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Enabling Wittig reaction on site-specific protein modification†

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An efficient aqueous Wittig reaction was enabled on protein bioconjugation for the first time. By investigating the reaction on small molecules, peptides, and proteins, a site-specific reaction targeting "aldehyde tag" was presented. A variety of functional groups could be introduced into the protein of interest.

The site-selective modification of proteins provides a powerful means to understand the protein dynamics and functions in living systems,[1] to improve the pharmacokinetics of protein-based drugs,[2] and to create new materials.[3] Lysine, cysteine, tryptophan and tyrosine, as the endogenous amino acids, are often chosen as the functionalized residues of proteins.[4] To this end, bioorthogonal reactions that occur selectively and rapidly under physiological conditions are important tools for protein modification. Although a number of bioorthogonal reactions such as the Staudinger ligation of azides and triarylphosphines,[5] the copper(I)-catalyzed[6] or strain-promoted[7] 1,3-dipolar cycloadditions of azides and alkynes, the photo-induced cycloaddition reaction between tetrazoles and alkenes[8] have been developed, the discovery of new bioorthogonal reactions is still in great demand. A major challenge in this respect is the dearth of unique bioorthogonal reactions that can be employed to functionalize chemical reporters in proteins of interest. The aldehyde functionality has been always an attractive chemical reporter for bioconjugation reactions. Several strategies have been developed for incorporating an aldehyde functional group into proteins. When treated with periodate, the N-terminal Ser or Thr residues of protein would generate an aldehyde,[9] which could be further labelled with hydrazide or aminooxy reagents, but only the oxime adducts are stable.[4c] The generated aldehyde would also react with tryptophan, known as the Pictet–Spengler reaction, though it suffered from slow reaction kinetics.[4c] Francis and co-workers developed a novel biomimetic transamination to introduce the aldehyde functional group at the N-terminus by PLP (pyridoxal-5-phosphate) oxidation.[10] Remarkably, the aldehyde reporter was also introduced at an interior position of the protein by installing the 6-amino acid motif (CXPXR) which was recognized by the formylglycine-generating enzyme.[11] In this report, our efforts are focused on the bioconjugation of the aldehyde reporter.

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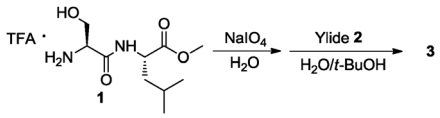
The Wittig reaction, discovered in 1954 by Georg Wittig,[12] is one of the most important approaches to construction of C–C bonds. Many efforts have been made to improve the reaction. Recently, it was found that water can serve as an effective solvent[13] in Wittig reaction. Inspired by this, we wish to investigate the possibility of this reaction on labelling the aldehyde functional group at the N-terminus of proteins.

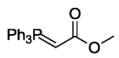
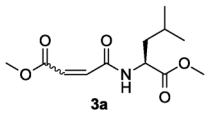
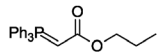
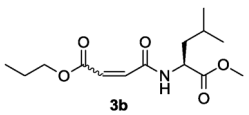
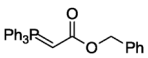
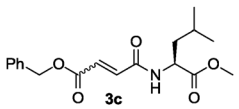
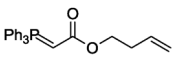
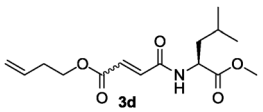
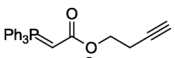
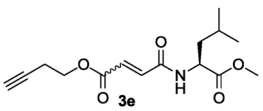
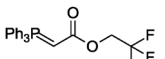
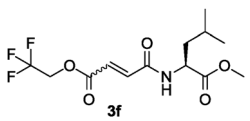
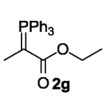
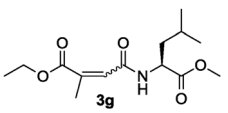
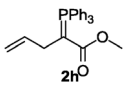
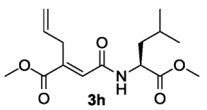
To determine the viability of Wittig reaction on proteins, the protected dipeptide **1** was firstly chosen as the substrate for the preliminary model reactions. As displayed in Table 1, dipeptide **1** was oxidized with sodium periodate ($NaIO_4$) in H_2O, which was followed by Wittig reaction with different ylides using H_2O–*t*-BuOH as solvent. It is delightful that the reactions proceeded very well, various functional groups were introduced, and the reaction yields were good. For instance, alkene (entry 4) and alkyne (entry 5) functionalities, which could be further functionalized by olefin metathesis[14] or [3 + 2] cycloadditions of azides and alkynes,[6,7] were introduced smoothly through this approach. Compound **3f** with the fluoro-substituted functional group (entry 6) that may have important applications in NMR, MRI, and position emission tomography (PET) techniques[15] was obtained in 92% isolated yield. Encouragingly, the ylides **2g** and **2h** with two substituents efficiently reacted with dipeptide **1** to produce compounds **3g** and **3h** (entries 7 and 8), respectively, implying that the peptide and protein could be conjugated by two different functional groups in a single process.

Then, the Wittig reaction was further applied to peptide modification. The hexapeptide **4a** (H_2N-Ser-Leu-Lys-Phe-Tyr-Gln-OH) with the N-terminal Ser residue was treated with $NaIO_4$. Without further purification, the generated aldehyde was subsequently subjected to the reaction with ylide **2h** in H_2O–*t*-BuOH (1 : 1) at room temperature. And the reaction was completed within 30 min, yielding the only desired product **4b** (Fig. 1) as detected by LC-MS/MS. Similarly, the reaction of pentapeptide **5a** (H_2N-Ser-Val-Thr-Arg-Ala-OH) with ylide **2h** under the same conditions provided **5b** as the only product. Our results demonstrated that the Wittig reaction is highly chemoselective and can be used to modify peptides in a site-selective manner, which implies the potential for applications in more complex protein modification.

To explore the potential of the Wittig reaction for protein modification, we tested this method on the chemokine interleukin-8 (IL-8) (8–79) which has a serine residue at the N-terminus. As reported in the previous literature,[16] IL-8 (8–79) (30 μM) was oxidized by $NaIO_4$ (2 eq.) for 0.5 h at room temperature to generate the N-terminal aldehyde successfully. Without further purification, the mixture was treated with

Table 1 The model reactions by using dipeptide **1**[a]



Entry	Ylide	Product	Yield[b]	Ratio[c]
1	 **2a**	 **3a**	77	3 : 1
2	 **2b**	 **3b**	74	16 : 5
3	 **2c**	 **3c**	90	2 : 1
4	 **2d**	 **3d**	84	16 : 9
5	 **2e**	 **3e**	85	18 : 7
6	 **2f**	 **3f**	92	17 : 12
7	 **2g**	 **3g**	67	15 : 4
8	 **2h**	 **3h**	67	*E*-only

[a] Conditions: dipeptide **1** (10 mM), $NaIO_4$ (2 eq.), ylides (2 eq.), H_2O : *t*-BuOH = 1 : 1, rt, 1.5 h. [b] Isolated yield. [c] Isomeric ratios (*Z* : *E*) determined by isolated products.

ylide **2h** (50 eq.) at 37 °C for 0.5 h. As expected, only the desired protein modification product was observed by MALDI-TOF analysis (Fig. 2). Various solution systems including buffers and co-solvents were screened, and H_2O–*t*-BuOH (4 : 1) was shown to be the best medium for this transformation. It is noteworthy that the conjugation yielded the complete conversion at a neutral and alkaline environment, no byproduct was detected at all.

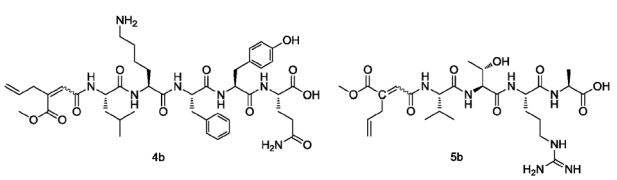
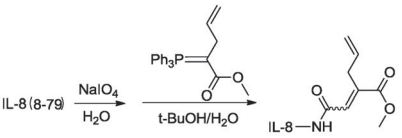


Fig. 1 The structures of modified peptides by Wittig reaction.



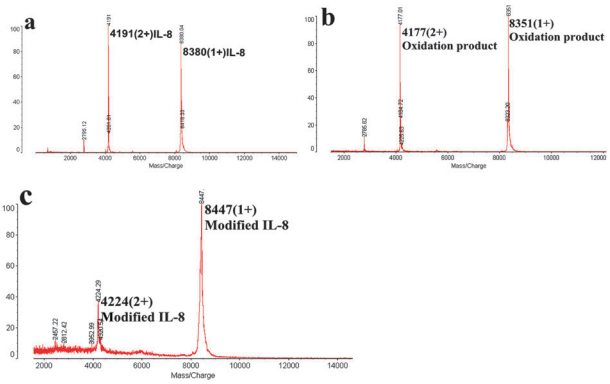


Fig. 2 MALDI-TOF analysis of IL-8 modification through Wittig reaction: (a) unmodified IL-8; (b) the oxidation product of IL-8; (c) modified IL-8.

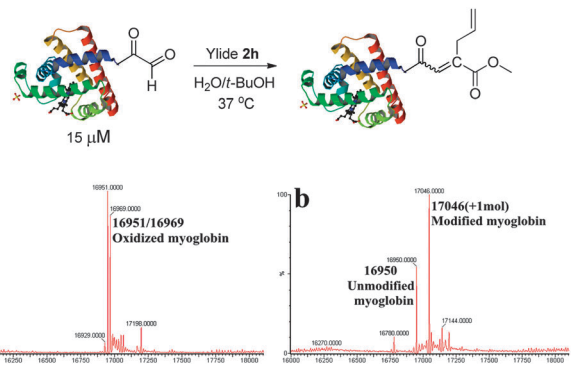


Fig. 3 The functionalization of myoglobin: (a) myoglobin after oxidation; (b) myoglobin after modification by Wittig reaction.

To further evaluate the scope of Wittig reaction for protein modification, myoglobin was selected as another protein model. Since myoglobin has a glycine residue at the N-terminus, we used the PLP oxidation method[10] for the aldehyde formation. In the same media as that used for modification of IL-8 (8–79), the intermediate aldehyde reacted with ylide **2h** at a concentration as low as 15 μM at 37 °C for 0.5 h. After the removal of small molecules, the reaction yielded 65% labelling of myoglobin determined by ESI-MS analysis (Fig. 3). To confirm the site-specific modification of protein, the modified myoglobin was subjected to trypsin digestion. Analysis of the resulting peptide fragments by MALDI-TOF-TOF showed that the Wittig reaction indeed only modified the protein at the N-terminus.

Upon comparing the spectrum of modified myoglobin with that of native myoglobin, it was found that the UV-Vis absorbance of heme was unchanged (Fig. 4), providing good evidence that the tertiary structure was undamaged through the modification process.[10a,17] The circular dichroism (CD) spectra of modified and unmodified proteins were in substantial agreement, which demonstrated that the modification through Wittig reaction did not destroy the secondary

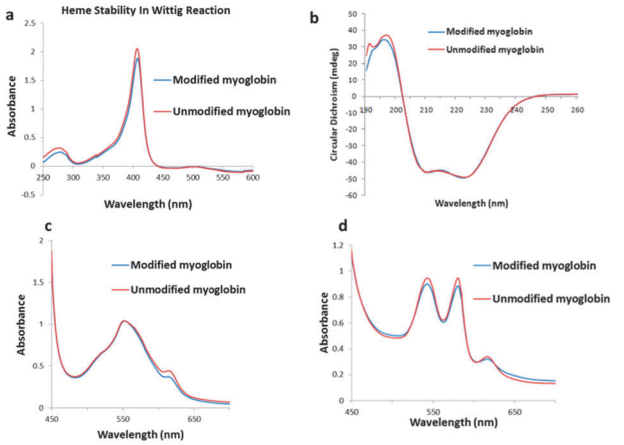


Fig. 4 Evaluation of the influence on structure and function of modified protein: (a) UV and (b) CD spectra of the modified and unmodified myoglobin; (c) releasing and (d) storing oxygen function of the modified and unmodified myoglobin.

structure of myoglobin. Furthermore, the visible spectra (450–700 nm) of myoglobin were also measured under both the oxidation and reduction conditions. The identical traces indicated that its function of storing and releasing oxygen was not influenced after the conjugation. Additionally, the colour changes of solution also drew the same conclusion (see the ESI†).

In conclusion, the Wittig reaction was explored on the protein modification for the first time. Various functional groups could be chemoselectively and efficiently introduced into peptides and proteins *via* Wittig reaction under mild conditions, offering a new member to the family of bioorthogonal reactions. Furthermore, this reaction could label proteins at a specific site in a di-substituted manner, which would make two different functionalities attached. Wittig reaction may become an important approach to the functionalization of biomolecules. Further applications of this reaction in bioconjugation are now under investigation.

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