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Templated alkylation of hexahistidine with Baylis–Hillman esters†

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Alkylation of one of the imidazole rings of hexahistidine with Baylis–Hillman esters tethered to nitrilotriacetate residue was achieved in aqueous solutions at neutral pH and at micromolar concentrations in the presence of Ni^{2+}, Cu^{2+}, or Zn^{2+} cations. The utility of the approach for selective functionalization of His-tagged recombinant proteins was demonstrated by attachment of a fluorescent label to recombinant protein A with an alkynyl group followed by a "click" 1,3-dipolar cycloaddition reaction.

Chemical functionalization of proteins is a common approach toward their utilization in a plethora of functional assemblies. It is extensively used in the study of protein structure,[1] labelling of proteins,[2] their immobilization,[3] drug targeting,[4] modification of immunological properties,[5] bioanalytics,[6] and other applications.[7] Availability of recombinant proteins that can be expressed and purified using standard methodologies makes essentially any protein with a known amino acid sequence available through routine protocols. In contrast, due to the presence of multiple residues of amino acids in any protein, conventional methods producing protein conjugates through reactions of side chains of cysteine[8] and lysine[9] rarely provide well-defined protein conjugates. To overcome this problem other approaches involving site-specific incorporation of unnatural amino acids,[10] introduction of aldehyde tags into proteins by the formylglycine generating enzyme,[11] and native chemical ligation[12] were tried. To increase versatility, protein bioconjugates are prepared in two stages through attachment of a reactive functionality that is capable of highly selective subsequent transformations such as biotinylation.[13] Alternatively, proteins are functionalized with groups capable of highly specific bioorthogonal reactions[14] such as cycloaddition or "click reactions" with complementary components attached to a small molecule or another protein.[15] However, preparation of well-defined conjugates from an arbitrary protein remains a considerable challenge.

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Separation of proteins expressed by bacteria from recombinant DNA vectors and native proteins is commonly done by affinity chromatography through encoding of a hexahistidine motif at the beginning or at the end of an amino acid sequence of a recombinant protein.[16] This method became the standard technology for preparation of recombinant proteins. The hexahistidine tag constitutes an excellent opportunity toward selective chemical covalent derivatization of these proteins. Such an approach can utilize the ability of the hexahistidine sequence to produce a metal complex that can serve as a template for subsequent intramolecular formation of a covalent bond with one of the histidine residues of the sequence. In many aspects this approach resembles the methodology of temporary tethering of two reactants through a covalent[17] or a coordinative[18] bond, which is followed by the intramolecular formation of a permanent covalent bond and final removal of the temporary tether.

Although there were recent attempts to use formation of Ni^{2+} nitrilotriacetate (NTA) complexes for facilitating alkylation of histidine residues in his-tagged proteins they suffered from low reactivity of imidazole rings towards conventional alkylation reactions.[19] An alternative can be found in Baylis–Hillman esters that are versatile reagents for alkylation of a variety of nucleophilic groups through a concerted addition–elimination process.[20] Particularly interesting is reported[21] high reactivity of Baylis–Hillman esters to imidazole in the presence of water, which can make these compounds suitable as histidine-reacting agents. Here we report on successful alkylation of hexahistidine using Baylis–Hillman esters templated by transition metal cations, and application of this methodology for derivatization of recombinant proteins (Fig. 1).

Our approach involved tethering of NTA functionality to the Baylis–Hillman group through the ester group using a flexible linker. In that case formation of the Ni^{2+} ternary complex between the hexahistidine fragment and NTA would bring the reactive double bond of the Baylis–Hillman ester group into the vicinity of one of the imidazole rings that do not form the coordination bond with Ni^{2+} cations. The resultant addition–elimination process would form a C–N covalent bond while breaking the linkage with NTA leaving the $ArCH{=}CH(CO_2Me)CH_2$– moiety attached to the hexahistidine residue.

Syntheses of NTA tethered Baylis–Hillman esters were done through conversion of aldehydes **1a,b** into Baylis–Hillman adducts

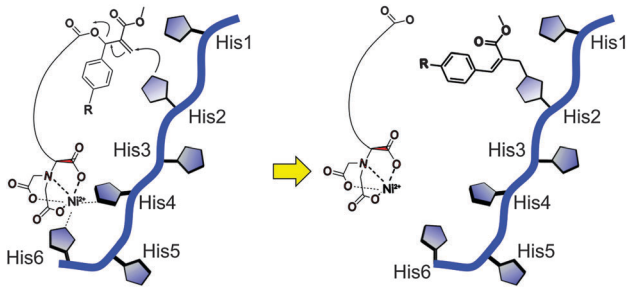
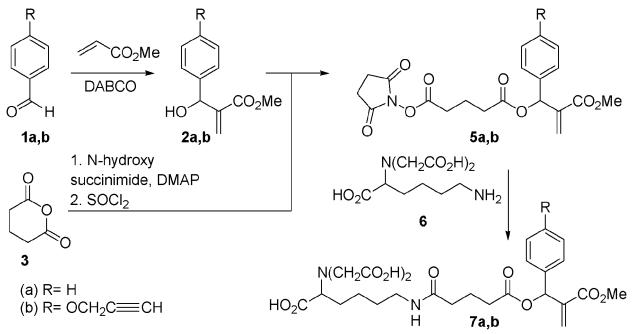


Fig. 1 General approach to alkylation of an imidazole residue in the hexahistidine tag through formation of a ternary metal complex with NTA function carrying a Baylis–Hillman ester alkylating moiety.



Scheme 1 Synthesis of NTA tethered Baylis–Hillman esters **7a,b**.

2a,b followed by their reaction with acyl chloride prepared from glutaric anhydride **3** through successive treatment with *N*-hydroxysuccinimide/DMAP and thionyl chloride. Reaction of the resultant active ester **5a,b** with L-lysine derived amine **6** provided the desired esters **7a,b** (Scheme 1).

The approach was initially studied on a model reaction of ester **7a** (0.25 mM), *N*-acetylhexahistidine (0.25 mM), and $Ni(OAc)_2$ (0.25 μM) in a mixture of methanol–water. To determine the contribution of an intermolecular alkylation of *N*-acetylhexahistidine **9** with ester **7a** a control experiment was conducted, which excluded $Ni(OAc)_2$. The reaction was analyzed by a direct injection into an ESI-TOF spectrometer (negative mode) after 16 h, and concentrations of *N*-acetylhexahistidine **9** and expected alkylation products of type **10** were estimated from relative intensities of $M - H^+$ peaks at 881 D and 1055 D (Fig. 2). The validity of ESI-MS for monitoring of the conversion was proved by comparison of a set of parallel experiments using analysis of the reaction mixture by HPLC.

The control experiment in the absence of Ni^{2+} showed only minor (<3%) amounts of alkylation products of type **10.** Initial experiments

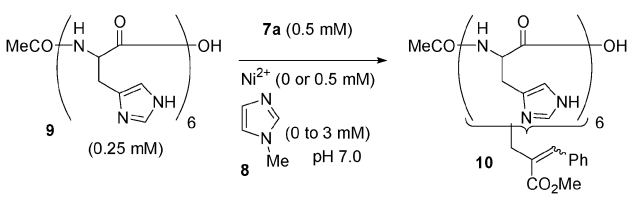


Fig. 2 Alkylation of *N*-acetylhexahistidine **9** with ester **7a** templated by formation of their ternary complex with Ni^{2+} cations.

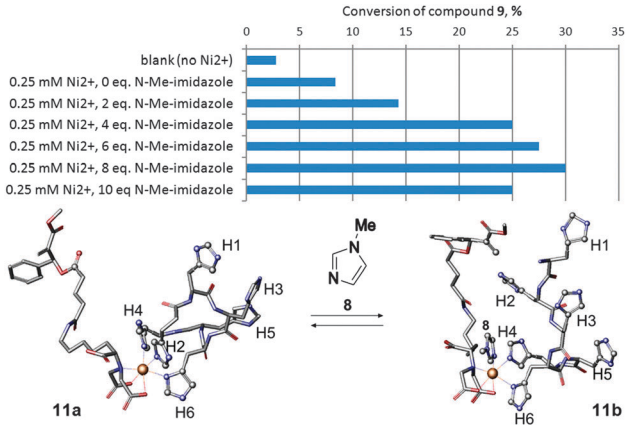


Fig. 3 Above: conversion in alkylation of *N*-acetylhexahistidine **9** with ester **7a** at different concentrations of *N*-methylimidazole **8** after 16 h. Below: schematic representation of transition of binding of Ni^{2+} by *N*-acetylhexahistidine **9** from the tridentate mode in ternary complex **11a** to the bidentate mode in the quaternary complex **11b** induced by *N*-methylimidazole (**8**).

with addition of 1 eq. of nickel salt to the reaction mixture provided only a marginal improvement of the conversion (Fig. 3). The observed low rates of the reaction were attributed to the formation of a rigid ternary complex of type **11a** through tridentate binding of Ni^{2+} cations by *N*-acetylhexahistidine. In such a complex, Ni^{2+} bound imidazole rings can sterically shield remaining non-coordinating imidazole rings from approaching the alkylating Baylis–Hillman ester group.

To overcome this shielding we added an additional monodentate ligand capable of binding Ni^{2+} cations to the reaction mixture to force switching of coordination of Ni^{2+} by *N*-acetylhexahistidine to a bidentate mode through formation of a quaternary complex of type **11b**. Indeed, addition of imidazole or *N*-methylimidazole **8** to the reaction mixture resulted in dramatic increase in the conversion. The maximal conversion of 30% in 16 h was achieved at 8 eq. of *N*-methylimidazole. Further increase of concentration of *N*-methylimidazole decreases reaction rates due to its competition with *N*-acetylhexahistidine for Ni^{2+} binding.

Further study showed that this templated alkylation can be further accelerated by replacing Ni^{2+} cations with Cu^{2+}, and Zn^{2+} in the presence of 6 eq. of *N*-methylimidazole **8** (Fig. 4). Further increase in conversion above 85% is possible through using two equivalents of alkylating agent **7a** or by increase of reaction time. However, under these conditions dialkylation of *N*-acetylhexahistidine with two molecules of ester **7a** was observed as evidenced by formation of a $M - H^+$ peak at 1229.

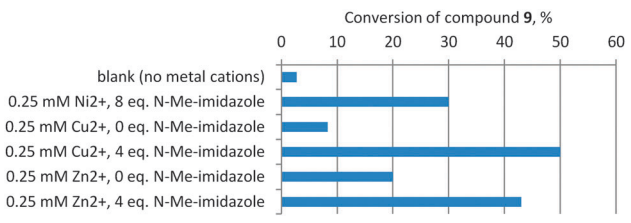


Fig. 4 Comparison of conversion alkylation of *N*-acetylhexahistidine **9** (250 μM) with ester **7a** (250 μM) templated by formation of their mixed Ni^{2+}, Cu^{2+} or Zn^{2+} (250 μM) complexes in the presence of **8** after 16 h.

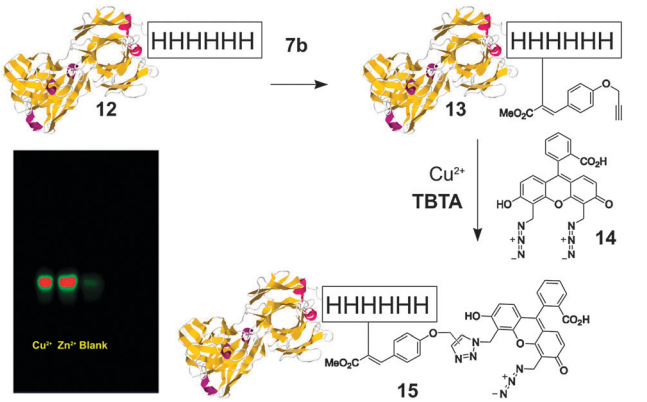


Fig. 5 Derivatizations of protein A **12** with an alkynyl function through the alkylation with ester **7b** followed by a ''click'' cycloaddition with fluorescein azide **14** to produce fluorescent protein **15**. Left bottom: scan of the fluorescent image of gel electrophoresis of modified protein A.

These results demonstrate the possibility of selective alkylation of hexahistidine in aqueous solution at micromolar concentrations, and suggest that this method can be used for selective derivatization of recombinant proteins possessing a hexahistidine tag. The reaction of alkylation can be used for introduction of a "sticky end" group such as alkynyl, which can be a versatile substrate for a subsequent bioorthogonal reaction to introduce variety of functionalities. To prove this approach a sample of recombinant protein A possessing a hexahistidine tag was alkylated with alkynyl carrying ester **7b** to perform two stage derivatization with a fluorescent functionality. On the first stage protein A (16 μM) was treated with 2 eq. of ester **7b**, 12 eq. of *N*-methylimidazole, and 2 eq. of either Cu^{2+} or Zn^{2+} cations to produce alkynyl functionalized protein **13**. To determine the rate of possible intermolecular alkylation of protein A of the hexahistidine sequence, or of any other reaction site, a control experiment in the absence of these metal cations was also performed. After incubation for 24 hours all three reaction mixtures were treated with diazidofluorescein **14** and TBTH/ascorbate for 5 h followed by separation of reaction products using SDS-PAGE (Fig. 5).

Strong fluorescence of the attached residue allowed visual observation of the derivatized product **15**. As can be seen in Fig. 5 both reactions in the presence of Cu^{2+} and Zn^{2+} cations showed successful derivatization of recombinant protein A. The otherwise identical control reaction in the absence of metal cations produced only trace amounts of protein A derivatized with the fluorescent label thus indicating a predictably extremely slow rates of intermolecular alkylation of protein A under the high dilution conditions employed. The ratio of spot fluorescence intensities was 18.6 (Zn^{2+}): 23.3 (Cu^{2+}): 1 (no metal cation). Quantitative measurement of the dye/protein ratio by UV-Vis absorption was found to be 35% for the reaction in the presence of Zn^{2+}, and 60% for the reaction with Cu^{2+}. These reaction rates were several times lower than reported alkylation of a protein containing a His10 tag,[19a] most likely due to substantially higher flexibility of His10 over the commonly used hexahistidine tag.

We demonstrated that the hexahistidine group can be alkylated in aqueous solutions at micromolar concentrations of reagents assembled from Baylis–Hillman esters tethered to nitrilotriacetate through a flexible linker. The reaction is templated by formation of the quaternary complex composed of these reactants, a monodentate ligand such as *N*-methylimidazole, and a metal cation such as Ni^{2+}, Cu^{2+}, or Zn^{2+}. While currently achieved alkylation time is higher than in the existing bioconjugation methods, this reaction can be a promising way for specific derivatization of recombinant proteins possessing a hexahistidine tail through introduction of an alkynyl function that can be used for subsequent bioorthogonal cycloaddition reactions. Studies of this versatile approach are currently being extended to development of new alkylating agents to perform derivatizations of recombinant proteins with quantitative yield using stoichiometric amounts of reagents at low micromolar concentrations *in vitro* and *in vivo*.

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