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Highly selective lysine acylation in proteins using a Lys-His tag sequence

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Abstract: Chemical modification of proteins has numerous applications, but it has been challenging to achieve the required high degree of selectivity on lysine amino groups. Recently, we described the highly selective acylation of proteins with an N-terminal Gly-His$_6$ segment. This tag promoted acylation of the N-terminal N$^{\alpha}$-amine resulting in stable conjugates. Here, we report the peptide sequences His$_n$-Lys-His$_m$, which we term Lys-His tags. In combination with simple acylating agents, they facilitate the acylation of the designated Lys N$^{\varepsilon}$-amine under mild conditions and with high selectivity over native Lys residues. We show that the Lys-His tags, which are 7 to 10 amino acids in length and still act as conventional His tags, can be inserted in proteins at the C-terminus or in loops, thus providing high flexibility regarding the site of modification. Finally, the selective and efficient acylation of the therapeutic antibody Rituximab, pure or mixed with other proteins, demonstrates the scope of the Lys-His tag acylation method.

The chemical modification of proteins to introduce fluorophores, half-life extending moieties, binders such as biotin, and more, is of great importance in chemical biology, biotechnology, and for the production of biopharmaceuticals. The introduction of new functionalities often relies on the modification of cysteine (Cys) and lysine (Lys). While Cys is a low abundant residue (Uniprot estimate ~1.4%),[1] mostly participating in disulfide linkages and typically buried inside wildtype proteins (8.9% relative surface accessibility, RSA),[2] Lys is an abundant residue (Uniprot estimate ~5.8%),[1] frequently found at the protein surface (~43.5% RSA).[2] Hence, the chemical, selective labeling of a single Lys residue is often challenging if not impossible.

For improved site-selectivity, small peptide tags have been developed with optimized local chemical environments, promoting the modification of a designated Cys residue using specific reagents.[3] Other chemical methods have been developed,[4] for example, those that target the N-terminus of proteins,[5,6] those which rely on the modification of specific amino acids such as Trp[7] or non-canonical amino acids,[8] and diazo transfer to amines.[9] Some peptide-tag based strategies have been developed enabling the modification of a specific Lys residue.[10] Some strategies rely on the use of enzymes, such as biotin ligase and lipoic acid ligase,[11,12] or the pre-formation of a complex between a metal-chelating reagent and the tagged protein of interest.[13, 14] While all these methods show creative use of different chemistries, they often have limited generality or require reagents prepared by multistep synthesis.

Recently, we introduced a strategy for the site-selective acylation of peptides and proteins, enabled by the combination of a simple N-terminal Gly-His$_6$ tag (GHHHHHH) and the use of 4-methoxyphenyl esters to react site-selectively with the N-terminal α-amine group.[15] During this study, we observed that a Lys C-terminal of a Gly-His$_6$ tag gave an increased amount (13%) of inadvertently diacylated byproduct. Inspired by this observation, we hypothesized that a short sequence containing multiple copies of His in close proximity to a target Lys would facilitate selective labeling of the respective ε-amine over the N-terminal α-amine and other Lys residues (see Figure 1).

Here, we describe Lys-His tags, all of which are able to undergo site-selective Lys acylation, while retaining their capacity to bind to immobilized metal ions. A site-selective protein modification method is provided, which due to its operational simplicity, robustness, mildness and anticipated scalability, we believe has the potential to become a highly attractive method for many protein scientists in the fields of biotechnology, biotherapeutics and chemical biology.

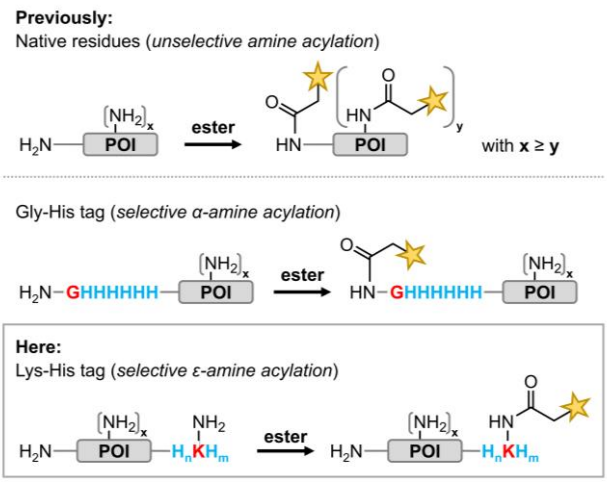

Figure 1. Schematic representation of (top) conventional NHS labeling, (middle) the Gly-His tag acylation method using 4-methoxyphenyl esters and (bottom) the Lys-His tag acylation method, presented in this work, with n,m = 0, 3, 6. POI: protein of interest. Amines are implicit in the 1-letter code for amino acids, but here they are shown explicitly (in back) for added clarity.

First, potential Lys-His tags were placed N-terminal of the 18-mer peptide DWLKAFYDKVAEKLKEAF (Beltide).[16] The peptides, which were N-terminally acetylated to allow us to focus on selective acylation among Lys residues, were reacted with 4-methoxyphenyl 2-azidoacetate (**1**) and analyzed by LC-MS (Table S1, entries 1–4). Ac-GH_6-Beltide showed little acylation, whereas placing a Lys directly N- or C-terminally to His_6 or in the middle to form H_3KH_3 were acylated to a significant degree. Noticing that the latter variation led to a considerably higher conversion, we extended the tag to H_3KH_6, which promoted the selective acylation even further (Table S1, entries 5–9). Next, we assessed the acylation of C-terminally tagged peptide sequences (Table S1, entries 10–17). The selectivity of the acylation reaction towards the target Lys over other Lys residues in the peptide was confirmed by MS analysis of a peptide treated with V8 protease (Figure S1–S4).

The selectivity for N^{ε}-acylation over acylation of the N-terminal N^{α}-amine was assessed on Beltide with a free N^{α}-amine and C-terminal H_3KH_6 tag (Table S1, entries 13–17, and Figures S1–S4). Rewardingly, high selectivity for N^{ε}-acylation was observed. In control experiments, using a version of the peptide with a free N^{α}-amine and the H_3KH_6 tag installed at the N-terminus, both N^{α}-acylation and N^{ε}-acylation were observed (Figure S5). MS/MS analysis confirmed that the N^{α}-amine was acylated before the N^{ε}-amine, which suggests the possibility for dual labelling (Figure S5). We did not notice any reversibility in the acylations. Overall, these results on model peptides gave a first indication that selective acylation of a Lys N^{ε}-amine in or directly adjacent to a poly-His sequence was promoted.

We then inserted the KH_6 and H_3KH_6 sequences at the C-terminus of three model proteins, small ubiquitin-related modifier (SUMO), super-folder green-fluorescent protein (sfGFP) and maltose-binding protein (MBP), all of which have multiple native, surface-exposed Lys residues (Figure 2A). Both tags facilitated purification by immobilized metal affinity chromatography (IMAC) (Figures S6 and S7). We compared tagged proteins with tag-free versions in reactions using acylating reagent **1** (Figure 2A). Mass spectrometric analysis showed that a significant degree of conversion occurred when the proteins were tagged, while acylation of the tag-free protein was very low (Table 1, and Figure 2B). We observed that H_3KH_6 consistently gave the highest degree of Lys acylation. Thrombin-mediated cleavage of the H_3KH_6 tag from acylated sfGFP-H_3KH_6 followed by MS analysis of both the cleaved peptide and the truncated protein proved that the acylation had taken place on the tag (Figure S8).

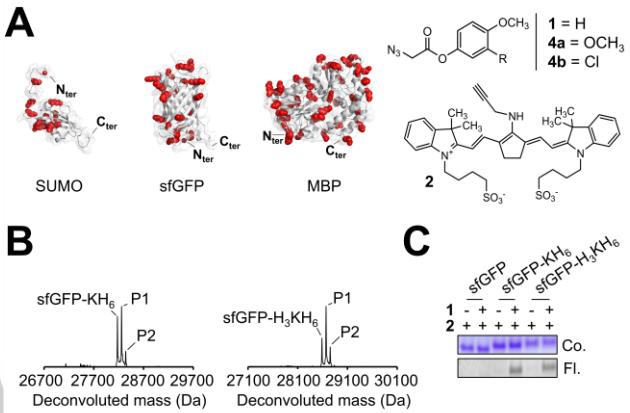

Figure 2. A) Models of SUMO (9 Lys; PDB 1A5R), sfGFP (20 Lys; PDB 2B3P), MBP (37 Lys; PDB 1ANF), and reagents **1**, **2**, **4a** and **4b**. Lys residues are depicted as red spheres. B) Deconvoluted MS spectra of reactions between Lys-His tagged sfGFPs (SM = starting material) and **1**, with corresponding products (P1 = mono-functionalized, and P2 = di-functionalized). C) In-gel fluorescence imaging depicting the outcome of the two-step fluorescent labeling of sfGFP.

Table 1. Acylation of three model proteins carrying a C-terminal Lys-His tag.

Protein	P1 (%)[a]	P2 (%)[a]
sfGFP	7 (± 1)	0
sfGFP-KH_6	43 (± 6)	9 (± 2)
sfGFP-H_3KH_6	55 (± 7)	17 (± 4)
SUMO	9 (± 3)	0
SUMO-KH_6	42 (± 5)	6 (± 3)
SUMO-H_3KH_6	51 (± 7)	6 (± 4)
MBP	0	0
MBP-KH_6	34 (± 6)	2 (± 1)
MBP-H_3KH_6	49 (± 5)	7 (± 3)

[a] Degree of acylation of proteins (29 μM) after treatment with 4-methoxyphenyl 2-azidoacetate **1** (0.6 mM) in 50 mM sodium phosphate and 150 mM NaCl (pH 7.5) at 4 °C for 16 h, as determined by ESI-TOF MS. The relative abundances of mono-functionalized (P1) and di-functionalized (P2) products were derived from deconvoluted mass spectra from 3-6 independent experiments. Mass spectra (before deconvolution) are shown in Figures S46-48.

The acylation was (partially) inhibited by divalent metal salts (Table S2). Introduction of the azide group facilitated conjugation of the alkyne-functionalized cyanine dye **2** by copper-catalyzed

azide-alkyne cycloaddition, as demonstrated by in-gel fluorescence imaging (Figure 2C, and Figure S9).

Next, we studied whether the acylation of Lys-His tags could also occur in protein loops. We tested a series of Lys-His sequences, including two with Glu (introducing a negative charge) and one with Pro (likely to promote a turn). When inserted in the loop connecting β-strands β10 and β11 in sfGFP (Figure S10), the sequences were found to be fully compatible with protein maturation (Figure S11)[17] and to have retained the ability to bind to Ni-NTA (Figure S12). Compared to tag-free sfGFP, all Lys-His tags promoted acylation (Table 2 and Figure S13) and facilitated fluorescent labeling with reagent **2** (Figure S14). In general, a higher concentration of **1** had to be used to achieve yields similar to those obtained when the same tag was positioned at the C-terminus. This might stem from a difference in conformational freedom between the restricted loop and the free terminal chain. Again, the H_3KH_6 tag yielded the highest degree of conversion.

Table 2. Acylation of sfGFP containing different versions of a Lys-His tag inserted in a loop.

Protein	P1 (%)[a]	P2 (%)[a]
sfGFP	10 (± 1)	0
sfGFP(KH_6)	46 (± 1)	12 (± 2)
sfGFP(H_3KH_3)	49 (± 3)	14 (± 3)
sfGFP(H_6K)	43 (± 3)	11 (± 2)
sfGFP(EKH_6)	44 (± 1)	12 (± 2)
sfGFP(H_3EKH_3)	45 (± 1)	10 (± 2)
sfGFP(H_3KH_6)	59 (± 1)	23 (± 4)
sfGFP(H_3PKH_3)	45 (± 2)	9 (± 2)

[a] Degree of acylation of sfGFP with respective sequences inserted in the β10 →β11 loop (29 µM) after treatment with 4-methoxy phenyl ester **1** (1.2 mM) in 50 mM sodium phosphate and 150 mM NaCl (pH 7.5) at 4 °C for 16 h, as determined by ESI-TOF MS. The relative abundances of mono-functionalized (P1) and di-functionalized (P2) products were derived from deconvoluted mass spectra from three independent experiments (see Figure S13 and Figure S49-50).

We studied the effect of pH on the conversion and selectivity of the acylation reaction. Eight different pH values between 6.75 and 8.25 were tested in reactions with sfGFP-H_3KH_6 (Figure S15). Below pH 7.25, less di-functionalized product was observed but more starting material had remained, indicating an increase in selectivity and a decrease in the reaction efficiency. Between 7.25 and 8.25 the effect of pH was less pronounced, though, a gradual increase in the amount of di-functionalized product was observable. An advantage of our Lys-His tag acylation is the use of near-neutral pH. This pH scan shows that small adjustments in pH can be used to direct product formation, which will be useful when optimizing the acylation for a protein of interest.

Next, we compared different ester derivatives in reactions with sfGFP variants to determine if the reaction could be directed by the choice of acylating reagent. In control experiments, even low concentrations of 2-azidoacetic acid NHS ester **3** remained too reactive and produced multiple conjugates with tag-free sfGFP, and more so with the tagged proteins (Figure S16). Phenyl esters 3,4-dimethoxyphenyl 2-azidoacetate (**4a**) and 3-chloro-4-methoxyphenyl 2-azidoacetate (**4b**), predicted to be less and more reactive analogues of **1**, respectively, were shown not to modify tag-free sfGFP at the concentrations employed here. Rewardingly, sfGFP-KH_6 and sfGFP-H_3KH_6 underwent uncomplicated conversion with **4a** and **4b** (Figure S16).

We further assessed different biotin ester derivatives (**5**, **6a**, and **6b**) to directly biotinylate sfGFP, SUMO and MBP. The biotinylated sfGFP products were incubated with streptavidin-coated resin to allow modified protein to be captured as visualized by fluorescence microscopy. We found that all three sfGFP versions bound to the resin after treatment with biotin NHS ester **5** (Figure 3B). In contrast, acylation with **6a** led to significant binding of sfGFP-KH_6 and sfGFP-H_3KH_6, whereas sfGFP produced only a weak fluorescence signal. These observations were supported by MS analysis (Figure S17). For example, a single addition of **6a** to a final concentration of 1.5 mM led to 31% biotinylated sfGFP-KH_6 and 63% biotinylated sfGFP-H_3KH_6. Adding the reagent (**6a**) twice gave 47% biotinylated sfGFP-KH_6 and 90% biotinylated sfGFP-H_3KH_6 (Figure 3C). In contrast, less than 3% of sfGFP was biotinylated under identical conditions (Figure S17). We also tested biotin 3-chloro-4-methoxy-phenyl ester **6b** and found it to be more reactive than **6a** (Figure 3D and Figure S18). Finally, we assessed the generality of this Lys-His tag-selective biotinylation method on SUMO and MBP. We found again that in contrast to tagged protein, tag-free protein remained largely unmodified by both **6a** and **6b**, which was easily identified by Western blot analysis (Figure S19).

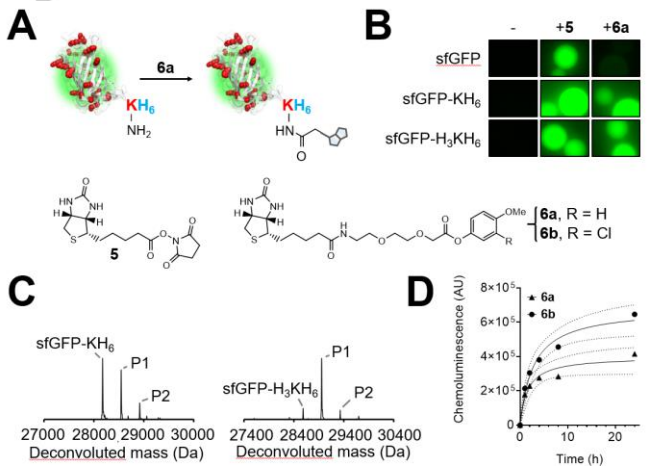

Figure 3. A) Representation of the biotinylation of sfGFP-KH_6 with biotin ester derivative 6a. B) Fluorescence microscopy images of streptavidin-coated beads exposed to solutions of three different versions of sfGFP (29 µM), which were either untreated or modified with biotin ester derivative **5** (0.29 mM) or **6a** (1.5 mM). C) MS spectra of reactions between Lys-His tagged sfGFPs (29 µM, SM) and **6a** (2 additions of 1.5 mM), with species corresponding to the product masses labeled P1 and P2. D) Biotinylation of sfGFP-H_3KH_6 (29 µM) with **6a** (1.5 mM) and **6b** (0.73 mM) followed in time and quantified by Western blotting (see Figure S18 for Western blot image).

In order to assess the applicability of the Lys-His tag acylation method on larger proteins, the sequences KH_6 and H_3KH_6 were

installed at the C-terminus of the heavy chain of the antibody Rituximab.[18] Both tagged antibody versions as well as the tag-free reference antibody (Figure 4A) were expressed in Chinese Hamster Ovary (CHO) cells. Lys-His tagged Rituximab retained binding to CD20 positive Ramos cells (Figure 4B). Upon purification, the tagged antibody variants were reacted with acylating reagent **1** under similar conditions as those used for sfGFP, SUMO and MBP. The reacted antibodies were reduced prior to MS analysis, such that modification of the light chain and heavy chain could be assessed separately. As expected, the heavy chains of both tagged antibody versions were found to be modified, whereas the light chains displayed the same mass before and after acylation (see Figure 4C and Figure S20). Consistent with the acylation of the three model proteins, the acylation was more efficient when Rituximab was tagged with H_3KH_6 instead of KH_6 (88% vs. 72% of acylated protein). Furthermore, the chemical modification was shown not to interfere with the antibody's ability to interact with a protein known to bind to the Fc region of human IgG's (Figure S21).

Using KH_6-tagged Rituximab as reference, we studied the effect of gradually reducing the number of His from 6 to 1. With the number of His residues in the tag being 4–6, similar degrees of acylation were obtained, while a decrease in the degree of acylation was observed with the number of His residues being ≤ 3 (Figure S22).

To assess whether the Lys-His tag acylation method could be employed to conjugate larger chemical entities, Rituximab modified with azide derivative **1** was reacted with 5 kDa poly(ethylene glycol) containing a dibenzocyclooctyne (DBCO) moiety. The crude reactions were analyzed by gel electrophoresis (see Figure 4D). In accordance with the MS data of the acylation reaction, the amount of PEGylated light chain (Lc) was negligible. On the contrary, a clear band representing mono-PEGylated heavy chain (Hc-PEG) was detected for the tagged antibodies. Smaller amounts of non-PEGylated (Hc) and di-PEGylated (Hc-PEG_2) species of the heavy chain were also present. The heavy chain of the tag-free antibody variant (Figure 4D, lane 3) was found to be PEGylated to a minor degree as well, caused by a combination of off-target acylation and unintended cyclooctyne–Cys conjugation. Nevertheless, the selectivity of the acylation reaction towards the Lys residue residing in the Lys-His tag remained significant, considering that each Rituximab molecule contains 96 native Lys residues.

The selectivity of the method was further assessed by addition of biotin ester derivative **6a** to a mixture of proteins containing Rituximab-H_3KH_6 as the only Lys-His tagged protein. Biotinylated protein species were detected by Western blot analysis (see Figure 4E). As anticipated, the heavy chain of tagged Rituximab was the most predominantly biotinylated product in the reaction mixture and only very faint bands corresponding to minor amounts of biotinylated, untagged proteins were detected. This result shows that the method is not restricted to pure, tagged proteins and suggests its applicability in more complex biological samples.

In summary, we have shown that positioning a Lys within the context of a histidine-rich peptide segment, termed a Lys-His tag, promotes the highly selective acylation of this specific Lys residue when using tuned phenyl ester derivatives as acylating reagents. Among the peptide sequences tested, H_3KH_6 emerged as the most effective Lys-His tag. The sequences H_3KH_3, KH_6, and H_6K were viable alternatives.

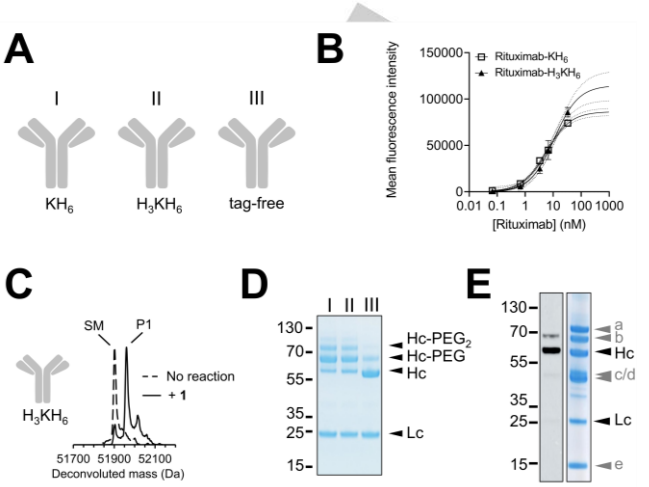

Figure 4. Acylation of the antibody Rituximab. A) Schematic representation of the three different versions of Rituximab (96 Lys) used in this study. The KH_6 (I) or H_3KH_6-tag (II) were installed at the C-terminus of the heavy chain. B) Binding of Rituximab-KH_6 and Rituximab-H_3KH_6 to CD20-positive Ramos cells. Cells were treated with increasing concentrations of Rituximab, stained with phycoerythrin-conjugated polyclonal goat F(ab')2 anti-hu**m**an IgG Fc and analyzed by flow cytometry. C) Deconvoluted MS spectra of the heavy chain of Rituximab-H_3KH_6 before and after acylation with **1** (see Figure S20 for MS spectra of the light chain of the same samples as well as MS spectra of the heavy and light chain of Rituximab-KH_6). SM = starting material, P1 = mono-functionalized product. D) PEGylation of the heavy chain of Rituximab, visualized by gel electrophoresis. Lc = light-chain, Hc = heavy chain. E) Biotinylation of Rituximab-H_3KH_6 with biotin derivative **6a** in a mixture of proteins, assessed by Western blot analysis with streptavidin-HRP (left) and Coomassie staining (right). Bands corresponding to the antibody are indicated as Hc and Lc. Non-tagged proteins a – e: a = conalbumin, b = BSA, c = ovalbumin, d = aldolase, and e = lysozyme.

We hypothesize that the Lys-His tags are autocatalytic in the sense that they promote their own acylation at the internal Lys side-chain. Studies on the exact mechanism behind the autocatalysis are on-going. The Lys-His tag proved efficient in the site-selective modification of a range of proteins, including an antibody. The selectivity in the Lys-His acylation appeared higher for some proteins than for the peptides. This may be due to the highly dynamic structures of linear peptides, which provide unhindered access to Lys side-chain amines.

Our Lys-His tags retain the ability to bind to immobilized metal ions and to act as epitopes recognized by anti-His antibodies (data not shown). Hence, they harbor a three-fold functionality, providing a handle for affinity purification, diagnostic and site-selective modification. They are an important addition to our previously developed Gly-His_6 tag,[15] which facilitates N-terminus selective protein modification. In cases where the N-terminus is insufficiently solvent-exposed or naturally modified (e.g. through methylation, acetylation, or cyclization of an N-terminal glutamine),[19] a C-terminal Lys-His tag can now be employed. Furthermore, when both termini are sequestered,[20] the Lys-His tag can be inserted in a loop within the protein structure. We previously demonstrated that the conjugates from N-terminal Gly-His_6 acylation were stable. We did not observe any reversibility of the Lys-His tag acylation either.

The highly selective acylation of our Lys-His tags relies on the use of phenyl ester derivatives as acylating agents. The reagents are easily synthesized and readily derivatized with various groups. Moreover, they display very low side-reactivities and remain stable for extended periods of time, also when stored as stock solutions (for example, when stored cold in DMSO, DMF or acetonitrile). The reactivity of these reagents can be finely adjusted by both the phenol leaving group and the electronic properties on the carbonyl side. In addition, reagents **1** are **6a** are commercially available.

Based on the popularity of the polyhistidine tag in general, the freedom in choice of where to install the Lys-His tag, the convenient access to the acylating agents, and finally the extremely mild and reductant-free reaction conditions, we foresee that our site-selective modification strategy will be of great benefit to researchers in the fields of protein science and chemical biology, especially when reaction times in the order of hours can be used.

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Keywords: acylation • antibodies • chemical biology • peptide tags • site-selective protein modification

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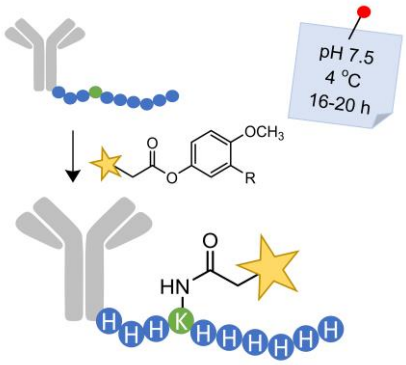

A short, designed Lys-His tag can be inserted into proteins where it autocatalyzes the highly selective acylation of the Lys side-chain amine with phenyl esters. The reaction conditions of this site-selective, chemical protein modification method are mild, and no oxidative reagents, reductants nor enzymes are required.