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Photocatalysis

A Water-Soluble Iridium Photocatalyst for Chemical Modification of Dehydroalanines in Peptides and Proteins

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Abstract: Dehydroalanine (Dha) residues are attractive noncanonical amino acids that occur naturally in ribosomally synthesised and post-translationally modified peptides (RiPPs). Dha residues are attractive targets for selective late-stage modification of these complex biomolecules. In this work, we show the selective photocatalytic modification of dehydroalanine residues in the antimicrobial peptide nisin and in the proteins small ubiquitin-like modifier (SUMO) and superfolder green fluorescent protein (sfGFP). For this purpose, a new water-soluble iridium(III) photoredox catalyst was used. The design and synthesis of this new photocatalyst, $[Ir(dF(CF_3)ppy)_2(dNMe_3bpy)]Cl_3$, is presented. In contrast to commonly used iridium photocatalysts, this complex is highly water soluble and allows peptides and proteins to be modified in water and aqueous solvents under physiologically relevant conditions, with short reaction times and with low reagent and catalyst loadings. This work suggests that photoredox catalysis using this newly designed catalyst is a promising strategy to modify dehydroalanine-containing natural products and thus could have great potential for novel bioconjugation strategies.

Introduction

Peptides and proteins are valuable for therapeutic intervention and research tools alike.[1–4] However, they rarely display all the desirable features for "unnatural" applications. For example, properties like activity, stability, and solubility are often not optimal for the desired application. Late-stage, site-selective chemical modification of peptides and proteins is a way to fine-tune the properties of biologics. However, this is challenging due to the large diversity of functionalities present in side chains of amino acids of which these peptides and proteins are comprised.

Dehydrated amino acid residues are targets for bio-orthogonal modifications, as the electron-deficient carbon–carbon double bond of these noncanonical amino acids shows unique electrophilic reactivity.[5] Dehydroamino acids naturally occur in many ribosomally synthesised and post-translationally modified peptides (RiPPs), which are of interest because of their antibiotic activity.[6,7] There, dehydroalanine (Dha) and dehydrobutyrine (Dhb) result from the post-translational enzymatic dehydration of serine (Ser) and threonine (Thr), respectively.[5] Moreover, Dha can also be easily introduced synthetically in proteins through a bis-alkylation-elimination reaction of cysteine (Cys).[8]

The unique reactivity of dehydrated amino acids allows for a plethora of reactions to modify these residues. 1,3-Dipolar cycloadditions,[9] cross-couplings,[10,11] cyclopropanations,[12] Diels–Alder reactions,[13] hydrogenations,[14] Michael additions,[8,15–22] radical carbon–carbon bond formations[23] and amidations,[24] have been developed to modify these unique noncanonical residues.

Recently, a number of photocatalytic methods for the modification of peptides have been developed.[25–39] Our group and others have reported photoredox catalysis for the modification of Dha.[40,41] We used the well-known iridium photocatalyst $[Ir(dF(CF_3)ppy)_2(dtbbpy)]PF_6$ and RBF_3K salts as radical precursors to achieve efficient modification of Dha in thiostrepton, a RiPP from the thiopeptide family, and nisin, a lanthipeptide. However, both the catalyst and the radical precursor require significant amounts of organic co-solvent, thus limiting the scope of peptides and proteins that can be modified.

Inspired by the photocatalytic radical alkylation of electrophilic olefins using zinc sulfinates developed by Gualandi and co-workers,[42] we considered these water-soluble zinc benzylsulfinates as attractive modification reagents. Aiming for a general method to modify dehydrated amino acids in both peptides and proteins, we report herein a novel designed water-soluble iridium(III) photoredox catalyst $[Ir(dF(CF_3)ppy)_2(dNMe_3bpy)]Cl_3$ that catalyses the benzylation of Dha residues in the antimicrobial peptide nisin and several proteins in aqueous solutions by using a variety of zinc benzylsulfinates as reagents.

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Results and Discussion

Initial studies were focused on the choice of a water-soluble photoredox catalyst. Organometallic complexes have been used and studied most as photoredox catalysts, as their physical and photophysical properties are easily tuneable.[43] However, recently, organic dyes have drawn much attention as they are cheaper and more abundant, even though that they are more difficult to tune.[44] Therefore, we decided to use the commercially available organic dye riboflavin, which is often used as water-soluble photoredox catalyst.[39,45–48]

However, as organometallic Ir complexes are generally more powerful photocatalysts, we also decided to investigate a water-soluble Ir complex. A heteroleptic Ir^{III} photoredox catalyst was chosen for this design, because its redox events take place in different areas of the molecule and can therefore be tuned separately (Figure 1).[49] Therefore, we hypothesised that the dative ligand of **1** (i.e., dtbbpy) could be exchanged with a water-soluble variant to increase the water-solubility of the complex (Figure 1). Furthermore, the use of chloride or bromide as counter-ion instead of acquiring the complex as PF_6 salt is expected to further enhance the water-solubility of the organometallic complex.

Initially, a similar approach as Li et al. was used to design and synthesise a glucose-decorated iridium(III) photoredox catalyst, but in this case containing the fluorinated dF(CF_3)ppy cyclometallated ligands (S1.12–S1.15 in the Supporting Information).[50] However, dissolving this catalyst in ethanol and subsequent diluting the mixture with water, caused the complex to precipitate immediately. Apparently, the glucose moieties are not enough to overcome the hydrophobicity of the dF(CF_3)ppy ligands. Therefore, this complex could not be further investigated and we designed another water-soluble photoredox catalyst **2** based on Ir^{III} as core element (Figure 1).

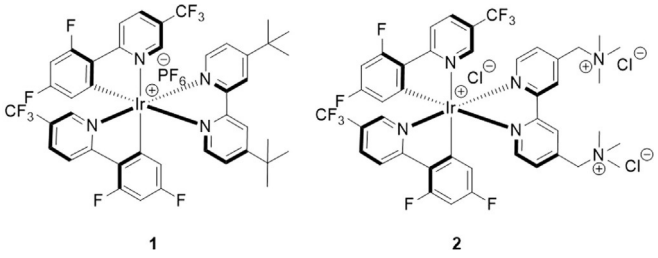
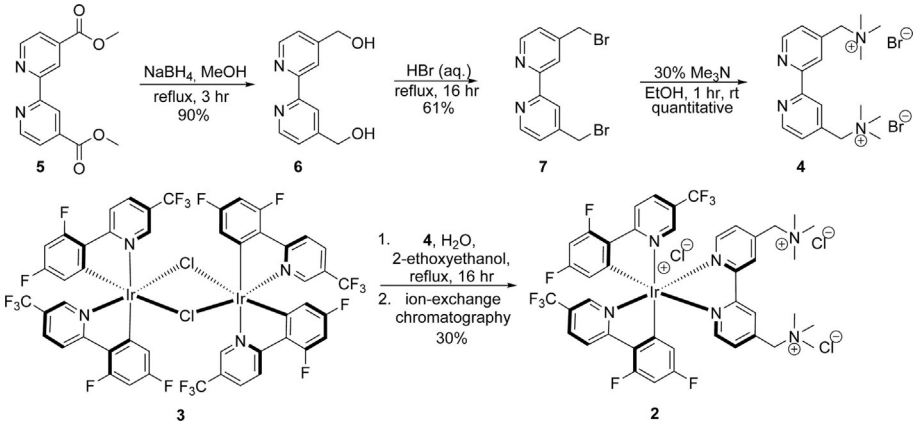


Figure 1. Schematic representation of a common commercially available photoredox catalyst [Ir(dF(CF_3)ppy)$_2$(dtbbpy)]PF_6 (**1**) and a designed, charged Ir^{III} photoredox catalyst [Ir(dF(CF_3)ppy)$_2$(dNMe$_3$bpy)]Cl_3 (**2**).

Inspired by Macmillan et al., who designed a carboxylate-containing iridium(III) photoredox catalyst, we also introduced charges to generate hydrophilic groups.[51] In our design, the dative ligand of the Ir^{III} complex is equipped with two permanently charged hydrophilic moieties over all pH ranges. The *tert*-butyl groups on the dative ligand were replaced with quaternary ammonium groups.

Synthesis of **2** was envisioned from a known iridium dimer intermediate (**3**) following procedures described by Singh et al.[52] and Tellis et al.[53] Synthesis of catalyst **2** was achieved by refluxing iridium dimer **3** with the bipyridine ligand, 4,4′-bis(trimethylammoniummethyl)-2,2′-bipyridine (**4**; Scheme 1). This ligand was synthesised in three steps from 4,4′-bis(methoxycarbonyl)-2,2′-bipyridine (**5**). Reduction of ester **5** with $NaBH_4$ gave 4,4′-bis(hydroxymethyl)-2,2′-bipyridine (**6**).[54] Substitution of the hydroxy groups with hydrogen bromide yielded 4,4′-bis(bromomethyl)-2,2′-bipyridine (**7**).[54] Subsequent nucleophilic substitution with trimethylamine gave **4**.[55] Reaction of ligand **4** with iridium dimer **3** gave complex **2** as a mixture with both chloride and bromide counter-ions. Ion-exchange chromatography provided the organometallic complex as the pure chloride salt. Iridium(III) complex **2** proved to be water-soluble and stock solutions of 1 mM in pure water could easily be prepared.

Next, the catalytic activity of **2** in water was investigated and compared to the water-soluble organic dye riboflavin using a protected Dha substrate **8** (Figure 2). Addition of either 2 mol% iridium(III) catalyst **2** or riboflavin and irradiation for 3 hours resulted in almost full conversion of Dha **8** into the homophenylalanine product **9**, both at pH 4 (0.1% AcOH in H_2O) and pH 7 (50 mM PBS), as determined by UPLC/MS TQD. Absence of photocatalyst, performance of the reactions without irradiation, and addition of TEMPO to the reaction mixture, to



Scheme 1. Synthesis of permanently charged iridium(III) photoredox catalyst **2**.

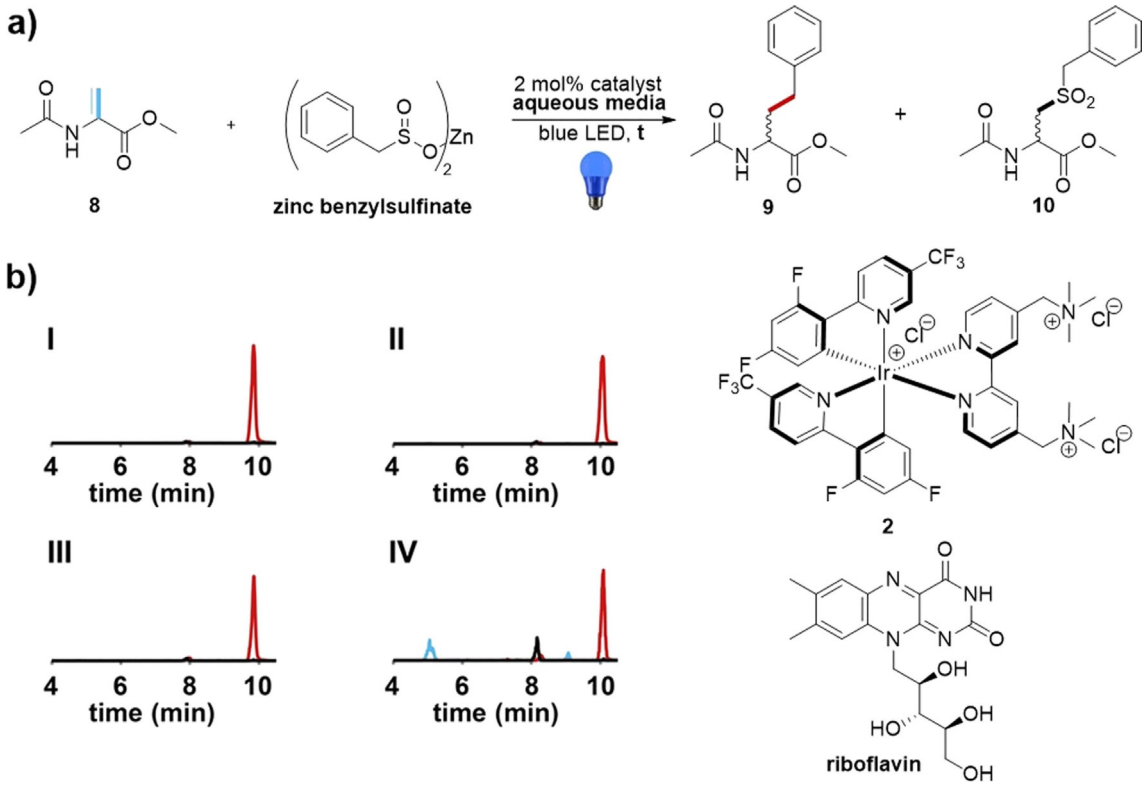


Figure 2. Comparison of the photocatalytic activity of **2** with riboflavin in the photoredox-catalysed modification of Dha **8** with zinc benzylsulfinate in various aqueous media. a) General reaction scheme for nisin. b) Extracted ion chromatograms (EICs) of **8** $[M+H]^+ = 144$ Da (blue), homophenylalanine **9** $[M+H]^+ = 236$ Da (red) and sulfonylated product **10** $[M+H]^+ = 300$ Da (black) from the crude reaction mixture catalysed with I: **2** for 3 h at pH 7 (50 mM PBS), II: **2** for 3 h at pH 4 (0.1 % AcOH in H_2O), III: riboflavin for 3 h at pH 7 (50 mM PBS) and IV: riboflavin for 3 h at pH 4 (0.1 % AcOH in H_2O).

trap formed radicals, all resulted in the recovery of **8** and formation of **10**, while no generation of **9** was detected (S1.21 in the Supporting Information). This proves that the presence of photoredox catalyst combined with blue LED light irradiation is required to induce catalysis through a single-electron-transfer (SET) reaction and accomplish the photoredox catalysed benzylation to give product **9**. Moreover, it shows that sulfonylation, giving rise to product **10**, is not a photocatalysed process and proceeds significantly slower than the photocatalytic benzylation.

The reaction was performed at preparative scale (50 mg **8**), using higher concentrations of catalyst and substrates. Product **9** was obtained with an isolated yield of 27 %, indicating that photoredox catalysis is possible at larger scale, but more efficient at a lower concentration. Tentatively, this is related to the formation of insoluble zinc salts during the reaction, which reduce the efficiency of irradiation.

Having established the photoredox catalysed modification of **8** in aqueous conditions, iridium(III) complex **2** and riboflavin were subsequently evaluated as catalysts for the modification of the lanthipeptide nisin (Figure 3 a). Nisin is an antimicrobial peptide containing three naturally occurring dehydroamino acids: Dhb-2, Dha-5, and Dha-33, making this peptide a suitable and attractive candidate for modification. Reactions were performed in 0.1 % AcOH in H_2O at pH 4, as nisin has proven to be more stable at acidic conditions.[56] Initial treatment of the peptide with 9 equivalents zinc benzylsulfinate, which amounts to 3 equivalents per dehydrated amino acid, 10 mol % **2** or riboflavin, and irradiation for 3 hours with 407.5 nm light resulted in a significant conversion of nisin into its modified variants, as determined by MALDI-TOF mass spectrometry (Figure 3 b and c). Reaction with catalyst **2** resulted in almost complete conversion of nisin into the singly and doubly modified peptide (Figure 3 b). Reaction of nisin catalysed by riboflavin showed a larger amount of unmodified nisin compared to the reaction catalysed by **2** (Figure 3 b and c), while mainly singly modified nisin and a negligible amount of doubly modified peptide was observed (Figure 3 c). Hence, both water-soluble photoredox catalysts are able to modify dehydroamino acids in the lanthipeptide nisin in aqueous acidic medium, but the designed iridium catalyst **2** is more active than riboflavin. Note, besides peaks corresponding to the modified nisin species, additional peaks are observed in the MALDI-TOF spectra. These peaks result from a commonly observed water addition to the double bond of Dha and Dhb.[57,58] Increased number of modifications and a decreased quantity of unmodified nisin upon increased catalyst loadings and irradiation times were observed for both catalysts (Figure 3 d and e). Excess zinc benzylsulfinate (36 or 54 equiv) in combination with 10 or 20 mol % iridium catalyst **2** resulted in increased amounts of triply modified nisin, up to 20–30 %. Further increase of catalyst or reagent loadings resulted in formation of a turbid suspension, which decreased the efficiency of photoredox catalysis and resulted in irreproducible results.

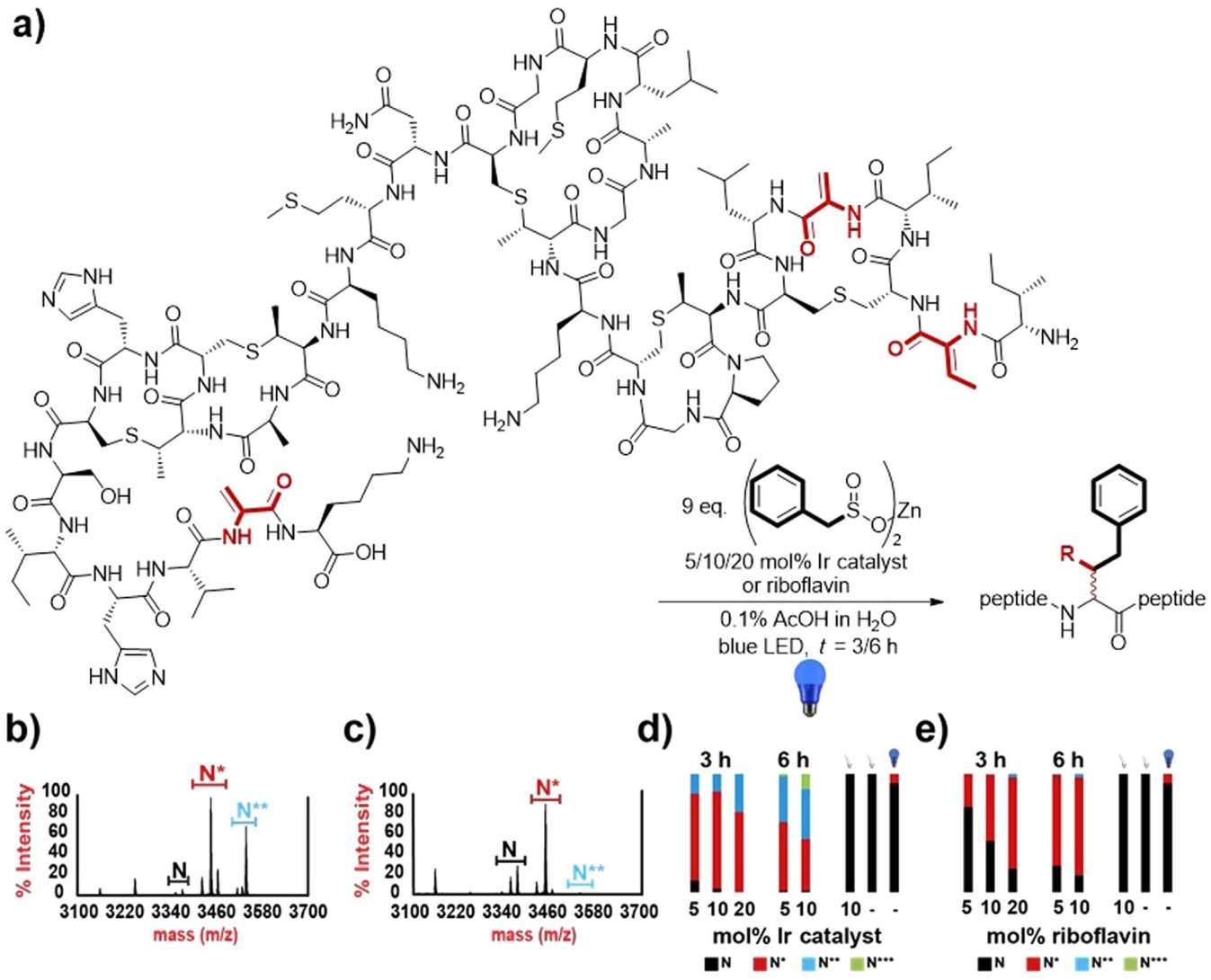


Figure 3. Photoredox catalysed benzylation reaction on nisin. a) General reaction scheme for nisin. MALDI-TOF spectra of the reaction on nisin using b) 10 mol% **2** with irradiation for 3 h and c) 10 mol% riboflavin with irradiation for 3 h. d) Reaction condition screening with **2**, bar charts represent the amount of (un)modified nisin detected for different loadings of **2**, different irradiation times and for the control reactions. e) Reaction condition screening with riboflavin, bar charts represent the amount of (un)modified nisin detected for different riboflavin loadings, different irradiation times and for the control reactions. N = unmodified, N* = singly modified, N** = doubly modified and N*** = triply modified nisin. Yields are based on the peak area obtained from MALDI-TOF mass spectrometry of the corresponding product divided by the sum of the areas of all compounds, assuming that ionisation is similar for all products, which are structurally very similar.[11,59] Water adducts are included in the yields of the respective (un)modified nisin peptides.

Control reactions, in which the photoredox catalyst, irradiation, or both were omitted from the reaction, resulted in no significant reaction, which demonstrates that a combination of photoredox catalyst and irradiation with 407.5 nm light is required for the desired reaction to take place.

Chemoselectivity of the reaction at the dehydroamino acid residues was determined by using Marfey's method.[60] This method was originally developed to quantitatively determine D-amino acids in protein hydrolysates by preparing diastereomers of amino acids and has been used previously to prove the selectivity of modification reactions in lanthipeptides.[11,40,61] Peptide reaction mixtures were hydrolysed in a microwave in 6 M HCl (aq.), concentrated to dryness, and the individual amino acids were derivatised with Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA)) under basic conditions. If photoredox catalysed modification occurs chemoselectively at a Dha residue, FDAA-derivatised homophenylalanine (FDAA-HomoPhe) should be detectable in the peptide hydrolysate. Analysis of the yellow mixture of FDAA-derivatised amino acids with UPLC/MS TQD at 340 nm using a non-chiral column showed the presence of both FDAA-L,L-HomoPhe and FDAA-D,L-HomoPhe at 13.2 and 14.8 minutes, respectively (Figure 4). This shows that the photoredox catalysed reaction takes place chemoselectively at the dehydrated amino acids. Detection of both Marfey-derivatised D- and L-HomoPhe indicates that the reaction is not enantioselective, as expected for these radical reactions. No FDAA-derivatised modified Dhb was detected under the reaction conditions used, indicating that the Dha

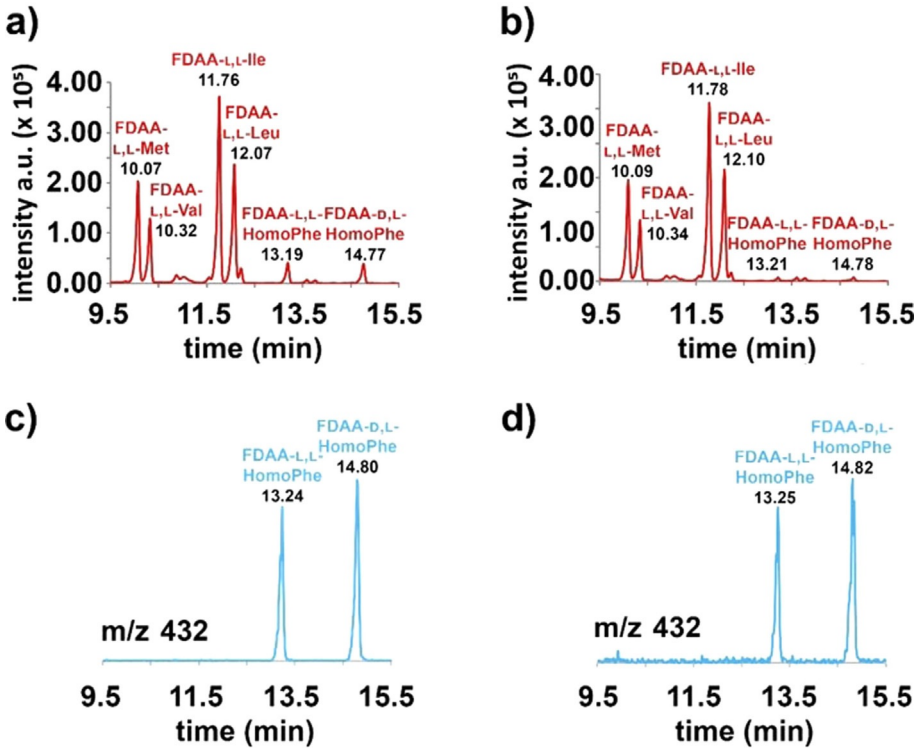


Figure 4. Analysis of the chemoselectivity of photoredox-catalysed modified nisin by using Marfey's method. UV trace (340 nm) of modified nisin hydrolysate generated by a reaction with a) 9 equiv zinc benzylsulfinate and 10 mol% **2**, with irradiation for 3 h and b) 9 equiv zinc benzylsulfinate and 10 mol% riboflavin, with irradiation for 3 h. EICs of $[M+H]^+ = 432$ Da, corresponding to Marfey-derivatised D- and L-HomoPhe; reaction catalysed by c) **2** and d) riboflavin.

residues are more reactive than Dhb, presumably as Dha's are less sterically hindered. In agreement with the MALDI-TOF mass spectrometry results, comparison of reactions catalysed by **2** and riboflavin (Figure 4a and b) show a significantly increased amount of FDAA-HomoPhe for **2**, which further demonstrates the higher activity of **2** over riboflavin in this transformation.

^{1}H NMR studies were performed to study the site selectivity of the reaction. Appearance of a multiplet around 7.4 ppm demonstrated the presence of an aromatic ring, consistent with the addition of the benzyl-group, both in the reaction catalysed by **2** and riboflavin (Figure 5 and S3.6 in the Supporting Information). Comparison of the areas under the distinct dehydroamino acid peaks between 5 and 7 ppm were used as measure for the site-specificity of the reaction. According to Marfey's analysis, Dhb residues were not reactive in this transformation and therefore the integral of Dhb-2 ($\delta = 6.65$ ppm)[62] could be set to 1. The decreased intensity of the integrals of the Dha residues (Dha-33, $\delta = 5.77$ ppm; Dha-5, $\delta = 5.47$ and 5.59 ppm)[62] shows that the reaction takes place at Dha, but that there is no substantial difference that suggests a preference for one of the two Dha's present.

The scope was investigated by the use of zinc benzylsulfinates with a variety of electron-withdrawing and electron-donating substituents in the *para* position. Various side chains (tolyl, 4-fluorobenzyl, and 4-trifluoromethylbenzyl) were introduced in nisin via this photoredox catalysed reaction (Figure 6). Successful addition was achieved for all *p*-substituted zinc benzylsulfinates with only 10 mol% **2** and irradiation for 3 hours, as determined by MALDI-TOF mass spectrometry (S3.7 in the Supporting Information). A decreased number of modifications and a less clean reaction was observed in case of the strong electron-withdrawing substituent CF_3. This substitu-

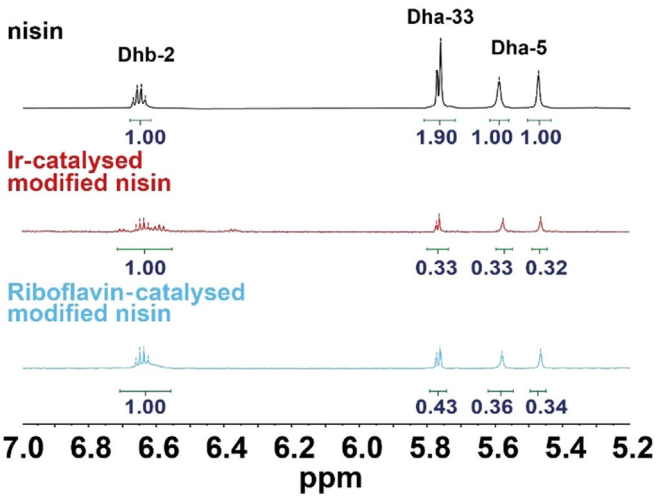


Figure 5. ^{1}H NMR studies to analyse the site selectivity of the photoredox-catalysed modification of nisin with 9 equiv zinc benzylsulfinate and 5 mol% **2** or 10 mol% riboflavin an irradiation for 6 h. Comparison of unmodified nisin (black) with Ir-catalysed modified nisin (red) and riboflavin-catalysed modified nisin (blue). Chemical shifts of dehydroamino acids: $\delta = 5.47$ (s, 1H, Dha-5), 5.59 (s, 1H, Dha-5), 5.77 (d, 2H, Dha-33) and 6.65 ppm (q, 1H, Dhb-2).[62]

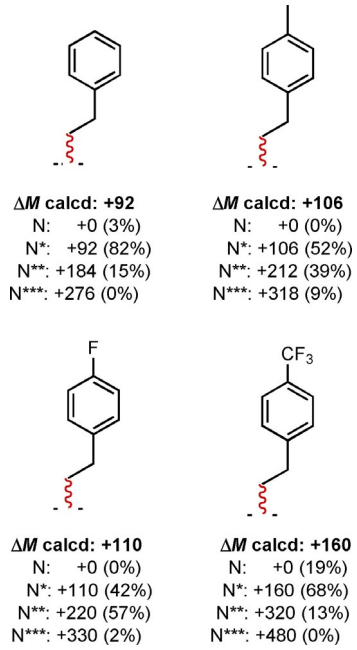


Figure 6. Scope of zinc benzylsulfinates in the photoredox-catalysed modification of nisin. Unmodified (N), singly modified (N*), doubly modified (N**), and triply modified (N***) nisin are observed. The relative yield is displayed in parentheses. This is based on the peak area obtained from MALDI-TOF mass spectrometry of the corresponding product divided by the sum of the areas of all compounds, assuming that ionisation is similar for all products, which are structurally very similar.[11,59] Note: the various zinc benzylsulfinates used for this modification were not purified and obtained as a mixture with the corresponding sulfone. Therefore, not many structure–reactivity relationships can be drawn from these results.

ent tentatively destabilises the generated nucleophilic benzylic radical. Nevertheless, using this method, it is shown to be possible to introduce a variety of benzyl groups to the Dha residues in nisin.

The generality of the photoredox catalysed benzylation was investigated by using the reaction for protein modification. As substrate, a 12.5 kDa small ubiquitin-like modifier (SUMO) protein was used. Two SUMO proteins with Cys residues at different positions were expressed and purified. SUMO_G98C contains a Cys residue near the C terminus to minimise steric effects and SUMO_M60C contains a Cys residue in one of the solvent exposed loops. Quantitative conversion of both Cys residues was achieved chemically, using the bis-alkylation-elimination reaction with 2,5-dibromohexanediamide.[8] The presence of Dha-containing protein was confirmed by UPLC/MS TQD analysis (S4.2 in the Supporting Information). Additionally, as thiols readily react with the electrophilic Dha residues, additional evidence of the presence of Dha in SUMO was obtained by thiol Michael addition (S4.3 in the Supporting Information). Treatment of both Dha-containing SUMO proteins with 50 equivalents zinc benzylsulfinate, 25 mol% **2**, and irradiation for 1 hour (Figure 7a and S4.4 in the Supporting Information) showed full conversion for both proteins and formation of the benzylated protein as only detectable product as determined by UPLC/MS TQD and subsequent deconvolution (Figure 7b and S4.4 in the Supporting Information). This shows that both low reagent and low catalyst loadings are achieved for the modification of the SUMO proteins using water-soluble iridium(III) catalyst **2**. Modification of Dha-containing SUMO proteins using riboflavin as water-soluble catalyst, resulted in formation of unknown by-product (S4.5 in the Supporting Information). Furthermore, solubility and efficiency of photoredox catalyst **2** at basic pH (50 mM PBS, pH 8) was confirmed by successful conversion of SUMO_M60Dha into its HomoPhe-derivative (S4.5 in the Supporting Information).

Furthermore, to investigate the generality of this approach, a larger protein, that is, superfolder green fluorescent protein (sfGFP), was used as substrate. A 30.8 kDa sfGFP protein was expressed and purified with a Cys residue near the C terminus

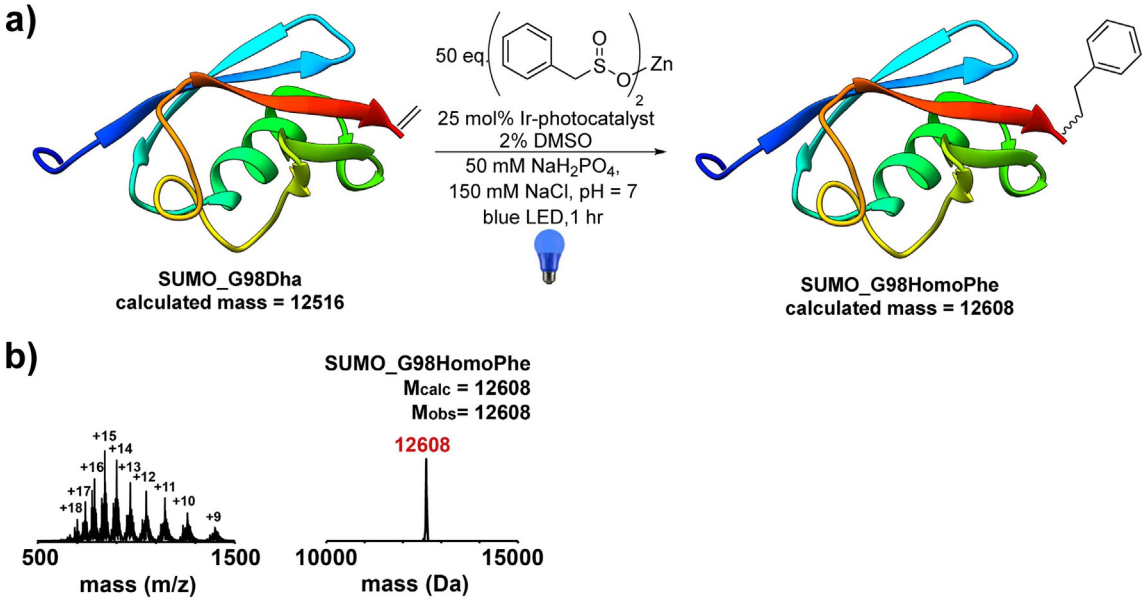


Figure 7. Photoredox-catalysed benzylation of SUMO containing a chemically introduced Dha residue. a) General reaction scheme of photoredox catalysis on SUMO_G98Dha. b) UPLC/MS TQD spectrum of SUMO_G98HomoPhe and deconvoluted spectrum.

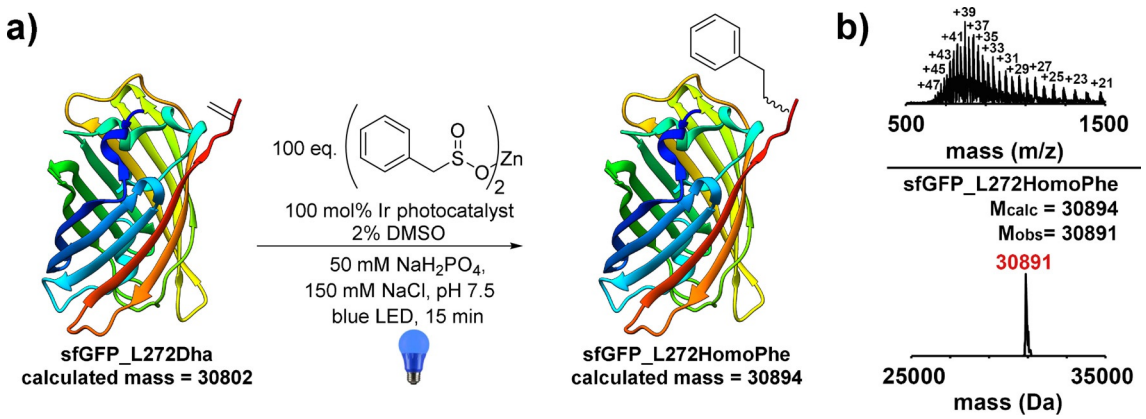


Figure 8. Photoredox-catalysed benzylation on sfGFP containing a chemically introduced Dha residue. a) General reaction scheme of photoredox catalysis on sfGFP_L272Dha. b) UPLC/MS TQD spectrum of sfGFP_L272HomoPhe and deconvoluted spectrum.

to minimise steric effects (sfGFP_L272C). Quantitative conversion of Cys at position 272 was achieved with 2,5-dibromohexanediamide in a bis-alkylation-elimination reaction (S5.2 and S5.3 in the Supporting Information).[63] Initial combination of 25 or 50 mol% **2** with 50 or 100 equivalents zinc benzylsulfinate and irradiation for 15 min without stirring (to prevent mechanical degradation) was insufficient to achieve a significant conversion of Dha to HomoPhe. Ultimately, treatment of sfGFP_L272Dha with 100 equivalents zinc benzylsulfinate, 100 mol% **2**, and irradiation for 15 min without stirring (Figure 8a) showed conversion of the Dha-containing protein into a HomoPhe-derivative (Figure 8b). Emission spectra, emission quantum yield, and emission lifetime of sfGFP_L272HomoPhe are indistinguishable from sfGFP_L272Dha, which indicates that the excited state properties of sfGFP_L272HomoPhe are not affected by the modification (supporting information S5.6).

The combined results shows that the newly designed water-soluble iridium(III) photoredox catalyst $[Ir(dF(CF_3)ppy)_2(dNMe_3bpy)]Cl_3$ can be used for bio-orthogonal modification of dehydroalanines in peptides, such as the antimicrobial peptide nisin, and proteins, as demonstrated for SUMO and sfGFP. In comparison with water-soluble riboflavin, the newly designed catalyst **2** shows better activity for the modification of nisin, SUMO, and sfGFP (Figure 3d–e, S4.5, and S5.5 in the Supporting Information), and less by-product formation in the modification of SUMO at pH 7 (S4.5 in the Supporting Information). In comparison with commercially available $[Ir(dF(CF_3)ppy)_2(dtbbpy)]PF_6$, the water-solubility of photoredox catalyst **2** allows modification of peptides and proteins in aqueous medium. Photoredox catalyst **2** allows modification of peptides and proteins under physiologically relevant conditions at a broad pH range, while maintaining the high activity of iridium(III) complexes.

Conclusions

In conclusion, we have designed and synthesised a new water-soluble iridium(III) photoredox catalyst $[Ir(dF(CF_3)ppy)_2(dNMe_3bpy)]Cl_3$. It was demonstrated that this novel designed catalyst is promising for the chemo- and site-selective late-stage bio-orthogonal modification of the noncanonical amino acid Dha in peptides and proteins in aqueous solvents under physiologically relevant conditions over a broad pH range. Dha reacts selectively with the radical precursor zinc benzylsulfinate in the presence of low catalyst loadings in a short reaction time. Even though that the reaction is not stereoselective, it could have great potential for novel bioconjugation strategies. This research shows the potential of this water-soluble iridium(III)complex in photoredox-catalysed bio-orthogonal late-stage modification of complex peptides and proteins in water and aqueous solutions.

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Conflict of Interests

The authors declare no conflict of interests.

Keywords: bio-orthogonal catalysis · dehydroalanine · nisin · photoredox catalysis · protein modifications

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