	71
I4M-M17I-M21V	Aha	95
	Hpg	93
	Nle	88
	Eth	71

[a]>99.5% means the peak of peptides containing methionine is undetectable. The incorporation efficiency indicates the percentage of the produced peptide with methionine analogues incorporated completely.

Antimicrobial Activity of Nisin and Its Derivatives. In consideration of a sufficient production yield of the fully modified peptides, 12 peptides containing methionine, Aha or Hpg were purified in large scale for antimicrobial activity tests (Figure 3). *M. flavus* was used as a first indicator strain in an agar-well diffusion assay to assess the antimicrobial activity. The results showed that M17Aha-M21Aha, M17Hpg-M21Hpg, M21V, M21V-M17Aha, and M21V-M17Hpg have higher antimicrobial activity compared to WT nisin, and mutant M21V showed the best activity with any of the three amino acids. However, in all the cases, the activity of mutants M17I and M17I-M21V-M35 decreased dramatically.

The MIC values were determined for *L. lactis* and six Gram-positive pathogenic strains. The tested strains included two Staphylococci, two Enterococci, *Bacillus cereus* and *Listeria monocytogenes* (Table 3). The results showed that the

https://dx.doi.org/10.1021/acssynbio.0c00308
ACS Synth. Biol. 2020, 9, 2525−2536

Table 2. MS Analysis of Prenisin and Its Derivatives[a]

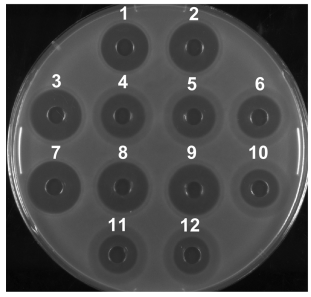
			predicted mass (Da)		measured mass (Da)									
					Met		Aha		Hpg		Nle		Eth	
peptide	methionine analogue	modification	+Met1	−Met1	+Met1	−Met1	+Met1	−Met1	+Met1	−Met1	+Met1	−Met1	+Met1	−Met1
Nisin	Met	$-8H_2O$	5818.85	5687.66	5818.80	5687.76								
		$-8H_2O$ + Oxi	5834.85	5703.66	5835.80	5704.76								
		$-8H_2O$ + 2Oxi	5850.85	5719.66	5850.79	5720.75								
	Aha	$-8H_2O$	5803.63	5677.51			5803.84	5677.79						
	Hpg	$-8H_2O$	5752.66	5643.53					5752.84	5643.79				
	Nle	$-8H_2O$	5764.75	5651.59							5764.93	5651.85		
		$-7H_2O$	5782.77	5669.61							5782.90	5669.81		
	Eth	$-8H_2O$	5860.93	5715.71									5861.84	5716.79
		$-8H_2O$ + Oxi	5876.93	5731.71									5877.84	5732.79
		$-8H_2O$ + 2Oxi	5892.93	5747.71									5893.83	5748.78
M17I	Met	$-8H_2O$	5800.81	5669.62	5800.85	5669.81								
		$-8H_2O$ + Oxi	5816.81	5685.62	5816.84	5685.80								
	Aha	$-8H_2O$	5790.66	5664.54			5790.87	5664.82						
	Hpg	$-8H_2O$	5756.68	5647.55					5756.87	5647.82				
	Nle	$-8H_2O$	5764.74	5651.58							5764.93	5651.85		
		$-7H_2O$	5782.76	5669.60							5782.90	5669.82		
	Eth	$-8H_2O$	5828.86	5683.64									5829.87	5683.82
		$-8H_2O$ + Oxi	5844.86	5699.64									5845.87	5700.82
		$-8H_2O$ + 2Oxi	5860.86										5861.87	
M21V	Met	$-8H_2O$	5786.79	5655.60	5786.83	5655.79								
		$-8H_2O$ + Oxi	5802.79	5671.60	5802.82	5672.78								
	Aha	$-8H_2O$	5776.64	5650.52			5776.85	5650.80						
	Hpg	$-8H_2O$	5742.66	5633.53					5742.85	5633.50				
	Nle	$-8H_2O$	5750.72	5637.56							5750.91	5637.83		
		$-7H_2O$	5768.74	5655.58							5768.89	5655.81		
	Eth	$-8H_2O$	5814.84	5669.62									5815.85	5670.80
		$-8H_2O$ + Oxi	5830.84	5685.62									5831.85	5685.80
		$-8H_2O$ + 2Oxi	5846.84										5846.85	
M17I-M21V-M35	Met	$-8H_2O$	5899.95	5768.76	5899.91	5768.87								
		$-7H_2O$	5917.97	5786.77	5917.81	5786.88								
		$-7H_2O$ + Oxi	5933.97	5802.77	5933.91	5802.87								
	Aha	$-8H_2O$	5889.80	5763.68			5889.94	5763.89						
		$-7H_2O$	5907.82	5781.70			5906.94	5781.89						
	Hpg	$-8H_2O$	5855.82	5746.69					5855.97	5746.88				
		$-7H_2O$	5873.84	5764.71					5873.94	5764.89				
	Nle	$-8H_2O$	5863.88	5750.72							5864.00	5750.91		
		$-7H_2O$	5881.90	5768.74							5882.01	5768.93		
	Eth	$-8H_2O$	5928.00	5782.78									5928.94	5782.89
		$-7H_2O$	5946.02	5800.80									5945.94	5800.89
		$-7H_2O$ + Oxi	5962.02	5816.80									5961.94	5816.89

https://dx.doi.org/10.1021/acssynbio.0c00308
ACS Synth. Biol. 2020, 9, 2525−2536

Table 2. continued

peptide	methionine analogue	modification	predicted mass (Da)		measured mass (Da)									
					Met		Aha		Hpg		Nle		Eth	
			+Met1	−Met1	+Met1	−Met1	+Met1	−Met1	+Met1	−Met1	+Met1	−Met1	+Met1	−Met1
I4M-M17I-M21V	Met	$-8H_2O$	5786.79	5655.60	5787.83	5655.79								
		$-7H_2O$	5804.81	5673.61	5804.82	5672.78								
		$-7H_2O$ + Oxi	5820.81	5689.61	5819.82	5689.78								
	Aha	$-8H_2O$	5776.64	5650.52			5776.85	5650.80						
		$-7H_2O$	5794.66				5792.85							
	Hpg	$-8H_2O$	5742.66	5633.53					5742.85	5633.80				
		$-7H_2O$	5760.68						5758.85					
	Nle	$-8H_2O$	5750.72	5637.56							5750.91	5637.83		
		$-7H_2O$	5768.74	5655.58							5767.90	5655.81		
	Eth	$-8H_2O$	5814.84	5669.62									5815.85	5670.80
		$-8H_2O$ + Oxi	5830.84	5685.62									5830.85	5686.80
		$-8H_2O$ + 2Oxi	5846.84										5846.84	

[a]+Met1, with N-terminal Met; −Met1, without N-terminal Met; $-8H_2O$, eight times dehydrated; $-8H_2O$ + Oxi, eight times dehydrated and one time oxidized; $-8H_2O$ + 2Oxi, eight times dehydrated and two times oxidized; $-7H_2O$, seven times dehydrated; $-7H_2O$ + Oxi, seven times dehydrated and one time oxidized.



Micrococcus flavus

	Peptide	Diameter
1	Nisin	22.0 mm
2	M17Aha-M21Aha	23.0 mm
3	M17Hpg-M21Hpg	23.9 mm
4	M17I	20.4 mm
5	M17I-M21Aha	19.8 mm
6	M17I-M21Hpg	20.1 mm
7	M21V	24.5 mm
8	M21V-M17Aha	23.6 mm
9	M21V-M17Hpg	24.0 mm
10	M17I-M21V-M35	18.0 mm
11	M17I-M21V-M35Aha	17.8 mm
12	M17I-M21V-M35Hpg	16.8 mm

Figure 3. Antimicrobial activity of nisin and its derivatives against *M. flavus*. In gray: values that are improved in comparison to nisin.

replacement of Met with Met analogues can alter the antimicrobial activity and spectrum. Certain peptides retained or even displayed higher activity against a specific strain, while showing reduced activities against others, suggesting a possible increase in selectivity. For example, M17Aha-M21Aha showed improved activity against *L. monocytogenes*, but the activity against the other strains was reduced when compared to nisin. M21V has been reported to have enhanced bioactivity and specific activity against all tested Gram-positive pathogens including four VRE strains compared to WT nisin.[26,27] In our study, M21V showed reduced activities against two Enterococci strains, but retained a high activity against others. Improving the antimicrobial activity of nisin turned out to be difficult. However, engineering nisin can generate new nisin derivatives that have different properties and can be used for specific targets. In addition, some nisin derivatives showed different inhibition activity in solid media tests compared to the broth MIC test. This phenomenon can be related to the difference in diffusion ability.

Production of Dimeric Nisin Constructs and Nisin Hybrids. The mode of action of nisin involves its binding to lipid II, followed by membrane insertion, which leads to pore formation. The pore-complex has a uniform and stable structure, consisting out of eight nisin and four lipid II molecules.[28] In a previous study, a nisin dimer was prepared by connecting two nisin molecules at the C-terminus through a linker, which led to slightly increased pore-forming activity.[13] As the nisin derivatives contain a clickable group (azide or alkyne) at positions 17, 21, and 35, a setup was devised to investigate how different orientations and multivalency patterns of nisin dimers affect antimicrobial activity.[29] M17I-M21Aha, M17I-M21Hpg, M17I-M21V-M35Aha, M17I-M21V-M35Hpg, M21V-M17Aha, and M21V-M17Hpg were coupled either mutually or with nisin ABC-azide to generate six dimeric nisin constructs and three nisin hybrids which were characterized by MALDI-TOF (Supplementary Figure S1). The antimicrobial activity of these dimers was tested against *M. flavus* by agar diffusion assays. The resulting growth inhibition halos indicated the retainment of at least some degree of activity in all variants. We found that the activity of dimeric nisin constructs increased in order as reactions are performed at the hinge region (position 21), the C-terminus (position 35), and ring C (position 17). M17I-M21Aha + M17I-M21Hpg is the least active dimeric nisin construct (Figure 4A and 4B). Coupling at the hinge region may result in increased steric hindrance and decreased flexibility and therefore hindering its lipid II binding and pore formation features. It again proves that the flexibility of the hinge region

https://dx.doi.org/10.1021/acssynbio.0c00308
ACS Synth. Biol. 2020, 9, 2525−2536

Table 3. MIC Values (μM) of Nisin and Its Derivatives

peptide	CAL-MRSA	MW2-MRSA	*B. cereus*	*E. faecalis*	*E. faecium*	*L. monocytogenes*	*L. lactis*
Nisin	10.39	5.19	5.19	2.60	0.32	2.60	0.020
M17Aha-M21Aha	19.99	13.33	6.66	3.33	0.42	1.67[a]	0.026
M17Hpg-M21Hpg	19.41	19.41	9.70	4.85	0.61	4.85	0.019[a]
M17I	>19.92	>19.92	>19.92	19.92	2.49	4.98	0.622
M17I-M21Aha	>17.42	>17.42	8.71	17.42	2.18	4.36	0.544
M17I-M21Hpg	>19.08	>19.08	19.08	19.08	2.38	4.77	0.596
M21V	9.81[a]	2.45[a]	4.90[a]	4.90	0.61	2.45[a]	0.019[a]
M21V-M17Aha	>19.72	19.72	19.72	9.86	0.62	4.93	0.039
M21V-M17Hpg	>19.74	19.74	19.74	9.87	0.62	4.93	0.019[a]
M17I-M21V-M35	>18.33	>18.33	>18.33	>18.33	2.29	>18.33	0.573
M17I-M21V-M35Aha	>19.96	>19.96	>19.96	>19.96	2.49	>19.96	0.624
M17I-M21V-M35Hpg	>16.82	>16.82	>16.82	16.82	2.10	>16.82	>0.526

[a]MIC values that are retained or improved in comparison to nisin.

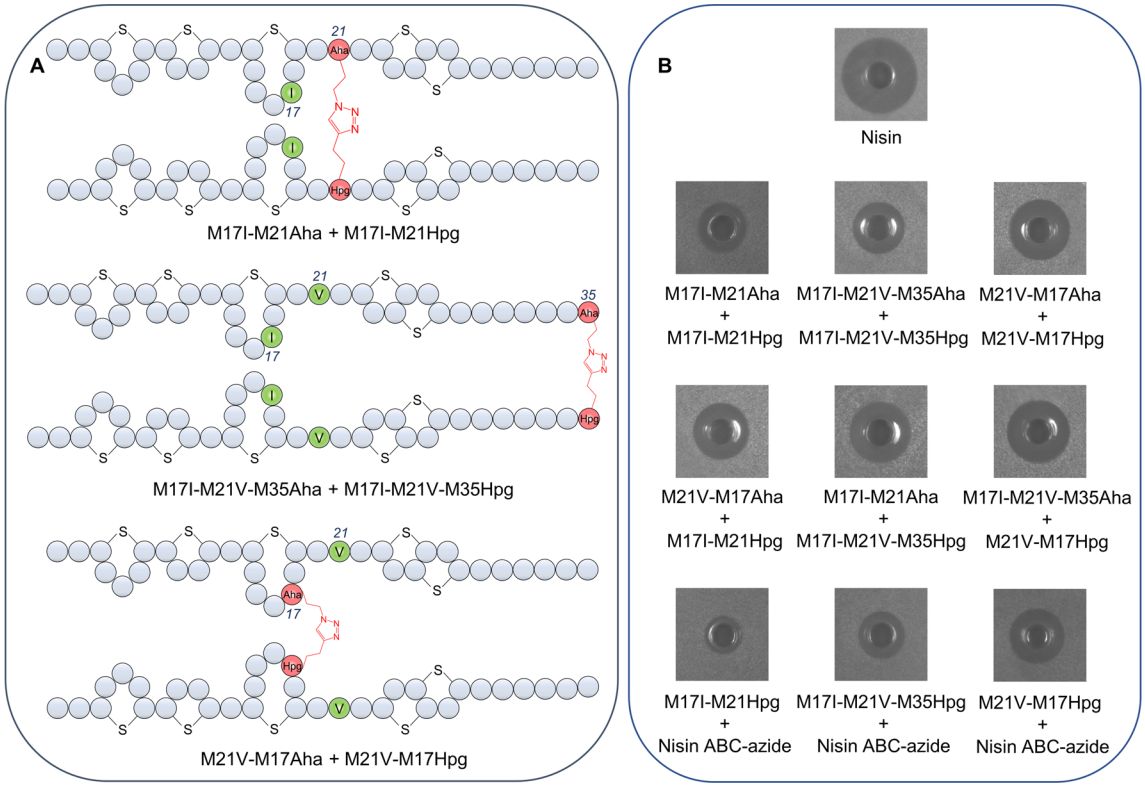


Figure 4. (A) Structure of three representative dimeric nisin constructs with reactions performed at the hinge region (position 21), the C-terminus (position 35), and ring C (position 17). (B) Antimicrobial activity of six dimeric nisin and three nisin hybrids at equimolar concentrations against *M. flavus* with nisin as positive control. M17I-M21Aha + M17I-M21Hpg is the least active dimeric nisin construct whereas M21VM17Aha + M21V-M17Hpg is the most active. Similarly, M17I-M21Hpg + Nisin ABC-azide is the least active nisin hybrid, whereas M21V-M17Hpg + Nisin ABC-azide is the most active.

is important for the activity, which is in accordance with previous studies.[30,31] Also M17I-M21V-M35Aha + M17I-M21V-M35Hpg showed lower activity. Coupling at the C-terminus of nisin gave rise to a dimeric nisin construct containing two lipid II binding sites. However, the pore formation ability may be weakened or abolished as the C-terminus of nisin was involved in the connection since both C-termini must flip simultaneously and insert in the membrane. This involves the movement and reorientation of a bulky set of amino acids, including the intertwined rings DE of each monomer, through the membrane. M21V-M17Aha + M21V-M17Hpg is the most active dimeric nisin construct. Coupling at ring C gave the best activity, which may be due to the fact that rings AB are still able to bind lipid II, while the hinge region, rings DE and the linear C-terminus keeps their individual flexibility, allowing the C-terminus of nisin to form pores. Therefore, position 17 is the optimal site for coupling moieties out of the 3 chosen positions. This study shows the great potential of this strategy for linking active modules from different peptides. Moreover, nisin ABC was obtained by enzymatic digestion of nisin using chymotrypsin and it was subsequently C-terminally functionalized with azidopropylamine to generate nisin ABC-azide. Coupling M17I-M21Hpg, M17I-M21V-M35Hpg, and M21V-M17Hpg with nisin ABC-azide showed the same antimicrobial activity pattern as above; *i.e.*, activity is altered in ascending order as reactions are

https://dx.doi.org/10.1021/acssynbio.0c00308
ACS Synth. Biol. 2020, 9, 2525−2536

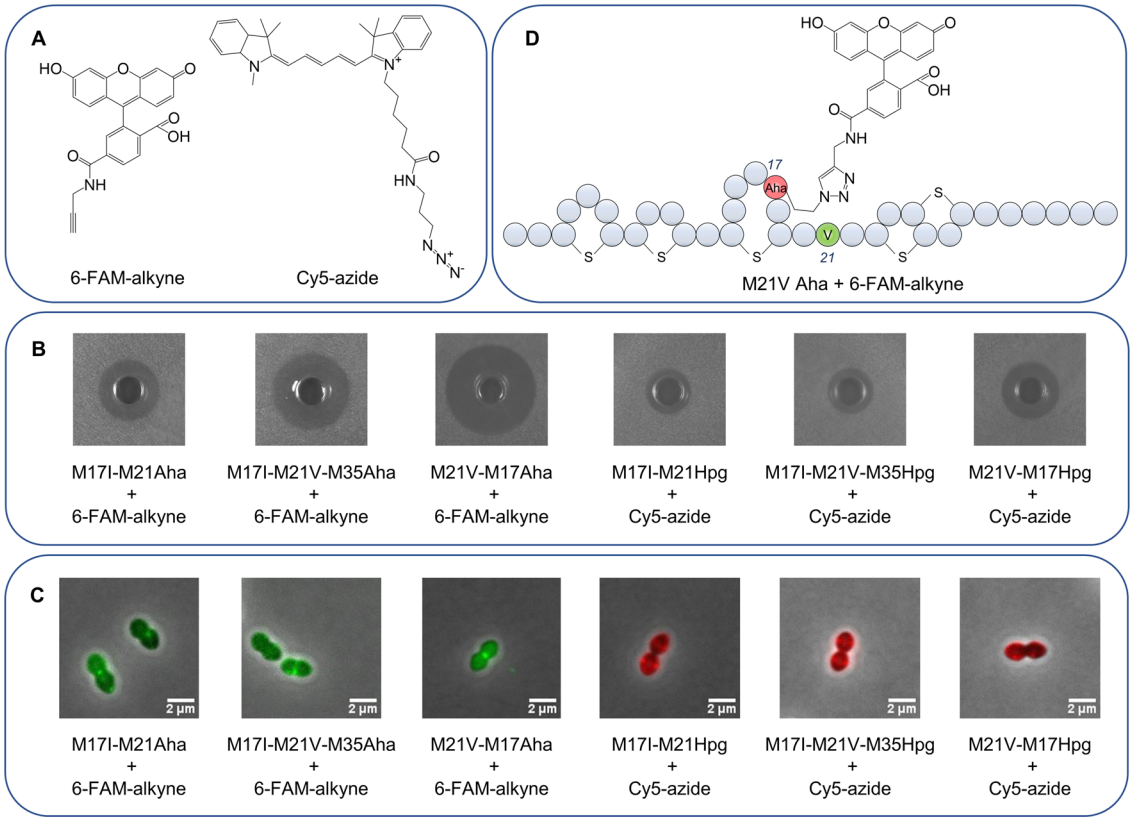


Figure 5. (A) Structure of fluorescent dyes 6-FAM-alkyne and Cy5-azide. (B) Antimicrobial activity of six fluorescently labeled nisin variants. (C) Localization of six fluorescently labeled nisin variants by fluorescence microscopy. (D) Structure of the most potent fluorescently labeled nisin variant M21V-M17Aha + 6-FAM-alkyne.

performed to the hinge region, the C-terminus, and ring C, respectively (Figure 4B).

Fluorescently Labeled Nisin Variants. Labeling of nisin with fluorescent probes has greatly contributed to understanding its mechanism of action as shown in studies by Scherer *et al.*[32] and Descobry *et al.*[33] The C-terminus of nisin is the common site for labeling. However, introduction of a tag in this position poses a considerable perturbation in the structure and activity of nisin. Here, 6-FAM-alkyne and Cy5-azide (Figure 5A) were successfully coupled at three different positions (positions 17, 21, and 35) of nisin, and the resulting compounds were characterized by MALDI-TOF (Supplementary Figure S2). The antimicrobial activity of six fluorescently labeled nisin variants was retained (Figure 5B). Coupling 6-FAM-alkyne and Cy5-azide at different positions of nisin showed the same activity pattern as that obtained with dimeric nisin constructs. Thus, M21V-M17Aha was found to be the most suitable derivative for labeling with both 6-FAM-alkyne and Cy5-azide. The localization of six fluorescently labeled nisin variants interacting with *E. faecium* were studied by fluorescence microscopy (Figure 5C). Fluorescence intensity detection indicated that the labeled nisin conjugates were all located at the cell membrane. Cy5-azide labeled nisin variants showed lower activity than their 6-FAM-alkyne labeled counterparts, and no aggregation was observed in cell division sites. This may be due to the fact that Cy5-azide would affect the binding of nisin conjugates to lipid II. M21V-M17Aha + 6-FAM-alkyne (Figure 5D) was found to be the most potent fluorescently labeled nisin variant, as it showed similar activity to nisin. It was located at the septum of cell division sites where the membrane-bound cell wall precursor lipid II concentration is maximal. These results are in accordance with previous studies using fluorescently labeled nisin A and nisin Z, which indicated that both molecules were accumulating at the cell division sites of *Bacillus subtilis* and *L. monocytogenes,* respectively.[33,34] M21V-M17Aha + 6-FAM-alkyne shows great potential as a tool to further study the antibacterial mechanism of action of nisin and for understanding the mechanism of synergy of nisin with other molecules on Gram-negative strain. Moreover, this strategy can be extended to modify other ribosomally synthesized and post-translationally modified peptides (RiPPs). While numerous novel RiPPs have been reported, little is known about the mechanism of action of these peptides. It would be highly appropriate to use this method to modify such RiPPs with biomarkers or fluorescence probes to investigate their mechanism of action.

CONCLUSIONS

In summary, we have demonstrated for the first time the incorporation of methionine analogues into RiPPs in *L. lactis*. Four methionine analogues were successfully installed at four distinct positions of the lantibiotic nisin. The genetic code of *L. lactis* can be regarded to be expanded by incorporating methionine analogues. The structural diversity was enhanced, while retaining or even improving antimicrobial activity against specific pathogens or Gram-positive bacteria. In addition, this study underlines that the bio-orthogonal reactive groups of ncAAs can serve as a platform for post-biosynthetic modifications, such as conjugating with peptides, or functional

https://dx.doi.org/10.1021/acssynbio.0c00308
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labels (e.g., fluorescence). The insertion of ncAAs during translation along with the possibility for their subsequent modification (postsynthetic conjugation) could further expand the chemical and functional space of RiPPs. Overall, our experiments further exemplify one of the most important applications of ncAA incorporation, that is, the functional, structural, and chemical diversification of RiPPs. This study provides an efficient method for RiPPs engineering by incorporation of ncAAs and chemical coupling.

METHODS

Bacterial Strains, Plasmids, and Growth Conditions. Strains and plasmids used in this study are listed in Supplementary Table S1. All *L. lactis* strains were grown in M17 broth supplemented with 0.5% (w/v) glucose at 30 °C for genetic manipulation. Five μg/mL erythromycin and/or chloramphenicol were added when it was necessary. Chemical defined medium lacking tryptone (CDM-P)[5] was specially used for peptide expression and methionine analogues incorporation.

Construction of Expression Vectors. The primers used in this study are listed in Supplementary Table S2. The molecular cloning techniques were performed following standard protocols.[35] The preparation of competent cells and transformation were performed according to Holo and Nes.[36] Fast digest restriction enzymes and ligase were used as recommended by the manufacturer. Nisin derivatives with one mutation in the core peptide (pCZ-nisA-M17I and pCZ-nisA-M21V) were produced by spice overlap extension PCR. For the construction of pCZ-nisA-M17I-M21V-M35 and pCZ-nisA-I4M-M17I-M21V, nested PCR of pCZ-nisA was used to introduce the mutation. The amplification was performed using Phusion Polymerase (Thermo Scientific) following the provider's instructions and the primers are listed in Supplementary Table S2. After amplification and digestion with NheI and PaeI, it was ligated in pCZ-nisA digested with the same enzymes. The ligation product was desalted and transformed into *L. lactis* NZ9000. The plasmid was isolated and sequenced to check the integrity of the sequence.

Methionine Analogues and Fluorescent Probes. The methionine analogue L-homopropargylglycine (Hpg) was purchased from Chiralix (Nijmegen, Netherlands). L-Azidohomoalanine (Aha), L-norleucine (Nle), L-norvaline (Nva), and L-allyglycine (Alg) were purchased from Iris Biotech GmbH (Marktredwitz, Germany). L-Ethionine (Eth) was purchased from Alfa Aesar (Karlsruhe, Germany). 6-FAM-alkyne and Cy5-azide were purchased from Jena Bioscience (Thuringia, Germany).

Precursor Peptide Precipitation. *L. lactis* strains harboring pIL3eryBTC and pCZ-nisA were grown overnight in CDM-P with 5 μg/mL erythromycin and 5 μg/mL chloramphenicol. Subsequently, the overnight culture was diluted in 20 mL fresh CDM-P back to OD_{600} = 0.1. When the OD_{600} reached 0.4−0.6, 10 ng/mL nisin was added to induce the expression of NisBTC. Three hours later, the cells were spun down at room temperature for 8 min at 5000 rpm and then washed three times with CDM-P lacking methionine and resuspended back in the initial volume of CDM-P lacking methionine. The medium was supplemented with either methionine (38 mg/L) or 50 mg/L methionine analogues, and 0.5 mM $ZnSO_4$ was added to induce peptide expression. After overnight growth, the supernatant was harvested by centrifugation at 8500 rpm for 20 min at 4 °C. The precursor peptides were precipitated by Trichloroacetic acid (TCA) for further analysis according to Link *et al.*[37] Briefly, an ice-cold 100% TCA solution was added to the supernatant to reach a final concentration of 10% TCA, and the solution was stored overnight at 4 °C. The precipitated peptide was pelleted by centrifugation at 8000 rpm for 60 min at 4 °C. The supernatant was discarded and the pellet was washed with ice-cold acetone in half the original culture volume by a second centrifugation (8000 rpm, 45 min, 4 °C). The acetone was discarded, and the remaining acetone was evaporated off over several hours at room temperature. Dried pellets were suspended in 300 μL 0.05% aqueous acetic acid solution.

Tricine-SDS-PAGE Analysis. The precipitated precursor peptides were analyzed by Tricine-SDS-PAGE according to Schagger *et al.*[38] 15 μL of each sample mixed with 4 μL loading dye was loaded on the gel. Coomassie brilliant blue G-250 was used to stain the gel.

LC-MS Analysis of Nisin Derivatives. The precipitated precursor peptides were injected into the LC-MS system consisting of an Ultimate 3000 UHPLC system coupled *via* a HESI-II electrospray source with a Q-Exactive Orbitrap-based mass spectrometer (all Thermo Scientific, San Jose, CA, USA). Three μL of each sample was loaded onto a Kinetex EVO-C18 column (2.6 μm particles, 100 × 2.1 mm, Phenomenex). The eluents for the LC separation were (A) water and (B) Acetonitrile both containing 0.1% formic acid. The following gradient was delivered at a flow rate of 0.5 mL/min: 10% B until 1 min; then linear to 40% B in 9 min; linear to 80% B in 2 min; hold in 80% B for 2 min, after which a switch back to 10% B was performed in 0.1 min. After 5 min of equilibration the next injection was performed. The LC column was kept at 60 °C. The HESI-II electrospray source was operated with the parameters recommended by the MS software for the LC flow rate used (Spray voltage 3.5 kV (positive mode)); other parameters were sheath gas 50 AU, auxiliary gas 10 AU, cone gas 2 AU; capillary temperature 275 °C; heater temperature 400 °C. The samples were measured in positive mode from m/z 500−2000 at a Resolution of 140 000 @ m/z 200. The instrument was calibrated in positive mode using the Pierce LTQ Velos ESI positive-ion calibration solution (Thermo Fisher Scientific, Rockford, USA) (containing caffeine, the tetrapeptide MRFA and a mixture of fluorinated phosphazines ultramark 1621). The system was controlled using the software packages Xcalibur 4.1, SII for Xcalibur 1.3 and Q-Exactive Tune 2.9 (Thermo Fisher Scientific). The Xtract-algorithm within Xcalibur was used for deconvolution of the isotopically resolved data.

Purification of Nisin and Its Derivatives. To obtain pure peptides for activity test, the supernatant of 1 L culture was first incubated with purified NisP[39] at 37 °C for 3 h to cleave off the nisin leader, and then the supernatant was loaded on a C18 open column (Spherical C18, particle size: 40−75 μm, Sigma-Aldrich). The column was washed and eluted with different concentrations of buffer B (buffer A, Milli-Q with 0.1% TFA; buffer B, acetonitrile with 0.1% TFA). The active fractions were lyophilized and further purified by HPLC using an Agilent 1200 series HPLC with a RP-C12 column (Jupiter 4 μm Proteo 90A, 250 × 4.6 mm, Phenomenex). The peak that is the fully modified peptide with the correct molecular weight was lyophilized and stored as powder until further use.

MALDI-TOF Mass Spectrometry Characterization. One μL of each sample was spotted, dried and washed with Milli-Q water on the target. Subsequently, 1 μL of 5 mg/mL a-

https://dx.doi.org/10.1021/acssynbio.0c00308
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cyano-4-hydroxycinnamic acid (Sigma-Aldrich) was spotted on top of the sample. An ABI Voyager DE Pro (Applied Biosystems) matrix-assisted laser desorption/ionization time-of-flight analyzer (MALDI-TOF) operating in linear mode using external calibration was used to obtain mass spectra.[40]

Agar Well Diffusion Assay. Antimicrobial activity was tested against *M. flavus* according to protocols described previously.[40] HPLC purified and lyophilized peptides were resuspended in 0.05% aqueous acetic acid solution and the peptide amount was quantified by HPLC according to Schmitt *et al.*[5] 0.15 nmol of sample was added to each well. The agar plate was incubated at 30 °C overnight, after which the zone of inhibition was measured.

Determination of the Minimal Inhibitory Concentration (MIC). For the MIC assay, the indicator strains CAL-MRSA, MW2-MRSA, *E. faecalis*, *E. faecium*, *B. cereus*, *L. monocytogenes*, and *L. lactis* were first streaked on GM17 plate and cultured overnight. The peptide samples were diluted with 0.05% acetic acid to a concentration of 4−128 μg/mL (depending on the estimated activity of the peptide and the strain tested). GM17 broth was used for the activity test against *E. faecium*, *L. monocytogenes*, and *L. lactis*. MHB was used for the activity test against CAL-MRSA, MW2-MRSA, *E. faecalis*, and *B. cereus*. The MIC value test was performed according to Wiegand *et al.*[41]

Preparation of Nisin ABC-Azide. Nisin was digested using chymotrypsin to generate nisin ABC. The truncated nisin molecule can be readily purified in accordance with protocols reported previously.[42] Nisin (180 mg) was dissolved in 150 mL Tris buffer (25 mmol Tris acetate, pH 7.5) and the solution was cooled on ice for 15 min. Then chymotrypsin (15 mg) was added and stirred at room temperature for 15 min. The reaction was performed at 30 °C for 16 h and an extra 15 mg chymotrypsin was added. After 24 h incubation, another 15 mg chymotrypsin was added and incubated for another 24 h. The reaction mixture was acidified with HCl (1 M) to pH 4.0 followed by adding 3 mL MeCN and concentrated *in vacuo*. The pure nisin ABC was purified from the mixture by RP-HPLC and lyophilized to obtain a white powder (20 mg). Nisin ABC (10 mg, 6.5 μmol) was dissolved in DMF (50 μL) and azidopropylamine (44 μL, 43.2 mg, 432 μmol), PyBOP (Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate) (9 mg, 17.2 μmol), and DIPEA (*N*,*N*-diisopropylethylamine) (6 μL, 34.8 μmol) were added. The reaction was vortexed for 20 min and subsequently quenched with 5 mL buffer (H_2O:MeCN, 95:5 + 0.1% TFA). The reaction mixture was purified by HPLC and pure nisin ABC-azide was lyophilized to obtain a white powder (8 mg).

Click Chemistry. A stock solution of $CuSO_4$ (10 mg, 100 mM), BTTAA (2-(4-((bis((1-(*tert*-butyl)-1*H*-1,2,3-triazol-4-yl)methyl)amino)methyl)-1*H*-1,2,3-triazol-1-yl)-acetic acid) (25 mg, 50 mM), and sodium ascorbate (200 mg, 1 M) in ddH_2O were prepared, aliquoted and stored at −20 °C for further use. M17I-M21Aha (70 μg, 0.02 μmol) and M17I-M21Hpg (70 μg, 0.02 μmol) were dissolved in 100 mM phosphate buffer (pH 7.0, final reaction volume: 200 μL). Then, $CuSO_4$ (4 μL, 0.4 μmol): BTTAA (40 μL, 2 μmol)-premix were added followed by the addition of sodium ascorbate (20 μL, 20 μmol). The reaction was performed at 37 °C for 1 h and purified directly by RP-HPLC. M17I-M21V-M35Aha, M17I-M21V-M35Hpg, M21V-M17Aha, and M21V-M17Hpg were reacted either mutually or with nisin ABC-azide (40 μg, 0.02 μmol), Cy5-azide (5 μL, 10 mg/mL), or 6-FAM-alkyne (4 μL, 10 mg/mL) at the above conditions. The reaction was further scaled up in ratio to obtain more products. The reaction products were purified directly by HPLC and the peak with the correct molecular weight was lyophilized and stored as powder until further use.

Fluorescence Microscopy. Cultures of overnight grown *E. faecium* were diluted 1:100 and incubated in GM17 at 37 °C for about 4 h to reach OD_{600} of 0.5. Then, 0.5 mL of culture were centrifuged at 7000 rpm for 3 min. Fluorescently labeled nisin variants were added into the Eppendorf tube with cells at desired concentration in 100 μL saline solution and cells were incubated at 37 °C for 30 min. After three other washes in saline buffer, 0.6 μL bacterial suspensions and 1% low-melting-point agar were added to a microscopy plate and the localization of nisin variants were inspected with a Delta Vision Elite inverted epifluorescence microscope (Applied Precision, GE Healthcare, Issaquah, WA, USA) equipped with a stage holder, a climate chamber, a seven-color combined set InsightSSI Solid-state Illumination module and an sCMOS camera (PCO AG, Kelheim, Germany). Excitation was set to 646 nm and emission to 662 nm to capture Cy5-azide fluorescence. For 6-FAM-alkyne fluorescence, we employed 490 nm for excitation and emission at 513 nm. Images were obtained by ImageJ software.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.0c00308.

Supporting figures that provide MALDI-TOF MS data of dimeric nisin constructs, nisin hybrids, and fluorescently labeled nisin variants; supporting tables that list strains, plasmids, and primers used in this study (PDF)

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Author Contributions

JD designed and carried out the experiments, obtained and analyzed data, and wrote the manuscript. JHV contributed to the manuscript. JC contributed to the data interpretation. OPK conceived and supervised the project and corrected the manuscript.

Notes

The authors declare no competing financial interest.

https://dx.doi.org/10.1021/acssynbio.0c00308
ACS Synth. Biol. 2020, 9, 2525−2536

■ ACKNOWLEDGMENTS

We thank Manuel Montalban-Lopez (Department of Microbiology, Faculty of Sciences, University of Granada, Spain) for his valuable suggestions during the project. JD and JC were supported by Chinese Scholarship Council (CSC). JHV was supported by The Netherlands Organization for Scientific Research (NWO, ALW OP.214).

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