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.

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2.1 - Introduction

Dehydroalanine (Dha) is a remarkably versatile non-canonical, yet naturally occurring α,β-unsaturated amino acid that features a unique \( sp^2 \) hybridised α-carbon. The resulting planar structure provides different structural properties and reactivity than conventional \( sp^3 \) 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,\(^9\) and piperidine moieties found in thiopeptides.\(^3\) Most of these peptides possess antimicrobial or antitumor activity,\(^4,5\) which make them interesting targets for new antibiotics and medicines. Yet, modification of these peptides via bio-engineering,\(^6-10\) or total synthesis\(^11, 12\) is challenging and is thus preferably done by late-stage site-selective chemical modification. The residual Dha residues in these peptides are excellent reactive sites for such transformations. Michael additions,\(^13-17\) 1,3-dipolar cycloadditions,\(^18\) radical carbon-carbon bond formations,\(^19-20\) and catalytic arylation of thiopeptides in organic solvent have been reported.\(^21\) In all these transformations the \( sp^2 \) 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\(^22-24\) cross coupling could leave the \( sp^2 \) hybridisation intact. 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.\(^25\)

![Scheme 2.1. Schematic representation of palladium cross coupling on dehydrated amino acids in peptides and proteins](image)

2.2 - Results & Discussion

Initial studies focused on the reaction of Dha monomer (1), with 4-methoxyphenylboronic acid (2a) (table 2.1). Neutral to slightly basic conditions (pH 7-8) proved necessary to obtain conversion of the Dha monomer, as was determined by \(^1\)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,\cite{26} and is difficult to avoid. 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 2.1). 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.

<table>
<thead>
<tr>
<th>entry</th>
<th>1[a]</th>
<th>2a[b]</th>
<th>Pd(EDTA)OAc_2 [c]</th>
<th>solvent</th>
<th>pH</th>
<th>conversion[d]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>10</td>
<td>5</td>
<td>H_2O</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>10</td>
<td>5</td>
<td>40 mM NaH_2PO_4</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>10</td>
<td>5</td>
<td>160 mM NaH_2PO_4</td>
<td>8</td>
<td>40</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>30</td>
<td>5</td>
<td>160 mM NaH_2PO_4</td>
<td>7</td>
<td>50</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>20</td>
<td>10</td>
<td>160 mM NaH_2PO_4</td>
<td>7</td>
<td>55</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>20</td>
<td>BIAN 10%</td>
<td>200 mM NaH_2PO_4</td>
<td>7</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>10</td>
<td>20</td>
<td>hydroxypyrimidine 10%</td>
<td>200 mM NaH_2PO_4</td>
<td>7</td>
<td>-</td>
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<tr>
<td>8</td>
<td>10</td>
<td>20</td>
<td>EDTH_4 10%</td>
<td>200 mM NaH_2PO_4</td>
<td>7</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2.1: Overview of screened conditions [a] final concentration (mM) of Dha monomer (1); [b] final concentration (mM) 4-methoxyphenylboronic acid (2a); [c] mol% catalyst; [d] conversion (%) as determined by \(^1\)H-NMR after extraction to DCM.
We focused on the palladium mediated cross coupling reaction of the lantipeptide nisin.\[^{10}\] 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.\[^{27}\] Moreover, conjugate addition of water to Dha, and hydrolytic cleavage at this site are well-known degradation reactions.\[^{28}\] 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.\[^{18, 29}\]

Nisin was reacted with phenylboronic acid (2b) using Pd(EDTA)(OAc)\(_2\) as catalyst (scheme 2.2). 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 2.1). 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.\[^{30, 31}\] 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 2.2). 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.

![Scheme 2.2: 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 NaH\(_2\)PO\(_4\) pH 7 2.2% DMF) shaken 16 hours at 37 °C. Prior to mass analysis 3 eq (w.r.t. Pd) 3-MPA (3-mercaptopropanoic acid), MTG (methyl thioglycolate) or APDTC (ammonium pyrrolidine dithiocarbamate) are added](image-url)
Therefore, alternative scavengers for the palladium catalyst were investigated, which included a variety of water soluble thiols, as well as resin-based scavengers (table 2.2). 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,\(^{[32-33]}\) 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). 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.

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
## Table 2.2: Overview of tested palladium scavengers for purification of protein after cross coupling reaction in order of trials.

<table>
<thead>
<tr>
<th>entry</th>
<th>Name</th>
<th>Structure</th>
<th>Efficiency[a]</th>
<th>Purification[b]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3-mercaptopropanoic acid (MPA)</td>
<td><img src="image1" alt="Structure" /></td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>3,3’-((propane-1,3-diylbis(sulfanediyl))dipropionic acid</td>
<td><img src="image2" alt="Structure" /></td>
<td>+</td>
<td>n.d.</td>
</tr>
<tr>
<td>3</td>
<td>thiourea</td>
<td><img src="image3" alt="Structure" /></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>sodium thiocyanate</td>
<td><img src="image4" alt="Structure" /></td>
<td>-</td>
<td>n.d.</td>
</tr>
<tr>
<td>5</td>
<td>MPA + charcoal</td>
<td><img src="image5" alt="Structure" /></td>
<td>-</td>
<td>n.d.</td>
</tr>
<tr>
<td>6</td>
<td>Chelex-resin</td>
<td><img src="image6" alt="Structure" /></td>
<td>-</td>
<td>n.d.</td>
</tr>
<tr>
<td>7</td>
<td>2,4,6-trimercaptotriazine</td>
<td><img src="image7" alt="Structure" /></td>
<td>-</td>
<td>n.d.</td>
</tr>
<tr>
<td>8</td>
<td>mercaptoethanol</td>
<td><img src="image8" alt="Structure" /></td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>Biotage ISOLUTE® Si-Thiol</td>
<td><img src="image9" alt="Structure" /></td>
<td>-</td>
<td>n.d.</td>
</tr>
<tr>
<td>10</td>
<td>SiliCycle SiliaMetS® dimercaptotriazine</td>
<td><img src="image10" alt="Structure" /></td>
<td>-</td>
<td>n.d.</td>
</tr>
<tr>
<td>11</td>
<td>Biotage® MP-TMT</td>
<td><img src="image11" alt="Structure" /></td>
<td>-</td>
<td>n.d.</td>
</tr>
<tr>
<td>12</td>
<td>SiliCycle SiliaMetS® cysteine</td>
<td><img src="image12" alt="Structure" /></td>
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<td>n.d.</td>
</tr>
<tr>
<td>13</td>
<td>thiocetic acid</td>
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<td>n.d.</td>
</tr>
<tr>
<td>14</td>
<td>MPA methylester</td>
<td><img src="image14" alt="Structure" /></td>
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<td>++</td>
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<tr>
<td>15</td>
<td>Smopex®-234 mercaptoethylacrylate grafted fibre</td>
<td><img src="image15" alt="Structure" /></td>
<td>+</td>
<td>n.d.</td>
</tr>
<tr>
<td>16</td>
<td>methylthioglycolate</td>
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<td>+++</td>
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<tr>
<td>17</td>
<td>pyrolidinethiocarbamate ammonium salt</td>
<td><img src="image17" alt="Structure" /></td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

[a]: - = no scavenging, +/++/+++ = scavenging efficiency from minor scavenging to full scavenging.  
[b]: - = purification not achieved, + = purification achieved with ion exchange, ++/+++ = purification achieved by filtering, n.d. = not done.  
[c] Prepared as described by Spicer et al.[9]
(FDAA)) which will react with phenylalanine to give FDAA-Phe.\textsuperscript{[64]} 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 2.3). Since nisin naturally does not contain phenylalanine, its presence in the hydrolysate 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)\textsubscript{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.

Marfey’s reagent does not reveal the presence of 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). The other half of the hydrolysate was therefore treated with dansylhydrazine, which reacts with α-keto-acids to form hydrazones.\textsuperscript{[35]} The reaction usually yields two isomers (E/Z), which separate during LC. Analysis with LC/MS and comparison with a sample of the hydrazone formed with PhPA, confirmed the presence of PhPA in the hydrolysate of 3b (figure 2.4). Also the α-keto-acid of the product of Heck reaction on Dhb was detected. So, the Heck pathway is also followed in the palladium mediated cross coupling reaction of peptides. Thus, product 3b has maintained partially its sp\textsuperscript{2} hybridised α-carbon and, as a result, its unique structural properties.
In an attempt to increase the rate of the reaction, the cross coupling reaction was performed at pH 8 (table 2.3). 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 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 aryl groups containing diverse functional groups to nisin (figure 2.5). 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.

**Figure 2.4:** UPLC/MS chromatograms of hydrazone analysis; a) EIC of [M+H] = 412 Da of phenylpyruvic acid treated with dansylhydrazine (black) and [M+H] = 426 Da (brown) of 3-methyl-3-phenylpyruvic acid treated with dansylhydrazine b) EIC of [M+H] = 412 Da (black) and [M+H] = 426 Da (orange) of hydrolysate of 3b treated with dansylhydrazine

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**Figure 2.5:** Scope of arylboronic acids in cross coupling reaction with nisin. Single modification (*), double modification (**) and triple modification (***) is observed. The conversion displayed in parentheses is based on integration of the 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.[36-38]
Next, the palladium cross coupling reaction was tested on a different Dha containing peptide: thiostrepton, a member of the thiopeptide family (table 2.4 in experimental section). This thiopeptide is more hydrophobic than nisin, so more DMF as co-solvent was required in the reaction. Thiostrepton contains three Dha residues and one Dhb. Treatment with the Pd(EDTA)(OAc)₂ catalyst and arylboronic acid resulted in singly- and doubly modified thiostrepton, albeit starting material was also still present after the reaction. A variety of arylboronic acids was coupled via this method, showing the cross coupling reaction is not limited to nisin, but is generally applicable to Dha containing peptides.

Finally, the generality of palladium mediated cross coupling reaction was investigated by using the reaction for protein modification. SUMO (Small Ubiquitin-like MODifer, ~11kDa) containing a chemically introduced Dha residue[16] was used as substrate. The Dha residue was introduced at two different positions: near the C-terminus of the protein, to minimise steric effects on the reaction (SUMO_G98Dha), and in one of the solvent exposed loops (SUMO_M60Dha) (figure 2.6). Treatment of the protein with 20 eq Pd(EDTA)(OAc)₂ catalyst and 100 eq of arylboronic acid showed full conversion to the cross coupled product for p-toluylboronic acid. Control experiments performed on SUMO_G98A, which lacks the Dha moiety, resulted in no reaction, which demonstrates that the reaction is also in proteins site-specific at the Dha residue. Reactions

Figure 2.6: Pd(EDTA)(OAc)₂ catalysed cross coupling reaction on SUMO. a) General reaction scheme for the chemical introduction of Dha in SUMO; b) General reaction scheme, optimised conditions: protein (45 μM), boronic acid (4.5 mM) and Pd(EDTA)(OAc)₂ (0.9 mM) in 22 μL buffer (50 mM NaH₂PO₄ 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.
with phenyl-, d₄-phenyl-, 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 (see figure 2.7). 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 2.6 in experimental section), while 4-fluorophenylboronic acid did not give rise to full conversion.

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 2.8).

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 2.9).
**Palladium mediated Cross Coupling**

**Figure 2.8:** SDS/PAGE analysis of SUMO before and after cross coupling and via CuAAC. All lanes are filled with 0.8 nmol protein; L: ThermoFisher PageRuler™ Prestained Protein Ladder 10-180kDa, weight given in kDa.; lane 1: SUMO_G98C; lane 2: SUMO_Dha; lane 3: 5d; lane 4: 5e; lane 5: 16.

**Figure 2.9:** Marfey’s analysis: I) EIC [M+H] = 432 Da of p-toluylalanine treated with Marfey’s reagent (blue); II) EIC [M+H] = 432 Da of hydrolysate of 5c treated with Marfey’s reagent; b) Hydrazine analysis: I) EIC [M+H] = 426 Da of p-toluylpyruvic acid treated with dansylhydrazine (blue); II) EIC [M+H] = 426 Da of hydrolysate of 5c treated with dansylhydrazine.
2.3 - Conclusions

In conclusion, here we have introduced the Pd(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 synthesis.\(^{[7]}\)

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 \(sp^2\)-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.

2.4 - Experimental

General remarks
Chemicals were purchased from Sigma-Aldrich, TCI Europe, Acros, Strem Chemical or Chem-Impex, solvents from Lab-Scan and were all used without further purification. Column chromatography was performed by hand on silica gel (Aldrich, 230-400 mesh) or automated on a Grace Reveileris Flash Chromatography system. Solvents were removed under reduced pressure at 40 °C (water bath). \(^1\)H-NMR and \(^{13}\)C-NMR spectra were recorded with Varian Mercury Plus 400, Agilent Technologies 400/54 Premium Shield or Varian VXR 300 at ambient temperature. HRMS ESI mass spectra of small organic molecules were recorded with Thermo Fisher Scientific Orbitrap XL. Melting points were recorded on a Büchi B-545 melting point apparatus. Elemental analysis were determined on a EuroVector S.P.A. model Euro EA 3000. E. coli strains XL1 Blue and BL21 DE3 C43 (Stratagene) were used for routine cloning and protein production, respectively. Streptag®-Strep-Tactin® purification columns were purchased from IBA. PCR reactions were carried out using an Eppendorf Mastercycler Personal apparatus. DNA sequencing was carried out by GATC-Biotech (Berlin, Germany). Primers were synthesised by Biotez (Berlin, Germany). Restriction endonucleases were purchased from New England biolabs. T4 DNA ligase, DNA gel Extraction Kit and Plasmid Purifying kit were purchased from Roche. Pfu Turbo polymerase was purchased from Stratagene. Plasmid pET17b was purchased from Novagen. Champion™ pET SUMO Expression System was purchased from Invitrogen. UPLC/MS analysis was done on Waters Acquity Ultra Performance LC with Acquity TQD detector. Separation of peptides and proteins was achieved with an Acquity UPLC BEH C8 1.7 \(\mu\)m 2.1x150 mm column and a linear gradient of 90% - 50% water (0.1%FA) in ACN (0.1%FA) in 10 minutes. Separation of small molecules was achieved with an Acquity UPLC HSS T3 C18 1.8 \(\mu\)m 2.1x150 mm column and linear gradient of 80% - 5% water (0.1%FA) in ACN (0.1%FA) in 15 minutes and monitored at 340 nm. rp-HPLC purification of peptides was done on a Shimadzu HPLC. Separation was achieved with an XBridge C8 3.5 \(\mu\)m 4.6x250mm column and a linear gradient of 80% - 30% water (0.1%FA) in ACN (0.1%FA) in 30 min. Charge density spectra were deconvoluted with the algorithm MagTran.\(^{[46]}\) ICP-OES was recorded with Perkin Elmer Optima 7000DV. Optical density of the bacterial cultures was measured with an Amersham Biosciences Ultrospec 10. Protein...
concentrations were measured on a Thermoscientific Nanodrop 2000. Peptide concentrations were determined with Pierce™ BCA Protein Assay Kit. Denaturing Polyacrylamide gel for SDS/PAGE were casted from stock solution of 30 % (w/v) 29:1 monomer:crosslinker acrylamide/N,N-methyl-bisacrylamide in Tris-Cl/SDS buffer (=3M Tris-base, 10 mM SDS pH 8.45) and 15 % glycerol (w/v). Polymerization was started with 10 % ammonium persulfate (APS) and N,N,N,N-tetraemethylthylene diamine (TEMED). Denaturing gels were run in Tris-Tricine-SDS buffer (cathode: 1M Tris-base, 1M Tricine, 3 mM SDS; anode: 200 mM Tris-base pH 8.9) at 150 V for 1 hour. Gel staining was done by Coomassie® stain with InstantBlue™. Vivaspin spinfilters were bought from Sartorius. Graphic representation of SUMO was taken from PDB 1L2N\[47\]. Microwave reactions were done in a CEM Discover SP microwave oven. Eppendorf vials were concentrated in Eppendorf® Concentrator Plus.

Methyl 2-acetamidoacrylate (1)
Prepared as described by Crestey et al.\[41\]. Acetamide (1000 mg, 16.9 mmol), methyl pyruvate (1.3 mL, 15.2 mmol) and 30 mL toluene were added to a round-bottom-flask equipped with magnetic stirrer and Dean-Stark-trap. A catalytic amount of p-toluenesulfonice acid (0.001 eq) and p-methoxyphenol (0.001 eq) were added. After heating under reflux for 24 hours, the solvent was evaporated. The crude yellow oil was redissolved in dichloromethane, washed with saturated NaHCO₃(aq) and water. Drying over MgSO₄, removal of the solvent and purification by column chromatography (SiO₂, pet ether / ethyl acetate 3:1, Rₙ=0.71 in EtOAc) gave 1 (805 mg, 37%) as a white solid. ¹H-NMR (CDCl₃, 400MHz) δ 2.13 (s, 3H), 3.84 (s, 3H), 5.88 (s, 1H), 6.60 (s, 1H), 7.71 (br, 1H) ppm; ¹³C-NMR (CDCl₃, 101 MHz) δ 24.8, 53.1, 108.9, 131.1, 164.7, 169.0 ppm; Calc: C: 50.35, H: 6.34, N:9.79, Found: C: 50.27, H: 6.35, N: 9.66. MS (ESI, HCOOH) m/z 144.0654 ([M+H]⁺, calc: 144.0655) mp: 51.4-52.3 ºC

Methyl (E)-2-acetamido-3-(4-methoxyphenyl)acrylate (6)
Prepared as described by Wagaw et al.\[42\]. 4-iodo-anisole (297 mg, 1.27 mmol) was dissolved in 1 mL triethylamine. Palladium(II)acetate (14 mg, 0.06 mmol) and 2-acetoamido acrylate (1, 200 mg, 1.4 mmol) were added and the mixture was heated to 100 ºC for 2.5 hours. After cooling to room temperature, the reaction mixture was diluted with dichloromethane and washed with water. After extraction of the aqueous layer with fresh dichloromethane, the combined organic layers were dried over MgSO₄ and concentrated. Purification by column chromatography (SiO₂, ethyl acetate / heptane 1:1, Rₙ=0.25) gave 6 (224 mg, 71%) as a white solid. ¹H-NMR (CDCl₃, 400MHz) δ 2.10 (s, 3H), 3.78 (s, 3H), 3.79 (s, 3H), 6.84 (d, 2H, J=8.2), 7.36 (s, 1H), 7.41 (d, 2H, J=8.8) ppm; ¹³C-NMR (CDCl₃, 101 MHz) δ 24.8, 53.1, 108.9, 131.1, 164.7, 169.0 ppm; Calc: C: 62.64, H: 6.07, N:5.62, Found: C: 62.63, H: 6.13, N: 5.55; MS (ESI, HCOOH) m/z 218.08133 ([M-OCH₃]⁺ calc: ), 250.10741 ([M+H]⁺, calc: 250.10738), 272.0894 ([M+Na]⁺, calc: 272.0893); mp: 133-135 ºC.

(S)-2-((5-fluoro-2,4-dinitrophenyl)amino)propanamide (Marfey’s reagent) (7)
Prepared as described by Sheppard et al.\[43\]. Alaninamide (473 mg, 3.8 mmol) is dissolved in 4 mL 1 M NaOH(aq) was added to 60 mL acetone. MgSO₄ (10 gram) was added and the mixture was stirred for 3 hours at room temperature, whereafter the MgSO₄ is filtered off and added dropwise to a mixture of 1,5-difluoro-2,4-dinitrobenzene (668 mg, 3.2 mmol) in 15 mL acetone. After addition the mixture is stirred for 30 minutes at room
temperature. Addition of water (80 mL) and cooling in ice results in precipitation of the product. Filtration and washing with acetone / water (v/v 1:1) gave 7 (505 mg, 57%) as yellow needles. 1H-NMR (DMSO-d$_6$, 400 MHz) 1.46 (d, 3H, J=6.84), 4.39 (quint, 1H, J=6.89), 6.94 (d, 1H, J=14.34), 7.50 (s, 1H), 7.72 (s, 1H), 8.89 (d, 1H, J=8.19), 9.11 (m, 1H) ppm; 13C-NMR (DMSO-d$_6$, 100 MHz) 21.5, 54.7, 105.1 (d, J=29.0), 128.2 (d, J=29.0), 130.5, 150.7 (d, J=14.6), 160.8, 163.5, 175.4 ppm; MS (ESI, HCOOH) m/z 295.045 ([M+Na]$^+$, calc: 295.0455); Calcd for C$_9$H$_9$FN$_4$O$_5$: C: 39.71, H: 3.33, N: 20.58, Found: C: 39.60, H: 3.38, N: 20.40.

2-(2-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenoxy)ethoxy)ethyl 4-methylbenzenesulfonate (8)
Prepared as described by Han et al.[44]: Oligo(ethyleneglycol)bistoluene sultanate (1865 mg, 4.5 mmol) was dissolved in 30 mL acetonitrile. (4-hydroxyphenylboronate pinacol ester (330 mg, 1.5 mmol) and K$_2$CO$_3$ (317 mg, 2.3 mmol) were added and the mixture was heated to reflux overnight. Removal of solvent and purification by column chromatography (SiO$_2$, petroleum ether / ethyl acetate 3:1, R$_f$=0.65) gave 8 (478 mg, 69%) as a clear oil. 1H-NMR (CDCl$_3$, 400 MHz) δ 1.33 (s, 12H), 2.40 (s, 3H), 3.76 (m, 4H), 4.06 (m, 2H), 4.19 (m, 2H), 6.87 (d, 2H, J=8.3) 7.30 (d, 2H, J=8.3), 7.74 (d, 2H, J=8.3), 7.80 (d, 2H, J=8.3) ppm; 13C-NMR (CDCl$_3$, 100 MHz) δ 21.6, 24.8, 67.1, 68.8, 68.3, 69.7, 83.5, 113.8, 127.9, 129.8, 132.9, 136.5, 144.8, 161.2 ppm; MS (ESI, HCOOH) m/z 501.1512 ([M+K]$^+$, calc: 501.15).

2-(4-(2-(2-azidoethoxy)ethoxy)phenyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (9)
8 (260 mg, 0.56 mmol) was dissolved in 5 mL ethanol. Sodium azide (182 mg, 2.8 mmol) was added and the mixture was heated to reflux overnight. After cooling to room temperature the mixture was filtered through celite. Removal of the solvent and purification by column chromatography (SiO$_2$, petroleum ether / ethyl acetate 3:1, R$_f$=0.71) gave 9 (160 mg, 85%) as a clear oil, which solidifies upon cooling o/n. 1H-NMR (CDCl$_3$, 400 MHz) δ 1.33 (s, 12H), 3.41 (m, 2H), 3.75 (m, 2H), 3.87 (m, 2H), 4.17 (m, 2H), 6.90 (d, 2H, J=8.6), 7.73 (d, 2H, J=8.4) ppm; 13C-NMR (CDCl$_3$, 101 MHz) δ 24.8, 50.7, 67.2, 69.7, 70.3, 83.5, 113.9, 136.5, 155.4 ppm; MS (ESI, HCOOH) m/z 334.1934 ([M+H]$^-$, calc: 334.1933) 356.1750 ([M+Na]$^+$ calc: 356.1752).

(4-(2-(2-azidoethoxy)ethoxy)phenyl)boronic acid (10)
9 (65 mg, 0.19 mmol) was dissolved in 4 mL THF / H$_2$O (3:1 v/v). Sodium periodate (125 mg, 0.56 mmol) was added and the mixture was stirred overnight at room temperature. 4 mL 1M HCl(aq) was added and after stirring for another 3 hours the mixture was diluted with ethyl acetate, washed with water and brine, dried over Na$_2$SO$_4$ and concentrated to give 10 (44 mg, 90%) as a white solid. 1H-NMR (CDCl$_3$, 400 MHz) δ 3.44 (m, 2H), 3.78 (m, 2H), 3.91 (m, 2H), 4.22 (m, 2H), 7.01 (d, 2H, J=8.6), 8.14 (d, 2H, J=8.6) ppm; 13C-NMR (CDCl$_3$, 101MHz) δ 50.7, 67.2, 69.7, 70.3, 114.1, 125.5, 137.5, 162.3 ppm; MS (ESI, NH$_4$OAc) m/z 250.1002 ([M-H]$^-$, calc: 250.1005) 264.1160 ([M+CH$_2$]$^+$ calc: 264.1161); mp.: 57.4 °C.

4-(prop-2-yn-1-yloxy)benzaldehyde (11)
Prepared as described by He et al.[45]: 4-hydroxy benzaldehyde (500 mg, 4.1 mmol) and propargyl bromide (975
mg, 8.2 mmol) were dissolved in 20 mL acetone. Potassium carbonate (787 mg, 5.7 mmol) was added and the mixture was heated to reflux for 2 hours. After cooling to room temperature and removal of the solvent, the crude mixture was dissolved in water and extracted to chloroform. Drying over Na₂SO₄, removal of solvent and purification by column chromatography (SiO₂, petroleum ether / chloroform 1:1, Rf=0.5) gave 11 (535 mg, 81%) as a white solid. ¹H-NMR (CDCl₃, 400 MHz) δ 2.57 (m, 1H), 4.78 (m, 2H), 7.09 (d, 2H, J=8.7), 7.85 (d, 2H, J=8.8), 9.91 (s, 1H) ppm; ¹³C-NMR (CDCl₃, 101 MHz) δ 55.9, 76.4, 77.6, 115.1, 130.5, 131.9, 162.3, 190.7 ppm; MS (ESI, HCOOH) m/z 161.0597 ([M+H]⁺, calc: 161.0597); Calc: C: 74.99, H: 5.03, N: 0, Found: C: 74.83, H: 5.01, N: <0.01. mp.: 77.9 °C.

5,5-difluoro-1,3,7,9-tetramethyl-10-(4-(prop-2-yn-1-yloxy)phenyl)-5H-4λ4-dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-5-uide (Bodipy-Alkyne, 12)
Prepared as described by He et al.⁵: 11 (320 mg, 2 mmol) was dissolved in dry dichloromethane. 2,4-dimethylpyrrole (418 mg, 4.4 mmol) and trifluoroacetic acid (5 drops) were added. After stirring at room temperature overnight, 2,3-dichloride-5,6-dicyano-p-benzoquinone (DDQ) (454 mg, 2 mmol) was added and the resulting mixture was stirred for 3 hours. Diisopropylethylamine (3.5 mL, 20 mmol) and borontrifluoride etherate (3.4 mL, 28 mmol) were added. After stirring for another 3 hours, the resulted mixture was washed with brine. Drying over Na₂SO₄, removal of the solvent and purification by column chromatography (SiO₂, petroleum ether / chloroform 1:1, Rf=0.35) gave 12 (48 mg, 6%) as a red solid. ¹H-NMR (CDCl₃, 400 MHz) δ 1.42 (s, 6H), 2.55 (m, 7H), 4.76 (s, 2H), 5.98 (s, 2H), 7.10 (d, 2H, J=8.0), 7.18 (d, 2H, J=8.1) ppm; ¹³C-NMR (CDCl₃, 101 MHz) δ 14.6, 14.6, 56.0, 75.9, 78.0, 115.6, 121.2, 128.0, 129.2, 131.8, 141.5, 143.1, 155.3, 158.1 ppm; MS (ESI, HCOOH) m/z 417.1344 ([M+K]⁺, calc: 417.14); Calc: C: 69.86, H: 5.60, N: 7.41, Found: C: 69.36, H: 5.94, N: 7.13; mp.: 210.1 °C.

Catalyst stock solution preparation (5 mM)
Palladium(II)acetate (56 mg, 0.25 mmol) and ethylenediaminetetraacetic acid (EDTA, 73 mg, 0.25 mmol) were dissolved in 40 mL buffer or water by stirring the mixture at 60 °C for at least 1 hour. After cooling down to room temperature the stock solution was supplemented with buffer or water to a volume of 50 mL to yield a bright yellow stock solution of 5 mM catalyst.

Catalyst stock solution preparation (10 mM)
Palladium(II)acetate (112 mg, 0.5 mmol) and ethylenediaminetetraacetic acid (EDTA, 146 mg, 0.5 mmol) were dissolved in 40 mL buffer or water by stirring the mixture at 60 °C for at least 1 hour. After cooling down to room temperature the stock solution was supplemented with buffer or water to a volume of 50 mL to yield a bright yellow stock solution of 10 mM catalyst.

General procedure for cross coupling reaction on small molecules
Catalysis was performed in phosphate buffer (160 mM NaH₂PO₄ pH 7 or pH 8) with a final concentration of 10 mM of dehydroalanine, 10-30 mM of the boronic acid and 5%-10% catalyst loading. A typical catalysis reaction was set up as follows: 1 (28.7 mg, 0.2 mmol) and 4-methoxyphenylboronic acid (30.4 mg, 0.2 mmol) were dissolved in 16 mL buffer. After addition of 4 mL of the 5 mM catalyst stock solution, the vial was closed and the mixture was stirred overnight at 37 °C. During the course of the reaction, the mixture turned black. After cooling to room temperature, the reaction mixture was filtered over celite and extracted to dichloromethane. After drying over Na₂SO₄ and removal of the solvent, conversions were determined by ¹H-NMR. The ratio between the peaks at 7.38 ppm (Heck product), 4.80 ppm (conjugated addition product) and 5.86 ppm (starting material) were compared.
Boronic acid stock solution preparation (10 mM)
Boronic acid was dissolved in DMF to a concentration of 200 mM. The solution was 20x diluted with phosphate buffer (50 mM NaH$_2$PO$_4$, pH 7) to obtain a final stock solution of 10 mM boronic acid.

General procedure of catalysis on Nisin without scavenger
Catalysis was preformed in 50 mM NaH$_2$PO$_4$ 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. 10 μL of 10 mM catalyst stock solution was added. The vial was shaken overnight at room temperature. The crude reaction mixture was analysed by UPLC/MS TQD.

Investigation of palladium scavengers
General procedure of catalysis was set up as described above (50 mM NaH$_2$PO$_4$ buffer pH 7 or pH 8 with a final concentration of 40 μM peptide, 2 mM boronic acid and 2 mM catalyst). After reaction overnight 50 mM stock solution of scavenger was added (3 eq w.r.t. palladium), or the sample was treated with resin based scavenger according to the suppliers manual. The sample was analysed by UPLC/MS to determine scavenging efficiency.

General procedure of catalysis on Nisin with scavenger
Catalysis was preformed in 50 mM NaH$_2$PO$_4$ 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. 10 μL of 10 mM catalyst stock solution was added. The vial was shaken overnight at room temperature. 5 μL 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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Table 2.3: Scope of arylboronic acids in the cross coupling reaction on nisin. Between (..) is given the percentage of product in the crude reaction mixture [a] single cross coupled product; [b] double cross coupled product; [c] triple cross coupled product. [d] starting material was still present; [e] reaction performed with 10 eq Pd; [f] reaction performed at pH 8.
Representative ICP measurement
An 500 μL aliquot (1.25 nmol in peptide) of 250 μM purified protein 3b was taken and diluted to 5 mL for ICP.
Measured concentration Pd: 0.011 ppm or 5.2*10^{-10} mol in aliquot.
Comparison with calculated amount of Pd in 500 μL reaction mixture = 1*10^{-4} mol Pd. -> 99.5% removal of Pd.

Marfey’s analysis
An aliquot of 0.1 mg amino acids (30 nmol for modified nisin) was added to 350 μL 6M HCl(aq) in a microwave tube equipped with stir bar. The sample was exposed to microwave irradiation for 10 minutes at 160 °C, with maximum 50 Watt power. The mixture is split in half and transferred to an eppendorf vial and concentrated to dryness in vacuo. The residue was dissolved in 25 μL 1M NaHCO₃(aq), and 5 μL 1% Marfey’s reagent (FDAA or 7) in acetone was added. After shaking for 1 hour at 40 °C, 15 μL 2M HCl(aq) and 150 μL methanol were added to obtain a clear bright yellow solution. The sample was analysed directly by UPLC/MS TQD. Signals obtained at 340 nm absorption were assigned to the corresponding FDAA-derivative.

Hydrazone analysis
An aliquot 0.1 mg amino acids (30 nmol for modified nisin) was added to 350 μL 6M HCl(aq) in a microwave tube equipped with stir bar. The sample was exposed to microwave irradiation for 10 minutes at 160 °C, with maximum 50 Watt power. The mixture is split in half and transferred to an eppendorf vial and concentrated to a volume of 100 μL. 200 μL of 2 mg/mL dansylhydrazine solution in methanol is added. After shaking for 1 hour at 40 °C, the sample was analysed directly by UPLC/MS TQD. Signals obtained at 340 nm absorption were assigned to the corresponding hydrazone.

General procedure for catalysis on Thiostrepton
Catalysis was preformed in 50 mM NaH₂PO₄ buffer pH 7 with 50% 1,4-dioxane or DMF with a final concentration of 45 μM peptide, 4.5 mM boronic acid and 2.7 mM catalyst. A typical catalysis reaction was set up as follows: Thiostrepton (1 nmol in 1 μL DMF) was diluted with 5 μL DMF. 10 μL of 10 mM boronic acid stock solution was added. 6 μL of 10 mM catalyst stock solution was added. The vial was shaken overnight at 37 °C. 1 μL of 150 mM mercaptopropanoic acid stopk solution was added to scavenge the palladium, the reaction mixture turned yellow instantly. The reaction was analysed by UPLC/MS TQD.

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Table 2.4: Scope of arylboronic acids in the cross coupling reaction on thiostrepton. [a] single cross coupled; [b] double cross coupled; [c] starting material was still present.

Construction of plasmid pET17b_SUMO_G98C
The pET17b_SUMO_G98C plasmid was derived from the commercially available plasmids pET17b (Novagen) and the Champion™ PET SUMO Expression System (Invitrogen). The SUMO gene, including a N-terminal Strep-tag® and C-terminal cysteine-alanine addition, was amplified by PCR using the following primers; primer 1: 5’ – TAC TAC CAT ATG TGG AGC CAC CCG CAG TTC GAA AAA ATG TCG GAC TCA GAA GTC AAT CAA GAA G–3’ (including Ndel restriction site underlined), primer 2: 5’ – GTA GTC ACC AAT CTG TTC TCT GTG AGC CT–3’ (including Xhol restriction site underlined). PCR cycles were as
following: initial denaturation at 94 °C for 5 min. Denaturation at 94 °C for 30 sec, annealing at 55 °C for 30 sec, extension at 72 °C for 90 sec., for 30 cycles. Final extension at 72 °C for 10 min. The obtained PCR product was digested with NdeI and XhoI, and inserted between the same sites of the expression vector pET17b.

**DNA sequence of pET17b_SUMO_G98C construct**

5’-CAT ATG TGG AGC CAC CCG CAG TTC GAA AAA ATG TCG GAC TCA GAA GTC AAT CAA GAA GCT AAG CCA GAG GTC AAG CCA GAA GTC AAG CCT GAG ACT CAC ATC AAT TTA AAG GTG TCC GAT GGA TCT TCA GAG ATC TTC TAT GAG ACC ACT CCT TTA AGA AGG CTG ATG GAA GCG TTC TCT GCT AAA AGA CAG GGT AAG GAA ATG GAC TCC TTA AGA TTC TITG TAC GAC GGT ATT AGA ATT CTG GCT CAC AGA GAA GCG TAC GGT GAA CAG ATT GTG TGC GCG TAA CTC GAG-3’

**Site-directed mutagenesis**

Site-directed mutagenesis was performed on the pET17b_SUMO_G98C plasmid and introduced sequentially. Primers used for preparing the mutants are shown in table 2.5. PCR cycles were as following: initial denaturation at 95 °C for 5 min. Denaturation at 95 °C for 30 sec., annealing at 55 °C for 1 min., extension at 68 °C for 4.5 min., for 16 cycles. The obtained PCR product was digested with DpnI and used for transformation to E. Coli BL21 DE3 C43 without further purification.

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**Table 2.5: PCR primers for site-directed mutagenesis**

**Protein expression and purification**

Protein expression plasmids of the SUMO constructs were transformed into *E. Coli* BL21 DE3 C43 and a single colony was used to inoculate a starter culture of 5 mL fresh LB medium containing 100 μg/mL ampicillin at 37 °C overnight. 1 mL of the starter culture was used to inoculate 250 mL of fresh LB medium containing 100 μg/mL ampicillin at 37 °C. When the culture reached the mid-log phase (optical density at 600 nm around 0.6-0.8) isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM to induce the expression of the target protein. Expressions were done at 37 °C overnight. Cells were harvested by centrifugation (6000 rpm, 20 min, 4 °C, Beckman JA-10), resuspended in phosphate buffer (50 mM NaHPO₄, 150 mM NaCl, 2.5 mM DL-dithiotreitol (DTT) (pH 7), and sonicated (75% 200W) for 10 min (15 sec on, 10 sec off). The disturbed cells were incubated with DNAsel (0.1 mg/mL), phenylmethanesulfonyl fluoride (PMSF, 1 mM) and MgCl₂ (10 mM) for 30 min at 30 °C. After centrifugation (15000 rpm, 45 min, 4 °C, Beckman JA-17.5) the supernatant was equilibrated with 4 mL slurry of Strep-Tactin® column material for 30 min (mixed on a rotary shaker) at room temperature. The column was washed with 3x 5 mL of washing buffer (50 mM NaHPO₄, 150 mM NaCl, 2.5 mM DTT pH 7) and eluted with 6x 2 mL eluting buffer (50 mM NaHPO₄, 150 mM NaCl, 2.5 mM DL-dithiotreitol (DTT), 2.5 mM D-desethiobiotin pH 7). Fractions were analysed on a 12% polyacrylamide SDS-Tris Tricine gel followed by Coomassie® staining with InstantBlue™. Fractions containing protein were concentrated using a spinfilter (Vivaspin-15). Concentration of protein was determined using the calculated extinction coefficient ε₂₈₀=6990 M⁻¹ cm⁻¹ (Espaxy Tool[46]). Expression yields typically were 20-30 mg/L.

**Protein sequence**

M WSHPQFEK MSDEVNOEA KPEVKPVEKP ETHINLKVSD GSSEIFFKIK KTTPLRLRME AFAKRQGKEM DSLRFLYDGI RIQADQTPED LD MEDNDII EAHREIQGC A; underlined = Strep-tag sequence; yellow = mutation sites
**General procedure for converting cysteine to dehydroalanine**

The cysteine containing protein was dissolved in phosphate buffer (50 mM Na₂HPO₄, 150 mM NaCl, 2.5 mM DTT pH 7) and diluted to a concentration of 2.5-5 mg/mL. An additional 1 mg DTT was added and the protein is incubated for 15 min. The sample was purified by PD MiniTrap™ G-25 size exclusion chromatography. 450 μL of protein solution was combined with 50 μL 2,5-dibromohexanediamide in DMF (20 mg/mL). The mixture was shaken at 750 rpm at 37 °C overnight. Purification by PD MiniTrap™ G-25 size exclusion chromatography and concentration using a spinfilter (Vivaspin-500) gave Dha-proteins in full conversion as was analysed by UPLC/MS.

**General procedure for the Ellman’s test**

An aliquot of 3 nmol protein (SUMO_G98C or SUMO_G98Dha) was taken and diluted with phosphate buffer (50 mM Na₂HPO₄, 150 mM NaCl, pH 8) to 50 μL. 5 μL 5,5′-Dithiobis(2-nitrobenzoic acid) (2 mg/mL in water, Ellman’s reagent) was added. The mixture was shaken at 750 rpm at 37 °C for 15 min, followed by direct analysis with UPLC/MS.

**Confirmation of Dha residue by thiol addition**

An aliquot of 3 nmol SUMO_G98Dha was taken and diluted with phosphate buffer (50 mM Na₂HPO₄, 150 mM NaCl, pH 7) to 50 μL. 5 μL mercaptoethanol was added. The mixture was shaken at 750 rpm at 37 °C for 15 min, followed by direct analysis with UPLC/MS TQD.

**General procedure for catalysis on proteins (without purification)**

Catalysis was performed in phosphate buffer (50 mM NaH₂PO₄, 150 mM NaCl, pH 7) with a final concentration of 40 μM protein, 4 mM boronic acid and 800 μM catalyst. A typical catalysis reaction was set up as follows: An aliquot of 1 nmol SUMO_G98Dha or SUMO_M60Dha was taken and diluted with phosphate buffer to 11 μL. 10 μL of 10 mM boronic acid stock solution was added, and 4 μL of 5 mM catalyst stock solution was added. The vial was shaken overnight at 37 °C. 5 μL mercaptopropanoic acid solution (50 mM in water) was added to scavenge the palladium, the reaction mixture turned yellow instantly. The crude reaction mixture was analysed by UPLC/MS TQD.

**General procedure for preparative scale catalysis on proteins with purification**

Catalysis was performed in phosphate buffer (50 mM NaH₂PO₄ buffer, 150 mM NaCl, pH 7) with a final concentration of 44 μM protein, 4.4 mM boronic acid and 889 μM catalyst. A typical catalysis reaction was set up as follows: An aliquot of 20 nmol SUMO_G98Dha was taken and diluted with phosphate buffer to 200 μL. 200 μL of 10 mM boronic acid stock solution was added, and 40 μL of 5 mM catalyst stock solution was added. The vial was shaken overnight at 37 °C. 8 μL methyl thioglycolate solution (150 mM in water) was added to scavenge the palladium. The mixture turned yellow instantly and a yellow precipitate appeared over time. After shaking at 750 rpm at 37 °C for another 3 hours, the sample was centrifuged 15 min at 134000 rpm. The supernatant was filtered over 0.45 μM syringe filter and loaded on PD MiniTrap™ G-25 size exclusion chromatography column. The protein was eluted with phosphate buffer and concentrated using a spinfilter (Vivaspin-500) and analysed by UPLC/MS TQD.

<table>
<thead>
<tr>
<th>#</th>
<th>R</th>
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</tr>
<tr>
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<td>H</td>
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<tr>
<td>15c</td>
<td>4-Me</td>
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<td>12550</td>
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<tr>
<td>15m</td>
<td>3-Me</td>
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Table 2.6: Scope of arylboronic acids in the cross coupling on SUMO_M60Dha. [a]: starting material still present.
Representative ICP measurement:
An 22 μL aliquot (1 nmol in protein) of the reaction mixture was taken and diluted to 5 mL for ICP. Calculated concentration Pd: 0.424 ppm; measured concentration Pd: 0.386 ppm.
An 40 μL aliquot (2.4 nmol in protein) of 60 μM purified protein 4b was taken and diluted to 5 mL for ICP. Measured concentration Pd: 0.015 ppm.
Comparison of the measured concentration per nmol protein before and after the reaction, shows a 98.4% removal of palladium.

Procedure for the click reaction to azide modified SUMO
Catalysis was performed in phosphate buffer (50 mM NaH₂PO₄ buffer, 150 mM NaCl pH 7) with a final concentration of 20 μM protein, 53 μM bodipy-alkyne (12), 800 μM CuSO₄, 1.6 mM tris(3-hydroxypropyltriazolylmethyl)amine (THPTA) and 5 mM sodium ascorbate. and set up as follows: An aliquot of 5 nmol 5e was taken and diluted with phosphate buffer to 125 μL. 50 μL of 12 (1 mg/mL in DMSO), and 75 μL of a premixed mixture of copper sulphate (32 μL, 1 mg/mL in water), THPTA (18 μL, 10 mg/mL in water) and sodium ascorbate (25 μL, 10 mg/mL in water) was added. The mixture was shaken at 750 rpm at 37 °C for 3 hours. Coupled product was purified using PD MiniTrap™ G-25 size exclusion chromatography column, concentrated with Vivaspin-500 spinfilter, and analysed by SDS-PAGE.

2.5 - Bibliography


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