Bioorthogonal Metalloporphyrin-Catalyzed Selective Methionine Alkylation in the Lanthipeptide Nisin

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Bioorthogonal catalytic modification of ribosomally synthesized and post-translationally modified peptides (RiPPs) is a promising approach to obtaining novel antimicrobial peptides with improved properties and/or activities. Here, we present the serendipitous discovery of a selective and rapid method for the alkylation of methionines in the lanthipeptide nisin. Using carbenes, formed from water-soluble metalloporphyrins and diazoacetates, methionines are alkylated to obtain sulfonium ions. The formed sulfonium ions are stable, but can be further reacted to obtain functionalized methionine analogues, expanding the toolbox of chemical posttranslational modification even further.

Peptide derived natural products such the ribosomally synthesized and post-translationally modified peptides (RiPPs) are of interest because of their biological activity, in particular for their potential as antibiotic.[1,2] Selective chemical modification of RiPPs offers the possibility of further improving their properties and/or activity. A plethora of selective, bioorthogonal reactions for the modification of specific amino acid residues in proteins and peptides have been developed,[3–6] some of which have been applied to RiPPs.[7–10] However, methods for selective methionine modification are rare.[11–13] Here, we report on a serendipitously discovered novel method for modification of methionines in the lanthipeptide nisin A to obtain the corresponding sulfonium ions, which can subsequently be reacted further to give the corresponding methionine analogues.

Nisin A is an antimicrobial peptide that contains several interesting structural features installed by post-translational modification of the canonical amino acids. These include the dehydroamino acids, formed by dehydration of serine or threonine, and lanthionine bridges (Figure 1).[14,15] Nisin derivatives have been obtained by bio-engineering.[16–19] For modifications not accessible by bioengineering, site-selective chemical modification of nisin is preferred, as its total synthesis is highly challenging.[20–22]

The dehydroamino acids, that is, dehydroalanine (Dha) and dehydrobutyrine (Dhb) present in nisin A are attractive target
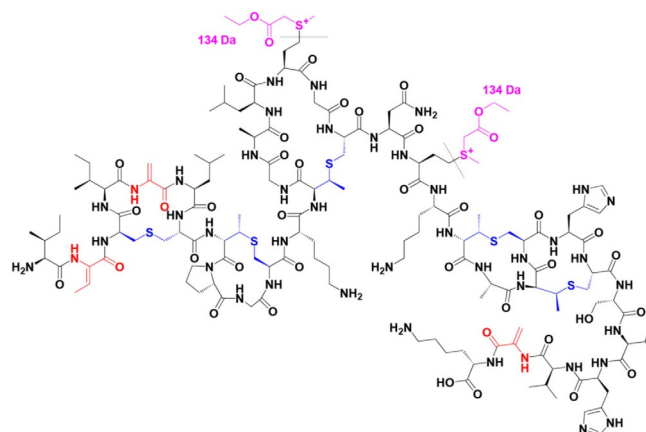

Figure 1. Structure of the modified lanthipeptide nisin A. Lanthionines are marked in blue. Dehydroamino acids (dehydroalanine and dehydrobutyrine) are marked in red. The modified methionine presented here is marked in pink. Bonds fragmented in the mass source are indicated with a dotted line.

for modification. A variety of method for the bioorthogonal modifications of dehydroalanine have been developed,[23–26] albeit that only a few of these are catalytic. This includes palladium- and rhodium-catalyzed arylations, P450-catalyzed cyclopropanations and photocatalytic additions.[27–30] We envisioned the cyclopropanation of dehydroalanine using water-soluble metalloporphyrins as catalyst and diazo reagents as carbene precursors.[31,32]
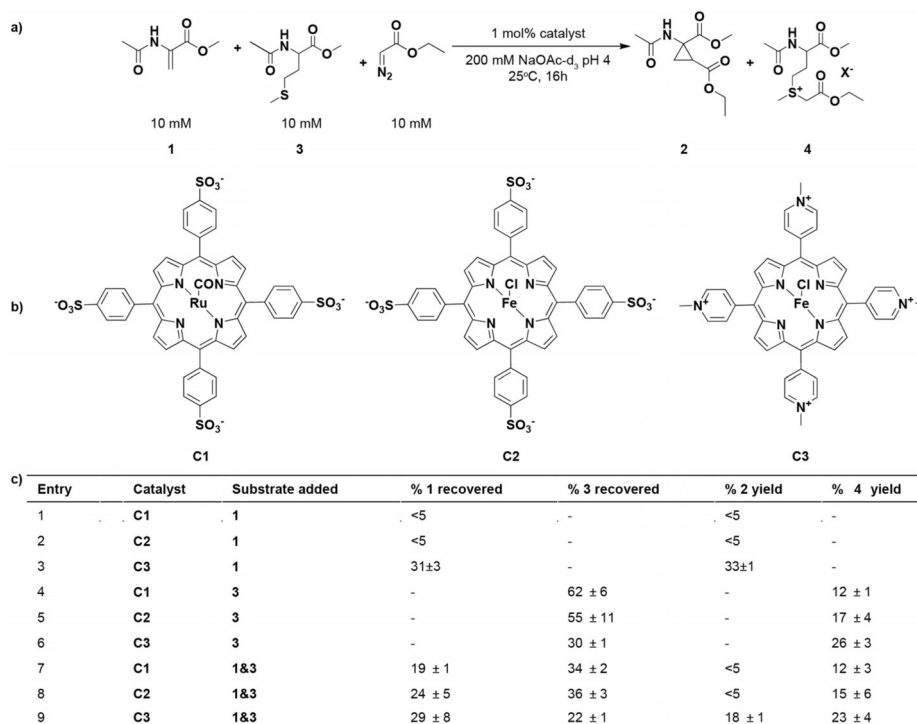
The reactivity of dehydroalanine analogue **1** towards cyclopropanation with ethyl diazoacetate (EDA) was investigated in [D$_3$]acetate-buffered solution at pH 4, as nisin itself is most stable at lower pH[33] (Scheme 1a, b). Deuterated buffer was employed to allow direct quantification of the reaction, both in the aqueous phase and the extracted organic phase, by ^{1}H NMR spectroscopy. Three metalloporphyrin catalysts were screened: anionic ruthenium(II) *meso*-tetra(4-sulfonatophenyl)-porphyrin ([Ru-TSPP], **C1**), anionic iron(III) *meso*-tetra(4-sulfonatophenyl)porphyrin ([Fe-TSPP], **C2**) and cationic iron(III) *meso*-tetra(*N*-methyl-4-pyridyl)porphyrin ([Fe-TMe4PyP], **C3**).[34,35] Neither the cyclopropane product nor the starting material was recovered from the reactions with anionic porphyrins **C1** and **C2** (Scheme 1c). Most likely **1** polymerized under these reaction conditions. In contrast, the reaction catalyzed by **C3** resulted in the formation of cyclopropane product **2** in a modest yield of 33% (Scheme 1c).

In view of the unexpected first results with nisin (vide infra), also the modification of methionine was studied. Methionine residues are known to be good nucleophiles in a wide range of pH values and as such they potentially could compete for reaction with the carbenes.[11,36] In the reaction of methionine

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Scheme 1. a) Reaction of ethyl diazoacetate with dehydroalanine analogue **1** and/or methionine analogue **3** to obtain, respectively cyclopropane **2** and sulfonium ion **4**. b) Catalysts employed: [Ru(CO)TSPP] (**C1**), [Fe(Cl)TSPP] (**C2**) and [Fe(Cl)TMe-4PyP] (**C3**). c) Yields from experiments with **1** and **3** as determined by qNMR spectroscopy of both organic and aqueous phases with internal standard (respectively, HMDSO and Me$_2$SO$_2$); %**1** and %**3** represent amounts of starting material recovered after reaction. Results are the average of quadruplicate experiments.

Entry	Catalyst	Substrate added	% 1 recovered	% 3 recovered	% 2 yield	% 4 yield
1	C1	1	<5	–	<5	–
2	C2	1	<5	–	<5	–
3	C3	1	31±3	–	33±1	–
4	C1	3	–	62 ±6	–	12 ±1
5	C2	3	–	55 ±11	–	17 ±4
6	C3	3	–	30 ± 1	–	26 ±3
7	C1	1&3	19 ± 1	34 ± 2	<5	12 ±3
8	C2	1&3	24 ± 5	36 ±3	<5	15 ±6
9	C3	1&3	29 ± 8	22 ± 1	18 ± 1	23 ± 4

analogue **3** to give sulfonium ion **4** moderate yields of 12 to 17 % were obtained with anionic catalysts **C1** and **C2**, respectively (Scheme 1 c). The cationic porphyrin **C3** gave a significantly higher yield of 26 %. Competition experiments between dehydroalanine **1** and methionine **3** showed that sulfonium ion **4** was the exclusive product formed when using **C1** and **C2** (Scheme 1 c). Using **C3**, the sulfonium ion **4** was obtained in similar yields. However, the yield of cyclopropane **2** nearly halved from 33 to 18 %.

Metalloporphyrins **C1**–**C3** were employed in a reaction on nisin (containing 72 % nisin A and 28 % nisin Z, Figure S8 in the Supporting Information) with ethyl diazoacetate under modified conditions: the concentration of nisin and catalyst was lowered to 600 and 60 μM, respectively, whereas the total concentration of EDA was increased to 15 mM (Scheme 2 a). The yield was determined by integration of the peaks in the UPLC chromatogram at 254 nm.[37] After 3 h, the reaction with **C1** resulted in 32 and 10 % yield to single and double modified nisin A, respectively, as judged by UPLC-MS.[38] **C2** gave slightly higher yield of 46 and 25 %, respectively. Increasing the reaction time to 24 h resulted in increased yields of single and double modified nisin in both the **C1**- and **C2**-catalyzed reactions: 53 % single and 47 % double modified nisin A in case of **C1** and 69 and 31 %, respectively, with **C2**. In all cases the conjugate addition of water to dehydroamino acids of unmodified nisin (both nisin A and nisin Z) and modified variants, a known side reaction, was also observed.[29] A reaction of nisin with **C1** in 0.2 M MES buffer pH 6 gave single modified product with excellent selectivity, albeit with a lower conversion than

in 0.2 M acetate buffer pH 4 (Figure S9). Reaction at pH 7 in phosphate or MOPS buffer resulted in rapid degradation of nisin (Figure S10).

In the reaction catalyzed by **C3**, after 3 h, full conversion to give exclusively double modified nisin A and nisin Z was achieved (Scheme 2 b, full UV and TIC traces in Figure S8). The fast reaction and excellent selectivity enabled us to carry out the reaction on a preparative scale (> 60 mg per batch of nisin), which could subsequently be purified by preparative HPLC (Figure S11). While the MS results confirm the single and double modification with a 2-ethoxy-2-oxoethyl moiety derived from EDA, they do not provide information on which position the modification occurred.

The exact positions of the modifications were established by ^{1}H NMR spectroscopy. Surprisingly, ^{1}H NMR spectroscopy showed that the signals of the alkenyl protons of the dehydroamino acids, which are well resolved in the NMR spectrum were still present, both in single and double modified nisin A (Figure 2 a, full spectra in Figure S12). This indicates that, contrary to our initial expectation, the dehydroamino acids were not converted to the corresponding cyclopropane products. Further analysis showed that the sharp singlets at 2 ppm, corresponding to S-methyl groups of methionines **17** and **21** had disappeared upon modification (Figure 2 b).[39,40] This strongly suggested that the methionines were modified by alkylation with the metallocarbenes (Supporting Information p. 5, Note 1, Figures S5 and S12).

This was supported by more detailed analysis of the mass spectra of the purified products: in addition to the peaks with
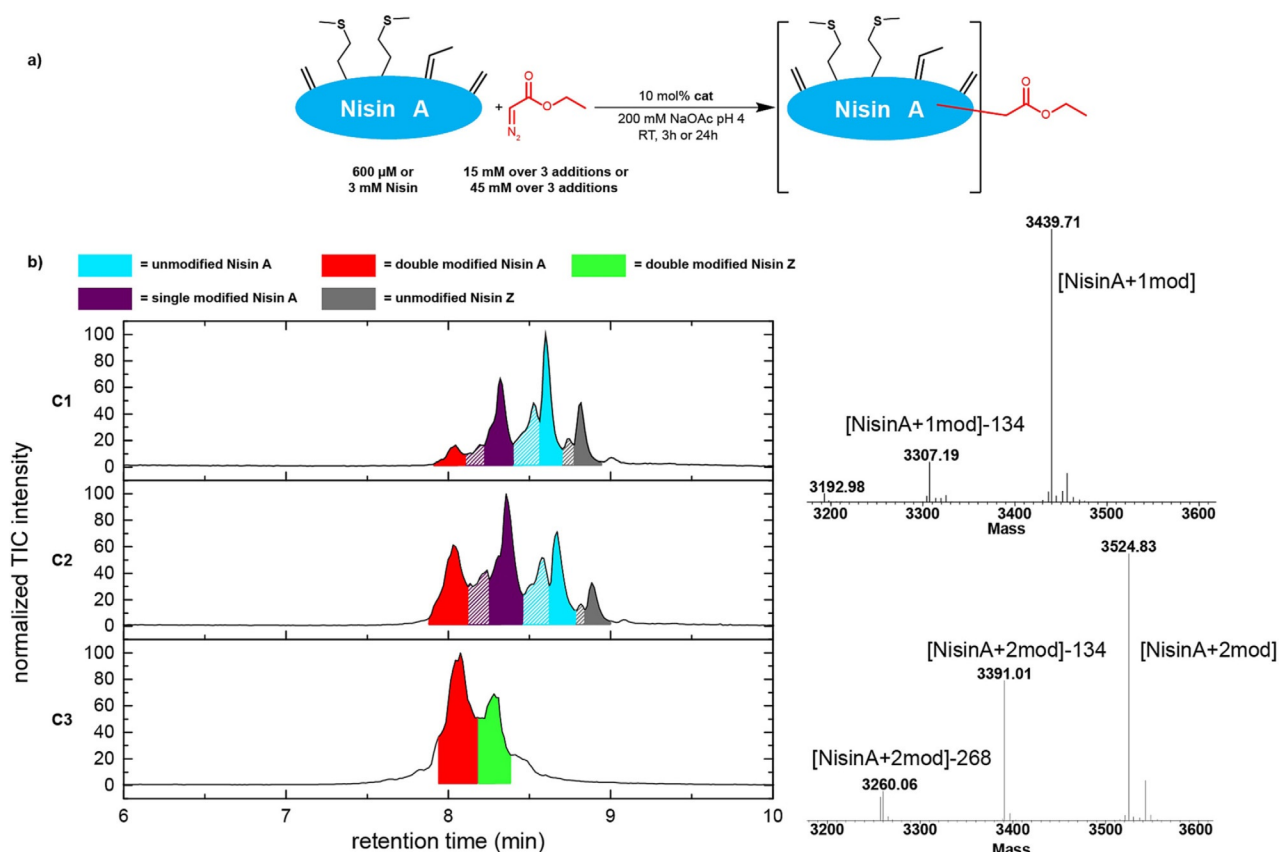
Scheme 2. a) Metalloporphyrin-catalyzed reaction of nisin (600 μм) with ethyl diazoacetate (15 mм added in equal portions over three additions). b) LC-ESI-MS total ion chromatogram with nisin A with up to two modification visible for **C1** and **C2** after 3 h. nisin A was fully converted by **C3** to the double modified species (6.0 min), with nisin Z also being double modified (6.3 min). Striped areas correspond to the respective species with one water molecule added by conjugate addition to a dehydroamino acid. Mass spectra of all species can be found in Figure S8. The observed masses corresponded to the expected masses for the modified product.
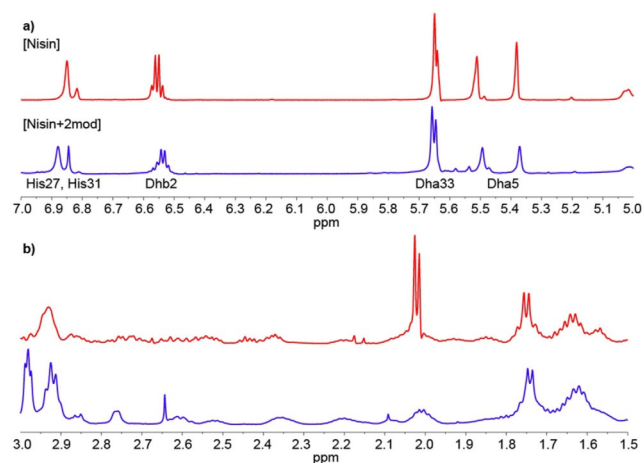

Figure 2. ^{1}H NMR spectra of unmodified and double modified nisin A. a) The signals of the dehydroamino acids are well resolved and can be integrated using signals from histidines as reference. No significant change is observed. b) Aliphatic region of the spectrum. The two singlets at 2.1 ppm are from the methionine methyl groups. These signals disappeared upon modification.

the expected *m/z* for the S-alkylated product, a satellite peak with a *m/z* 134 lower than unmodified nisin A was observed

consistently (Figure 2). It has been reported that in the mass source sulfonium ions cleave between C$_\gamma$ and the sulfur atom, eliminating a sulfide.[41,42] The observed difference in mass between the satellite peak and the parent peak matched the expected mass loss following elimination of ethyl 2-(methyl-thio)acetate (Figure 1). Additionally, the number of modifications corresponded to the number of satellite peaks observed.

In addition to ethyl diazoacetate, two other commercially available diazo reagents were employed under the same reaction conditions. Using *tert*-butyl diazoacetate and **C3** as catalyst, a single modified species was detected after 3 h, albeit with much lower conversion compared to the reaction with ethyl diazoacetate (Figure S13). A reaction with benzyl diazoacetate resulted in rapid degradation of nisin A; after 3 h nisin A was not detected anymore (Figure S14).

It has been reported by Deming and co-workers that sulfonium ions can be selectively dealkylated using nucleophilic thiols, resulting in the formation of the corresponding thioethers (Scheme 3 a).[11,12] Treatment of double modified nisin A with ammonium pyrrolidinedithiocarbamate under various conditions resulted in degradation. Reaction of double modified nisin A with 2-mercaptopyridine gave rise to complete dealkylation of both sulfonium ions, resulting in the corresponding thioethers. Three species were obtained: **6** (nisin A,
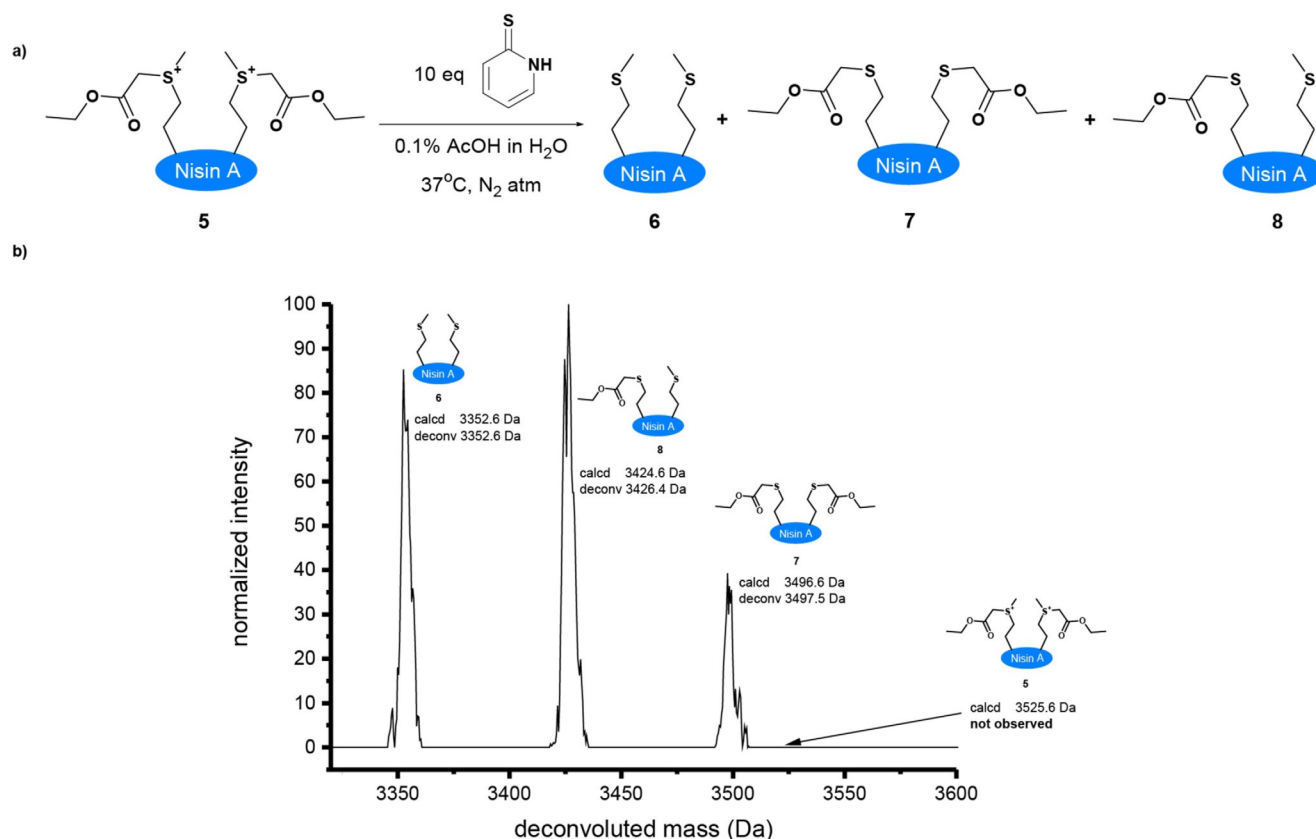
Scheme 3. a) Dealkylation of sulfonium ions using thionucleophiles to obtain a methionine analogue (by demethylation) or methionine (by removal of the ethyl acetate group). An adaptation of a procedure developed by Deming and co-workers was used.[11] b) Deconvolution of mass spectra after the reaction show species belonging to double demethylation (**7**), double dealkylation (**6**) and mixed dealkylation/demethylation (**8**). No single or double modified nisin A (**5**) is observed, indicating that modified peptide only contained sulfonium ions.

by double dealkylation, that is, removal of the 2-ethoxy-2-oxoethyl moiety), **7** containing two methionine analogues (by double demethylation) and **8** containing one methionine and one methionine analogue (Scheme 3 b). These results are also consistent with the conclusion that the metalloporphyrin-catalyzed alkylation reaction is fully selective for methionine, as products from other reactions (e.g., N-alkylation or cyclopropanation) are not susceptible to dealkylation by nucleophilic thiols.

In conclusion, here we reported the serendipitous discovery of a novel catalytic method for the fast and selective alkylation of methionines in nisin A, catalyzed by water soluble metalloporphyrins. The method presented here is the first transition-metal-catalyzed method for selective methionine alkylation under biocompatible conditions. Currently there are only few methods to selectively functionalize methionines in peptides,[11–13] of these methods only one allows for alkylation of methionines under biocompatible (aqueous solvent, neutral pH) conditions.[13] Finally, the possibility to convert methionine into methionine analogues via intermediate formation of the sulfonium ions further expands the toolbox of chemical posttranslational selective modification of RiPPs.

Experimental Section

Representative procedure for the alkylation of methionines in nisin (620 μM): To a 4 mL glass vial with stirring bar was added 3.35 mg (1 μmol, final concentration 620 μM) nisin, 0.2 M NaOAc buffer pH 4 (to a total volume of 1620 μL, corrected for volume of catalyst solution) and 0.1 μmol catalyst stock solution (10 mol%, final concentration 62 μM). The mixture was stirred at room temperature for 5 min, after which 3.1 μL (25 μmol, 25 equiv, corrected for dichloromethane content) ethyl 2-diazoacetate was added. The mixture was stirred at room temperature for 1 h. After 1 and 2 h additional aliquots (3.1 μL, 25 μmol) of ethyl 2-diazoacetate were added (3 additions in total). After 3 h, 5 μL of the reaction mixture was withdrawn and transferred to a vial with 45 μL double distilled water for analysis by UPLC-TQD or UPLC-LCQ. Prolongation of the reaction time to 24 h (total time from the moment of addition) resulted in a higher conversion in case of C1 and C2.

Representative procedure for the dealkylation of sulfonium ions in nisin with 2-mercaptopyridine (PyS): Procedure adapted from refs. [11] and [12].

To 1.5 mL Eppendorf cups was added 1 mg double modified nisin A, 1 mg (9 μmol, 30 equiv) 2-mercaptopyridine and 30 μL solvent (see note). The Eppendorf cups were purged with dried N₂, closed and shaken at 700 rpm for 24 h at 37 °C. After 8 and 24 h, 5 μL was transferred to an UPLC vial with 95 μL water for UPLC-TQD.

Acknowledgements

Conflict of Interest

The authors declare no conflict of interest.

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[36] At this low pH it was not expected that the lysine ε-NH$_2$ groups or the N-terminal amine in nisin would be nucleophilic enough to compete with dehydroalanine for the carbene.
[37] Although nisin does not have a large extinction coefficient at any UV wavelength, the emergence of the sulfonium cation makes integration of TIC signals (which is used for non-fluorescent protein quantification) unreliable.
[38] The two methionines in nisin Z were also alkylated in the same reaction, as shown in the reaction catalyzed by **C3**. As nisin Z was a minor constituent of the nisin mixture, the amounts of modified product were never quantified or isolated.
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