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Synthetic Methods |*Hot Paper*|

Catalytic Modification of Dehydroalanine in Peptides and Proteins by Palladium-Mediated Cross-Coupling

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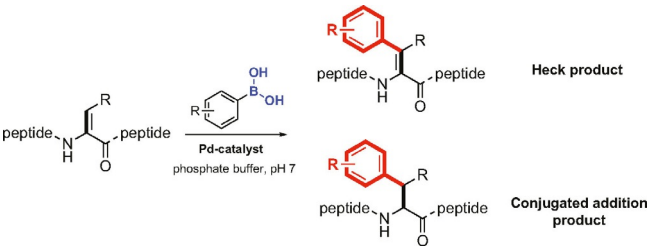
Abstract: Dehydroalanine (Dha) is a remarkably versatile non-canonical amino acid often found in antimicrobial peptides. Herein, we present the catalytic modification of Dha by a palladium-mediated cross-coupling reaction. By using $Pd(EDTA)(OAc)_2$ as water-soluble catalyst, a variety of arylboronic acids was coupled to the dehydrated residues in proteins and peptides, such as Nisin. The cross-coupling reaction gave both the Heck product, in which the sp^2-hybridisation of the α-carbon is retained, as well as the conjugated addition product. The reaction can be performed under mild aqueous conditions, which makes this method an attractive addition to the palette of bio-orthogonal catalytic methods.

Introduction

Dehydroalanine (Dha) is a remarkably versatile non-canonical, yet naturally occurring α,β-unsaturated amino acid that features a unique sp^2 hybridized α-carbon. The resulting planar structure provides different structural properties and reactivity than conventional sp^3 hybridized amino acids.[1] In Nature, dehydrated amino acids are installed via posttranslational dehydration of serine and threonine, and used to create lanthionine bridges found in lantipeptides,[2] and piperidine moieties found in thiopeptides.[3] Most of these peptides possess antimicrobial or antitumor activity,[4] which make them interesting targets for new antibiotics and medicines. Yet, modification of these peptides through bio-engineering[5] or total synthesis[6] is challenging and is thus preferably done by late-stage site-selective chemical modification.[7] The residual Dha residues in these peptides are excellent reactive sites for such transformations. Michael additions,[8] 1,3-dipolar cycloadditions,[9] radical carbon–carbon bond formations,[10] and catalytic arylation of peptides in organic solvents have been reported.[11] In all these transformations, the sp^2 hybridization of the α-carbon is lost, which may be of importance to preserve the structure and biological activity of the proteins and peptides. Palladium-mediated Heck-type[12] cross-coupling could leave the sp^2 hybridization intact, although competition of the conjugate addition product is also to be expected for the conjugate alkene in Dha.[12] Choosing a water-soluble organometallic complex contributes to the versatility of the approach: a requirement for protein modification over peptide modification is that the reaction has to take place under physiological conditions (e.g., in water at neutral pH at 37 °C; Scheme 1). Therefore, we sought a water-soluble palladium complex, which can carry out this transformation. Herein, we present the palladium-catalyzed cross-coupling reaction for the site-selective modification of Dha with arylboronic acids in peptides and proteins by a complex based on ethylenediaminetetraacetic acid (EDTA), a commonly used water-soluble metal chelator.[13]



Scheme 1. Schematic representation of palladium-catalyzed cross-coupling on dehydrated amino acids in peptides and proteins.

Results and Discussion

Initial studies were focused on the reaction of Dha monomer (**1**), with 4-methoxyphenylboronic acid (**2a**; Figure 1a). Neutral to slightly basic conditions (pH 7–8) proved to be necessary to obtain conversion of the Dha monomer, as was determined by ^1H NMR spectroscopy. Two products were obtained, and identified to be the Heck product (HP) and the conjugate addition product (CAP). A mixture of these products is commonly observed for cross-coupling of conjugated alkenes, and is difficult to avoid.[12] The HP was found to be the main product of the reaction (HP/CAP 80:20). Carrying out the reaction under oxygen atmosphere did not improve the conversion, which

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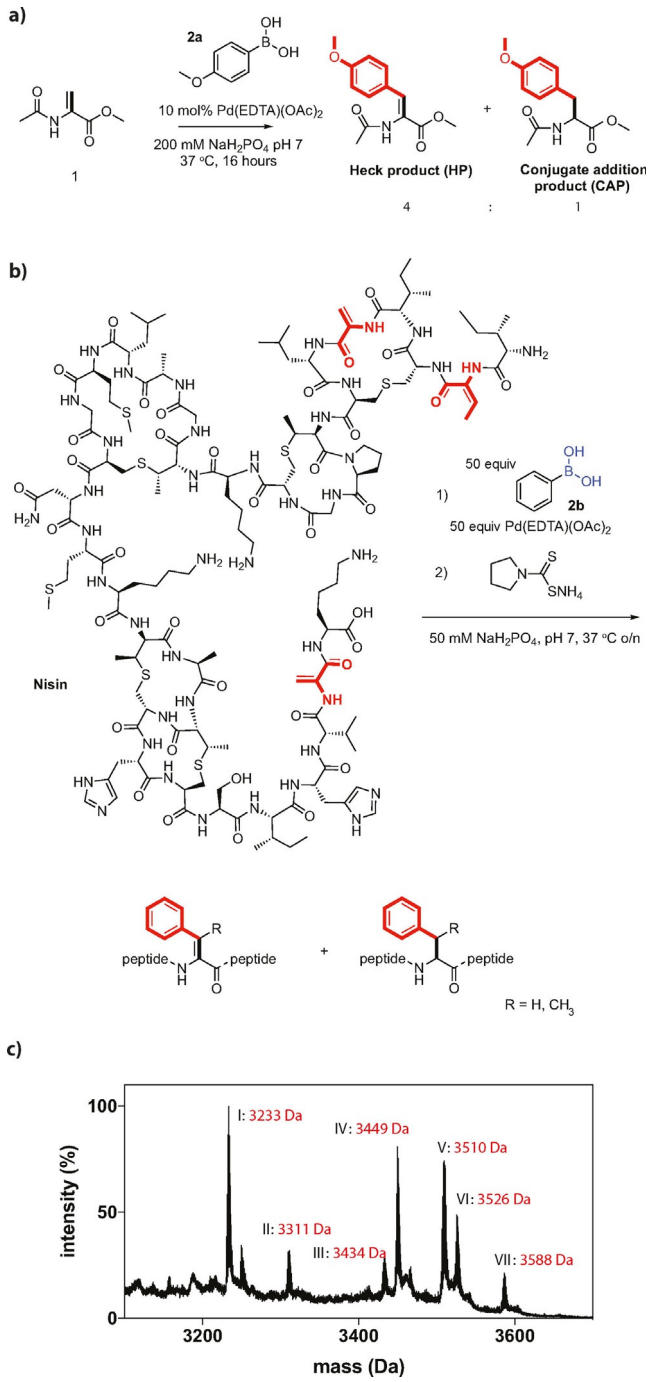

Figure 1. Cross-coupling reaction on Dha: a) General reaction scheme for the Dha monomer; b) General reaction scheme, optimized conditions: Nisin (40 μM), boronic acid (2 mM), and $Pd(EDTA(OAc)_2$ (2 mM in 25 μL buffer (50 mM NaH_2PO_4 pH 7 2.2% DMF) shaken 16 hours at 37 °C. Prior to mass analysis 3 equiv (w.r.t. Pd) 3-MPA, MTG or APDTC are added; c) Representative MALDI TOF spectrum of reaction mixture **3b**. (I) degraded (degr.) Nisin(Ph); (II) degr. $Nisin(Ph)_2$; (III) Nisin(Ph); (IV) $Nisin(Ph)(H_2O)$; (V) $Nisin(Ph)_2$; (VI) $Nisin(Ph)_2(H_2O)$; (VII) $Nisin(Ph)_3$.

means ambient atmosphere provides enough molecular oxygen for the Pd^0 to Pd^{II} oxidation to occur, thereby closing the catalytic cycle. The highest conversion was obtained with 10 mol% catalyst, an excess of arylboronic acid (2 equiv), in phosphate buffer at 37 °C. Interestingly, other commonly used water-soluble palladium complexes did not result in any conversion of Dha (Table S1 in the Supporting Information). The reaction conditions for the modification of the Dha monomer were not further optimized, because the main focus is on modification of Dha in proteins and peptides. The $Pd(EDTA)(OAc)_2$ catalyst, an excess of arylboronic acid, and phosphate buffer pH 7 were selected for our subsequent studies on protein and peptide modification.

We focused on the palladium-mediated cross-coupling reaction of the lantipeptide Nisin.[5e] Nisin naturally contains three dehydrated amino acids: Dhb-2 (dehydrobutyrine), Dha-5, and Dha-33. Hence, a maximum of three modifications is expected. The peptide is hydrophobic in nature, which gives rise to solubility problems in aqueous solution, and Nisin is less stable at pH > 5.[14] Moreover, conjugate addition of water to Dha, and hydrolytic cleavage at this site are known degradation reactions.[15] Despite the potential of Nisin as an antibiotic, to the best of our knowledge, no catalytic methods for modification have been reported, and stoichiometric chemical modifications are scarce.[9,16]

Nisin was reacted with phenylboronic acid (**2b**) by using $Pd(EDTA)(OAc)_2$ as catalyst (Figure 1b). The crude reaction mixture was analyzed directly by UPLC/MS. When more than one equivalent of palladium catalyst was used, no peptide signal was observed in the UPLC/MS chromatogram (Figure S5 in the Supporting Information). This was attributed to non-specific coordination of the palladium catalyst to the backbone or side chains of the peptide, a frequently observed limitation of palladium-mediated protein reactions.[17] This was addressed by addition of 3-mercaptopropanoic acid (3-MPA), a commonly used palladium scavenger, prior to mass analysis. To overcome the loss of catalyst due to unspecific coordination, a 50-fold excess of the catalyst was used, together with a 50-fold excess of arylboronic acid. Subsequent scavenging with 3-MPA gave **3b** as a mixture of singly and doubly modified Nisin (Figure 1c). However, purification of the peptide from the in situ formed palladium-[3-MPA]-complex proved to be difficult. The formed palladium complex is > 2 kDa, making removal by size-exclusion chromatography or dialysis inefficient.

Therefore, alternative scavengers for the palladium catalyst were investigated, which included a variety of water-soluble thiols, as well as resin-based scavengers (Table S3 in the Supporting Information). In most cases, these gave rise to either insufficient scavenging or purification difficulties similar to what was encountered with 3-MPA. Good results were obtained with methylthioglycolate (MTG) and ammonium pyrrolidine dithiocarbamate (APDTC), because these form insoluble palladium complexes,[18] which precipitate from the solution. The precipitate is readily removed by centrifugation or filtration over 0.45 μm pore filters. By using this method, 99% of the palladium was removed, as was measured by inductively coupled plasma optical emission spectrometry (ICP OES; see the Supporting Information, Section 3.5). Purification from starting materials and by-products was then achieved by size-exclusion column chromatography (PD Minitrap G25) or rp-HPLC. In this way, modified Nisin, as a mixture of 48% singly modified, 46% doubly modified, and 3% triply modified pep-

tide was obtained. Control experiments, in which either the arylboronic acid or palladium catalyst were omitted from the reaction mixture, resulted in no reaction, which demonstrates that the reaction is indeed mediated by the palladium catalyst (see the Supporting Information, Section 3.6).

To determine whether the cross-coupling reaction takes place at the expected dehydrated amino acids, and to determine whether for Nisin also the conjugated addition product is formed, besides the HP, modified Nisin (**3b**) was hydrolyzed in a microwave oven in 6 M HCl(aq.), and the individual amino acids were identified. Cross-coupling reaction at a Dha residue with **2b** results in either dehydrophenylalanine (the HP), or phenylalanine (the conjugate addition product), which should be detectable in the hydrolysate. Therefore, one half of the hydrolysate was derivatized with Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA)), which will react with phenylalanine to give FDAA-Phe.[19] Analysis with LC/MS and comparison with FDAA derivatized D/L-phenylalanine samples showed the presence of both enantiomers of phenylalanine in the hydrolysate of **3b** (Figure 2a). Because Nisin naturally does not contain phenylalanine, the presence of **3b** proves that the cross-coupling indeed takes place at a Dha residue and, moreover, that the reaction partly followed the conjugated addition pathway, similar to the reaction on the Dha monomer.

Interestingly, an excess of L-Phe was observed. Because the $Pd(EDTA)(OAc)_2$ catalyst is not chiral, the enantiomeric excess (*ee*) must be induced by the chirality of the peptide (i.e., substrate control). Furthermore, Dhb is also subjected to the cross-coupling reaction, as the product of conjugate addition of **2b** to Dhb derivatized with FDAA was also observed in the LC/MS chromatogram (Figure S8 in the Supporting Information).

Marfey's reagent did not reveal the presence of the dehydrophenylalanine (i.e., the HP), since unprotected dehydrated amino acids equal a primary enamine, and therefore quickly tautomerize, followed by hydrolysis to their corresponding α-keto-acid, that is, phenylpyruvic acid (PhPA). Therefore, the other half of the hydrolysate was treated with dansylhydrazine, which reacts with α-keto-acids to form hydrazones.[20] The reaction usually gives two isomers (*E*/*Z*), which separate during LC. Analysis with LC/MS and comparison with a sample the hydrazine formed between dansylhydrazine and PhPA, confirmed the presence of PhPA in the hydrolysate of **3b** (Figure 2b). Also, the α-keto-acid of the HP of Dhb was detected (Figure S9 in the Supporting Information), which supports that the Heck pathway was also followed in the palladium-mediated cross-coupling reaction of peptides. Thus, product **3b** has partially maintained its sp^2-hybridised α-carbon and, as a result, its unique structural properties.

To increase the rate of the reaction, the cross-coupling reaction was performed at pH 8 (Table S4 in the Supporting Information). Although an increased amount of double cross-coupled product was obtained, also a higher amount of degraded Nisin was observed due to the competing water addition to the Dha. The competition of the cross-coupling reaction with the spontaneous water addition in Nisin might explain the predominant formation of single cross-coupled product. Nevertheless, by using this method, it is possible to introduce a variety of different aryl groups containing diverse functional groups to Nisin (Figure 3). This includes an azide functionality (**3e**), which can subsequently be modified by alkyne-azide click reactions to conjugate the peptide further, and an carboxylic acid functionality (**3g**), which may enhance the water solubility of such peptides. Finally, the generality of palladium mediated cross-coupling reaction was investigated by using the reaction for protein modification. Small ubiquitin-like modifier (SUMO, ≈11 kDa)[22] containing a chemically introduced Dha residue was used as substrate.[8d,23] The Dha residue was introduced at two different positions: near the C-terminus of protein, to minimize steric effects on the reaction (SUMO_G98Dha), and in

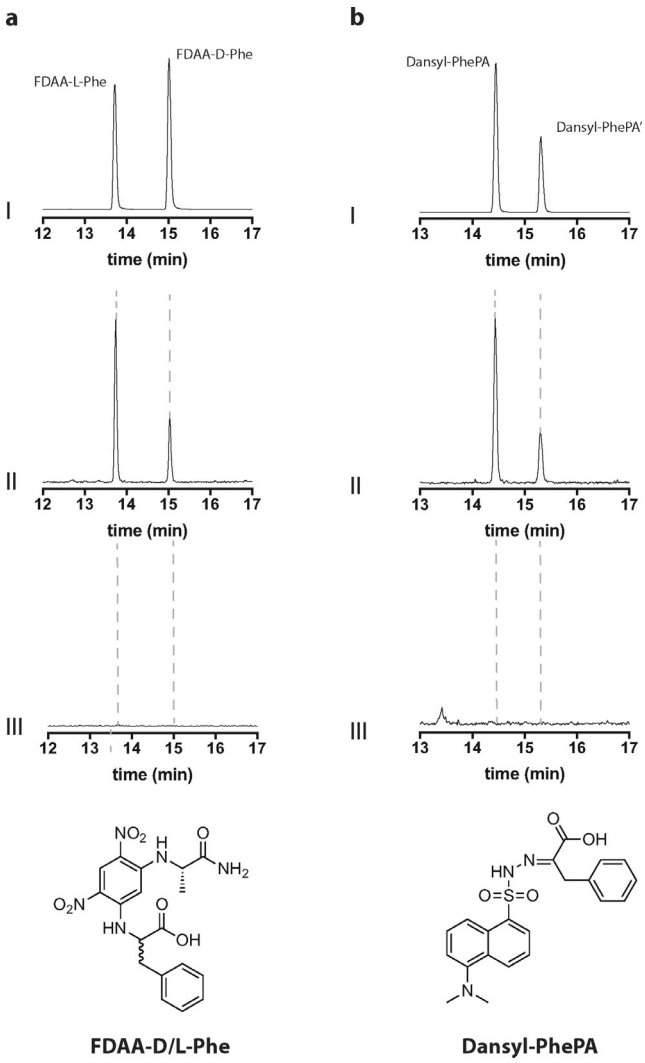

Figure 2. Analysis of the site-selectivity of cross-coupled Nisin and determination of Heck product and conjugated addition product. a) Analysis of introduced phenylalanine using Marfey's method: (I) EIC of $[M+H]=418$ Da corresponding to D/L phenylalanine derivatized with FDAA; (II) EIC of hydrolysate of **3b** derivatized with FDAA; (III) EIC of hydrolysate of Nisin derivatized with FDAA. b) Analysis of introduced dehydrophenylalanine using hydrazine formation: (I) Extracted ion chromatogram (EIC) of $[M+H]=412$ Da corresponding to phenylpyruvic acid derivatized with dansylhydrazine; (II) EIC of hydrolysate of **3b** derivatized with dansylhydrazine; (III) EIC of hydolysate of Nisin derivatized with dansylhydrazine.

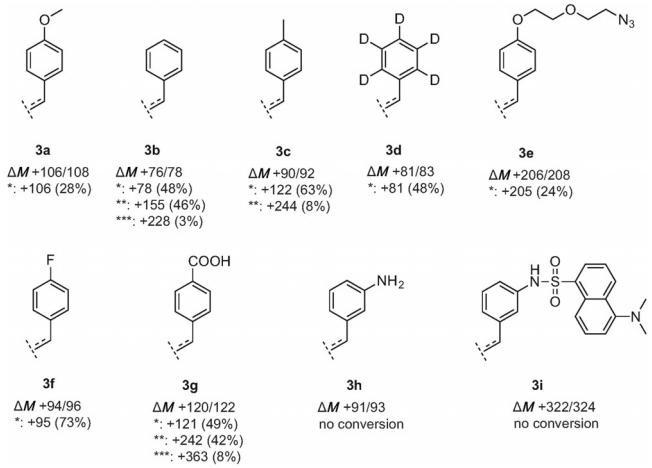

Figure 3. Scope of arylboronic acids in cross-coupling reaction with Nisin. Single modification (*), double modification (**), and triple modification (***) is observed. The conversion is displayed in parentheses based on integration of EIC of corresponding product. Conversion is calculated based on integration of the EIC of the corresponding product divided by sum of the areas of all compounds, assuming that ionization is similar for all products, which are structurally very similar.[21]

one of the solvent exposed loops (SUMO_M60Dha; see the Supporting Information, Section 3.2, 3.11–13). Treatment of the protein with 20 equivalents of $Pd(EDTA)(OAc)_2$ catalyst and 100 equivalents of arylboronic acid showed full conversion to the cross-coupled product for *p*-toluylboronic acid (Figure 4). Control experiments performed on SUMO_G98A, which lacks the Dha moiety, resulted in no reaction, which demonstrates that the reaction is also in proteins specific at the Dha residue (Figure S16 in the Supporting Information). Reactions with phenyl-, phenyl[D_5]-, and methoxyphenyl-substituted boronic acids (**5a–d**) resulted in full conversion of the cross-coupled product, too. Carboxylic acid-, fluorine-, and amine-substituted phenylboronic acids were coupled as well, although not with full conversion (**5f–h**). Neither an increase of palladium catalyst, nor an increase in arylboronic acid resulted in full conversion being achieved. Attempts to cross-couple dansyl substituted arylboronic acid **5i**, or a pyrene boronic acid **5j**, did not result in any conversion. Most likely, this is due to the poor water solubility of these reagents. Azide-substituted arylboronic acid **5e** was cross-coupled successfully, albeit that a fraction of the azide moieties was reduced to the corresponding amine during the treatment with the palladium scavenger. The azide was subsequently reacted in a copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) with an alkyne substituted bodipy (**12**; Figure S17).

SUMO_M60Dha showed a similar trend when applied in the cross-coupling reaction: full conversion was achieved with deuterium-, *p*-methyl-, and *p*-methoxy-substituted phenylboronic acids (Table S6b in the Supporting Information), whereas 4-fluorophenylboronic acid did not give rise to full conversion.

Further investigation of the modified protein by microwave-assisted hydrolysis of **5c** and subsequent derivatization with Marfey's reagent or dansylhydrazine revealed that the cross-coupling on proteins also follows both the conjugate addition

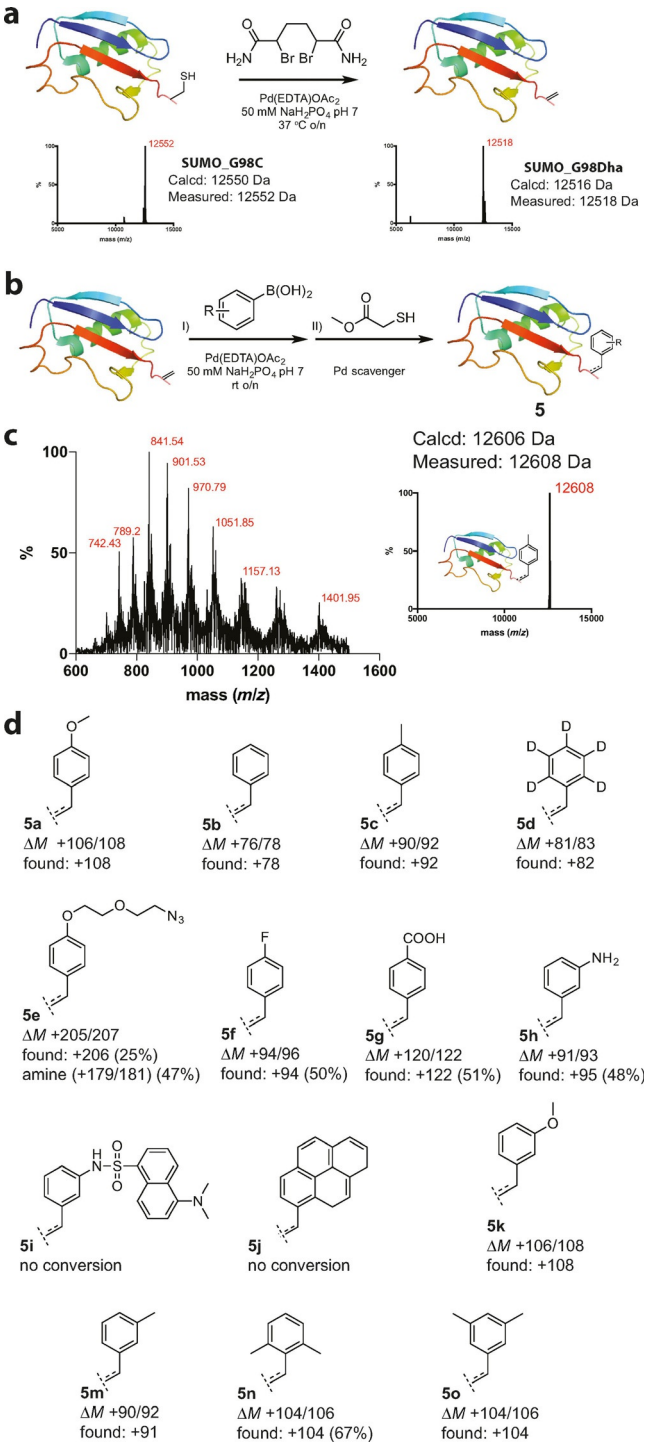

Figure 4. $Pd(EDTA)(OAc)_2$ catalyzed cross-coupling reaction on SUMO. a) General reaction scheme for the chemical introduction of Dha in SUMO;[8d] b) General reaction scheme, optimized conditions: protein (45 μM), boronic acid (4.5 mM), and $Pd(EDTA)(OAc)_2$ (0.9 mM) in 22 μL buffer (50 mM NaH_2PO_4 pH 7 2.2% DMF) shaken 16 hours 37 °C. Prior to UPLC/MS analysis 3 equiv (w.r.t. Pd) 3-MPA, MTG or ADPTC are added; c) Representative UPLC/MS spectrum of reaction mixture **5c** and deconvoluted spectrum; d) Scope of arylboronic acids in cross-coupling reaction. The conversion is given in parentheses if the reaction did proceed in full conversion. Conversion is calculated based on integration of the EIC of the corresponding product divided by sum of the areas of all compounds, assuming that ionization is similar for all products, which are structurally very similar.[21]

and Heck pathways, because both *p*-toluylalanine as *p*-toluylpyruvic acid were observed (Figure S18 in the Supporting Information).

Conclusion

We have introduced the $Pd(EDTA)(OAc)_2$ catalyzed cross-coupling reaction as a method for the modification of the non-canonical amino acid dehydroalanine in proteins and peptides. Although no full conversion was achieved for Nisin, it has to be emphasized that such a late-stage modification approach is far more efficient than the alternatives, such as total synthesis.[7] Detailed analysis of the individual amino acids of the product shows that the cross-coupling reaction is specific for the dehydrated residues, and follows two mechanistic pathways giving the Heck product and the conjugate addition product. In the Heck product, the sp^2 hybridization of the α-carbon is maintained, thus leaving the geometry of the backbone of the biomolecules intact, which may be of particular importance for natural Dha/Dhb containing compounds. Although an excess of the catalyst is necessary to obtain high conversions, purification by precipitation of the palladium catalyst with methylthioglycolate or pyrrolidine dithiocarbamate as new scavengers removes up to 98–99% of the catalyst. The unique product of the reaction on Dha, combined with the fact that the reactions can be performed under mild, aqueous, and pH-neutral conditions at 37 °C, makes this method an attractive addition to the palette of bio-orthogonal catalytic methods.

Experimental Section

General procedure of catalysis on Nisin

Catalysis was performed in 50 mM NaH_2PO_4 buffer pH 7 or pH 8 with a final concentration of 40 μM peptide, 2 mM boronic acid, and 2 mM catalyst. A typical catalysis reaction was set up as follows: Nisin (2 nmol in 30 μL buffer) and 10 μL of 10 mM boronic acid stock solution were combined. Catalyst stock solution (10 μL of 10 mM) was added. The vial was shaken for 16 h at room temperature. Methylthioglycolate stock solution (5 μL of 250 mM) was added to scavenge the palladium, and the reaction mixture turned yellow instantly. The reaction mixture was shaken at 37 °C for an additional hour. The precipitate was removed by centrifugation for 10 min at 13.4 Krpm. The supernatant was analyzed by UPLC/MS TQD and purified by rp-HPLC.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: bio-orthogonal catalysis · cross-coupling · dehydroalanine · Nisin · palladium

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