Master Thesis

Studies on α-Nucleophiles for Efficient Protein Labeling

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Abstract

Specific and efficient incorporation of covalent tags into a protein is of great significance in chemical biology. One common strategy involves targeting electrophilic functional groups in proteins with specific nucleophiles harboring fluorophores or affinity tags. We designed some novel nucleophiles with neighboring groups for intramolecular catalytic effects for more efficient bioconjugation under mild conditions in aqueous buffers. Our nucleophiles are based on hydrazine and aminooxy groups, which are known for their enhanced nucleophilicity.

Chapter 1 describes our investigation on these nucleophiles for reactions with acyl phosphates. Phosphoaspartate (pAsp), an acyl phosphate, is a key element in two-component systems (TCSs), which are crucial in bacterial virulence, survival, and pathogenicity. Fast and specific labeling of pAsp-containing proteins will enable facile detection, isolation, and identification of these modified proteins from cell lysates, and it will lay the foundation for the further studies on TCSs, as well as the development of novel antibacterial strategies. We tested a number of nucleophiles on various conditions using a model acyl phosphate and the results are described herein.

Chapter 2 discusses our progress on the utilization of novel alpha-nucleophiles for bioconjugation to aldehydes/ketones and thioesters. Aldehyde and ketone handles are commonly used for bioconjugation via the formation of hydrazones and oximes, but their slow kinetics with nucleophiles in neutral conditions hampered their widespread applications. Recently, it was found that aniline catalyst can accelerate these reactions, but very high concentration (>100 mM) of these catalysts are required for practically useful reaction rates. To address this issue, we aimed at the development of novel nucleophiles for fast bioconjugation without the need of catalysts. Moreover, our nucleophiles can yield cyclic stable products with aldehydes. So further studies on our nucleophiles can lead to superior nucleophiles for aldehyde/ketone bioconjugation. Thioesters are another group of electrophiles utilized for protein bioconjugation. Hydrazinylation and aminoxylation of thioesters have been reported to site-specifically label proteins. However, these reactions are very slow in neutral conditions and large excess (>100 mM) of nucleophiles are typically used. We tested our nucleophiles to thioesters, and our novel alpha-nucleophiles might turn out be a solution to this issue.

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List of Abbreviations

ADCs	Antibody-drug conjugates
ADP	Adenosine diphosphate
Asp	Aspartate
ATP	Adenosine triphosphate
Bn	Benzyl
CuAAC	Copper(I)-catalyzed alkyne-azide cycloaddition
CV	Column volume
Cys	Cysteine
DCC	*N,N'*-Dicyclohexylcarbodiimide
DCM	Dichloromethane
DMF	Dimethylformamide
EA	Ethyl acetate
EPL	Expressed protein ligation
Et_3N	Triethylamine
FACS	Fatty acyl-coenzyme A synthetase
FGE	Formylglycine generating enzyme
FTIR	Fourier transform infrared spectroscopy
HK	Histidine kinase
His	Histidine
HAD	Haloacid dehalogenase
HOAt	1-Hydroxy-7-azabenzotriazole
HOBt	Hydroxybenzotriazole
IMAC	Immobilized metal affinity chromatography
K_{eq}	Equilibrium constant
LplA	Lipoic acid ligase
MeOH	Methanol

MPAA	4-Mercaptophenylacetic acid
MESNA	2-Mercaptoethanolsulfonate sodium
NCL	Native chemical ligation
NMR	Nuclear magnetic resonance
NTA	Nitrilotriacetic acid
Nu	Nucleophile
O/N	Overnight
Oxyma pure	Ethyl 2-cyano-2-(hydroximino)acetate
PBS	Phosphate-buffered saline
PFTase	Protein farnesyl-transferase
PLP	Pyridoxal-5-phosphate
pAsp	Phosphoaspartate
pSer	Phosphoserine
POI	Protein of interest
PTMs	Post-translational modifications
PTS	Protein trans-splicing
pThr	Phosphothreonine
pTyr	Phosphotyrosine
pGlu	Phosphoglutamate
RP-HPLC	Reversed-phase high-performance liquid chromatography
RR	Response receptor
RS	Rapoport’s salt
rt	Room temperature
Ser	Serine
SAHA	Suberoylanilide hydroxamic acid
SPPS	Solid phase peptide synthesis
TCEP	Tris(2-carboxyethyl) phosphine
THF	Tetrahydrofuran
Thr	Threonine
TCSs	Two-component systems

TBD	1,5,7-triazabicyclo[4.4.0]dec-5-ene
TFA	Trifluoroacetic acid
TS	Transition state
TTL	Tubulin tyrosine ligase
TSA	Trichostatin A
Tris	Tris(hydroxymethyl)aminomethane
tRNA	Transfer RNA
UV	Ultraviolet

Chapter 1: Studies on Covalent Labeling of pAsp-Proteins

I. Introduction

1.1 Biology of phosphoaspartate (pAsp)

Protein phosphorylation takes an important position among post-translational modifications (PTMs). It is involved in almost every aspect of cell physiology[1], and recognized as major signaling mechanism responding to external physiological stimuli.[2] Accordingly, abnormal phosphorylation has been linked to many human diseases.[3]

Among several types of phosphoamino acids, phosphoesters, such as phosphoserine (pSer), phosphothreonine (pThr) and phosphotyrosine (pTyr), have drawn the most attention.[4] In contrast, acylphosphates, including phosphoaspartate (pAsp) and phosphoglutamate (pGlu), are much less studied, mostly owing to their chemical lability.[5] As pGlu is of less occurrence than pAsp in nature,[6] we will focus our discussion on pAsp here. (Fig. 1)

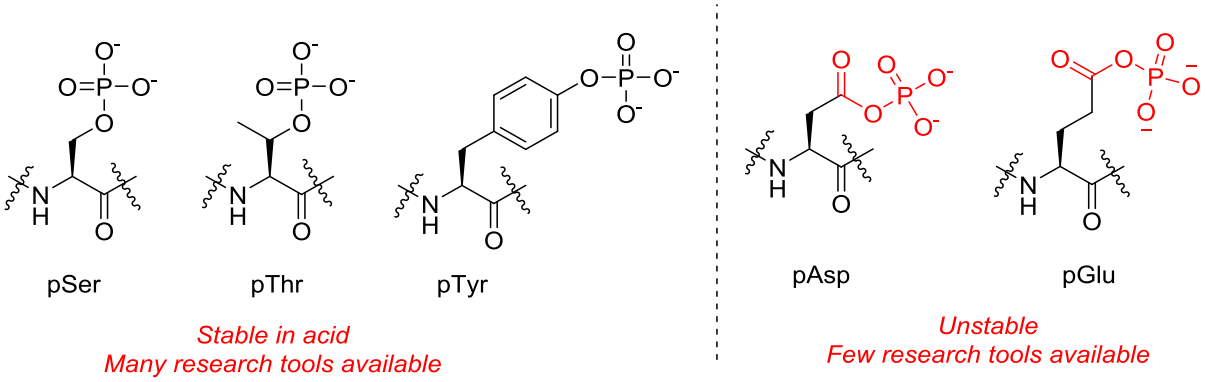


Fig. 1. Structures of phosphoesters (left) and acyl phosphates (right).

pAsp is known to be a crucial element in two-component systems (TCSs), which are sensory-response circuits in response to environment changes by phosphorelay between histidine and aspartate residues.[7] TCSs are commonly found in bacteria (up to ~200 TCSs per species), being crucial to bacterial virulence[8,9], survival[10], and pathogenicity[11]. However, TCSs are absent in mammals, making them attractive targets for novel antibacterial therapeutic strategies.[9,12]

As the major components in TCSs, a histidine kinase (HK) and the response regulator (RR) work together to control the appropriate responses to extracellular stimuli. A membrane-bound homodimeric HK autophosphorylates its histidine residue(s) with ATP in response to extracellular stimuli, then a cognate RR is activated through phosphor-transfer from phosphorylated-HK to its aspartate residue. The activated RR can then modulate the expression of target genes (Fig. 2).[13,6,14] Although the mechanism of activating the RR is still under debate, it cannot be unrelated to the phosphorylation of aspartate in RR.[6] Accordingly, knowledge of TCSs is essential for potential antibacterial drug development, and Utsumi and co-workers discussed the characteristics of HK/ RR pairs in TCSs, providing guidelines for the development of antibacterial strategies.[11]

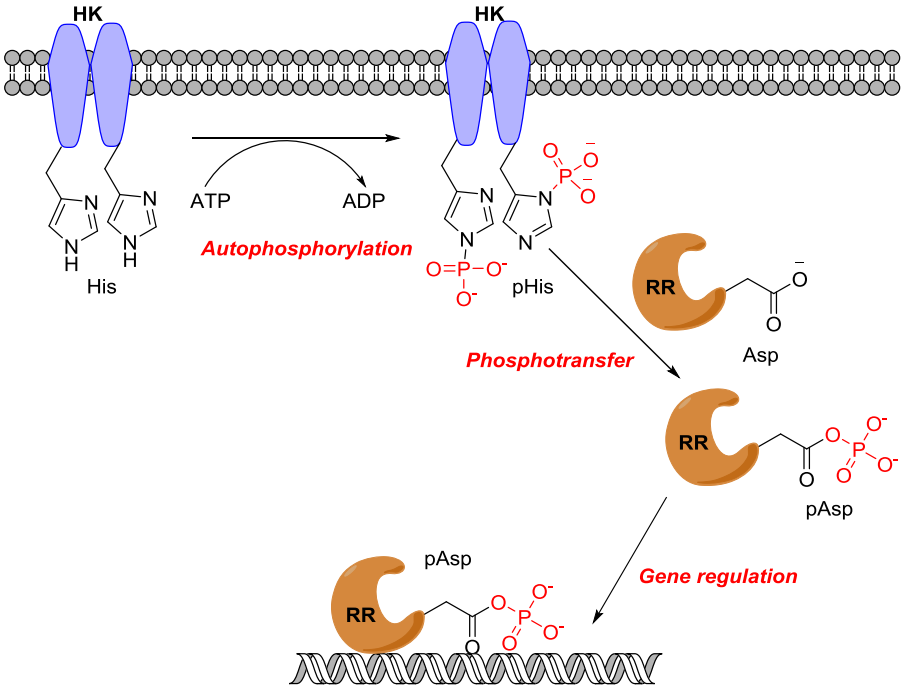
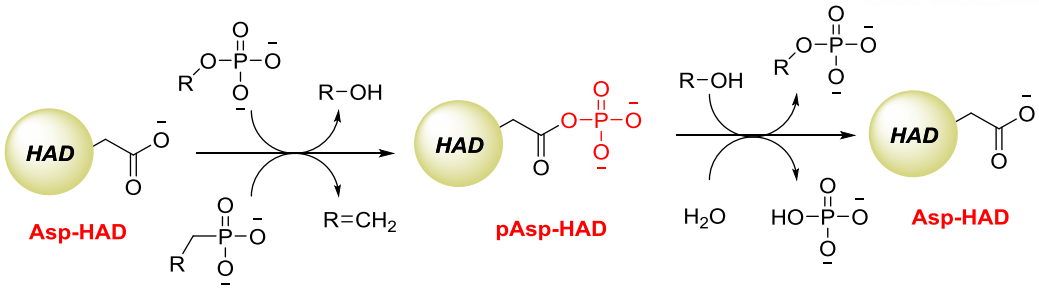


Fig. 2. Two-component system signaling pathway. HK: histidine kinase. RR: response regulator.

pAsp-proteins also occur as enzymatic intermediates, mostly in the haloacid dehalogenase (HAD) superfamily. HAD superfamily consists of P-type ATPases, phosphatases, phosphonoacetaldehyde hydrolase enzymes and β-phosphoglucomutase.[6] HAD enzymes transfer phosphoryl groups of many substrates with high specificity via a catalytic nucleophilic aspartate residue (Scheme 1)[6], where a Mg^{2+} cofactor is necessary.[15] HAD superfamily members are characterized by Rossmann-like fold and active site sequence DXDX (T/V), where the first aspartate residue functions as the nucleophilic catalyst in phosphoryl-transfer.[6,16]



Scheme 1. Asp-HAD enzymes catalyze phosphoryl transfer on various substrates.

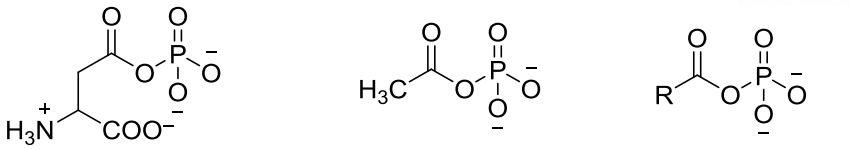
1.2 Chemistry of pAsp (acyl phosphate)

Table 1. Chemical stability of phosphoamino acids.

Natural phosphoamino acid		Stability in	
		Acid	**Base**
O-Phosphates	Phosphoserine (pSer)	+	-
	Phosphothreonine (pThr)	+	±
	Phosphotyrosine (pTyr)	+	+
N-Phosphates	Phosphoarginine (pArg)	-	-
	Phosphohistidine (pHis)	-	+
	Phospholysine (pLys)	-	+
Acyl phosphates	Phosphoaspartate (pAsp)	-	-
	Phosphoglutamate (pGlu)	-	-
S-Phosphates	Phosphocysteine (pCys)	(+)	+

Note: +: stable. -: unstable. (+): moderately stable.

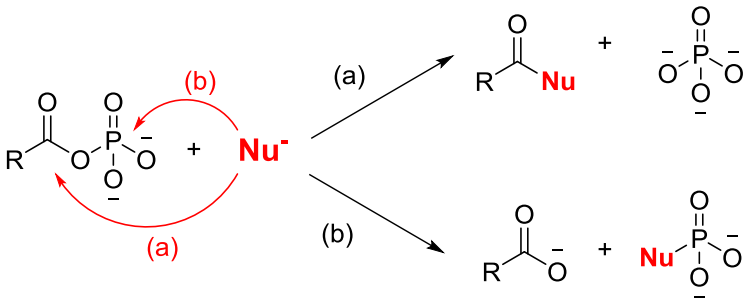
For pAsp (Scheme 2, a), the lack of appropriate research tools makes it much less known than acid-stable O-phosphates (pSer, pThr, and pTyr). It is because pAsp is an acyl phosphate, which is unstable under both acidic and basic conditions (Table 1).[5] Therefore most of biochemical and analytical tools developed for O-phosphates are not compatible with pAsp. Hence, it is necessary to discuss the chemical characteristics of acyl phosphate first.



(a) Phosphoaspartate (pAsp) (b) Acetyl phosphate (c) Acyl phosphate

Scheme 2. Chemical structures of pAsp, acetyl phosphate, acyl phosphate.

Two types of substitution reactions on acyl phosphate (Scheme 2, c) are possible. One reaction is acyl substitution. Electrophilic phosphorus draws the electron density of the bridging oxygen, making bridging oxygen electron delocalization onto the carbonyl group less efficient, thus resulting in more reactive carbonyl group. Moreover, the phosphoryl group is a good leaving group (Scheme 3, a). The other reaction is phosphoryl substitution. The existence of electrophilic acyl group, makes the phosphoryl group more electrophilic (Scheme 3, b).



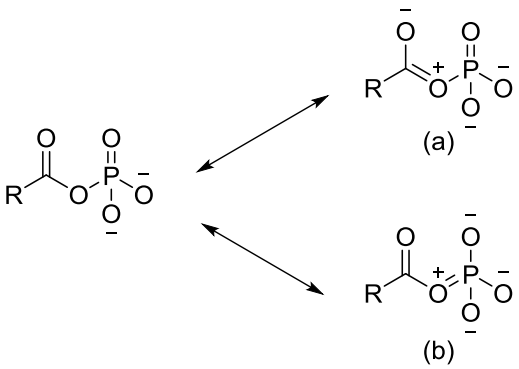
Scheme 3. Substitutions on acyl phosphate. (a) acyl substitution. (b) phosphoryl substitution.

1.2.1 Thermodynamic stability of acyl phosphates

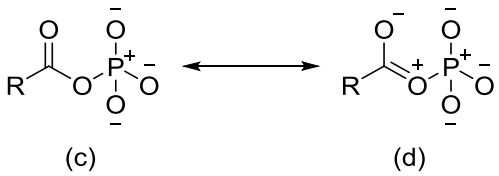
Thermodynamic studies of acyl phosphates were conducted on acetyl phosphate (Scheme 2, b), to directly estimate the lability of acyl phosphates. The standard free energy of acetyl phosphate hydrolysis is −10.3 kcal/mol,[17] while for ATP, the standard free energy of hydrolysis is about −7.3 kcal/mol,[6] indicating that acetyl phosphate holds stronger phosphoryl transfer capacity than ATP, i.e. more facile hydrolysis. Two factors can contribute to the large negative free energy of acetyl phosphate, the low electron delocalization efficiency of the bridging oxygen, and the good solvation of its hydrolysis products.[6]

For the low electron delocalization efficiency of the bridging oxygen in acyl phosphate, there are some explanations about it. One is the opposing resonance (Scheme 4), the lone pair of electrons on

bridging oxygen can delocalize to both carbonyl (a) and phosphoryl oxygens (b), thus, the competition between (a) and (b) makes the electron delocalization of bridging oxygen to either direction less efficient.[6] Another explanation is the highly polarization in phosphoryl group, namely, little π-character of phosphorus atom.[18] Accordingly, little resonance exists between phosphorus and oxygens in acyl phosphate (Scheme 5, c). Thus, the positively charged phosphorus atom will weaken the electron delocalization of bridging oxygen onto carbonyl oxygen (Scheme 5, d).[6] An anomeric effect has been also proposed to explain the low electron delocalization of bridging oxygen. The anomeric effect, i.e. stereoelectronic effect, makes the lone pair of electrons on phosphoryl negatively charged oxygen transfer from n(O) orbital to σ*(P-O) orbital, resulting weaker bridging P-O bond.[17]

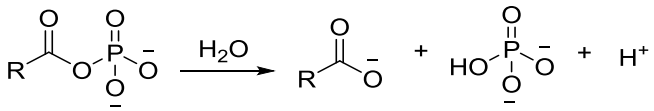


Scheme 4. Opposing resonances in acyl phosphate.



Scheme 5. Resonance of acyl phosphate response to little π-character of phosphorus atom.

The solvation effect is also related to the free energy of hydrolysis.[19] The enhanced solvation of the hydrolysis products contributes to the large negative free energy of acyl phosphate hydrolysis. That is, the well-soluble hydrolysis products of acyl phosphate lead to the large free energy of hydrolysis (Scheme 6).



Scheme 6. Ionization of acyl phosphate hydrolysis products at neutral pH.

1.2.2 Hydrolysis of acyl phosphate

For the hydrolysis of acyl phosphate, acetyl phosphate (Scheme 2, b) had been studied as a model system. The hydrolysis rate is pH dependent, as shown in Figure 3. In the pH range of 5-10, acetyl phosphate is relatively stable, and the hydrolysis rate is mostly changeless. Whereas, outside that pH range, very fast hydrolysis is observed.[20] For the mechanism of acetyl phosphate hydrolysis, the bond cleavage preference switches in different pH conditions. Acidic and basic conditions are prone to leading to C-O bond cleavage, while neutral hydrolysis of acetyl phosphate, P-O bond cleavage is more favored.[21,22]

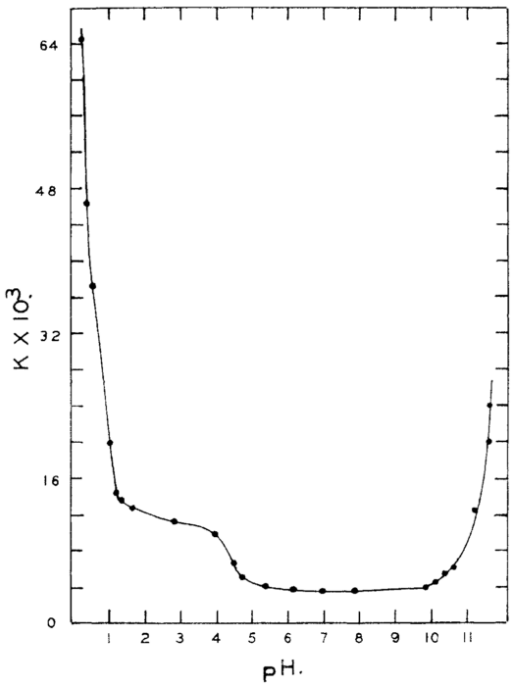
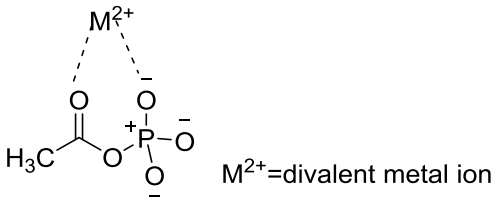


Fig. 3. Hydrolysis rate of acetyl phosphate with respect to pH. k unit: L/(mol·min). Copied from ref.20.

Some metal ions can accelerate the hydrolysis of acetyl phosphate dramatically. Divalent metal cations can bind to the acetyl phosphate by forming stable six-membered complexes between the carbonyl oxygen and phosphoryl oxygen (Scheme 7).[23] The stability of the complexes is dependent on

the polarity of solvents,[23] and highly polarized solvents can destabilize the complexes. Moreover, buffer species also brings some effect to the metal catalyst activity, since some buffer media are potential to form insoluble compounds with metal ions.[24]



Scheme 7. The complex formed between divalent metal and acetyl phosphate.

Different divalent metal ions exhibit different stabilizing ability to acetyl phosphate through six-membered complexes, where Cu^{2+} ions perform better than Mg^{2+} and Ca^{2+}, Zn^{2+} ions, which involve enzymatic transphosphorylation.[23] Herein, the Mg^{2+} ions catalyze better than Ca^{2+} in transphosphorylation process, which was confirmed by FT-IR studies.[25] And Ca^{2+} ions can precipitate some phospho-species. Divalent metal cations can enhance the reaction of water and acetyl phosphate. Because the metal cations can neutralize the negative charges of phosphoryl group, thereby decreasing acetyl phosphate's electrostatic repulsion to water molecules.[22]

1.3 Current methods for pAsp detection

There are several methods documented for identifying phosphoamino acids in proteins. One of the most classical method is radioactive labeling of phosphorylated proteins with ^{32}P. This method is very sensitive, and the identity of the phosphoamino acid can be inferred by using TLC after total hydrolysis of the target protein. But it is costly and dangerous to use radioactive materials. ^{31}P-NMR method can be also very useful, but this method always requires specific enrichment of phosphorylated proteins in lysate or cell, owing to the low content of phosphoamino acids in proteins.[5] Strategies involving antibody recognition (Western blot) can be used to identify phosphoamino acids, and they have been extremely valuable in the detection and enrichment of protein phosphorylation research. [5] Unfortunately, there is no specific antibody towards pAsp-containing proteins and peptides. For specific enrichment of phosphopeptides or –proteins, immobilized metal affinity chromatography (IMAC), associated with mass spectrometry, has been extensively employed to identify phospho-peptides or proteins. However, such IMAC uses acidic conditions for elution, making it unsuitable for pAsp-proteins. Edman degradation and phosphoamino acid analysis were utilized to localize phosphoamino

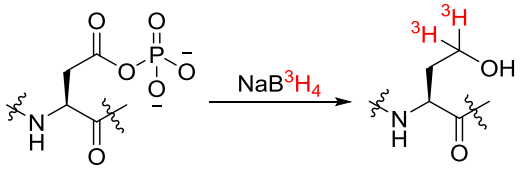
acid residues in peptide and protein before the development of mass spectrometry.[5] Still, the reaction conditions are incompatible with pAsp.

As shown above, these traditional phosphoamino acid detection methods cannot be directly applied to pAsp-protein. Therefore, new tools for studying pAsp-proteins are in demand. It is because the chemical lability of the phosphoanhydride has severely hampered the pAsp research. Currently, only a few detection methods of pAsp (or other acyl phosphates) have been documented.

One method utilizes calcium ions to precipitate acetyl phosphate, as well as inorganic phosphate. Subsequent phosphate determination could quantify acetyl phosphate and inorganic phosphate separately. However, this method cannot be applied to other phosphoanhydrides.[25]

Second documented determination method is the colorimetric assay, which allows quantitative and qualitative measurements of chromogentic complex formed between Fe^{3+} ions and hydroxamic acids. The hydroxamic acid can be obtained through treating the acyl phosphate with hydroxylamine.[25] However, this method was demonstrated only for small acyl phosphate molecules, and its applicability to acyl phosphate-containing proteins has not been reported. This method was specific to acyl phosphates to a degree, only in the absence of water-soluble organic anhydrides. In addition, the color intensity of hydroxamic acid-Fe(III) complexes was dependent on the pH, reaction time, as well as such anions as fluoride and phosphate, which presumably also formed complexes with Fe^{3+} ions.[25]

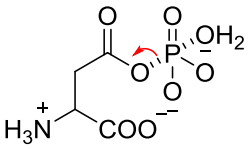
The third method involves radiolabeling acyl phosphates with tritium. pAsp can give a radiolabeled homoserine residue with NaB^3H_4 reduction (Scheme 8). Subsequent detection and characterization can be realized with electrophoresis, radioimmunoassay, NMR spectroscopy, HPLC, as well as mass spectrometry.[26,27,28] This method has also been used to identify the pAsp in the active site of phosphoserine phosphatase, combining with tandem mass spectrometry.[29]



Scheme 8. Reductive cleavage of pAsp-protein to give a tritiated homoserine protein.

The fourth way studies the pAsp in peptides or proteins directly by FT-IR.[6] That is through studying the vibration of C=O and PO_3^{2-} group's bonds of pAsp in different environments, to speculate pAsp states in the active sites of phosphoenzymes. The hydrolysis mechanism of pAsp in peptides or proteins

was proposed according to these researches, pAsp proceeded the dissociative transition state (Scheme 9) when undergoing hydrolysis, where the bridging P-O bond was longer, and the angle between the terminal P-O bond was larger, as well as more electron density of phosphoryl group delocalized to the bridging oxygen. And the state of pAsp in the active site of phosphoenzyme, was found similar to the transition state.[30,31] The FT-IR method also demonstrated the Mg^{2+} ions made carbonyl C=O bond weaker, conforming the interaction between carbonyl oxygen and Mg^{2+}.[31]



Scheme 9. The dissociative transition state of pAsp hydrolysis.

There are some other direct detection methods for pAsp, for example, β-phosphoglucomutase was observed by X-ray crystallography[32], and a pAsp-containing model peptide was identified by ^{31}P-NMR spectroscopy[33]. Although these detection methods of pAsp have been valuable in previous pAsp research, more convenient and efficient methods are still in demand, mainly because most of these methods are only applicable to purified pAsp-proteins in vitro. Accordingly, novel methods for the isolation and identification of pAsp proteins from proteomic samples will lead to discovery of novel pAsp-proteins, opening up many new research opportunities. Here, we would introduce a covalent labeling method for pAsp-proteins.

II. Preparation of pAsp derivatives

The lability of pAsp makes it more difficult to study pAsp-proteins. On the other hand, the highly unstable nature of pAsp can be tapped into fast chemical reactions. Herein, we aimed to take advantage of pAsp's high reactivity to form a covalent bond with a nucleophile, which contains a tag to achieve covalent labeling of pAsp-proteins (Fig.4).

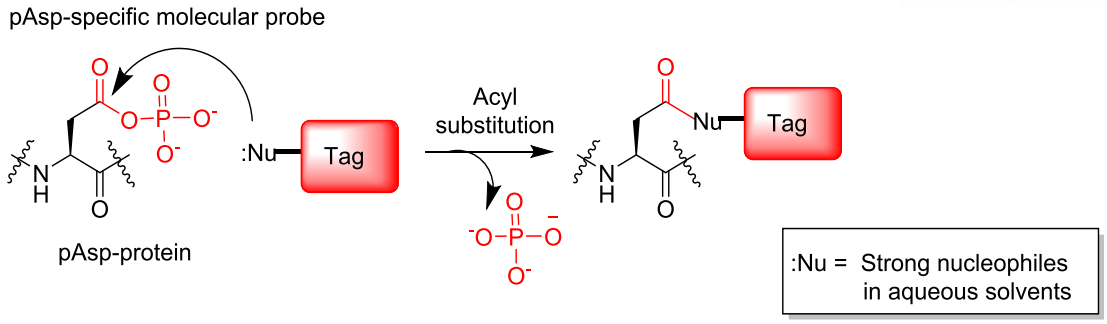


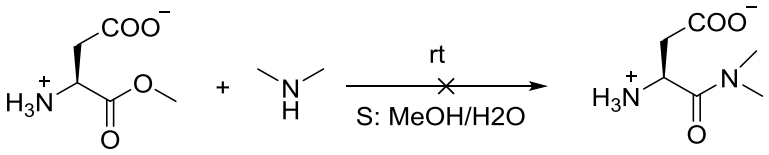
Fig. 4. Covalent nucleophilic labeling of pAsp-protein.

Although both carbonyl carbon and phosphorus atom in pAsp are electrophilic, when treating it with a nucleophile, substitution will mostly happen on the carbonyl carbon. Therefore, the nucleophile attached with a tag is incorporated into pAsp-proteins via acyl substitution reaction. The nucleophile needs to be efficient in aqueous solvents, and this requirement excludes a lot of organic and organometallic nucleophiles known to react with carbonyl groups, due to their low solubility in aqueous media or low stability in aqueous solvents. Once we identify such strong nucleophiles, they will be attached with small tags such as fluorophores and biotin for facile detection and affinity enrichment of pAsp-proteins.

2.1 Synthesis of pAsp model systems

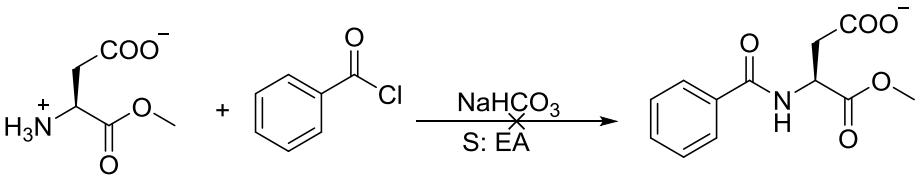
In order to estimate the labeling strategy, we planned to carry out on small pAsp derivatives firstly, which as model systems for pAsp-proteins. To mimic pAsp in proteins, we elected to block both α-amino and α-carboxyl groups of aspartate, then phosphorylate the side chain carboxyl group, to form pAsp derivative.

2.1.1 1st generation of the pAsp derivative



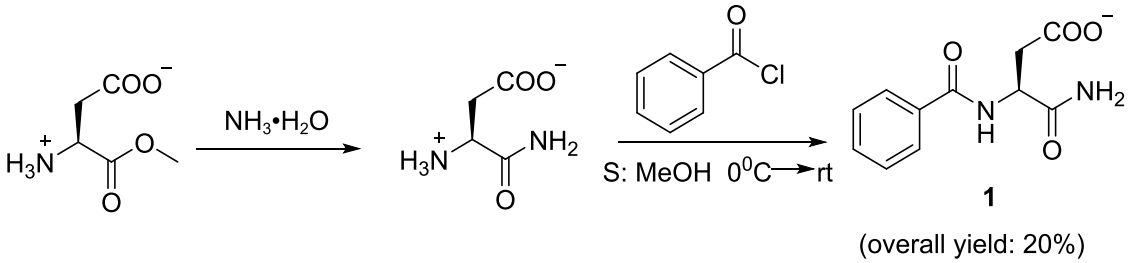
Scheme 10. Dimethylaminolysis of L-aspartic acid 1-methyl ester.

To synthesize a pAsp derivative, we started from L-aspartic acid 1-methyl ester, and selectively converted the C-terminus ester to amide with dimethylamine. The low reactivity of secondary amine contributed to the slow reaction here (Scheme 10). Although elevating the reaction temperature might be feasible to improve the reaction rate, the dimerization of the starting material would also be enhanced.



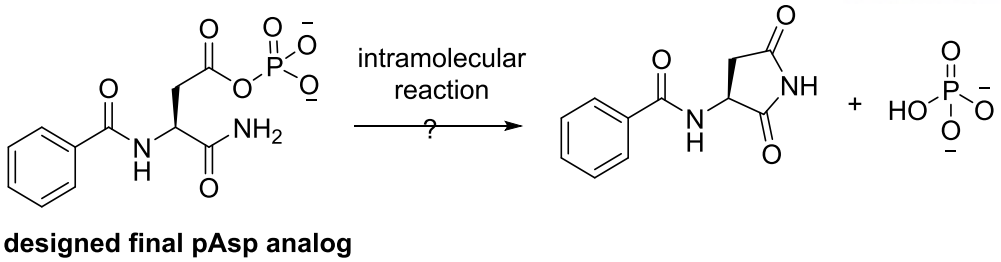
Scheme 11. Benzoylation of N-terminus of L-aspartic acid 1-methyl ester.

In order to eliminate the dimerization of starting material, we tried to protect the α-amino group first with benzoyl group (Scheme 11). However, due to the instability of the starting material under the reaction condition, we failed to get the desired product.



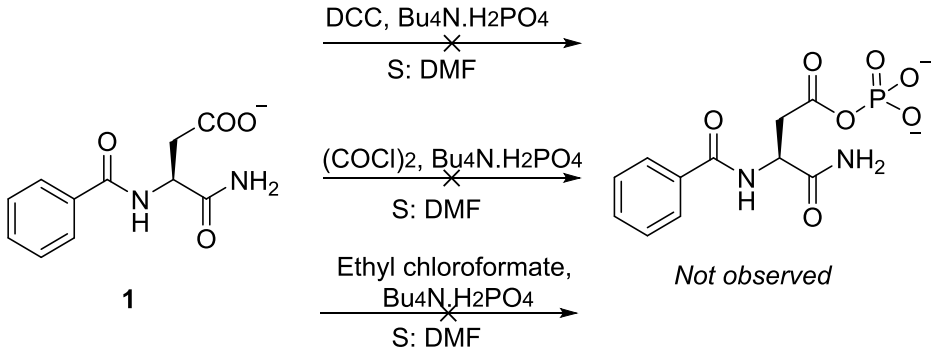
Scheme 12. Amidation followed by benzoylation.

This time, we reacted the methyl ester with ammonia first, and fast amidation was achieved. Then utilizing benzoyl chloride to block the α-amino group, we successfully obtained the desired product **1** (Scheme 12). However, once compound **1** was phosphorylated to give the designed final pAsp analog, the amide contained some potential risks, since it could undergo intramolecular cyclization reaction with phosphoanhydride (Scheme 13). Although the amino group of amide might not be so nucleophilic, the intramolecular reaction could not be ignored, especially when there existed some metal ions. So we needed to seek for other functional group to block the α-carboxyl group instead of ammonia.



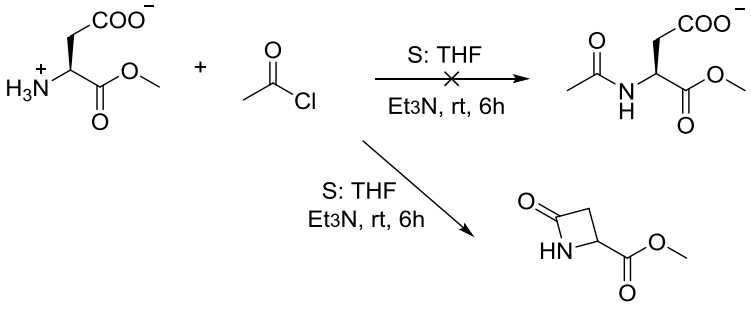
Scheme 13. Potential intramolecular cyclization.

Nevertheless, we tried to phosphorylate compound **1** with tetra-n-butylammonium phosphate, under the existence of some coupling reagents, such as DCC, ethyl chloroformate and oxalyl chloride, shown as follows (Scheme 14). However, the designed final pAsp analog was not obtained.



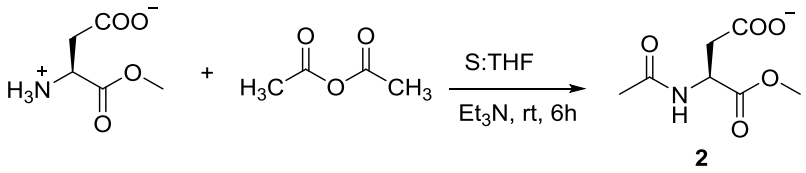
Scheme 14. Attempted phosphorylation conditions.

2.1.2 2nd generation of the pAsp derivative



Scheme 15. Unexpected lactam formation.

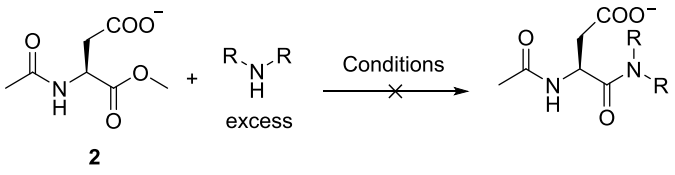
Based on the failure in Scheme 11, we chose more reactive acetyl chloride to avoid the dimer formation. However, unexpectedly, we only got the lactam product (Scheme 15). The mechanism of the reaction was proposed that the side chain carboxylate rather than the α-amino group, reacted with acetyl chloride to form an acid anhydride intermediate, ultimately an unstable lactam was formed through amino group intramolecularly attacking the anhydride. We suspected that the high reactivity of acetyl chloride promoted the formation of cyclic product.



Scheme 16. Successful acetylation without lactam formation.

To circumvent this problem, we used less reactive acetic anhydride to acetylate α-amino group, and obtained desired compound **2** (Scheme 16). With the N-terminus protected, we next tried to amidate the α-carboxyl group, making no side reaction give rise from the C-terminus. Therefore, we chose to amidate the carboxyl group with primary or secondary amines. We first carried out amidation with different secondary amines in different conditions (Table 2). With the N-terminus protected, we operated the reactions at elevated reaction temperatures, with different solvents, as well as different reaction time, to enhance the reactivity of secondary amine towards ester. However, we still failed to obtain the desired product.

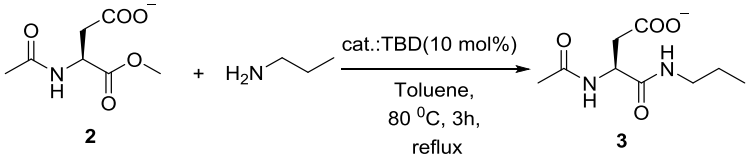
Table 2. Conditions for amidations.



Entry	Amino reagent	Solvent	Condition
1	Dimethylamine	THF	40 ^{0}C
2	Dimethylamine	MeOH	50 ^{0}C, 24h
3	Piperidine	MeOH	96 ^{0}C, 48h, reflux

The low reactivity of the secondary amines contributed to the failure of amidation on α-carboxylic group. So we turned to the primary amines. Some cyclic guanidine organic catalyst was reported to accelerate the formation of amides from esters and primary amines.[34] Therefore, we carried out the

aminolysis using propylamine and TBD. TBD did catalyze the formation of amide **3**. But the TBD was hard to remove from the product. Moreover, there were also some difficulties in detection of product **3**.

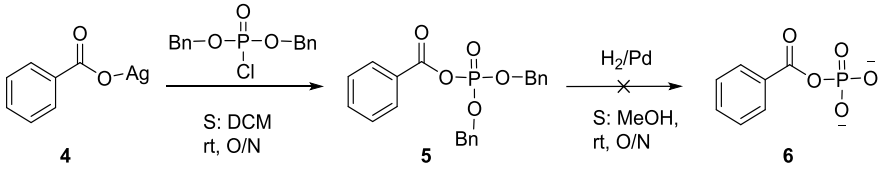


TBD: 1, 5, 7-triazabicyclo[4.4.0]dec-5-ene

Scheme 17. Aminolysis with propylamine.

2.1.3 Phosphorylating carboxylic acid

At the same time, we were also looking for the feasible method of phosphorylating the side chain carboxylic acid. Referring to the literature[35], we tried to phosphorylate benzoic acid as a simple model system. As shown below (Scheme 18), the silver salt of benzoic acid **4** was synthesized at first. Then product **4** was supposed to give product **5** when treated with dibenzyl phosphorylchloride (prepared via the literature method[36]), associated with silver chloride precipitate formation. The desired benzoyl phosphate **6** was expected to form upon the deprotection of benzyl groups.

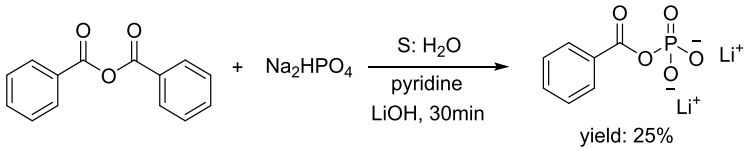


Scheme 18. Attempted preparation of benzoyl phosphate.

The phosphoanhydride **5** with two benzyl groups was more labile than phosphoanhydride, so column chromatography did not work, neither identification by MALDI-TOF. However, the final product benzoyl phosphate **6** could be prepared using alternative synthetic methods. Therefore, without purification, we went on to the deprotection of crude product **5**, but we still failed to observe the desired benzoyl phosphate **6**. Since this phosphorylation method[35] carried out on benzoic acid was not successful in our hands, we concluded that this method was not feasible for phosphorylating Asp derivative.

As a result of the difficulties in preparing acyl phosphoanhydride, we turned to synthesize aryl phosphoanhydride as pAsp model. Herein, we synthesized benzoyl phosphate following the literature[37] (Scheme19). Benzoyl phosphate is advantageous since it can be monitored by UV detector in the HPLC,

in addition to its easy synthesis. Its hydrolysis product can also be distinguished by different UV-absorption spectrum. Additionally, benzoyl phosphate was also identified as a good substrate of acyl phosphatase.[37]



Scheme 19. Preparation of benzoyl phosphate.

III. Covalent labeling of acyl phosphates

Highly reactive nucleophiles are indispensable for the efficient and specific labeling of pAsp-proteins. Then we set out to access various nucleophiles on benzoyl phosphate in aqueous solvents. For the reaction conditions, the following factors were taken into account. Firstly, due to the instability of benzyl phosphate, the pH effect cannot be ignored. In order to explore the pH effect to benzoyl phosphate substitution, we firstly incubated benzoyl phosphate with or without nucleophiles in pH 6.5, 7.0, 7.5 buffers. The reactions carried out in pH 7.0 buffer turned out to be the slowest, no matter whether the nucleophiles existed or not. And more hydrolysis product benzoic acid was observed in both pH 6.5 and 7.5 buffers. For minimizing the pH effect, we chose pH 7.0 as the reaction condition. Secondly, we avoided using phosphate-containing buffers, since the high concentration of phosphate exerted an influence on dephosphorylation of phosphospecies.[25] At last, we chose 0.1 M Tris buffer, pH 7.0 as our reaction buffer.

3.1 1st generation of nucleophiles

α-Nucleophiles are known for their high nucleophilicity, which can be attributed to the "α-effect" caused by the delocalization of the unshared electrons of the proximal heteroatom to the nucleophilic center[38]. Due to difficulties in preparing α-nucleophiles, we firstly paid attention to four kinds of simple α-nucleophile derivatives, namely, hydrazide, hydroxamic acid, hydrazone, aldoxime (Table 3, entries 2, 3, 4, 5, 6). Moreover, some H-bond donors/acceptors were also placed proximal to the reactive sites in nucleophiles (entries 3, 4, 5, 6), they were expected to exert their acid catalyst role in acyl substitution on benzoyl phosphate (Fig. 5).

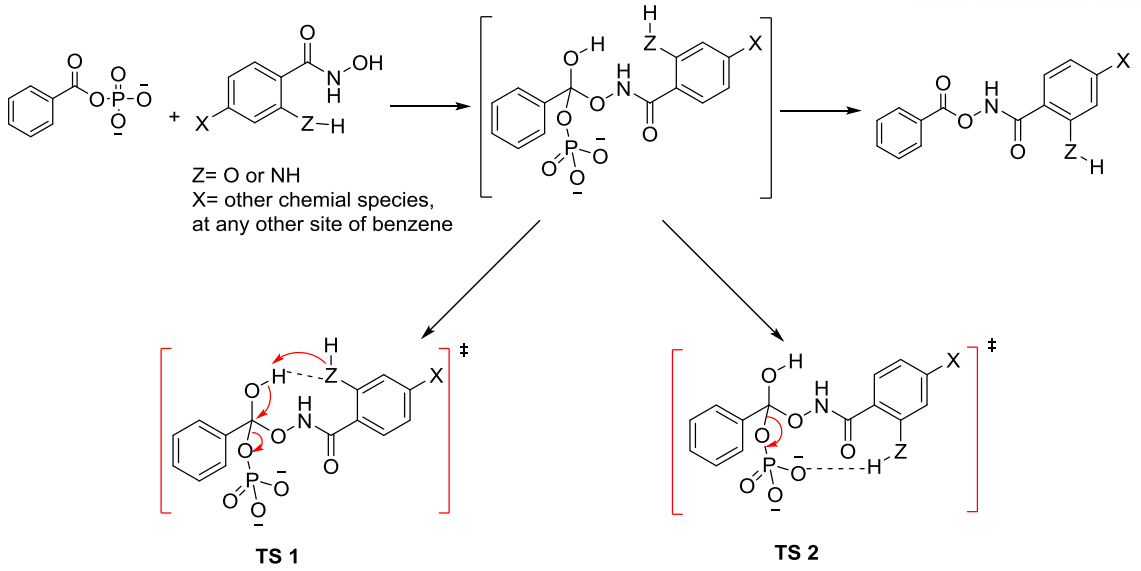
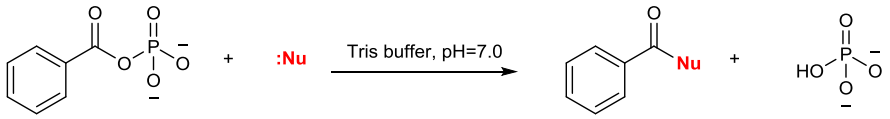
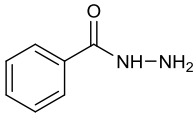
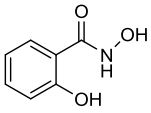
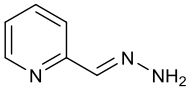
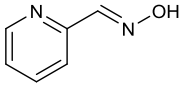
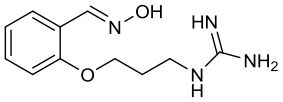
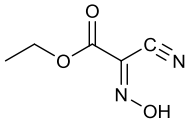
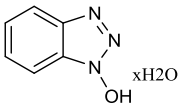
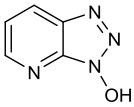
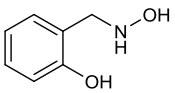
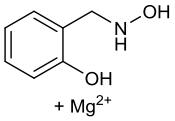
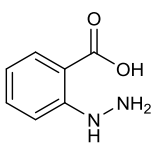


Fig. 5. Hypothetical mechanism of acyl substitution on benzoyl phosphate, taking hydroxamic acid nucleophile as example. Top: Acyl substitution on benzoyl phosphate attacked by nucleophiles at neutral pH. Bottom: Hypothetical transition states during the acyl substitution. TS: transition state.

Two scenarios were postulated in the hypothetical acyl substitution mechanism (Fig. 5). In **TS 1**, H-bonding, was proposed to facilitate the deprotonation of hydroxyl group, thus accelerate the phosphate cleavage. Similarly, in **TS 2**, the H-bonding with phosphoryl oxygen, was speculated to accelerate the acyl substitution, supported by the fact that H-bonding could neutralize the negative charges of phosphoryl group, thereby reducing its electrostatic repulsion toward nucleophiles, and facilitate the phosphate being a good leaving group.

Table 3. Testing various nucleophiles on benzoyl phosphate at neutral pH.[a]



Entry	Nuleophiles(24mM)	Structure	pKa	Reaction rate[b,c]
1			1.3	-37.4
2	Benzhydrazide		3.03	-48.7
3	Salicylhydroxamic acid		8.27	-44.4
4	Pyridine-2-carbaldehyde hydrazone			-20.7
5	Pyridine-2-aldoxime		7.8	-36.9
6	Guanidylated oxime			-18.3
7	Oxyma pure		<3	-21.6
8	HOBt		3.40[d]	-21.1
9	HOAt		Acidic	-18.8
10	Salicylhydroxylamine		10.02	-71.0
11	Salicylhydroxylamine + Mg2+ 24mM			-93.7
12	2-Hydrazinobenzoic acid			-55.3

[a]conditions: benzoyl phosphate 5 mM, nucleophiles 24 mM(catalyst 24 mM) in 0.1 M Tris buffer, pH=7.0. [b]all the reaction rates were based on duplicate measurements on benzoyl phosphate, and took the average values as the final reaction rates.[c]unit: mAU*s/h. [d]the pKa was obtained from ref 39.

Comparing with the background dephosphorylation rate of benzoyl phosphate, the hydrazide and hydroxamic acid exhibited some nucleophilic effect toward benzoyl phosphate (Table 3, entries 2, 3). Hydrazone and aldoximes, however, showed lower reactivity (entries 4, 5, 6). Their relative instability in aqueous solvent was suspected to influence their performance on benzoyl phosphate. And here, positioning H-bond donor/acceptor species seemed not so effective (entries 3, 4, 5). We also tested guanidine-assisted oxime nucleophile on benzoyl phosphate (entry 6), where the guanidine was designed to act as acid-catalyst with a linker. Unfortunately, slower reaction was observed for this nucleophile.

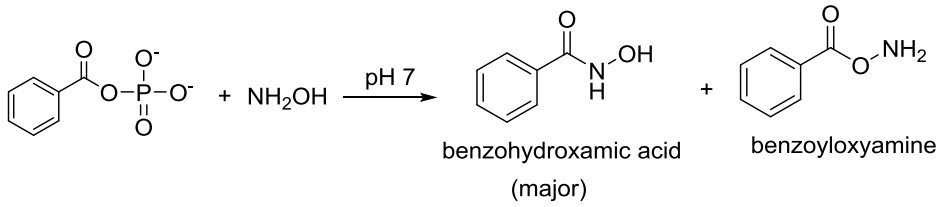
Therefore, we turned to some coupling reagents, i.e. oxyma pure, HOBt, HOAt, whose outstanding nucleophilicity was utilized for the synthesis of acyl derivatives in aqueous media.[40,41] Hence, we applied these coupling reagents as nucleophiles to benzoyl phosphate (Table 3, entries 7, 8, 9). However, all of these coupling reagents showed inferior reactivity towards benzoyl phosphate.

Therefore, we paid attention to α-nucleophiles, namely hydroxylamine- or hydrazine- derivatives again, since they are more nucleophilic than hydrazides and hydroxamic acids, and more stable products are expected to form. The high reactivity of α-nucleophiles could be also explained as the instability, which poses some obstacles to their synthesis. In our case, the salicylhydroxylamine was obtained through reducing salicylaldoxime with sodium cyanoborohydride.[42] And salicylhydroxylamine, in keeping with our hypothesis, performed better than previous nucleophiles (Table 3, entry 10). In addition, magnesium ions enhanced the reaction rate further (entry 11). But 2-hydrazinobenzoic acid, did not perform as well as salicylhydroxylamine (entry 12). We speculated that the conjugation between benzene ring and its proximal nitrogen's lone pair decreased the α-effect in 2-hydrazinobenzoic acid. Encouraged by the good performance of salicylhydroxylamine, we decided to explore other α-nucleophiles and catalysts, to achieve efficient and specific covalent conjugation with benzoyl phosphate.

3.2 2nd generation of nucleophiles

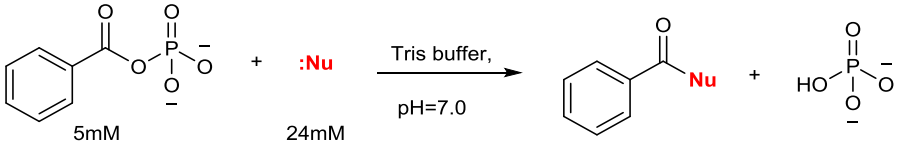
Hydrazine and hydroxylamine are the simplest α-nucleophiles, without the steric hindrance. We firstly tested them on benzoyl phosphate, respectively (Table 4, entries 1, 3). Both of them quickly reacted with benzoyl phosphate in aqueous solvent, and the reaction rates were dramatically enhanced by Mg^{2+} ions (entries 2, 4). For hydrazine, as one amine was protonated in pH 7 Tris buffer, its nucleophilic ability was much lower than hydroxylamine. Only one nucleophilic center in hydrazine at pH=7, while for hydroxylamine, both amino group and hydroxyl group functioned as the nucleophilic

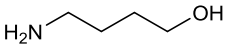
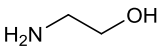
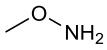
centers. Hence, two products were supposed to produce (Scheme 20), where the benzohydroxamic acid was major.



Scheme 20. The products of hydroxylamine reacting with benzoyl phosphate.

Table 4. Assessing a-nucleophiles and catalysts' performance towards benzoyl phosphate.[a]



Entry	Nuleophiles(24mM)	Structure	pKa	Reaction rate[b,c]
1	Hydrazine	H_2NNH_2	8.1[d]	-55.3
2	Hydrazine + Mg^{2+} 24mM	H_2NNH_2 + Mg^{2+}		-140.7
3	Hydroxylamine	NH_2OH	6[e]	-123.4
4	Hydroxylamine + Mg^{2+} 24mM	NH_2OH + Mg^{2+}		-274.0
5	4-Amino-1-butanol		10.3	-40.4
6	Monoethanolamine		9.50	-36.9
7	Methoxyamine		4.6[e]	-38.6
8	N-Methylhydroxylamine		6[e]	-91.3

[a]conditions: benzoyl phosphate 5 mM, nucleophiles 24 mM (catalysts 24 mM) in 0.1 M Tris buffer, pH=7.0. [b]all reaction rates were based on dulpicate measurements on benzoyl phosphate, and took the average values as the final dephosphorylation rates. [c]unit: mAU*s/h. [d]the pKa was obtained from ref 43. [e]the pKa was obtained from ref 44.

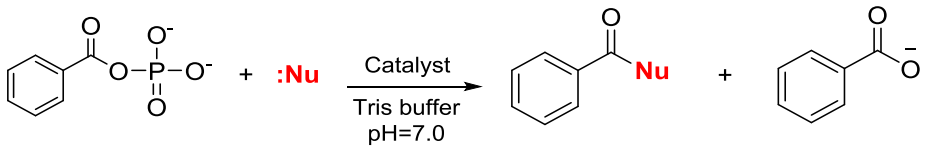
We also tested two analogs of hydroxylamine, 4-amino-1-butanol and monoethanolamine, on benzoyl phosphate (Table 4, entries 5, 6). However, slower reactions were observed in both cases compared with hydroxylamine, which confirmed the α-effect in good nucleophiles. In order to investigate which nucleophilic center in hydroxylamine was more effective in pH 7 buffer, we blocked each nucleophilic center with methyl group, by testing methoxyamine and N-methylhydroxylamine (entries 7, 8). Comparing with hydroxylamine, both of them were slower. The lower reactivity of methoxyamine could confirm two nucleophilic centers existed in hydroxylamine. Actually, for N-methylhydroxylamine, there has been some literature reported that the methyl group could enhance the amino group's nucleophilic ability in organic solvent.[46] But here, the reactivity of N-methylhydroxylamine was inferior than hydroxylamine.

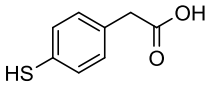
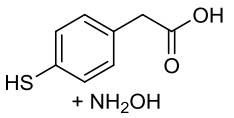
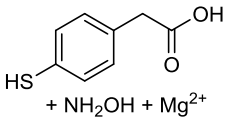
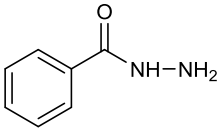
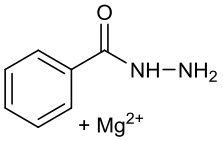
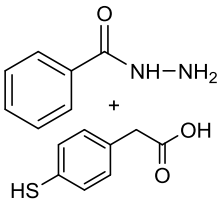
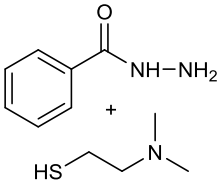
3.3 Catalytic effect on covalent labeling

In order to estimate catalyst performances on the covalent bond formation between nucleophiles and benzoyl phosphate, we tested nucleophiles benzhydrazide and hydroxylamine. The fastest reaction was obtained by the combination of hydroxylamine with Mg^{2+}. Then in order to improve the rates further, we tried some organic catalysts as well. We first tested sodium azide catalyst, due to the good nucleophilicity and small size of azide. In fact, acyl azides are efficient acylating agents in aqueous media.[47,48] However, the azide was found to decrease the hydrolysis of benzoyl phosphate (Table 5, entry 1 vs. Table 3, entry 1), and no catalytic effect on hydroxylamine addition was observed (Table 5, entry 2 vs. Table 4, entry 3).

Similarly, we also tested MPAA, the standard nucleophilic catalyst for native chemical ligation (NCL).[49] MPAA only (Table 5, entry 3) showed low reaction rate and no hydrolysis detected. However, it could catalyze the reaction of hydroxylamine and benzoyl phosphate (Table 5, entry 4 vs. Table 4, entry 3).

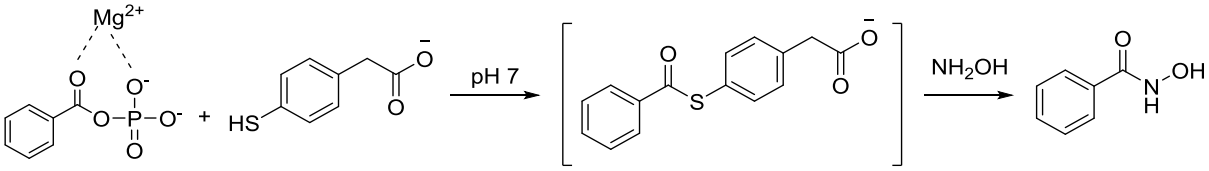
Table 5. Catalyst performances in Tris buffer.[a]



Entry	Nuleophiles(24mM)	Structure	pKa	Reaction rate[b]	Benzoic acid rate[c,e]
1	Sodium azide	NaN_3	4.0[f]	-14.3	6.2
2	Hydroxylamine + Sodium azide 24mM	$NH_2OH + NaN_3$		-78.8	6.6
3	MPAA		6.6[d]	-27.3	ND[g]
4	Hydroxylamine + MPAA 24mM			-196.8	55
5	Hydroxylamine+ MPAA 24mM + Mg^{2+} 24mM			-245.3	ND[g]
6	Benzhydrazide		3.03	-48.7	17.5
7	Benzhydrazide + Mg^{2+} 24mM			-87.5	31.7
8	Benzhydrazide + MPAA 24mM		3.03 6.6[d]	-58.2	22.9
9	Benzhydrazide + 2-dimethylamino-ethanethiol 24mM		3.03	-49.8	21.6

[a]conditions: benzoyl phosphate 5 mM, nucleophiles 24 mM (catalysts 24 mM) in 0.1 M Tris buffer pH=7.0. [b]all reaction rates were based on dulpicate measurements on benzoyl phosphate, and took the average values as the final dephosphorylation rates. [c]hydrolysis product benzoic acid. [d]the pKa was obtained from ref 45.[e]unit: mAU*s/h. [f]the pKa was obtained from ref 43.[g]No detection.

The catalytic ability of MPAA was inferior to Mg^{2+} on the reaction of hydroxylamine and benzoyl phosphate (Table 4, entry 4 vs. Table 3. entry 4), but we envisioned the collaboration of these two catalysts could enhance the reaction rate much further, following the mechanism below: the Mg^{2+} ions can stabilize the benzoyl phosphate, making the nucleophilic attack by MPAA easier, to produce the more reactive thioester intermediate, which reacts with hydroxylamine to yield the final product (Scheme 21). However, the reaction catalyzed with both catalysts was slower than that of only Mg^{2+} catalyst (Table 5, entry 5 vs. Table 4, entry 4), not in line with our expectation.



Scheme 21. The putative mechanism of MPAA and Mg^{2+} catalyzing benzohydroxamic acid formation.

In order to test whether the catalysts' catalytic effect depends on the substrates, we also tested on benzhydrazide nucleophile. And we also tried dimethylaminoethanethiol catalyst, which exhibited comparable catalytic ability to MPAA on thioesters (Hoyoung Jung, unpublished results). In pH 7 Tris buffer, benzhydrazide showed some reactivity towards benzoyl phosphate (Table 5, entry 6. vs. Table 3, entry 1), and the reaction was catalyzed by different catalysts. For example, Mg^{2+}, MPAA and 2-diemethylaminoethanethiol, different catalytic abilities were observed.

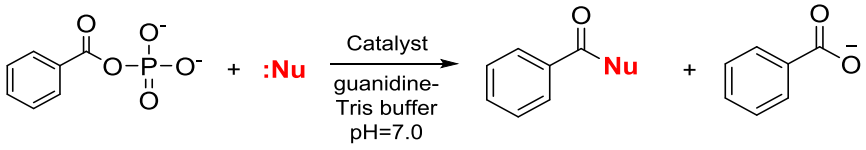
For Mg^{2+}, it catalyzed the acyl substitution, as well as the hydrolysis of benzoyl phosphate (Table 5, entries 6, 7). However, comparing with the hydroxylamine reaction, Mg^{2+} showed lower catalytic effect on benzhydrazide nucleophile (Table 5, entries 6, 7 vs. Table 4, entries 3, 4). For MPAA catalyst, it only showed slight catalytic effect on the substitution with benzhydrazide, as well as the hydrolysis (Table 5, entries 6, 8), whereas it catalyzed the hydroxylamine reaction more effectively (Table 5, entries 6, 8 vs. Table 4, entry 3 and Table 5, entry 4). At last, we tested 2-dimethylaminoethanethiol as catalyst on benzhydrazide and benzoyl phosphate reaction, however, no catalytic effect was observed (Table 5, entries 6, 9).

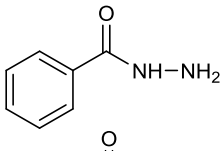
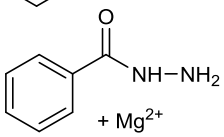
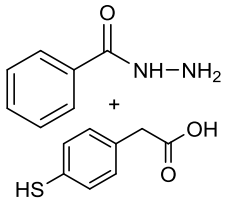
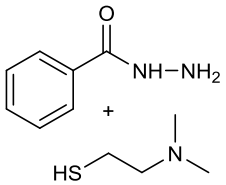
In conclusion, both MPAA and Mg^{2+} catalysts showed preference for the hydroxylamine nucleophile in acyl substitution of benzoyl phosphate. And Mg^{2+} catalyzed better than MPAA on different nucleophile reactions.

3.4 Solvent effect on the covalent labeling

The small α-nucleophile hydroxylamine exhibited excellent nucleophilic ability on benzoyl phosphate in aqueous media. For the application of hydroxylamine labeling of pAsp proteins in vitro, the buffer should contain some denaturing reagents, such as urea or guanidine, to make the pAsp residue exposed to nucleophiles. Here, we chose 0.1 M Tris buffer containing 6 M guanidine as the buffer for our tests in vitro. To figure out the guanidine effects, same reactions were run in the presence or absence of guanidine.

Table 6. Assessing α-nucleophiles and catalysts towards benzoyl phosphate in guanidine-containing buffer.[a]



Entry	Nuleophiles(24mM)	Structure	pKa	Reaction rate[b,e]	Benzoic acid rate[b,c,e]
1				-46.5	17.4
2	Hydrazine	H_2NNH_2	8.1[f]	-56.3	18.7
3	Benzhydrazide		3.03	-50.1	19.0
4	Benzhydrazide + Mg^{2+} 24mM		3.03	-50.3	23.3
5	Benzhydrazide + MPAA 24mM		3.03 6.6[d]	-54.8	20.4
6	Benzhydrazide + 2-dimethylamino-ethanethiol 24mM		3.03	-58.4	21.2

[a]conditions: benzoyl phosphate 5 mM, nucleophiles 24 mM (catalysts 24 mM) in 0.1 M Tris buffer containing 6 M guanidine, pH=7.0. [b]all the reaction rates were based on dulpicate measurements on benzoyl phosphate, and took the average values as the final reaction rates. [c]hydrolysis product benzoic acid. [d]the pKa was obtained from ref 45.[e]unit: mAU*s/h.[f] the pKa was obtained from ref 43.

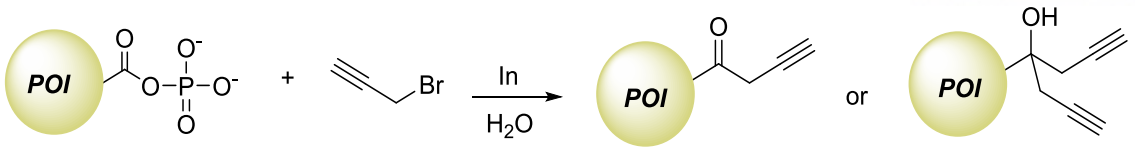
Guanidine accelerated the hydrolysis of benzoyl phosphate at pH 7 (Table 6, entry 1 vs. Table 3, entry 1). And guanidine is reported to denature proteins, owing to its polarization effect on aqueous solvent, where the dipolar moment generated by protonated guanidine cations and chloride anions.[50] We suspected that the polarization effect introduced by guanidine was related to the higher hydrolysis rate of benzoyl phosphate.

For hydrazine and benzhydrazide nucleophiles, the guanidine seemed no effect on them (Table 6, entries 2, 3 vs. Table 4, entry 1; Table 5, entry 6). However, unexpectedly, the Mg^{2+} ions seemed no catalytic effect in the guanidine-containing buffer (Table 6, entries 3, 4). The reason may also be attributed to the guanidine resulting in less electrostatic interaction between Mg^{2+} and benzoyl phosphate oxygens, thereby inhibiting the Mg^{2+} catalysis. And for MPAA and 2-dimethylaminoethanolthiol catalysts, they showed little catalytic effect in guanidine-containing buffer (Table 6, entries 5, 6, 3). However, due to their originally poor catalytic effect on the reactions between benzoyl phosphate and benzhydrazide (Table 5, entries 8, 9), it was hard to estimate guanidine effect on these reactions.

By now, we found the combination of hydroxylamine and Mg^{2+} were the fastest to react with benzoyl phosphate in pH 7 Tris buffer. However, attaching a fluorophore to hydroxylamine could decrease its nucleophilic ability to pAsp, based on methylamine and N-methylhydroxylamine results (Table 4, entries 7, 8). So in addition to the initial idea of labeling the pAsp-protein with a tag, we also decided to explore immobilized metal affinity chromatography (IMAC) to purify pAsp-protein by first converting pAsp into a hydroxamic acid (Section IV).

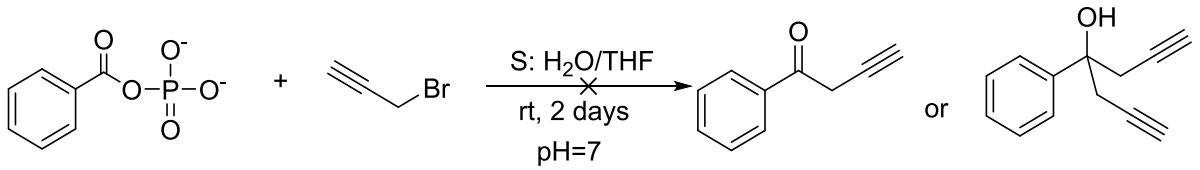
3.5 Indium-mediated labeling of pAsp

Indium metal is famous for its ability of mediating organometallic reactions in aqueous media. And indium-mediated allylation and propargylation of aldehydes or ketones have been well studied.[51,52,53] In these reactions, the organometallic nucleophiles formed between indium and alkyl bromide attack the electrophilic aldehydes or ketones. Accordingly, we hypothesized the pAsp's acyl phosphate group could be the target of indium complexes, to proceed indium-mediated propargylation as below, the propargylation can occur twice based on the formed ketone product (Scheme 22). The alkyne-containing products can be utilized for the "click reaction" with azides for labeling.



Scheme 22. Labeling pAsp-proteins using indium.

Cu (I)-catalyzed alkyne-azide cycloaddition, also called 'click reaction', a popular method in labeling protein of interest (POI) and drug discovery, due to its bioorthogonality and fast reaction rate[54]. However, the cytotoxicity of copper(I) hampers it application to living systems.[55] Because our design was to operate the reaction in vitro to label pAsp-containing proteins, thus the issue of cytotoxicity could be ignored in our case. As a model study, we attempted the indium-mediated reaction between benzoyl phosphate and propargyl bromide, to insert alkynyl group(s), as shown below (Scheme 23). However, we failed to get the desired product(s) despite long incubation.



Scheme 23. Attempted indium-mediated propargylation.

The indium catalyzed propargylation was slow in aqueous media. Since the long-time reaction could result in significant hydrolysis of pAsp, this method appeared less useful for labeling pAsp-proteins. Moreover, formed indium complex species is dependent on the reaction solvents.[51,52] The indium complexes can be propargylindium and allenylindium in water and THF mixture, when reacting with propargyl bromide.[52] And the chemical reactivity difference between phosphoanhydrides and aldehydes or ketones may account for the failure of the propargylation on benzoyl phosphate with indium.

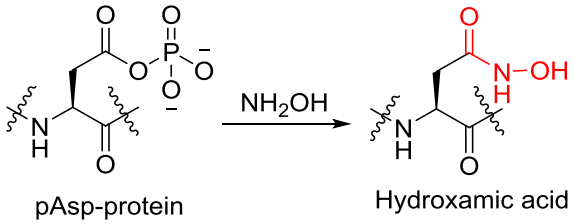
IV. IMAC for hydroxamic acid

4.1 Introduction of IMAC tactic and hydroxamic acids

Affinity purification or enrichment of proteins or peptides based on specific noncovalent interactions, such as biotin-(strept)avidin[56,57], His_6 tag-Ni(II)[58], and Zn(II)-DpaTyrs complex-D4 tag[59], has been

widely used in biochemical studies.[58,59,60] Among those methods, immobilized metal affinity chromatography (IMAC) makes the proteins or peptides enrichment easy.

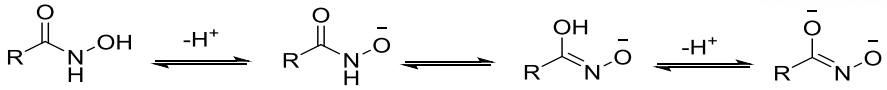
Based on our previous experiment, hydroxylamine can undergo fast acyl substitution with benzoyl phosphate in aqueous media, and form stable hydroxamic acid product, which is an excellent substrate for IMAC. If we apply this method to pAsp-proteins, treatment of pAsp-proteins with hydroxylamines will produce hydroxamic acid-containing proteins (Scheme 24), which can be enriched by IMAC**.** This labeling and enrichment method will be very helpful to discovery of novel pAsp-proteins, when combined with mass-spec based protein identification. In fact, Fritz et al. obtained the hydroxamic acid formed between acyl phosphate and hydroxylamine in pigeon liver, and they successfully identified and quantified the hydroxamic acid product through colorimetric assay.[61]



Scheme 24. Hydroxamic acid obtained from pAsp-protein.

Hydroxamic acids are known for their excellent chelating properties to some transition metal ions, especially Fe(III) ions.[62] This ability makes hydroxamic acids, such as desferrioxamine B and suberoylanilide hydroxamic acid (SAHA), trichostatin A (TSA),[63] competent to be environmental modulators involving metal ion dyshomeostasis and inhibitors of metalloenzymes. Moreover, hydroxamic acids have extensive application in extraction and separation metal ions,[64,65,66,67] as well as in colorimetric assay, due to their ability of forming colored complexes with ferric ions.[68,25] Interestingly, the hydroxamic acid is a major constituent of siderophores, low molecular-weight Fe(III)-sequestering compounds secreted by microorganisms[69].

Hydroxamic acids possess several sites for potential H-bonds, which can account for their strong chelating ability with metal cations. Hydroxamic acids form O,O- bidentate coordination with such ions as Ni(II), Fe(III), and Co(II),[70,71] where the ligands can be singly deprotonated or doubly deprotonated (Scheme 25).[62] Thus, hydroxamic acids chelate transition metals through deprotonated hydroxamates and carbonyl oxygen atoms, forming stable five-membered rings (Fig.6).[70]



Scheme 25. Singly or doubly deprotonated hydroxamates as ligands.

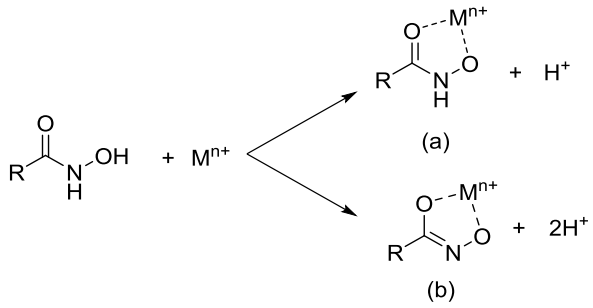


Fig. 6. Coordination complexes between transition metal (M^{n+}) and hydroxamic acid.

Importantly, the reactions between hydroxylamine and pAsp-proteins are considered specific in lysate. Amides and esters can undergo similar reactions with hydroxylamine only under concentrated solution, and elevated temperature conditions. And other phosphoamino acids will not form covalent adducts with hydroxylamine.[25]

4.2 Results and discussions

4.2.1 Utilization of Ni(II)-NTA resin

Now we aimed to enrich the hydroxamic acid-containing proteins selectively from lysate through IMAC. Nitrilotriacetic acid (NTA) is a tetradentate chelator which occupies four of six coordination sites of Ni(II), and the remaining two coordination sites are occupied with H_2O molecule or hydroxyl ion.[72] Through replacing the H_2O or hydroxyl ion on Ni^{2+} ligation sites, hydroxamate ligands can bind to Ni(II)-NTA resin, forming a stable octahedral complex (Fig. 7). Hence, the Ni-NTA resin could selectively enrich benzohydroxamic acid.

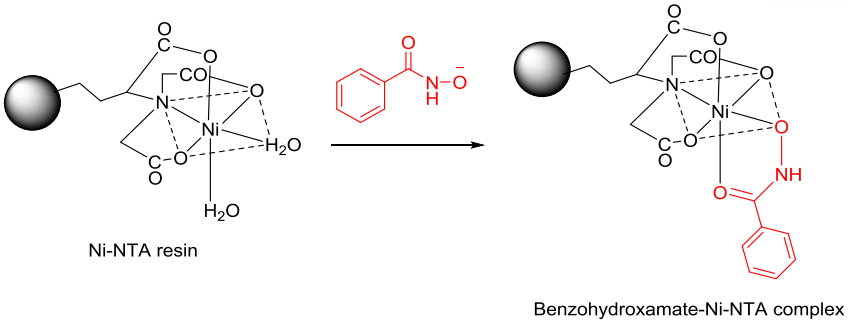


Fig. 7. Chelating benzohydroxamate with Ni(II)-NTA resin.

Hydroxamic acids are weak acids, and benzohydroxamic acid's –NHOH group pKa is approximately 8.6.[73] And effective coordination with metal ions only happens on the singly deprotonated or doubly deprotonated benzohydroxamic acids (Scheme 25), namely favored at high pH. However, there is also an equilibrium between water molecule and hydroxyl ion in the two remaining Ni(II) binding sites (Scheme 26). Herein, the water (aqua) ligands can be more easily displaced by the hydroxamate than hydroxyl ligands, where acidic condition can facilitate the water formation. Accordingly, the pH is crucial for hydroxamic acid binding to transition metals.

$$Ni(NTA)(OH_2)_2 \rightleftharpoons Ni(NTA)(OH_2)(OH)^- \rightleftharpoons Ni(NTA)(OH)_2^{2-}$$

Scheme 26. Equilibrium between water and hydroxyl ion in Ni(II) binding sites.

With this information in hand, we first performed relevant experiment with Ni-NTA resin. Assuming the existence of produced hydroxamic acid, other phosphorylated residues, as well as other carboxylates in vitro after treating cell lysates with hydroxylamine, we prepared a mixture of benzohydroxamic acid, benzoyl phosphate and benzoic acid (Fig. 8). Then we tested the ability of Ni-NTA resin to selectively affinity enrich benzohydroxamic acid from the mixture.

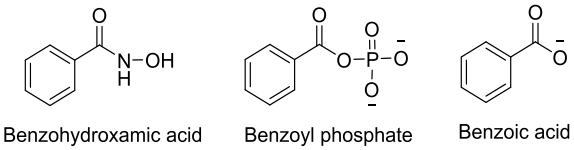


Fig. 8. Mixture constituents for Ni-NTA resin tests.

Condition 1[74]:

Substrates: benzohydroxamic acid, benzoyl phosphate and benzoic acid (100 μmol each)

Binding buffer: 20 mM NaH_2PO_4/ Na_2HPO_4 buffer + 500 mM NaCl, pH=8

Elution buffer: binding buffer + 0.5 M imidazole, pH=8

We conducted the IMAC experiment following a literature protocol[74], and analyzed each fraction with RP-HPLC. In this case, benzohydroxamic acid did not wash out until the 2nd wash with binding buffer, while benzoic acid and benzoyl phosphate were washed out at first. However, no benzohydroxamic acid was detected when washing with elution buffer after several wash with binding buffer. Benzohydroxamic acid exhibited slightly stronger affinity to Ni-NTA resin than benzoyl phosphate and benzoic acid in weakly basic phosphate buffer. However, it could not withstand frequent wash with binding buffer. Hence, we decided to look for other efficient binding buffers.

Condition 2[63]:

Substrates: benzohydroxamic acid, pTyr and benzoic acid (pTyr instead of benzoyl phosphate. 100 μmol each)

Binding buffer: 1 μM NaOH, pH=8

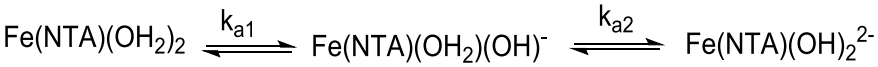
Elution buffer: 20 mM NaH_2PO_4/ Na_2HPO_4 buffer + 500 mM NaCl, pH=6.5

Unfortunately, all substrates flowed out when the resin was washed with the binding buffer, although a part of benzohydroxamic acid was retained better on the resin than benzoic acid and pTyr. In this case, the weakly basic binding buffer did not lead to strong binding between benzohydroxamic acid and Ni-NTA resin. Therefore, we sought for other transition metal binding.

4.2.2 Utilization of Fe(III)-NTA resin

The affinity of hydroxamic acid to Fe^{3+} ions was initially discovered, due to its particular relevance in the biological siderophores. Siderophores can chelate trace soluble Fe^{3+} in biological system with extremely high affinities ($K_{aff} \sim 10^{40}\ M^{-1}$).[74] Therefore, we set out to test the Fe(III) -NTA resin. Similar to the Ni(II)-NTA resin, Fe^{3+} ion's coordination sites are occupied by one tetradentate NTA, the remaining two coordination sites are occupied by water or hydroxyl ion in Fe(III) -NTA resin. The equilibrium of H_2O-Fe^{3+}-NTA complex is pH dependent (Scheme 27), and different reactivity of these complexes was reported. The $Fe(NTA)(OH_2)_2$ complex could accomplish faster substitution by ligand than $Fe(NTA)(OH_2)(OH)^-$.[75] Accordingly, lower pH can facilitate the formation of $Fe(NTA)(OH_2)_2$, thus resulting faster replacement of water by hydroxamate ligand. On the other hand, the low pH will prohibit

the deprotonation of hydroxamic acids, resulting low affinity to the Fe^{3+} ions. Therefore, balancing the pH effect is important.



Scheme 27. The equilibrium of H_2O-Fe^{3+}-NTA complex.

Taking all factors above into account, we performed the following experiments on Fe(III) -NTA resin. The Fe(III)-NTA resin was prepared following a literature protocol[76]. We tested a mixture of benzohydroxamic acid, benzoic acid and phosphotyrosine (pTyr), which replaced benzoyl phosphate to minimize the hydrolysis effect of substrates (Fig. 9).

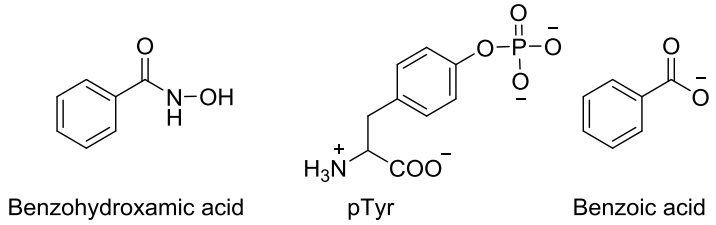


Fig. 9. Substrates for Fe(III) -NTA resin tests.

Condition 1[74]:

Each substrate loading amount: 0.50 μmol

Binding buffer: 20 mM NaH_2PO_4/ Na_2HPO_4 buffer + 500 mM NaCl, pH=8

Elution buffer: binding buffer + 0.5 M imidazole, pH=8

In this case, all substrates showed low affinity in this weakly basic phosphate binding buffer. And we also tried the binding experiment in water to exclude the phosphate effect on substrates' affinity to Fe(III)-NTA resin, no benzohydroxamic acid was retained on the resin. So we speculated the $Fe(NTA)(OH_2)_2$ formation might be the predominant factor for the effective coordination between the benzohydroxamic acid and Fe(III). Therefore, we tried more acidic binding buffer.

Condition 2: to facilitate $Fe(NTA)(OH_2)_2$ formation

Each substrate loading amount: 0.30 μmol

Binding buffer[77,76]: 0.1 M acetic acid, pH=3

Elution buffer: Ammonia in H_2O, pH=10.5 (replacing imidazole, because the imidazole has some interaction with Fe(III)[78,79])

Benzohydroxamic acid and benzoic acid were washed out with the acidic binding buffer, while pTyr was retained on the resin and then eluted with the elution buffer. Accordingly, pTyr, rather than benzohydroxamic acid was selectively enriched from the mixture by Fe(III) -NTA resin. Actually, the result was consistent with some studies which utilized Fe(III)-NTA resin to enrich phosphopeptides[80,81], and the putative complex was shown below (Fig. 10).

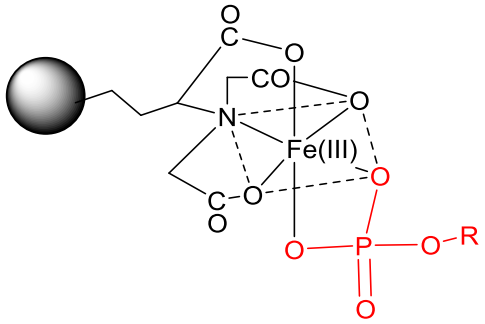


Fig. 10. The complex of phosphocompound and Fe(III)-NTA resin.

So we failed to extract the benzohydroxamic acid from the mixture with Fe(III)-NTA resin. But the pTyr exhibited strong affinity to Fe(III)-NTA resin in acidic or neutral conditions (results not shown). In contrast, the benzohydroxamic acid showed low affinity to Fe(III)-NTA resin in acetic, neutral and basic conditions. Therefore, we decided to look for other transition metals to capture benzohydroxamic acid.

4.2.3 Coordination with different transition metal ions

There have been some reports about the polyhydroxamic acid resin's capability to chelate different transition metals. In those reports, lead (II) ions showed the highest affinity to polyhydroxamic acid resin, better than Fe(III) and Ni(II) ions.[83] Therefore, we tried to extract benzohydroxamic acid with Pb(II)-NTA resin under acidic condition. Unfortunately, the benzohydroxamic acid did not bind to the resin. So here, we failed to enrich benzohydroxamic acid (a model for aspartyl hydroxamic acid generated form pAsp) with Ni(II)-NTA resin, Fe(III)-NTA resin and Pb(II)-NTA resin. And in these metal-NTA resins, only two open coordination sites exist for ligand binding. Therefore, switching resin can also be a feasible method to increase the open coordination sites for ligands.

V. Conclusions

To test the feasibility of covalently labeling pAsp-proteins, we have tested a variety of nucleophiles for the reaction with benzoyl phosphate, a model for pAsp, in aqueous buffers. Of those nucleophiles, hydroxylamine was the best nucleophile and their reaction rates were affected by pH and catalysts. Mg^{2+} accelerated the reaction to some degree, but their efficiency was reduced by guanidine.

To enrich the hydroxamic acids, the reaction product of the hydroxylamine and pAsp-proteins, we tried various IMAC strategies. However, we failed to enrich benzohydroxamic acid, a model compound, with Fe(III)-NTA resin or Ni(II)-NTA resin, as well as Pb(II)-NTA resin. In the future, it may be worth trying other metal-binding resins, such as IDA resin, which might have higher affinity for hydroxamic acids. And for the nucleophiles, although the hydroxylamine is the fastest so far, we still need stronger nucleophiles and better catalysts for practical applications to pAsp. Once the highly reactive nucleophile and catalyst are identified, fast and efficient covalent labeling of pAsp-proteins can be achieved.

VI. Experimental Section

6.1 General Information

Chemicals:

L-Aspartic acid 1-methyl ester (98%), dimethylamine, piperidine (99%), 1-propylamine (98%), silver nitrate (99.9+%), palladium (10% on activated carbon powder), dibenzyl phosphite (90+%), 2-hydrazinobenzoic acid hydrochloride. sodium cyanoborohydride (95%), diisobutylaluminium hydride, 4-amino-1-butanol (98%), N-methylhydroxylamine hydrochloride (98%), 4-mercaptophenylacetic acid (97%), indium powder, (99.9%), propargyl bromide (97%), iron (III) chloride hexahydrate (97%), lead (II) chloride (99%) were purchased from and Alfa Aesar. Benzoyl chloride (99+%), sodium Bicarbonate (99.0%), ammonia solution (28.0-30.0%), triethylamine (99.0%), acetyl chloride (98.0%), toluene (99.5%), hydrochloric acid soln (1N), sodium phosphate (98%), benzoic anhydride (98.0%), pyridine (99.5%), lithium hydroxide monohydrate (98.0%), sodium chloride, trifluoroacetic acid (99.0%), imidazole (99.0%) were purchased from SAMCHUN. Methanol, ethyl acetate, acetic anhydride, dichloromethane, sodium hydroxide(1N), ethyl ether, acetic acid glacial, ethanol, acetonitrile, hydroxylamine hydrochloride, monoethanolamine were purchased from DAEJUNG. Hydrazine monohydrate (80%) and sodium azide were purchased from JUNSEI. 1-Hydroxybenzotriazole hydrate (HOBt), 1-hydroxy-7-azabenzotriazole (HOAt), 1,5,7-triazabicyclo[4,4,0]dec-5-ene (TBD), methoxyamine hydrochloride (98%), benzoic acid (99%), HOAt were purchased from Aldrich.

Pyridine-2-aldoxime methochloride, benzhydrazide (98%), salicylhydroxamic acid, pyridine-2-aldoxime methochloride, O-phospho-L-tyrosine were purchased from Sigma Aldrich. Chemical tetrahydrofuran was purchased from Avantor Performance Materials, Inc. And Magnesium chloride hexahydrate was purchased from BIOSESANG. Tris Hydrochloride was purchased from Fisher. HisPur Ni-NTA resin was purchased from Thermo Scientific.

Instruments:

pHs of buffers were measured with a pH meter (Thermo Scientific, Orion Star A211). RP-HPLC analyses were carried out on Agilent 1200 Infinity instrument with an Agilent C18 column (5 μm, 4.6 x 150 mm) using a gradient of solvent A (0.1% trifluoroacetic acid in water) and solvent B: (90% acetonitrile in H_2O, 0.1% trifluoroacetic acid). NMR spectra were recorded on an Agilent 400 MR DD2.

6.2 Kinetic experiments

Procedures for the reaction between benzoyl phosphate and nucleophiles:

Stock solutions of benzoyl phosphate and nucleophiles were prepared in 0.1 M Tris buffer (pH=7.0), respectively. The pH was adjusted to 7.0. The reaction mixture (a total volume 1 mL) was prepared by adding the nucleophile (and catalyst) to Tris buffer and the reaction was started by adding benzoyl phosphate. The final concentration of the benzoyl phosphate was 5 mM, and the nucleophiles or catalyst was 24 mM. Then adjusting the reaction mixture pH to 7.0 with hydrochloric acid or sodium hydroxide by pH meter. The reactions were monitored with RP-HPLC (5 μL injected, detected at 254 nm. Running Method: 0-30B over 15 min) over time. From the graphs of the reaction time and the HPLC peak areas, reaction rates could be calculated.

6.3 IMAC procedures for enriching benzohydroxamic acid

Metal-NTA resin (~0.5 mL) was first washed with elution buffer (3 x CV) to remove non-specific binding to Metal-NTA resin, and followed by binding buffer (3 x CV) to equilibrate the resin. A mixture of substrates in binding buffer was loaded to metal-NTA resin. After incubation at RT for designated time, the resin was washed with binding buffer (10 x CV) and finally eluted with elution buffer (5 x CV). All the flow-out fractions were analyzed with RP-HPLC. (CV: column volume)

VII. References

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Chapter 2: Nucleophilic Labeling of Aldehydes/Ketones and Thioesters for Bioconjugation Applications

I. Introduction

1.1 Importance of bioconjugation

Bioconjugation technology for site-specific chemical modifications of biomolecules is critically important in the field of chemical biology. A variety of new tools are emerging in response to the great importance of biomolecule studies. Bioconjugation is usually achieved through inserting or modifying some functional moieties into the biomolecules, laying the foundation for the conjugation with complementary reactive functional groups. Among these functional groups, the carbonyl, alkene, alkyne, azide, thioester groups have attracted a great deal of interest. The well-known aldehyde/ketone condensation, Diels-Alder, CuAAC, Staudinger ligation, as well as the well-established native chemical ligation strategy, involve these functional groups.[1,2] Equipped with these powerful tools, great success on biomolecular studies has been achieved. For example, site-specific labeling of biomolecules in vivo has been realized,[3,4,5,6] as well as the covalent attachment of poly(ethylene glycol) to therapeutic proteins, i.e. PEGylation, which plays an important role in improving the pharmacokinetics of biologic drugs.[7,8] Recent development of antibody-drug conjugates (ADCs), covalent combination of target-specific antibodies with potent drug molecules, has enabled specific drug delivery to cancer cells.[9] These successful applications encourage further investigations in the bioconjugation strategies, and we will focus on aldehydes/ketones and thioesters handles in this thesis.

1.2 Previous labeling methods for aldehydes/ketones

Aldehyde and ketone handles are useful moieties for bioconjugation. Aldehydes can be introduced into biomolecules by various methods including sodium periodate-mediated chemical oxidation of N-terminal Ser/Thr residues[15] (Fig 1. a), transamination using pyridoxal-5-phosphate[16] (PLP) (Fig 1. b) or Rapoport's salt[17] (Fig 1. c). Aldehydes can also be catalytically incorporated into the side-chains of proteins with enzymes such as formylglycine generating enzyme (FGE), protein farnesyl-transferase (PFTase), lipoic acid ligase (LplA), and tubulin tyrosine ligase (TTL). In addition, taking advantage of

evolved tRNA/tRNA synthetase, it is possible to genetically introduce aldehyde-containing unnatural amino acids.[10,11,12] For ketones, enzymatic incorporation and the genetically coding unnatural amino acids have been reported.[13,14]

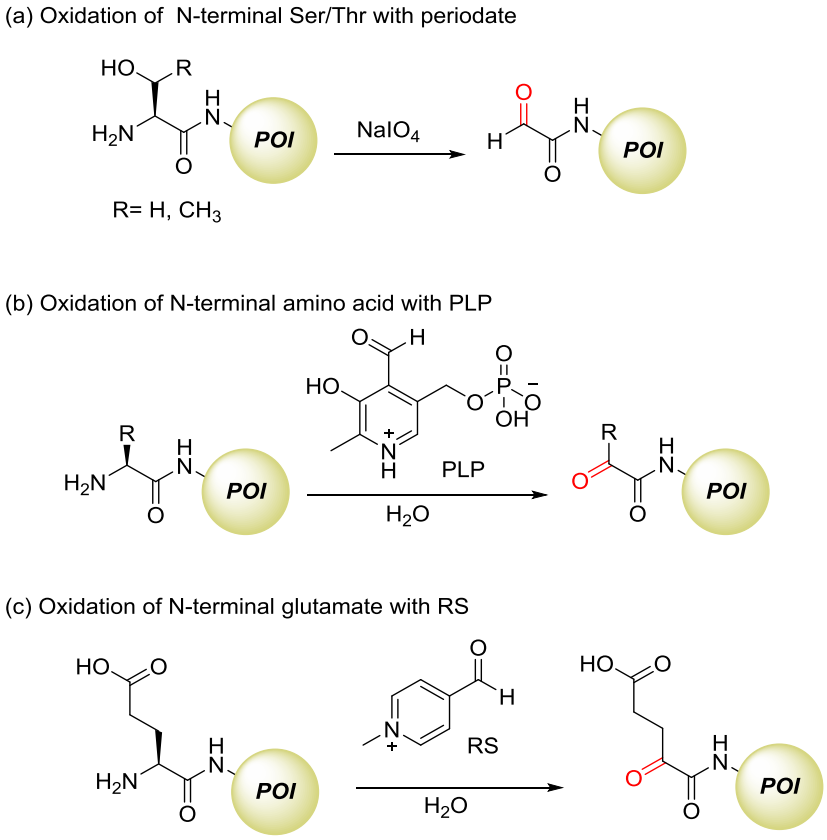
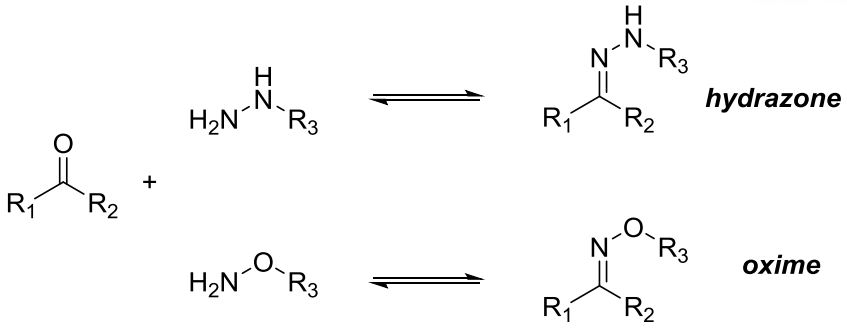


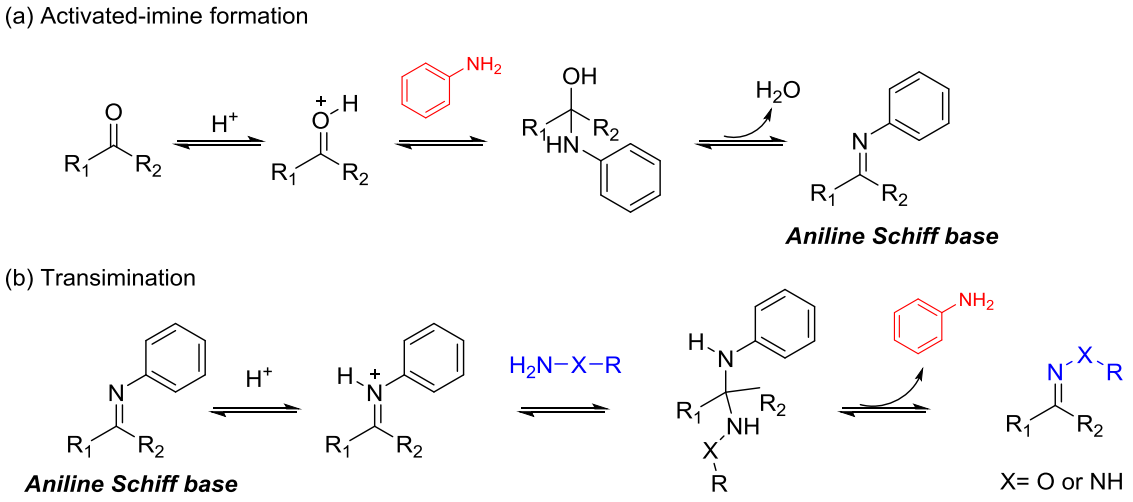
Fig. 1. Chemical incorporation of aldehyde/ketone groups into proteins.

Aldehydes and ketones can chemoselectively react with hydrazine- and aminooxy-derivatives in aqueous solution and yield hydrazones and oximes, respectively (Scheme 1).[18] The resulting hydrazones and oximes belong to Schiff bases, and they possess greater hydrolytic stability than imines. In addition, differences of hydrazones and oximes in hydrolytic stability and equilibration kinetics have been identified, and the lower pK_a of imine moiety in oximes compared to hydrazones contributes to their superior hydrolytic stability.[19] And the equilibrium constant (K_{eq}) of formation is 10^4—10^6 M^{-1} for hydrazones and >10^8 M^{-1} for oximes.[20] However, both hydrazone and oxime ligations proceed with unsatisfactory reaction rates (the second-order rate constant k_2: 10^{-4}—10^{-3} $M^{-1}s^{-1}$)[68], particularly at neutral pH. This slow kinetics hampered their widespread applications in biological systems until the advent of nucleophilic aniline catalyst initially reported by Jencks and re-introduced by Dawson.[21,22,23]



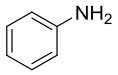
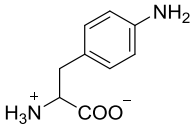
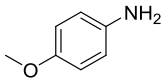
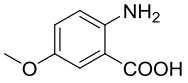
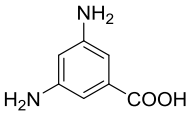
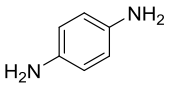
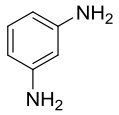
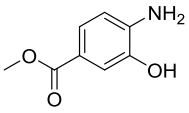
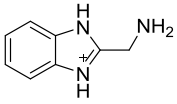
Scheme 1. Hydrazone and oxime formation from aldehyde or ketone.

The nucleophilic aniline catalyst can dramatically accelerate the hydrazone and oxime ligations, since protonated Aniline Schiff base intermediates are formed, which are much more reactive than the parent aldehydes and ketones. [21,22] Aniline is also a good leaving group in the transimination step (Scheme 2).[23] The small equilibrium constant of aniline Schiff base formation requires high concentration of anilines (typically ~100 mM) and acidic pH for efficient catalysis.[24,25] Such conditions can be incompatible with proteins, demonstrated with tubulin protein,[24] and they pose challenges for extensive applications in physiological conditions. Therefore, many new aniline derivatives have been studied to achieve efficient catalysis at neutral pH.



Scheme 2. The mechanism of aniline-catalyzed hydrazone or oxime formation in acidic condition.

Table 1. Overview of nucleophilic catalysts on hydrazone/oxime formation.

Entry	Catalyst	Structure	pKa	Properties	Ref
1	Aniline		4.6	Most active at acidic conditions, limited water solubility	13, 27, 25
2	4-Amino phenylalanine			Better biocompatibility, slightly lower activity than aniline,	24
3	4-Methoxyaniline		5.3	Higher pKa than aniline, more active at neutral pH.	23, 28
4	5-Methoxyanthranilic acid			High water solubility, more active than aniline at neutral pH	29
5	3,5-Diaminobenzoic acid			High water solubility, more active than aniline at neutral pH	29
6	p-Phenylenediamine		6.08, 3.29	More active than aniline over pH 4-7, but prone to oxidation	27
7	m-Phenylenediamine		4.88, 2.65	More active than aniline at neutral pH, prone to oxidation, inhibitory effect in a protein PEGylation	30, 27
8	Methyl 3-amino-4-hydro xybenzoate			More acitive than aniline at neutral pH, containing a H^+ donor	31
9	2-(Aminomethyl)benz imidazole		7.85	More acitive than aniline at neutral pH, containing a H^+ donor, catalyze ketones effectively	31

To accelerate the hydrazone and oxime formation at neutral pH, optimization of aldehydes/ ketones, α-nucleophiles, as well as the catalysts has been carried out by the Kool group. For the substrates, they found alkyl aldehydes reacted faster than aryl aldehydes. More importantly, neighboring groups in the substrates were crucial in enhancing the reaction rates via H-bonding.[26,18] For efficient catalysis, the catalyst should be more reactive towards aldehydes or ketones than the α-nucleophiles, and should form more electrophilic intermediates for the α-nucleophiles. Then the intermediates are supposed to have no effect on the final products. Some effective catalysts have been reported to accelerate the reaction at neutral pH (Table 1).

Para-methoxy aniline (pKa = 5.3) shows better catalytic ability than aniline (pKa = 4.6) at neutral pH (Table 1, entries 1, 3), since the Schiff base intermediate with elevated pKa is more easily protonated and leads to faster reaction. More hydrophilic zwitterionic catalysts are speculated to impose less effect on protein denaturation. Thus the amino acid-assisted aniline catalyst is more compatible with biomolecules, with slightly lower catalytic efficiency than aniline (entry 2). The catalysts assisted by ortho-carboxylate group catalyze more effectively than aniline at neutral pH, and possess better aqueous solubility[29] (entries 4, 5). Interestingly, phenylenediamine regioisomers exhibited quite different catalytic properties. O-phenylenediamine was demonstrated to inhibit hydrazone and oxime formation in some cases, same with m-phenylenediamine. While p-phenylenediamine can catalyze the hydrazone and oxime formation more efficiently than aniline throughout pH 4-7, owing to a second amino group assisting, making amino group(s) unprotonated[27] (entries 6, 7). And Kool *et al*. also developed other effective organocatalyst scaffolds (entries 8, 9). In these scaffolds, a proton donor was incorporated for intramolecular proton donation via a 7-membered transition state.[31]

1.3 Previous bioconjugation methods for thioesters

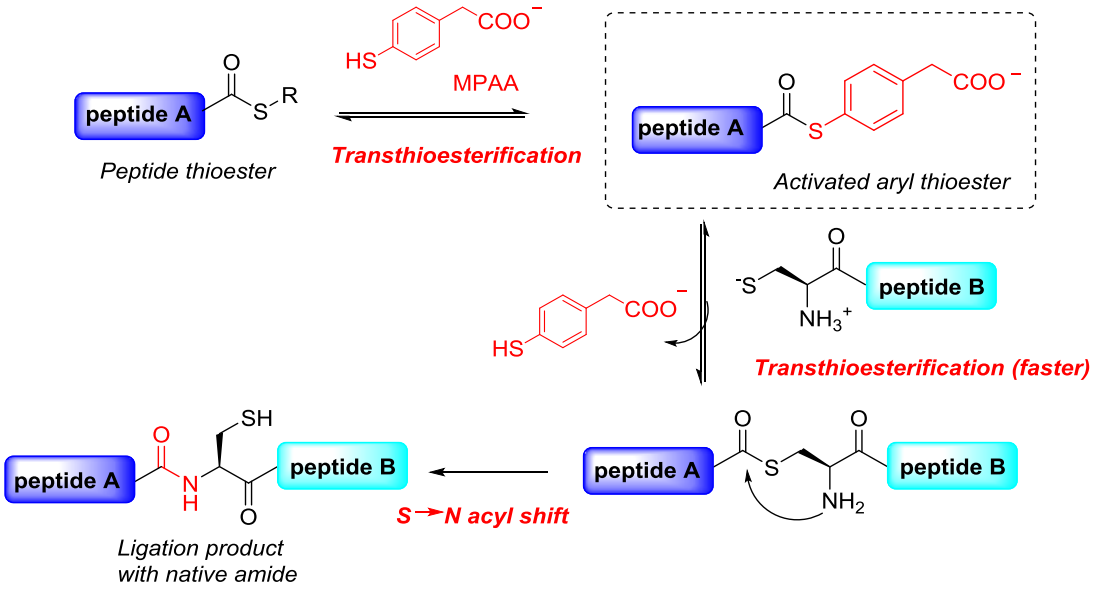
Thioesters have been utilized widely not only in organic synthesis as building blocks, [32,33,34,35] but also in the acyl transfer reactions. Thioesters are also involved in biological process such as in the form of acyl-coenzyme A,[36,37] as well as the intermediates of ubiquitination.[66,67]

To date, various thioester synthesis methods have been introduced.[38] The most common methods rely on the condensation reactions between thiols and carboxylic acids,[39,40,41] or other carbonyl groups in the presence of activating reagents.[42,43] [44] Thioesters also occur in inteins, which are the internal segments of precursor proteins for protein self-splicing. The self-splicing of inteins gives the mature proteins, and this process involves trans-thioesterification and subsequent N-to-S acyl shift, yielding a native cysteine amide bond linking two extein segments.[45] Peptide and protein thioesters can be prepared by the capturing these intein thioester intermediates with exogenous thiols, or synthetically by SPPS.[46,47]

Native chemical ligation (NCL) is a well-known peptide-forming method based on thioesters. In NCL, the thioester moiety is targeted by an N-terminal cysteine via chemoselective trans-thioesterification, and followed by the spontaneous S-to-N acyl shift, thus ligating two moieties by a native amide bond.[48] Nowadays this strategy is being widely used in combination with solid phase peptide synthesis (SPPS), effectively enhancing the peptide synthesis capacity.[49,50] More recently, the expressed protein ligation (EPL) and protein trans-splicing (PTS) technologies have been developed,

by taking advantage of intein-mediated expression of recombinant thioesters, to assemble semisynthetic proteins harboring covalent modifications.[46]

NCL enables chemoselective ligation of two unprotected peptides in neutral aqueous solvents.[48] However, one major drawback of NCL is its slow reaction rate, requiring multimillimolar concentrations for practical use. The ligation rates can be significantly enhanced by thiol catalysts such as 4-mercaptobenzoacetic acid (MPAA). MPAA can transiently generate more active aryl thioesters from alkyl thioesters, and the activated MPAA thioesters undergo faster transesterification with N-terminal Cys-peptide, since MPAA is a better leaving group than alkyl thiols (Scheme 3). It also exhibits better aqueous solubility than general aryl thiol catalysts.



Scheme 3. MPAA-catalyzed native chemical ligation (NCL).

The electrophilic nature of thioesters has also been exploited for bioconjugation via hydrazinolysis and aminoxylysis.[54] However, the ligations between thioesters and the hydrazine or aminooxy derivatives show even slower kinetics than NCL, and very high concentrations (>100 mM) of nucleophiles are needed.[54,55] Therefore, based on the chemoselective reaction between thiol groups and thioesters, we developed novel α-nucleophiles assisted with thiols, which could accelerate thioester ligation as well as the hydrazone and oxime ligations in aqueous solutions.

II. Design of thiol-assisted α-nucleophiles

To improve the thioester- or aldehyde/ketone-based bioconjugations, we designed novel nucleophiles with neighboring thiol groups (Fig. 2). Like N-terminal cysteine peptides, these thiol-assisted hydrazine and hydroxylamine should be in zwitterion forms at neutral pH, giving more nucleophilic thiolates.

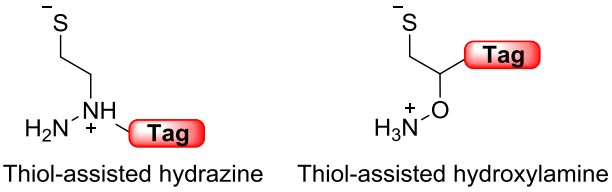
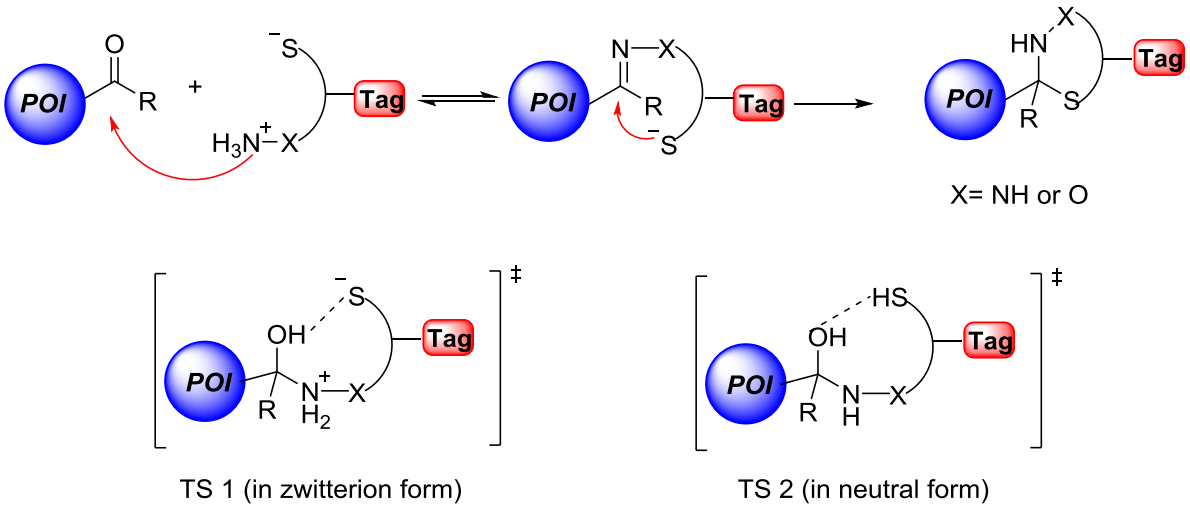


Fig. 2. Thiol-assisted α-nucleophiles.

2.1 Thiol-assisted α-nucleophiles for aldehydes/ketones

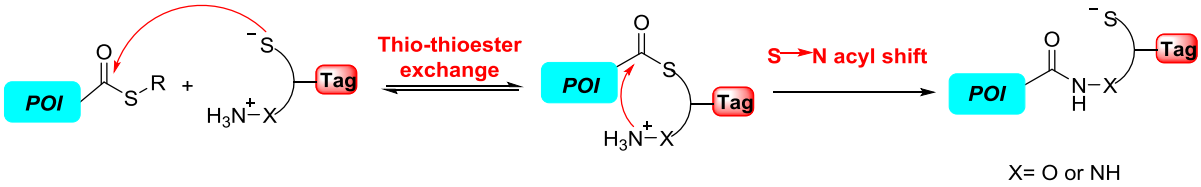
In the ligations between aldehydes/ketones and hydrazine and aminooxy derivatives, the resultant hydrazones and oximes are rather susceptible to hydrolysis, limiting their applications. Therefore, we designed nucleophiles that can address this problem by converting the labile hydrazones and oximes to more stable cyclic products with the pendant thiol groups. Moreover, the thiol groups have the potential to act as proton acceptor/donor in the transition states (TS 1, TS 2), thereby promoting the formation of hydrazones and oximes (Scheme 4). Therefore, we envisioned that thiol-assisted α-nucleophiles could lead to faster and more stable bioconjugation to aldehydes/ketones.



Scheme 4. Design of thiol-assisted α-nucleophiles to aldehydes/ketones.

2.2 Thiol-assisted α-nucleophiles for thioesters

The slow hydrazinolysis and aminoxylysis of thioesters make the bioconjugation on thioesters less attractive. However, if we take advantage of the chemoselective trans-thioesterification reactions between the thiol groups and thioesters, we could achieve intramolecular hydrazinolysis and aminoxylysis of thioesters for faster bioconjugation. Similar to the NCL mechanism, the thiol-assisted α-nucleophiles are expected to proceed the initial attack of the thioesters by the thiol moiety, and the intermediate thioesters would undergo spontaneous S-to-N acyl shifts to ultimately form amide bonds between tags and the proteins of interest (POI) (Scheme 5). In addition, these α-nucleophiles could enhance the ligation rates compared to NCL.

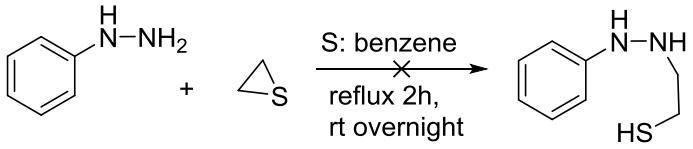


Scheme 5. Proposed reactions between thiol-assisted α-nucleophiles and thioesters.

III. Results and discussions

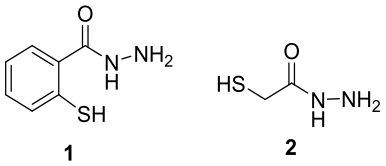
3.1 Results of thiol-assisted nucleophiles on aldehydes

We set out to synthesize alkyl thiol-assisted hydrazine nucleophiles on an aromatic ring for facile HPLC detection. We first tried to synthesize alkyl thiol-assisted hydrazine nucleophile, known in the literature[56], but we failed to obtain the desired product, due to the instability of the hydrazine (Scheme 6).

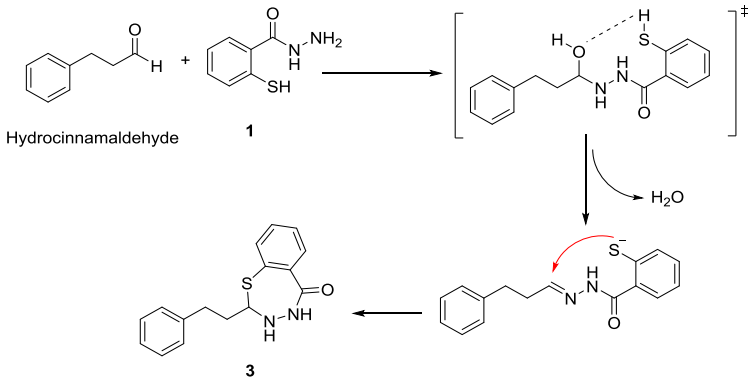


Scheme 6. Attempted reaction between phenylhydrazine and thiirane.

Then we turned to acyl hydrazides, namely 2-mercaptobenzohydrazide **1** and 2-mercaptoacetohydrazide **2**, shown below (see Experimental Section 5.2, 5.3), and applied them on the following experiments.



We first tested 2-mercaptobenzohydrazide **1** on hydrocinnamaldehyde to assess the efficiency of thiol-assisted nucleophiles on aldehydes.[57] As in Scheme 7, we envisioned that the neighboring thiol group had the potential to accelerate the hydrazone formation via H-bonding. The resulting hydrazone could then be attacked by the thiolate, yielding the final cyclic product (Scheme 7). In fact, the thiol-assisted acyl hydrazide is known to form a cyclic product when reacting with aldehydes, but the reaction rate and product stability in aqueous buffers were not reported.[58,59,60]



Scheme 7. Hypothetical reaction pathway for the reaction between 2-mercaptobenzohydrazide **1** and hydrocinnamaldehyde.

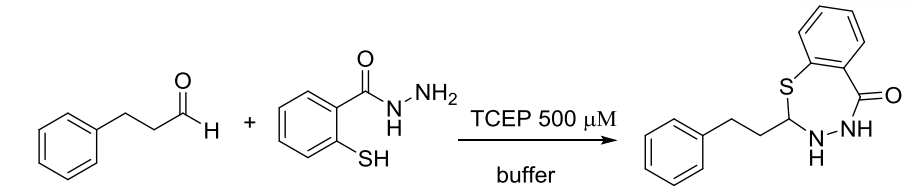
The reaction was initiated by adding 2-mercaptobenzohydrazide **1** to the mixture of hydrocinnamaldehyde and TCEP in PBS: Methanol=4:1 buffer (pH=7.4). However, white precipitates were observed as soon as **1** was added (intended final concentration 3 mM) to the mixture. NMR analysis confirmed this precipitate was indeed the desired product **3** (see Experimental Section 5.5). We also tested the stability of product **3** in different conditions, found that the product **3** was intact when incubated at pH 3 for about 3 days at room temperature, but decomposed to some degree at pH 5 (see Experimental Section 5.6).

3.1.1 Kinetic experiment of 2-mercaptobnezohydrazide **1** on hydrocinnamaldehyde

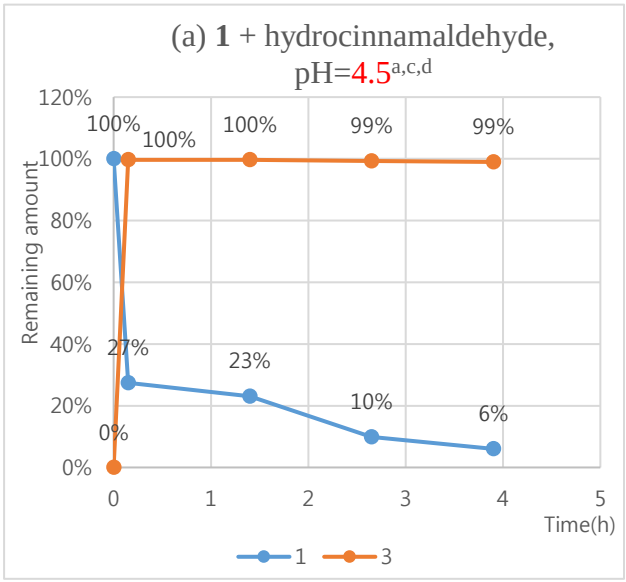
In order to analyze the mixture with RP-HPLC, we lowered the intial concentration of **1** from 3 mM to 50 μM to avoid precipitation. We also compared the reaction with benzhydrazide to explore the thiol

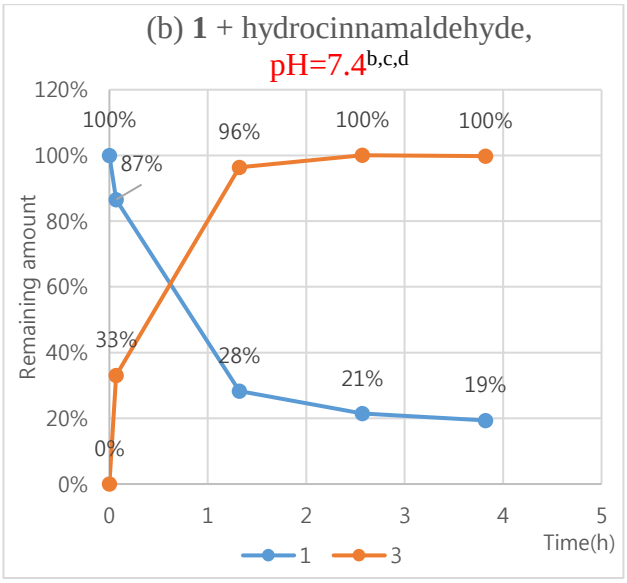
effect. And we carried out the reaction at pH 4.5 and 7.4, to explore the pH effect on the reaction rates (see Experimental Section 5.4.1).

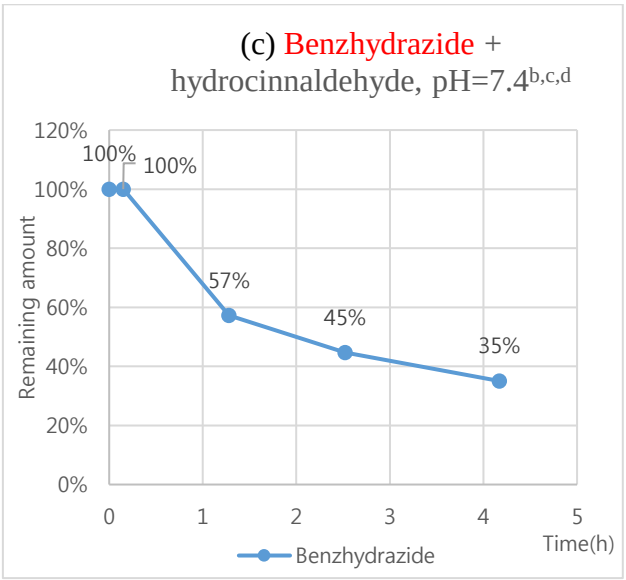
Fig. 3. (a) Reaction between hydrocinnamaldehyde and **1** at pH 4.5. (b) Reaction between hydrocinnamaldehyde and **1** at pH 7.4. (c) Reaction between hydrocinnamaldehyde and benzhydrazide.



Hydrocinnamaldehyde 2.5 mM + **1** 50 μM → **3**



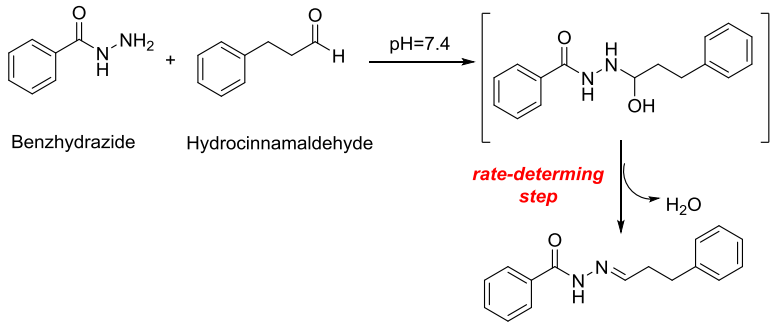




[a]50 mM acetic acid: methanol= 4: 1, pH 4.5 as buffer. [b]1 x PBS: methanol= 4: 1, pH 7.4 as buffer. [c] The initial concentration of nucleophile was 50 μM, hydrocinnamaldehyde 2.5 mM, (TCEP 500 μM). [d] The reactions were monitored by RP-HPLC at 254 nm wavelength.

According the results above, the reaction was faster in acidic condition (Fig. 3 (a) and (b)). The product reached its saturated concentration as soon as the mixture was analyzed by RP-HPLC at pH 4.5. And some precipitate came out as reaction proceeded further. Moreover, hydrazide **1** was almost consumed in the monitoring process (graph a). While at pH 7.4, it took approximate 1.3 hours for the product to reach its saturated concentration in buffer, and about 20% of **1** remained after the monitor process (graph b).

In order to test the thiol's catalytic effect (Scheme 7), we carried out benzhydrazide on hydrocinnamaldehyde in pH 7.4 buffer (Scheme 8), where the dehydration step was the rate-determining step.[21] Comparing the conversion of the nucleophiles (graphs b, c), higher conversion of **1** was observed, which indicated that the thiol in **1** facilitated its reaction with aldehyde.

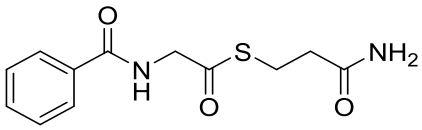


Scheme 8. Reaction between benzhydrazide and hydrocinnamaldehyde.

In conclusion, hydrazide **1** performed better than benzhydrazide on the reaction rates, and it resulted relatively stable cyclic product with hydrocinnamaldehyde. And it will be also meaningful to try the alkyl thiol 2-mercaptoacetohydrazide **2** on aldehydes in the future.

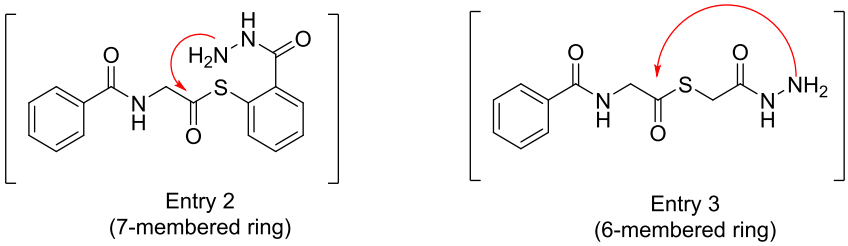
3.2 Results of thiol-assisted α-nucleophiles on thioesters

Taking the solubility, steric hindrance, as well as the facile HPLC detection into account, we chose a glycine-thioester **4** as the model system, shown below. Thiol-assisted α-nucleophile and control nucleophiles were assessed on this thioester.



Glycine-thioester(**4**)

The reactions between thiol-assisted nucleophiles (**1** and **2**) and the glycine-thioester **4** were monitored using RP-HPLC (See Experimental Section 5.4.2). Alkyl thiol-assisted hydrazide **2** was more reactive than aryl thiol-assisted hydrazide **1** towards glycine-thioester **4** at pH 7. And for **1**, the glycine-thioester reaction rate was similar to the background hydrolysis rate (Table 2, entries 1, 2). Much better performance of hydrazide **2** on glycine-thioester **4** was observed, presumably because the alkyl thiol is more nucleophilic than aryl thiol. In addition, the S-to-N acyl shift pathways were also suspected to partly contribute to the differential results, where Entry 2 was supposed to proceed a 7-membered transition state, entry 3 via relatively favored 6-membered transition state (Scheme 9).



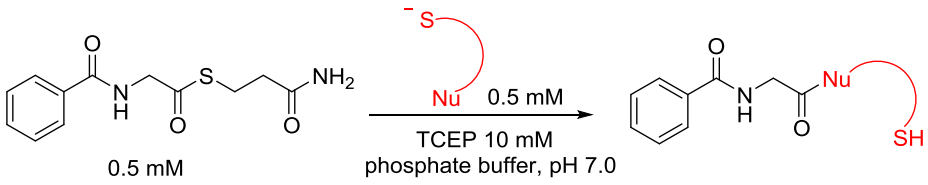
Entry 2
(7-membered ring)

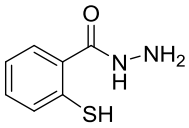
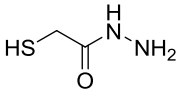
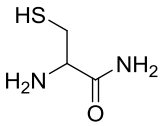
Entry 3
(6-membered ring)

Scheme 9. Hypothetical thioester intermediates of the entries 2, 3.

In order to check the influence of nitrogen nucleophiles on the thioester ligation rates, we switched the hydrazide nucleophile to primary amine (L-cysteinamide **5**, entry 4). However, both **2** and **5** exhibited similar reactivity towards glycine-thioester (entries 3, 4), which further supports the S-to-N acyl shift is not the rate-determining step. Interestingly, no thioester hydrolysis product was observed in the presence of these nucleophiles.

Table 2. Assessing thiol-assisted nucleophiles on glycine-thioester **4**.[a,b]



Entry	Nucleophile	Structure	Glycine-thioester rate[c]
1			-29.2
2	2-Mercaptobenzohydrazide (**1**)		-29.4
3	2-Mercaptoacetohydrazide (**2**)		-213.5
4	L-Cysteinamide (**5**)		-212.5

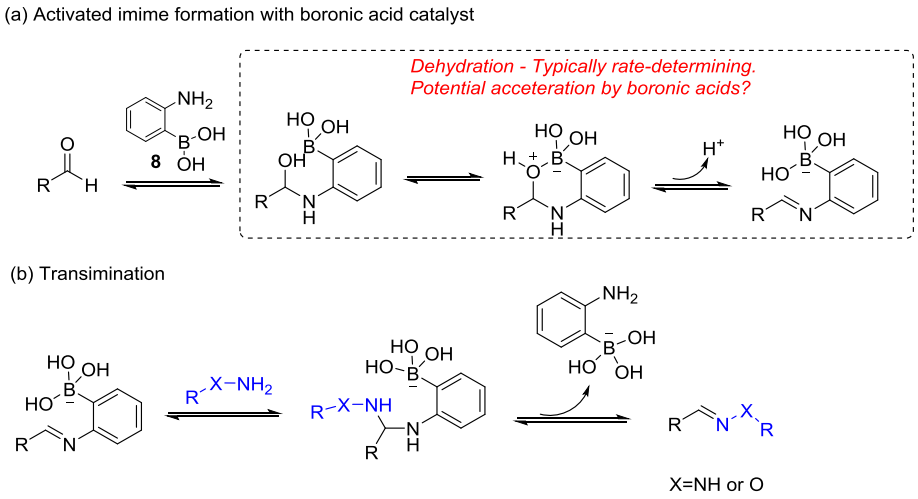
[a]Glycine-thioester 0.5 mM, TCEP 10 mM, (nucleophile 0.5 mM) in 0.1 M phosphate buffer (pH=7.0) [b]all the glycine thioester rates were based on duplicate measurements, and took the average values as the final glycine-thioester rates.[c]unit:mAU*s/h

In conclusion, it is the thiol species in thiol-assisted nucleophiles that decides the rates of thioester ligation. Alkyl thiol-assisted nucleophiles were more reactive than aryl thiols in thioester ligations. We envisioned that the ligation rates could be further enhanced by elevating the nucleophile amount, or using aryl thiol catalyst, based on the same mechanism to NCL.

3.3 Boronic acid-assisted catalyst results

In the formation processes of hydrazone and oxime from aldehydes/ketones and α-nucleophiles, dehydration of the intermediates is the rate-determining step. Therefore, we proposed inserting some neighboring groups in the catalysts was able to accelerate the dehydration. Lewis acidic nature of boronic acids makes them attractive, and the use of boronic acids to accelerate hydrazone and oxime formation has already been reported. However, they used boronic acid-containing aldehydes or ketones, which limited the reaction's scope.[61,62,63] For example, such boronic acid-aldehydes cannot be easily incorporated into proteins. Accordingly, we envisioned that the boronic acid-assisted aniline catalyst

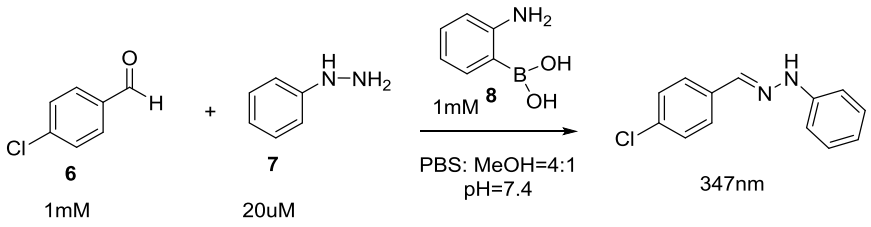
could accelerate the hydrazone and oxime ligations with regular aldehydes and ketones. The reaction design is shown below, in which boronic acid can facilitate the dehydration to form the activated imine intermediate, which is the rate-determining step for aniline-catalyzed conjugations. The formed imine is subject to fast transimination, and giving the final hydrazone product (Scheme 10).



Scheme 10. Design of boronic acid-assisted catalyst **8**.

Therefore, we tested 2-aminophenylboronic acid catalyst **8** on the reaction between 4-chlorobenzaldehyde **6** and phenylhydrazine **7** (Table 3). According to the literature[31], the formed hydrazone product has λ_{max}=347 nm, so the product formation was monitored with a UV-Vis spectrometer at 347 nm (See Experimental Section 5.4.3).

Table 3. Kinetic assessment of 2-aminophenylboronic acid **8**.



Entry	catalyst	$k_2(M^{-1}s^{-1})$	R-square
1	none	0.32697	0.99807
2	2-aminophenylboronic acid	1.01086	0.1019
3[a]	2-aminophenylboronic acid	0.84998	0.93736

[a]subtracting the initial boronic acid catalyst absorbance at 347nm.

In our case, catalyst **8** had some absorbance at 347nm, so we subtracted the initial 347 nm absorbance of **8**, and it resulted better fit to the kinetic equation[31] (Table 3, entries 2, 3). In comparison with the background, catalyst **8** did accelerate the hydrazone formation about 2.6-fold at neutral pH. However, its catalytic efficacy was not as good as the best aniline catalyst reported in the literature (Table 1, entry 8), where their catalyst accelerated the reaction 13-fold[31].

We think the following factors contributed to modest catalytic effect of 2-aminophenylboronic acid catalyst **8**. The compound **8** was kind of unstable in PBS/MeOH buffer. And in the mechanistic hypothesis (Scheme 10), the activated imine was formed via a six-membered ring, whereas the relatively stable six-membered ring might hamper its formation.[63]

IV. Conclusions

Due to the significance of efficient bioconjugation methods for aldehydes/ketones, as well as the thioesters, we tested novel nucleophiles and catalyst for these reactions. Particularly, we prepared novel thiol-assisted nucleophiles, and tested them for reactions with aldehydes and thioesters. The 2-mercaptobenzhydrazide **1** resulted relatively stable cyclic product with aldehyde, and the thiol also slightly accelerated the reaction rate. We envision that the cyclic product formation can be utilized for bioconjugation, due to their enhanced stability in aqueous solution. For thioesters, the alkyl thiol-assisted nucleophiles were much more efficient than aryl thiol-assisted nucleophiles on the ligation to glycine-thioester **4**. Therefore, increasing the amount of alkyl thiol-assisted nucleophiles or catalyzed by aryl thiol can realize fast thioester ligation. We also tested some boronic acid-assisted aniline catalyst for hydrazone formation in aqueous solution. However, the 2-aminophenylboroic acid catalyst **8** was susceptible to decomposition. We expect further studies in these areas will lead to superior nucleophiles and catalysts to achieve faster and more stable bioconjugations.

V. Experimental Section

5.1 General information

Chemicals:

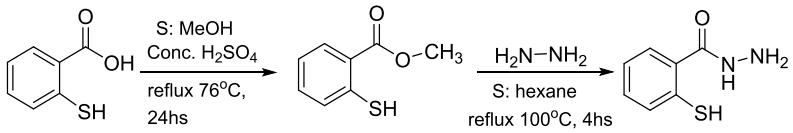
Thiosalicylic acid (98%), Methyl mercaptoacetate (98%), and tris(2-carboxyethyl) phosphine hydrochloride (TCEP, 98 %), 3-phenylpropionaldehyde (hydrocinnamaldehyde, 95%), 4-chlorobenzaldehyde (98%), phenylhydrazine (97%) were purchased from Alfa Aesar. Methanol,

sulfuric acid, n-hexane, N, N-dimethylformamide (DMF), ethyl acetate and acetonitrile were purchased from Daejung. Sodium bicarbonate (99.0%), trifluoroacetic acid (99%) were from Samchun. Benzhydrazide (98%) and ethylene sulfide (thiirane) were purchased from Sigma Aldrich. Hydrazine monohydrate (80%) and benzene were purchased from JUNSEI, and (2-aminophenyl) boronic acid was purchased from OXCHEM.

Instruments:

pHs were measured with a pH meter (Thermo Scientific, Orion Star A211). RP-HPLC analyses were carried out on Agilent 1200 Infinity instrument with an Agilent C18 column (5 μm, 4.6 x 150 mm) using a gradient of solvent A (0.1% trifluoroacetic acid in water) and solvent B: (90% Acetonitrile in H_2O, 0.1% trifluoroacetic acid). NMR spectra were recorded on an Agilent 400 MR DD2. The 347 nm absorbance of 2-amniophenylboronic acid catalyzed hydrazone formation reaction was detected by a UV-Vis spectrometer (Nanodrop® 2000c, Thermo Scientific).

5.2 Synthesis of 2-mercaptobenzohydrazide **1**[64]

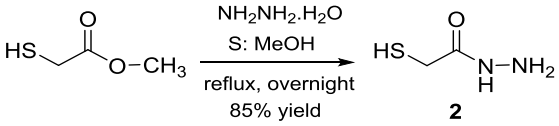


1

In a round bottom flask equipped with a reflux condenser, thiosalicylic acid (5 g, 0.03 mol) was dissolved in methanol (30 mL) and 98% conc. sulfuric acid (4 mL) was added, and the mixture was refluxed overnight. Then methanol was evaporated and the residue was dissolved in brine, and adjusted pH to 7 with $NaHCO_3$, then extracting with hexane to remove unreacted thiosalicylic acid, collecting the organic phase and evaporate hexane solvent. The crude ester product was dissolved in 80% hydrazine monohydrate (10 mL, 0.15 mol), refluxing at 100 ^{O}C for 4 hours. The mixture was cooled down to room temperature and the pH was adjusted to ~4 with concentrated HCl. Some yellow precipitate formation was observed. The solid was filtered through a Whatman filter paper, washed with cold water, and lyophilized to give the yellow solid product (0.46 g, overall yield 9%).

^{1}H NMR (400MHz, CD_3OD): δ 7.15-7.17(m, 1H), 7.27-7.29 (m, 1H), 7.36-7.43 (m, 2H). **^{13}C NMR** (400MHz, $CDCl_3$): δ 124.58, 128.03, 130.06, 130.44, 132.07, 133.14, 169.16.

5.3 Synthesis of 2-mercaptoacetohydrazide**2**[65]



Methyl thioglycolate (9.3 mL, 0.1 mol) was dissolved in methanol (30 mL) in a round bottom flask equipped with a reflux condenser. 80% hydrazine monohydrate (7.36 mL, 0.12 mol) was added and the reaction mix was refluxed overnight. The reaction was monitored with RP-HPLC. (If necessary, additional 0.2 equiv. of hydrazine was added to consume all methyl thioglycolate). The reaction mixture was evaporated and lyophilized to give a yellow solid. (9g, yield 85%).

^{1}H NMR (400MHz, $CDCl_3$): δ 1.67 (bs, 1H), 3.26 (s, 2H), 3.91 (bs, 2H), 7.71(bs, 1H). **^{13}C NMR** (400MHz, $CDCl_3$): δ 26.60, 170.01.

5.4 Kinetic experiments

5.4.1 Kinetic study of thiol-assisted nucleophiles on aldehyde

Stock solutions of hydrocinnamaldehyde, TCEP and nucleophiles were prepared in corresponding pH buffers, and their pHs were adjusted to the final pH with hydrochloric acid or sodium hydroxide. The reaction mixture (a total volume of 1mL) was prepared by adding the hydrocinnamaldehyde, (TCEP) to the corresponding buffer and the reaction was started by adding the nucleophile. The final concentration of the nucleophile was 50 μM, and hydrocinnamaldehyde was 2.5 mM, (TCEP 500 μM). The final pH was adjusted with hydrochloric acid or sodium hydroxide. The pH 4.5 buffer was made of 50 mM acetic acid: methanol= 4: 1. And pH 7.4 buffer was made of 1 x PBS: methanol= 4: 1. The reaction mixtures were monitored with RP-HPLC (50 μL injected, detected at 254nm. Running Method: 0-40-70B over 8 min) over time. The remaining amounts of nucleophile were calculated from its HPLC peak areas, using a calibration equation between the peak area and concentration. The relative amounts of cyclic product 3 were based on its saturated concentration in buffer solution.

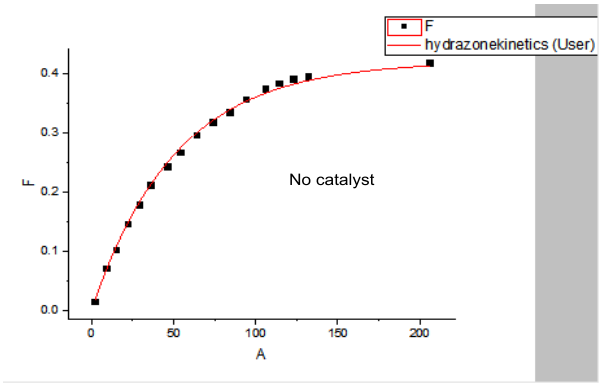
5.4.2 Kinetic study of thiol-assisted nucleophiles on glycine-thioester **4**

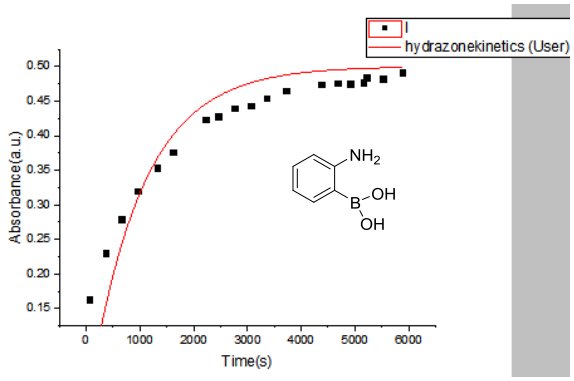
Stock solutions of nucleophiles, TCEP were pre-prepared in 0.1 M sodium phosphate buffer (pH=7.0), respectively. The glycine-thioester was pre-prepared in a mixture of 0.1M sodium phosphate/ acetonitrile (adding as less as acetonitrile to dissolve it). Their pHs were adjusted to 7 with hydrochloric acid and sodium hydroxide. The reaction mixture (a total volume of 1 mL) was prepared by adding the nucleophile, TCEP to phosphate buffer (pH=7) and the reaction was started

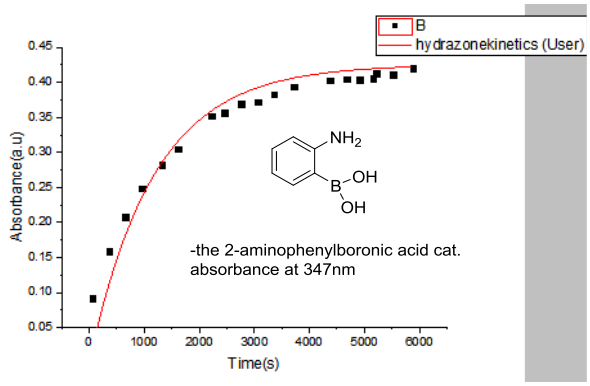
by adding the glycine-thioester 4. The final concentration of the glycine-thioester **4** was 0.5 mM, TCEP was 10 mM, and nucleophile was 0.5 mM. Then the final reaction mixture pH was adjusted to 7 with hydrochloric acid or sodium hydroxide by pH meter. The reaction mixture was subjected to analyzing with RP-HPLC (20 μL injected, detected at 254nm. Running Method: 20-45B over 8 min). The glycine-thioester rates were calculated based on the graphs of glycine-thioester HPLC peak areas and reaction time.

5.4.3 Kinetic study of 2-aminophenylboronic acid catalyst on aldehyde with UV-Vis spectrometer

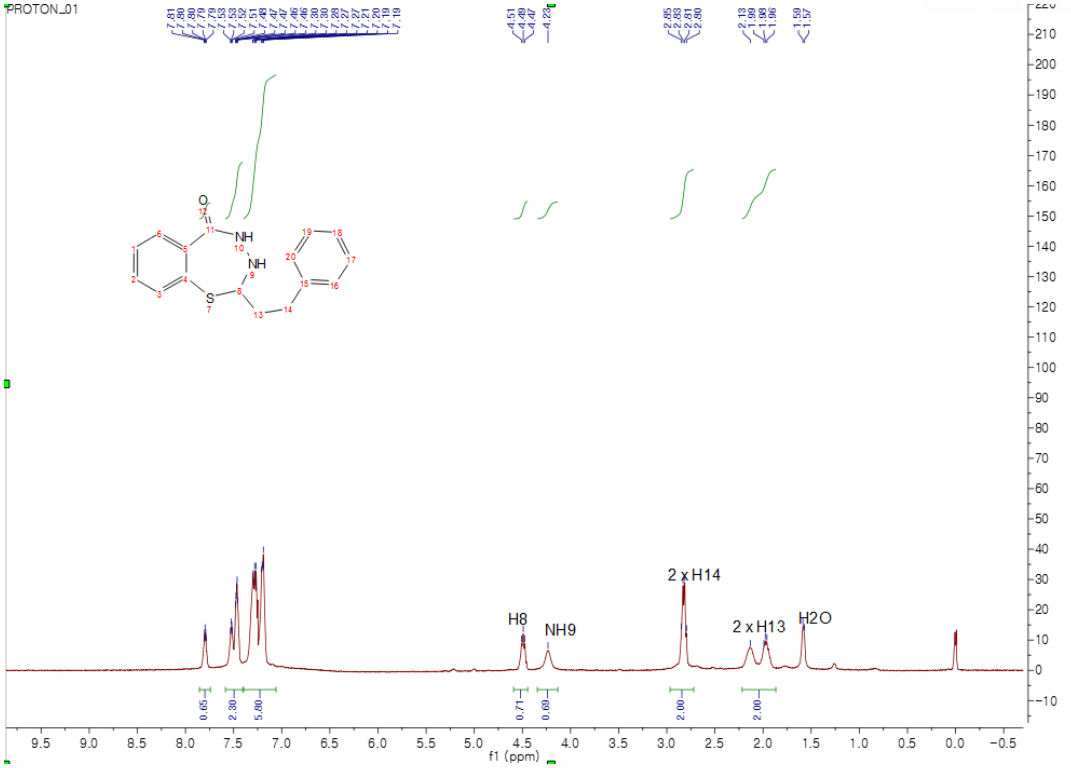
Stock solutions of 4-chlorobenzaldhyde and phenylhydrazine, as well as 2-aminophenylboronic acid **8** were pre-preparing in PBS: MeOH =4: 1 buffer (pH 7.4). Their pHs were adjusted to 7.4 with hydrochloric acid or sodium hydroxide. (Phenylhydrazine was discarded after 3 hours and made freshly). The reaction mixture (total volume of 1 mL) was prepared by adding the 2-aminophenylboronic acid and 4-chlorobenzaldhyde to PBS: MeOH =4: 1 buffer (pH 7.4) and the reaction was started by adding phenylhydrazine. The final concentration of phenylhydrazine was 20 μM, 4-chlorobenzaldhyde was 1 mM, and 2-aminophenylboronic acid was 1 mM. The final reaction mixture pH was adjusted to 7.4 with hydrochloric acid or sodium hydroxide by pH meter. The reaction mixture (500 μL) was loaded into the UV-Vis spectrometer vial, and was subject to monitoring with UV-Vis spectrometer at 347 nm wavelength. We set PBS: MeOH=4: 1 buffer (pH 7.4) as blank here. We calculated the secondary kinetic constants of the reaction by OriginPro 2015 program, and referred to the equation in the literature[26].

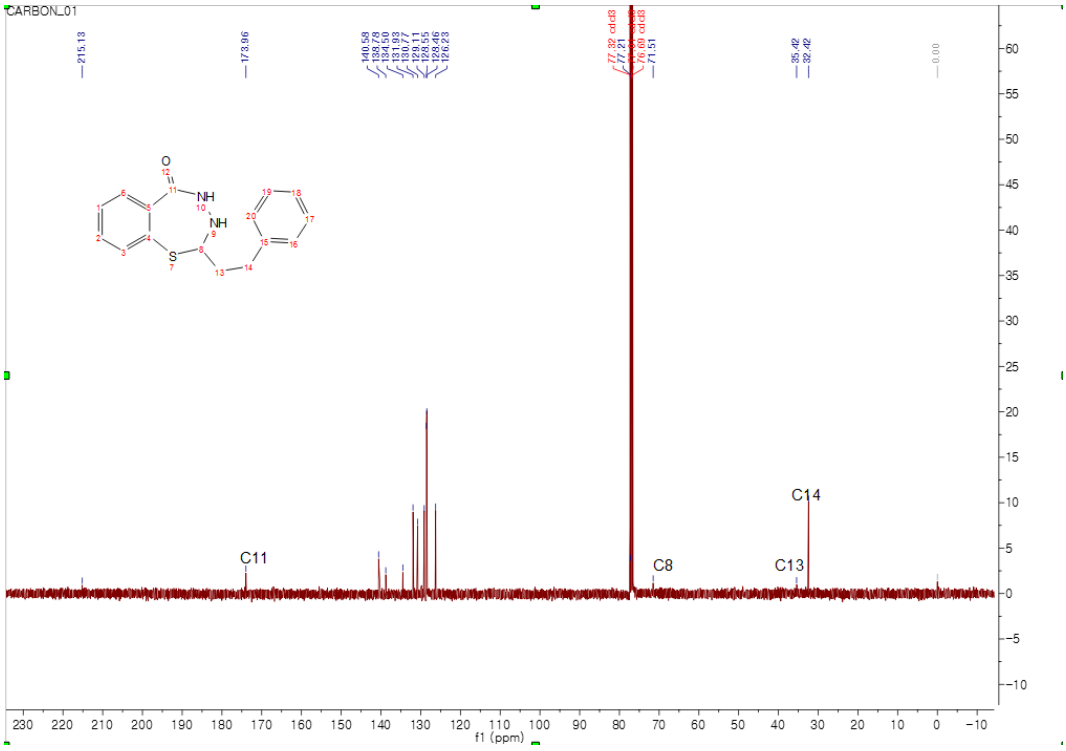


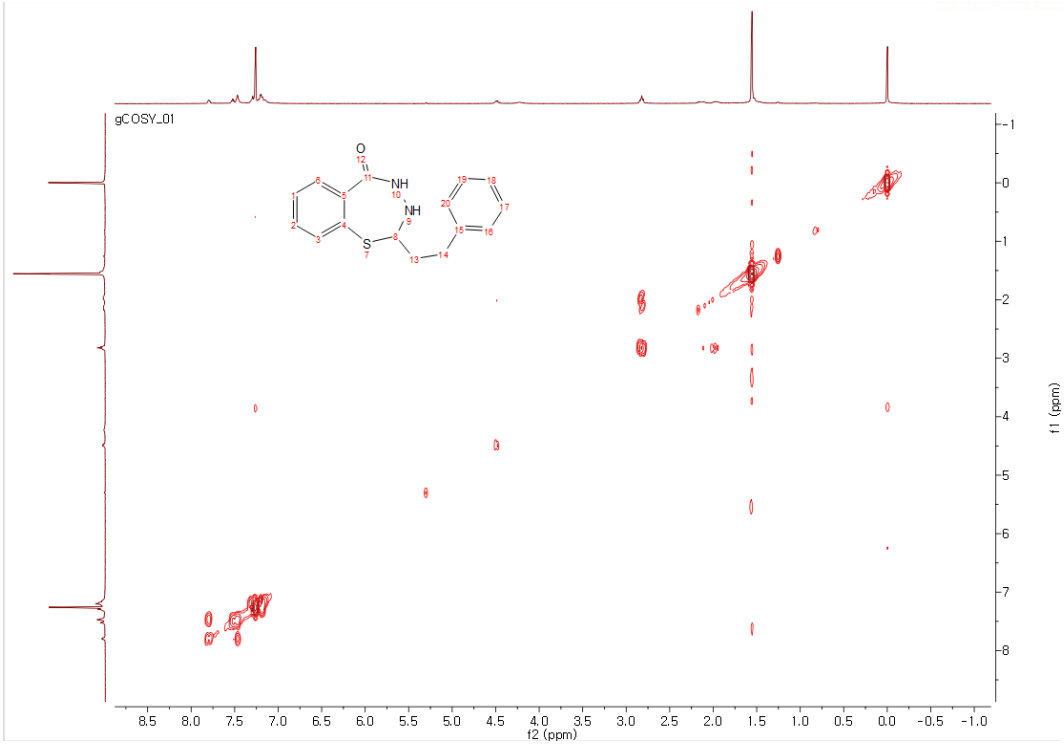


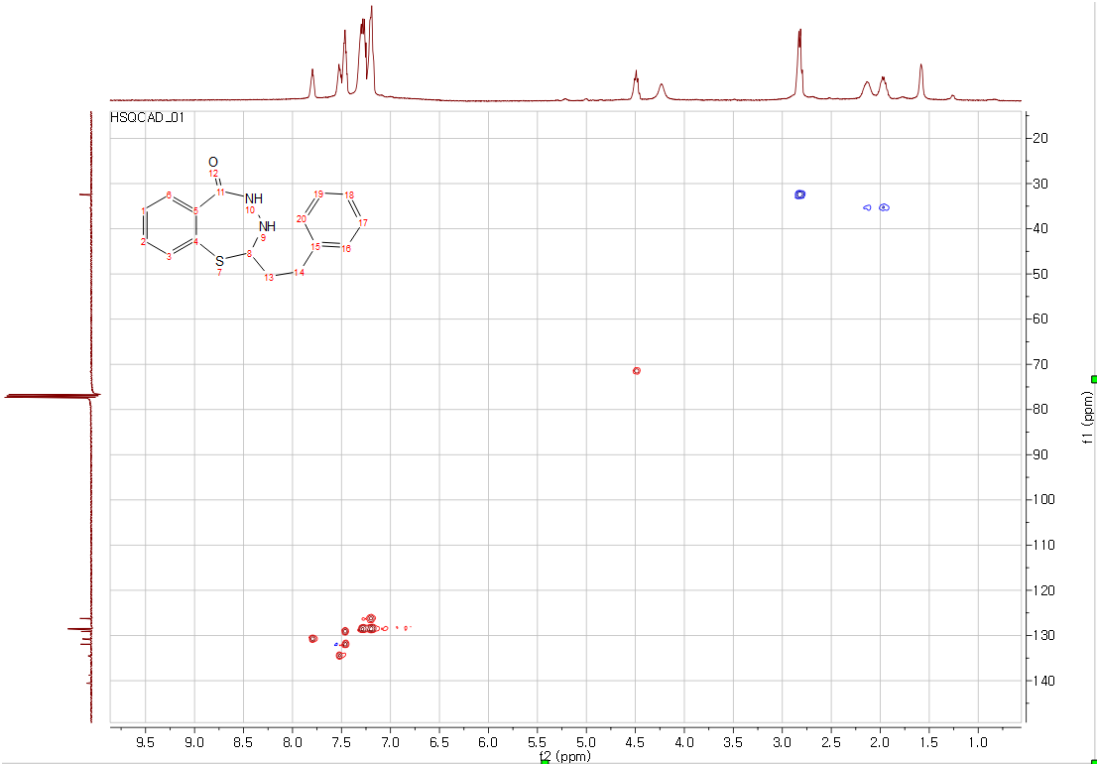


5.5 NMR spectra of the cyclic product **3**



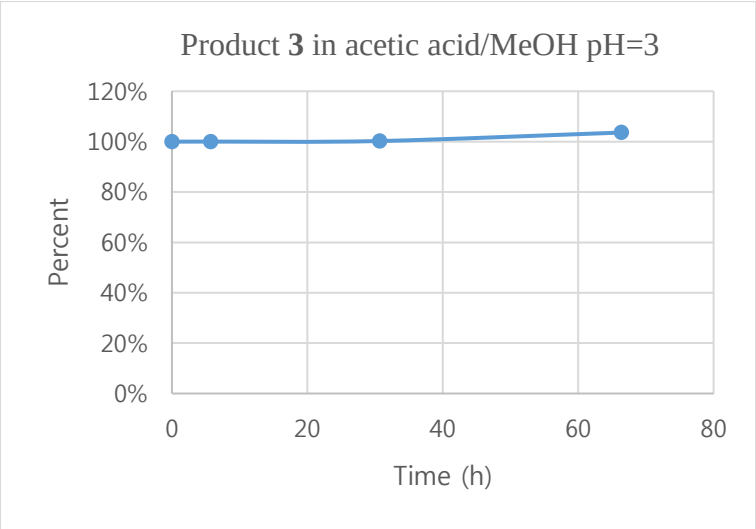


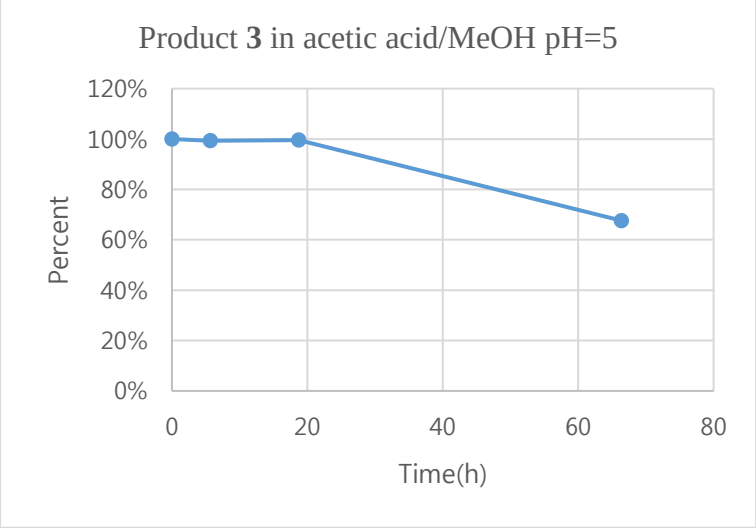




5.6. Testing the cyclic product 3 stability[57]

Solid product **3** was dissolved in pH=3 or pH=5 acetic acid/methanol buffer, and the solution was incubated at rt. The reaction was analyzed by RP-HPLC at 254 nm. (10 μL injected, analysis method: 20-45B over 8 min.)





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