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To be cited as: Chem. Eur. J. 10.1002/chem.201802846

Link to VoR: http://dx.doi.org/10.1002/chem.201802846
Catalytic modification of dehydroalanine in peptides and proteins via palladium mediated cross coupling

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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)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 yields both the Heck product, in which the sp²-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 sp² hybridised α-carbon. The resulting planar structure provides different structural properties and reactivity than conventional sp³ hybridised 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 via 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 sp² configuration 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 sp² hybridisation intact, although competition of the conjugate addition product is also to be expected for the conjugated alkenes in Dha.[13] 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 and at 37°C). Therefore, we sought a water soluble palladium complex which can carry out this transformation. Here, we present the palladium catalysed 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.[13]

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 ¹H-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.[12] 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, 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)₂ 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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Supporting information for this article is given via a link at the end of the document.((Please delete this text if not appropriate))
We focused on the palladium mediated cross coupling reaction of the lantipeptide nisin. 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. Moreover, conjugate addition of water to Dha, and hydrolytic cleavage at this site are well-known degradation reactions. 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.

Nisin was reacted with phenylboronic acid (2b) using Pd(EDTA)(OAc)2 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. 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 unspecified 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, which precipitate from the solution. The precipitate is readily removed by centrifugation, or filtration over 0.45 μm 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 Minitrapp 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 1:** Cross coupling reaction on Dha a) General reaction scheme on Dha monomer; b) General reaction scheme, optimised conditions: nisin (40 μM), boronic acid (2 mM) and Pd(EDTA)(OAc)2 (2 mM in 25 μL buffer (50 mM NaH2PO4 pH 7.2% 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)2; III: nisin(Ph); IV: nisin(Ph)(H2O); V: nisin(Ph)2; VI: nisin(Ph)2(H2O); VII: nisin(Ph).
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.[19] 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)₂ 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 α-keto-acid, i.e. 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 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 α-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 sp² hybridised α-carbon and, as a result, its unique structural properties.
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 aryl groups 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 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. SUMO (Small Ubiquitin-like MODifier, ~11kDa22) containing a chemically introduced Dha residue8d, 23 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)2 catalyst and 100 eq of aryboronic 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-, d5-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 aryboronic acid resulted in full conversion being achieved. Attempts to cross couple dansyl substituted aryboronic 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 aryboronic 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-tolylalanine as p-tolulpyruvic acid were observed (figure S18).
Conclusions

In conclusion, here we have introduced the Pt(EDTA)(OAc)₂ 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 nisin, it has to be emphasized that such a late stage modification of the non-canonical amino acid dehydroalanine in catalysed cross coupling reaction as a method for the backbone of the biomolecules intact, which may be of particular importance for natural Dha/Dhb containing compounds.

Experimental Section

General procedure of catalysis on Nisin

Catalysis was performed in 50 mM NaH₂PO₄ 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 added. The vial was shaken for 16 hours at room temperature. 5 μL of 250 mM methylthioglycolate stock solution was added to scavenge the catalyst. The reaction mixture turned yellow instantly. The reaction mixture was combined and centrifuged for 10 minutes at 13.4k rpm. The supernatant was analysed by UPLC/MS TQD and purified by rp-HPLC.

Acknowledgements

The authors thank Prof. Dr. Adri Minnaard and Prof. Dr. Frank Dekker for advice on palladium catalysis, Prof. Dr. Oscar Kuipers and Dr. Manuel Montalbán López for useful discussion and for providing the initial batches of nisin. Financial support from the Netherlands Ministry of Education, Culture, and Science (Gravitatie program no. 024.001.035) and the Netherlands Organisation for Scientific Research (NWO, vici grant 724.013.003) is gratefully acknowledged.

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


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