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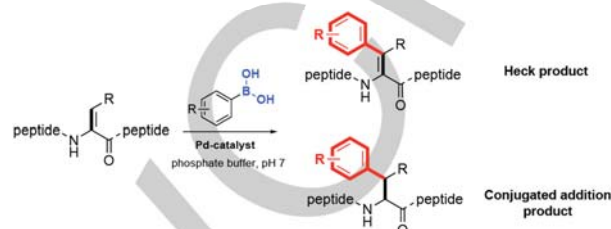
# Catalytic modification of dehydroalanine in peptides and proteins via palladium mediated cross coupling

A. Dowine de Bruijn<sup>[a]</sup> and Gerard Roelfes<sup>[a]\*</sup>

**Abstract:** Dehydroalanine (Dha) is a remarkably versatile non-canonical amino acid often found in antimicrobial peptides. Here we present the catalytic modification of Dha via a palladium mediated cross coupling reaction. Using Pd(EDTA)(OAc)<sub>2</sub> 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 yields both the Heck product, in which the *sp*<sup>2</sup>-hybridisation of the  $\alpha$ -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  $\alpha,\beta$ -unsaturated amino acid that features a unique *sp*<sup>2</sup> hybridised  $\alpha$ -carbon. The resulting planar structure provides different structural properties and reactivity than conventional *sp*<sup>3</sup> hybridised amino acids.<sup>[1]</sup> In nature, dehydrated amino acids are installed via posttranslational dehydration of serine and threonine, and used to create lanthionine bridges found in lantipeptides,<sup>[2]</sup> and piperidine moieties found in thiopeptides.<sup>[3]</sup> Most of these peptides possess antimicrobial or antitumor activity,<sup>[4]</sup> which make them interesting targets for new antibiotics and medicines. Yet, modification of these peptides via bio-engineering,<sup>[5]</sup> or total synthesis<sup>[6]</sup> is challenging and is thus preferably done by late-stage site-selective chemical modification.<sup>[7]</sup> The residual Dha residues in these peptides are excellent reactive sites for such transformations. Michael additions<sup>[8]</sup>, 1,3-dipolar cycloadditions,<sup>[9]</sup> radical carbon-carbon bond formations,<sup>[10]</sup> and catalytic arylation of peptides in organic solvents have been reported.<sup>[11]</sup> In all these transformations the *sp*<sup>2</sup> configuration of the  $\alpha$ -carbon is lost, which may be of importance to preserve the structure and biological activity of the proteins and peptides. Palladium mediated Heck-type<sup>[12]</sup> cross coupling could leave the *sp*<sup>2</sup> hybridisation intact, although competition of the conjugate addition product is also to be expected for the conjugate alkene in Dha.<sup>[12]</sup> Choosing a water soluble organometallic complex contributes to the versatility of the approach: a requirement for protein modification over peptide



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

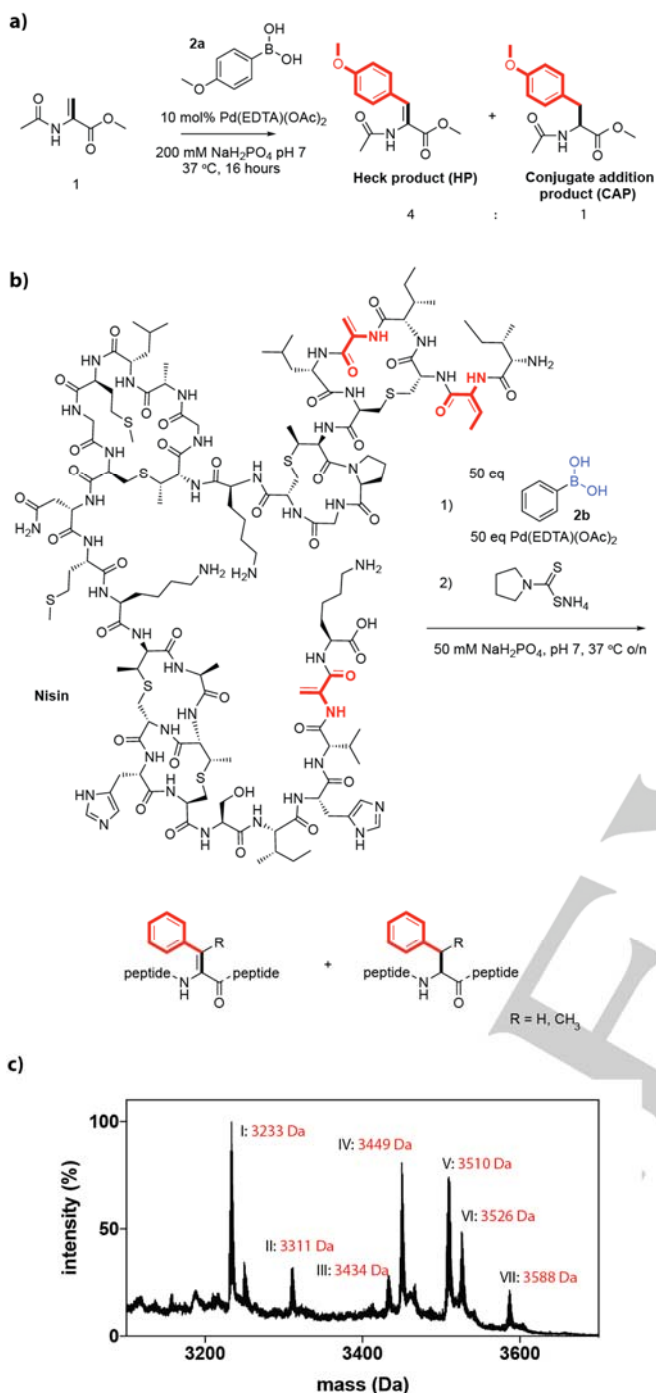
modification is that the reaction has to take place under physiological conditions (e.g. in water at neutral pH and at 37°C). Therefore, we sought a water soluble palladium complex which can carry out this transformation. Here, we present the palladium catalyzed cross coupling reaction for the site-selective modification of Dha with arylboronic acids in peptides and proteins by a complex based on ethylene-diamine-tetraacetic acid (EDTA), a commonly used water soluble metal chelator.<sup>[13]</sup>

## Results and Discussion

Initial studies focused on the reaction of Dha monomer (**1**), with 4-methoxyphenylboronic acid (**2a**) (figure 1a). Neutral to slightly basic conditions (pH 7-8) proved necessary to obtain conversion of the Dha monomer, as was determined by <sup>1</sup>H-NMR. 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.<sup>[12]</sup> The Heck product was found to be the main product of the reaction (ratio HP:CAP 80:20). Carrying out the reaction under oxygen atmosphere did not improve the conversion, which 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 eq), in phosphate buffer at 37 °C. Interestingly other commonly used water soluble palladium complexes did not result in any conversion of Dha (table S1). The reaction conditions for the modification of the Dha monomer were not further optimized since the main focus is on modification of Dha in proteins and peptides. The Pd(EDTA)(OAc)<sub>2</sub> catalyst, an excess of arylboronic acid, and phosphate buffer pH 7 were selected for our subsequent studies on protein and peptide modification.

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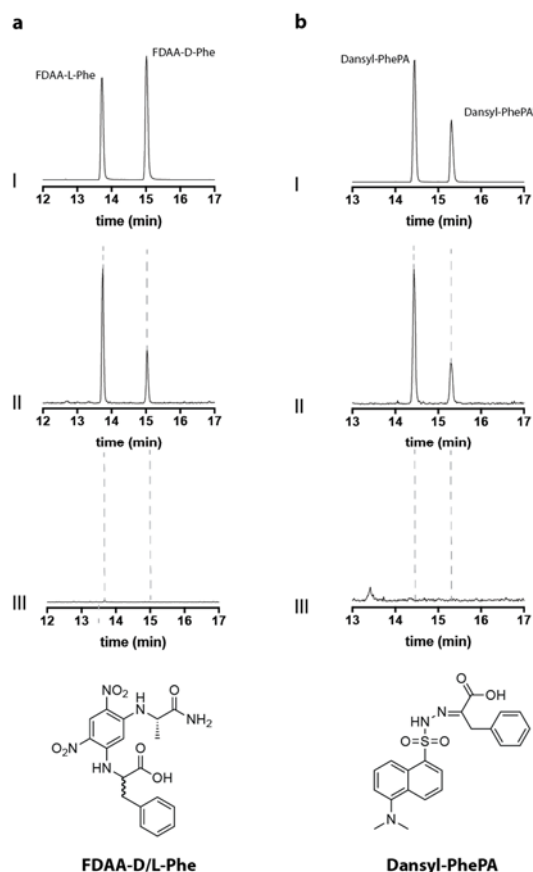


**Figure 1:** Cross coupling reaction on Dha **a)** General reaction scheme on Dha monomer; **b)** General reaction scheme, optimised conditions: nisin (40  $\mu$ M), boronic acid (2 mM) and Pd(EDTA)(OAc)<sub>2</sub> (2 mM) in 25  $\mu$ L buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub> pH 7 2.2% DMF) shaken 16 hours at 37°C. Prior to mass analysis 3 eq (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)<sub>2</sub>; III: nisin(Ph); IV: nisin(Ph)(H<sub>2</sub>O); V: nisin(Ph)<sub>2</sub>; VI: nisin(Ph)<sub>2</sub>(H<sub>2</sub>O); VII: nisin(Ph)<sub>3</sub>.

We focused on the palladium mediated cross coupling reaction of the lantipeptide nisin.<sup>[5e]</sup> Nisin naturally contains three dehydrated amino acids: Dhb-2 (dehydrobutyrine), Dha-5 and Dha-33, a maximum of three modifications is thus expected. The peptide is hydrophobic in nature, which gives rise to solubility problems in aqueous solution, and nisin is less stable at pH > 5.<sup>[14]</sup> Moreover, conjugate addition of water to Dha, and hydrolytic cleavage at this site are well-known degradation reactions.<sup>[15]</sup> 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.<sup>[9, 16]</sup>

Nisin was reacted with phenylboronic acid (**2b**) using Pd(EDTA)(OAc)<sub>2</sub> as catalyst (figure 1b). The crude reaction mixture was analysed 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). 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.<sup>[17]</sup> 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 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 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) since these form insoluble palladium-complexes,<sup>[18]</sup> which precipitate from the solution. The precipitate is readily removed by centrifugation, or filtration over 0.45  $\mu$ m pore filters. Using this method, 99% of the palladium was removed, as measured by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) (Supplementary Information S3.5). Purification from starting materials and byproducts 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 peptide, was obtained. Control experiments where 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 (Supplementary Information S3.6).



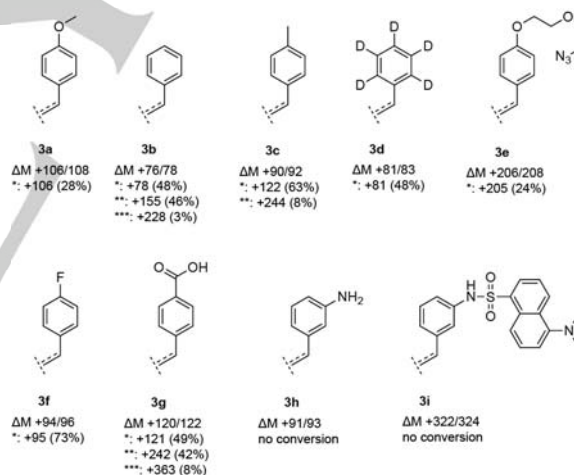
**Figure 2:** Analysis of the site selectivity of cross coupled nisin, and determination of Heck product and conjugated addition product. **a)** Analysis of introduced phenylalanine using Marfey's method: (I) EIC of  $[M+H] = 418$  Da corresponding to D/L phenylalanine derivatised with FDAA; (II) EIC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

To determine whether the cross coupling reaction takes place at the expected dehydrated amino acids, and to determine whether for nisin besides the Heck product also the conjugated addition product is formed, modified nisin (**3b**) was hydrolysed 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 Heck product), or phenylalanine (the conjugate addition product), which should be detectable in the hydrolysate. One half of the hydrolysate was therefore derivatised with Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA)) which will react with phenylalanine to give FDAA-Phe.<sup>[19]</sup> Analysis with LC/MS and comparison with FDAA derivatised D/L-phenylalanine samples showed the presence of both enantiomers of phenylalanine in the hydrolysate of **3b** (figure 2a). Since nisin naturally does not contain phenylalanine, the presence of **3b** proves 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. Since the Pd(EDTA)(OAc)<sub>2</sub> catalyst is not chiral, the enantiomeric excess 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 derivatised with FDAA was also observed in the LC/MS chromatogram (Figure S8).

Marfey's reagent does not reveal the presence of the dehydrophenylalanine (i.e. the Heck product), since unprotected dehydrated amino acids equal a primary enamine, and therefore quickly tautomerise, followed by hydrolysis to their corresponding  $\alpha$ -keto-acid, i.e. phenylpyruvic acid (PhPA). Therefore, the other half of the hydrolysate was treated with dansylhydrazine, which reacts with  $\alpha$ -keto-acids to form hydrazones.<sup>[20]</sup> The reaction usually yields 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** nisin (figure 2b). Also the  $\alpha$ -keto-acid of the product of Heck reaction on Dhb was detected (Figure S9). So, the Heck pathway is also followed in the palladium mediated cross coupling reaction of peptides. Thus, product **3b** has partially maintained its  $sp^2$  hybridised  $\alpha$ -carbon and, as a result, its unique structural properties.



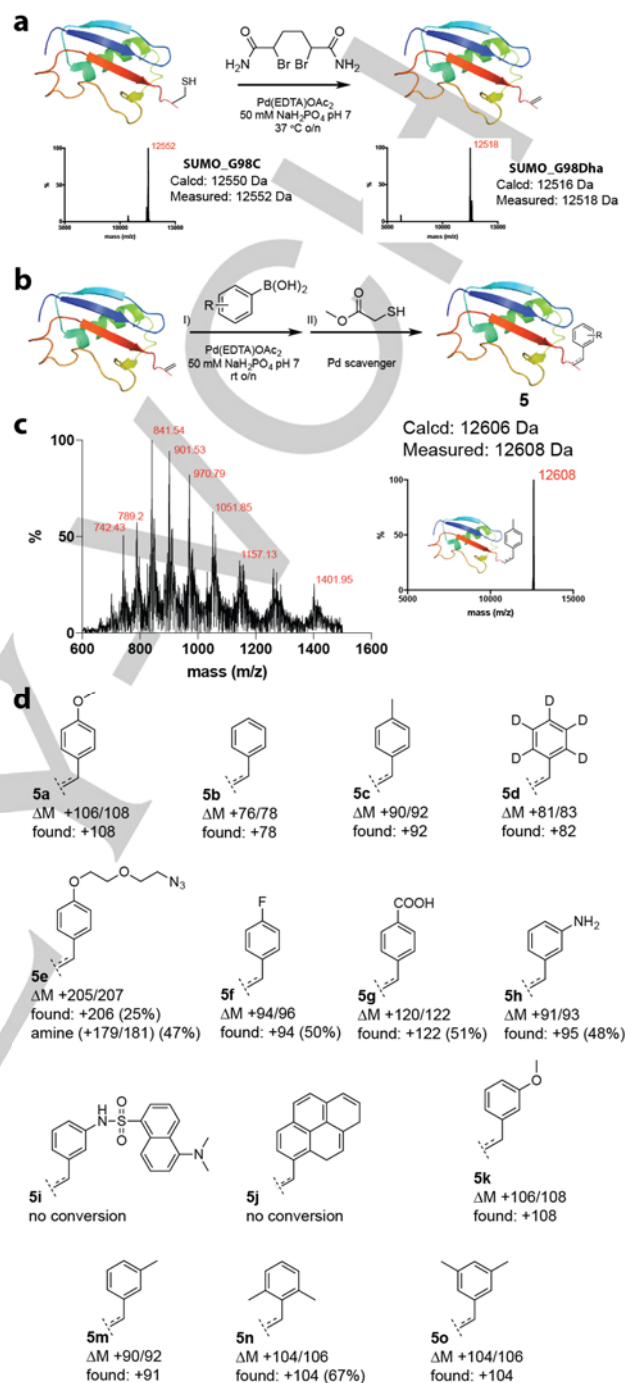
**Figure 3:** Scope of arylboronic acids in cross coupling reaction with nisin. Single modification (\*), double modification (\*\*) and triple modification (\*\*\*) is observed. In (..) conversion is displayed based on integration of EIC of corresponding product. Conversion is calculated based on integration of the EIC of the corresponding product divided by sum of the areas of all compounds, assuming that ionisation is similar for all products, which are structurally very similar.<sup>[21]</sup>

In an attempt to increase the rate of the reaction, the cross coupling reaction was performed at pH 8 (table S4). 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, using this method it is possible to introduce a variety of different arylgroups containing diverse functional groups to nisin (figure 3). This includes an azide functionality (**3e**) which can subsequently be modified via alkyne-azide click reactions to conjugate the peptide further, and a 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. SUMO (Small Ubiquitin-like MOdifier, ~11kDa)<sup>[22]</sup> containing a chemically introduced Dha residue<sup>[8d, 23]</sup> was used as substrate. The Dha residue was introduced at two different positions: near the C-terminus of protein, to minimise steric effects on the reaction (SUMO\_G98Dha), and in one of the solvent exposed loops (SUMO\_M60Dha) (Supplementary Information S3.11-13). Treatment of the protein with 20 eq Pd(EDTA)(OAc)<sub>2</sub> catalyst and 100 eq 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). Reactions with phenyl-, *d*<sub>5</sub>-phenyl-, and methoxyphenyl substituted boronic acids (**5a-d**) resulted 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), while 4-fluorophenylboronic acid did not give rise to full conversion.

Further investigation of the modified protein by microwave assisted hydrolysis of **5c** and subsequent derivatisation with Marfey's reagent or dansylhydrazine revealed that the cross coupling on proteins also follows both the conjugate addition and Heck pathways, as both *p*-toluylalanine as *p*-toluylpyruvic acid were observed (figure S18).



**Figure 4:** Pd(EDTA)(OAc)<sub>2</sub> catalysed cross coupling reaction on SUMO. **a**) General reaction scheme for the chemical introduction of Dha in SUMO;<sup>[8d]</sup> **b**) General reaction scheme, optimised conditions: protein (45 μM), boronic acid (4.5 mM) and Pd(EDTA)(OAc)<sub>2</sub> (0.9 mM) in 22 μL buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub> pH 7 2.2% DMF) shaken 16 hours 37 °C. Prior to UPLC/MS analysis 3 eq (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. Between (..) the conversion is given 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 ionisation is similar for all products, which are structurally very similar.<sup>[21]</sup>

## Conclusions

In conclusion, here we have introduced the Pd(EDTA)(OAc)<sub>2</sub> catalysed cross coupling reaction as a method for the modification of the non-canonical amino acid dehydroalanine in proteins and peptides. While 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.<sup>[7]</sup> Detailed analysis of the individual amino acids of the product shows the cross coupling reaction is specific for the dehydrated residues, and follows two mechanistic pathways yielding the Heck product and the conjugate addition product. In the Heck product the *sp*<sup>2</sup>-hybridisation of the  $\alpha$ -carbon in the Heck product 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 novel 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 bio-orthogonal catalytic methods.

## Experimental Section

### General procedure of catalysis on Nisin

Catalysis was performed in 50 mM NaH<sub>2</sub>PO<sub>4</sub> buffer pH 7 or pH 8 with a final concentration of 40  $\mu$ M peptide, 2 mM boronic acid and 2 mM catalyst. A typical catalysis reaction was set up as follows: Nisin (2 nmol in 30  $\mu$ L buffer) and 10  $\mu$ L of 10 mM boronic acid stock solution were combined. 10  $\mu$ L of 10 mM catalyst stock solution was added. The vial was shaken for 16 hours at room temperature. 5  $\mu$ L of 250 mM methylthioglycolate stock solution was added to scavenge the palladium, 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 minutes at 13.4k rpm. The supernatant was analysed by UPLC/MS TQD and purified by rp-HPLC.

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**Keywords:** bio-orthogonal catalysis • dehydroalanine • palladium • nisin • cross coupling

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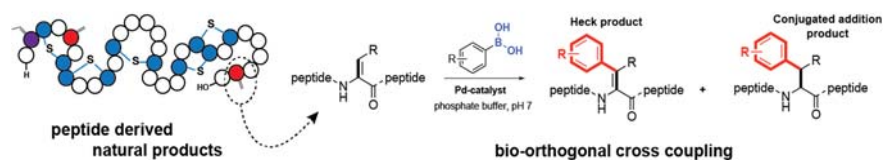
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## FULL PAPER



A. Dowine de Bruijn and Gerard Roelfes\*

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Catalytic modification of dehydroalanine in peptides and proteins via palladium mediated cross coupling

Dehydroamino acids are conveniently modified in Pd catalyzed bio-orthogonal cross-coupling reaction.