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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 carbones, 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 as the ribosomally synthesized and post-translationally modified peptides (RiPPs) are of interest because of their biological activity, in particular for their potential as antibiotic. 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, some of which have been applied to RiPPs. However, methods for selective methionine modification are rare. 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). Nisin derivatives have been obtained by bio-engineering. For modifications not accessible by bioengineering, site-selective chemical modification of nisin is preferred, as its total synthesis is highly challenging.

The dehydroamino acids, that is, dehydroalanine (Dha) and dehydrobutyryne (Dhb) present in nisin A are attractive target for modification. A variety of method for the bioorthogonal modifications of dehydroalanine have been developed albeit that only a few of these are catalytic. This includes palladium- and rhodium-catalyzed alylations, P450-catalyzed cyclopropanations and photocatalytic additions. We envisioned the cyclopropanation of dehydroalanine using water-soluble metalloporphyrins as catalyst and diazo reagents as carbene precursors.

The reactivity of dehydroalanine analogue 1 towards cyclopropanation with ethyl diazoacetate (EDA) was investigated in [D₄]acetate-buffered solution at pH 4, as nisin itself is most stable at lower pH (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 ¹H 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).

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. In the reaction of methionine
analogue 3 to give sulfonium ion 4 moderate yields of 12 to 17% were obtained with anionic catalysts C1 and C2, respectively (Scheme 1c). 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 1c). 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 2a). The yield was determined by integration of the peaks in the UPLC chromatogram at 254 nm. 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. 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. A reaction of nisin with C1 in 0.2 mM MES buffer pH 6 gave single modified product with excellent selectivity, albeit with a lower conversion than in 0.2 mM 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 2b, 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 1H NMR spectroscopy. Surprisingly, 1H NMR spectroscopy showed that the signals of the alkyl groups of the dehydroamino acids, which are well resolved in the NMR spectrum were still present, both in single and double modified nisin A and nisin Z (Figure 2a, 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 2b). This strongly suggested that the methionines were modified by alkylation with the metalocarbene (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

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\text{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)TM-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); %1 and %3 represent amounts of starting material recovered after reaction. Results are the average of quadruplicate experiments.}
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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 C₃ 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-(methylthio)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 3a).⁴¹,⁴² Treatment of double modified nisin A with ammonium pyrrolidinedithiocarbamate under various conditions resulted in degradation. Reaction of double modified nisin A with 2-mercaptopuridine gave rise to complete dealkylation of both sulfonium ions, resulting in the corresponding thioethers. Three species were obtained: 6 (nisin A,
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 3b). 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, of which only one allows for alkylation of methionines under biocompatible (aqueous solvent, neutral pH) conditions. Finally, the possibility to convert methionine into methionine analogues via intermediate formation of the sulfonium ions further expands the toolbox of chemical post-translational 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 M catalyst stock solution (10 mol%, final concentration 62 μM). The mixture was stirred at room temperature for 5 min, after which 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.
Note: Four different solvents were used (50 mM NaHPO₄ buffer pH 7, 0.1% AcOH in H₂O, 0.2 mM NaOAc pH 4 and H₂O), which all gave equal reaction rates and selectivities according to UPLC-TQD. The stability of the peptide highly depends on the solvent, immediate degradation was observed for the reaction in phosphate buffer. Acidic solvents gave the highest stability.

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**Conflict of Interest**

The authors declare no conflict of interest.

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