STUDIES AND APPLICATIONS OF ALDEHYDE CHEMISTRY FOR PEPTIDE MODIFICATION AND FORMALDEHYDE DETECTION

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Studies and Applications of Aldehyde Chemistry for Peptide Modification and Formaldehyde Detection

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CERTIFICATE OF ORIGINALITY

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ABSTRACT

Aldehyde is a versatile functional group and synthetic building block in organic synthesis. Significant advancement has been made to demonstrate the key role of aldehydes in synthetic chemistry through diverse organic transformation reactions including C-C, C-O, and C-N bond formations. In this work, we would like to expand the impact of aldehyde chemistry to the interdisciplinary research areas on chemical biology and food safety.

Selective modification of peptides and proteins has attracted great attention in chemical biology, as it allows the generation of novel bioconjugates used in the study of complex biological systems. The N-terminal α-amino group of peptides/proteins is utilized for site-specific modification as most proteins have only one N-terminus. In my work, site-specific N-terminal α-amino group modification of peptides by an oxime formation/exchange sequence was developed. We found that oxone ($2KHSO_5 \cdot KHSO_4 \cdot K_2SO_4$) is a convenient and chemoselective reagent for oxidation of *N*-terminal α-amino group to oximes. The substrate scope of the present oxime formation reaction was studied by using a series of unprotected peptides XSKFR (X = 20 natural amino acids). The

N-terminal modifications were found in the peptides in a range of conversions (21-99%) as confirmed by ESI-MS/MS analyses. With the above promising results, subsequent functionalization of the oxime-modified peptides via an oxime exchange with *O*-substituted hydroxylamines was studied. Moreover, incorporation of biophysical probes such as fluorescent dansyl and PEG-linked hydroxylamine by the oxime exchange reaction was performed, leading to the corresponding functionalized peptides in 99% conversion. These findings indicated that N-terminal α-amino group modification by using an oxime formation/exchange reaction sequence could be performed under mild reaction conditions and the present method demonstrates the potential of oxone towards peptide/protein modification.

Formaldehyde is known to be a natural metabolite in various living organisms, and is thus present in many fresh and preserved foods as a normal component. Formaldehyde is not permitted for food use in Hong Kong and China. However, formaldehyde was recently found to be deliberately used in food processing in China for bleaching, protein coagulation and preservation purposes, causing a serious hazard to public health. In my work, a gold(III) complex-catalyzed three component coupling reaction of formaldehyde, an amine and an alkyne for

propargylamine synthesis was established to allow formaldehyde detection within 1 hour at 50 ^{o}C. The high selectivity of the coupling reaction to detect formaldehyde is achieved by the steric bulkiness around the amino group of the amine, as formaldehyde is the smallest aldehyde in nature. Different parameters such as catalysts, reagent ratios and solvents were optimized using a HPLC-based assay, with up to 56% yield achieved in 1 hour. Different fluorophores such as coumarin and dansyl were employed for fluorescent detection. The reaction system could also be immobilized on solid supports such as 2-chlorotrityl chloride resin for washing of excess reagents. Measurement of fluorescent intensity on the resin showed excellent linearity (R^2 = 0.99). The reaction was applicable to detect formaldehyde in dried shiitake mushroom samples.

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LIST OF ABBREVIATIONS

A3-Coupling reaction	Three-component coupling of an aldehyde, an amine and an alkyne catalyzed by a transition-metal
AHMT	4-Amino-3-hydrazino-5-mercapto-1,2,4-triazole
AQSIQ	General Administration of Quality Supervision, Inspection and Quarantine
BODIPY	Boron-dipyrromethene
***m*-CPBA**	*m*-Chloroperoxybenzoic acid
DMF	Dimethylformamide
2,4-DNPH	2,4-Dinitrophenylhydrazine
EDTA	Ethylenediaminetetraacetic acid
ESI	Electrospray ionization
FET	Fluorescence energy transfer
GC	Gas chromatography
GDCIQ	Guangdong Entry-Exit Inspection and Quarantine Bureau
HBTU	N,N,N',N'-Tetramethyl-O-(1H-benzotriazol-1-yl)uroniu m hexafluorophosphate
HPLC	High performance liquid chromatography
IARC	International Agency for Research on Cancer

LC	Liquid chromatography
MALDI	Matrix-assisted laser desorption/ionization
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NHS	N-Hydroxysuccinimide
PBS	Phosphate buffered saline
PDA	Photodiode array
PEG	Polyethylene glycol
PET	Polyethylene terephthalate
PLP	Pyridoxal-5-phosphate
ppb	Parts-per-billion
ppm	Parts-per-million
ppt	Parts-per-trillion
R^2	Coefficient of determination
RfD	Reference dose
SPOS	Solid phase organic synthesis
Tb-SHMP	Terbium(III)-sodium hexametaphosphate
TDI	Tolerable daily intake
TFA	Trifluoroacetic acid

TOF	Time of flight
USEPA	United States Environmental Protection Agency
UV	Ultraviolet
WHO	World Health Organization

CHAPTER 1: INTRODUCTION

Chemistry is the study of structure, composition and property of matter. It is classified as a branch of physical science, and is frequently called the "central science" because it connects and incorporates both biology and physics for medicinal studies and engineering applications. In line with the theme of the International Year of Chemistry 2011 "achievements of chemistry and its contributions to the well-being of humankind" [1], we would like to study and apply chemistry on the multidisciplinary areas of chemical biology and food safety [2].

Organic chemistry is a sub-discipline of chemistry which is the study of structures and properties of functional groups of organic compounds. In particular, aldehyde is a versatile functional group and synthetic building block in organic synthesis. Significant advancement has been made to demonstrate the key roles of aldehydes in synthetic chemistry through diverse organic transformation reactions including C-C, C-O, and C-N bond formations. Besides organic synthesis, aldehydes have many applications in industries such as disinfectants, food flavourants, fungicides and perfumes [3]. In this work, we

would like to expand the impact of aldehyde chemistry to chemical biology and food safety.

Our group has extensive research experience on the studies and applications of aldehyde chemistry on organic synthesis [4]. One of our research focuses is the synthesis of propargylamines by a three-component coupling reaction of aldehydes, amines and alkynes using gold catalysts owing to the high reactivity, exceptional selectivity and high tolerance under air and aqueous reaction conditions. Propargylamines are versatile synthetic intermediates for further modifications to nitrogen-containing heterocycles, natural products and therapeutic drug molecules. For examples, propargylamines can be further modified to allenes which are synthetic intermediates in organic synthesis and structural elements of bioactive natural products and pharmaceutical compounds [4].

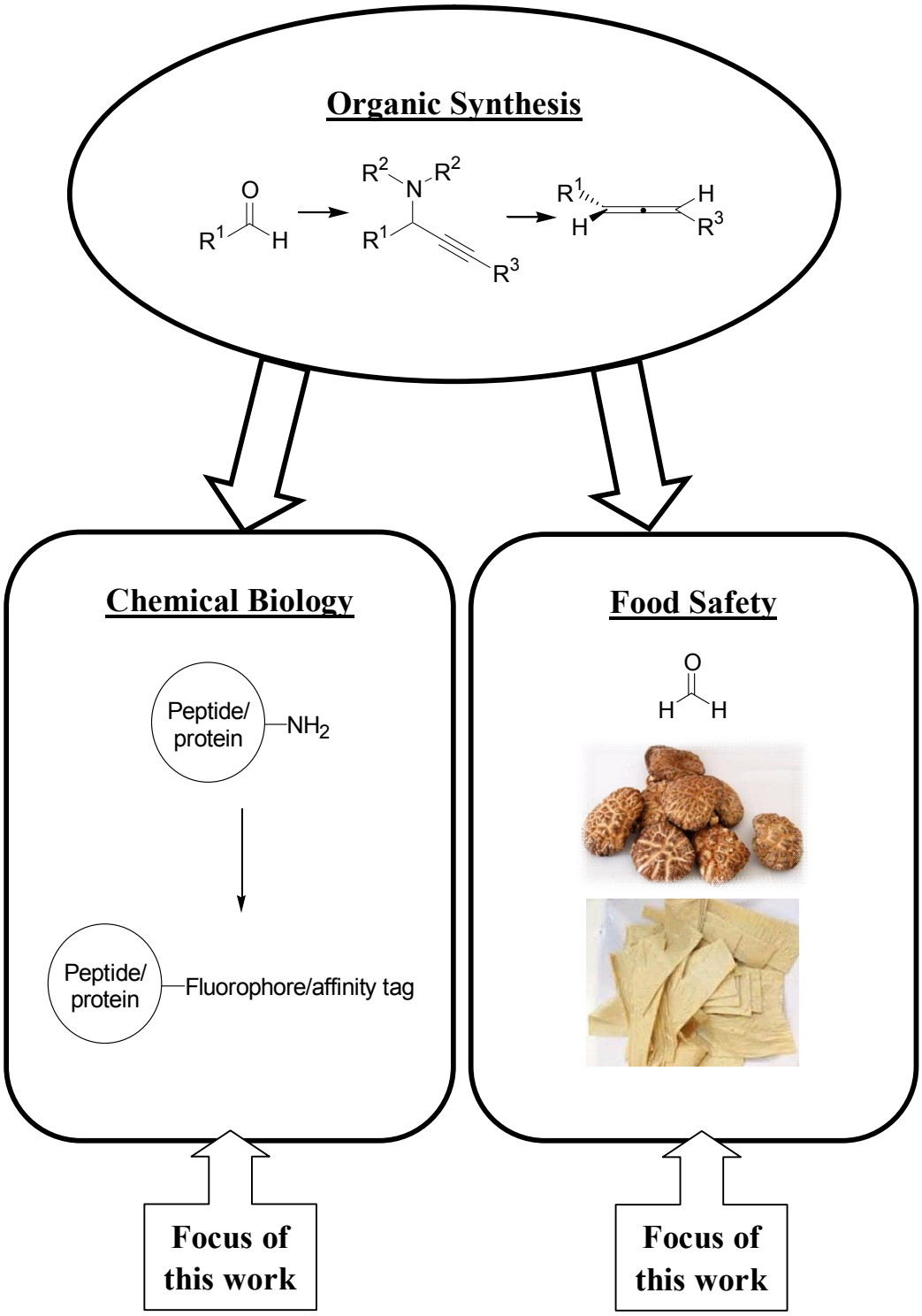
In recent decades, chemical biology, which is the study between the interface of chemistry and biology, has attracted a great deal of research interest as it provides essential tools for the synthesis of novel bioconjugates that are useful for studies on manipulation of complex biological processes and live cell

imaging [2b]. Built upon our past record and experiences on aldehyde-based synthetic chemistry, we plan to employ it as a core technology to create new molecules for the sake of understanding complex biological systems at the molecular level and providing unique molecular solutions to biomedical problems. Some of our previous works include modifications of oligosaccharides, peptides and proteins [5]. In this work, we plan to develop a mild and convenient method for N-terminal α-amino group modification of peptides. Since peptides and proteins have only one N-terminal α-amino group, modification at this site generally causes no significant influence on their biological activities.

In addition to chemical biology, we would like to expand the impact of aldehyde chemistry to the interdisciplinary research areas on food safety. Reports on "food poisoning" and "unsafe food" have repeatedly made the news headline in recent years. Food incidents can result in significant economic loss due to substantial amount of medical expenses and loss of productivity. Owing to rising living standards, globalization of food trade and advances in food technology, food safety is currently one of the most important issues need to be addressed. Being the smallest aldehyde in the nature, formaldehyde is inappropriately or illegally added to foods for bleaching, protein coagulation and preservation purposes,

causing a serious hazard to public health. Based on our previous studies and understanding of aldehyde chemistry, we plan to develop a method for fast and specific detection of formaldehyde in foods by fluorescence in this work. It is envisioned that the novel fluorescent probes exclusive for formaldehyde detection can be further developed into a simple and inexpensive rapid detection kit, which will not only enable the trade to implement front-line quality control, but also could assist the government agencies in high-throughput on-site food safety inspection.

A brief summary of our works is presented in the diagram below.



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CHAPTER 2: N-TERMINAL α-AMINO GROUP MODIFICATION OF PEPTIDES BY AN OXIME FORMATION / EXCHANGE REACTION SEQUENCE

2.1 Introduction

In the field of chemical biology, formation of bioconjugates by site-specific modification of peptides and proteins via covalent attachment of synthetic molecules under mild reaction conditions is a useful technique [1]. However, peptide and protein modification as well as other bioconjugations are a challenging task. To design the reaction conditions compatible with biomolecules, physiological conditions including a mild pH range of 6-8, a temperature range of 4-37 ^{o}C, and an aqueous medium should be used. Owing to the low concentrations (usually lower than 100 μM) of the biomolecules used, the bioconjugation reaction should be fast and efficient to minimize the use of additional reagents which may cause degradation to the biomolecules. Moreover, the reactions for modifications should be bioorthogonal which means the reagents do not cross-react with other biomolecules in the reaction medium [2].

One of the appealing approaches is site-specific modification of peptides and

proteins by using N-terminal α-amino group ligation [3-4]. In general, peptides and proteins have only one N-terminal α-amino group. Modification at this site usually causes no significant influence on their biological activities and does not produce a mixture of products. Therefore, N-terminal modification has many potential applications such as immobilization [5], bioconjugation [6], and positional proteomics studies [7].

2.1.1 Common N-Terminal Peptide Modification Approaches

2.1.1.1 Periodate Oxidation

The 2-amino alcohol of N-terminal serine (S) or threonine (T) could be oxidized by sodium periodate ($NaIO_4$) at pH 7 to form an aldehyde group, which is a bioorthogonal handle and could be further modified by hydrazides to give hydrazone-peptide conjugates [8], as shown in Figure 1 below.

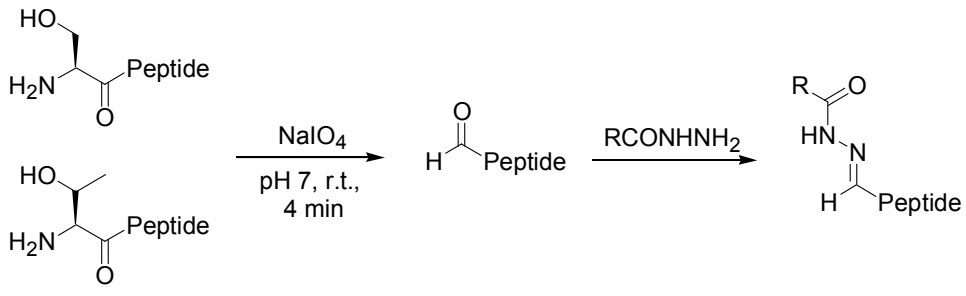


Figure 1. Oxidation of N-terminal serine or threonine by periodate and further modification by hydrazide.

For N-terminal serine residue, only a primary hydroxyl group is present. The oxidation reaction gives formaldehyde and forms an aldehyde group at the N-terminus. As long as there is no other group within the peptide or protein molecule which can be oxidized by periodate, this reaction is useful for the direct bioconjugation to a specific site on the biomolecules. If there is other periodate-oxidizable group present in the peptide or protein molecules such as carbohydrates, this method should not be used as it will lead to multi-site modification besides the N-terminus. To employ this strategy, a synthetic peptide segment bearing an N-terminal serine (S) or threonine (T) residue can be used to provide a site for conjugation [8].

If the N-terminal serine or threonine of a protein is to be oxidized by sodium periodate, dialysis or size exclusion chromatography can be used to remove the excess oxidizing agent easily. One the other hand, if a peptide is oxidized by periodate, the excess periodate is not easy to be removed from the reaction mixture. Reducing agents can be used in such case to quench the periodate oxidation. It should be noted that the reducing agent chosen should not reduce the aldehyde group formed. N-Acetylmethionine is one of the examples of

reducing agents [8]. The thioether group on the methionine side chain can easily be oxidized by periodate to form sulfoxide or sulfone. However, it does not affect the aldehyde formed at the N-terminus and it does not interfere with the subsequent conjugation. Sodium sulfite (Na_2SO_3) can be used as an alternative and a cheaper reducing agent to scavenge the remaining oxidant [9].

The oxidation reaction was rapid and mild as it required 4 minutes at room temperature only. Molecules with hydrazine or hydrazide groups can be incorporated to the peptides or proteins with the periodate-oxidized N-terminal aldehyde group. By doing so, potential side-reactions with the amino groups on lysine residue in the peptides or protein molecules can be avoided. The aldehyde group has a higher priority to react with the hydrazine or hydrazide group to form a hydrazone linkage even if other amines are present in the reaction mixtures [8].

The hydrazone products were stable at pH 6-8 which made them suitable for many biochemical applications. It should be noted that oxidation of the thioether group of internal methionine (M) to its sulfoxide was more favoured at low pHs. Although the oxidation reaction is compatible with internal histidine (H), tyrosine (Y) and tryptophan (W), peptides with internal cysteine (C) should have

it protected owing to its high sensitivity to oxidation [8]. Note that the aldehyde handle can also be modified by hydroxylamines in addition to hydrazones and hydrazides [1].

2.1.1.2 Pictet-Spengler Reaction

Pictet-Spengler reaction refers to the intramolecular condensation of an aromatic C-nucleophile and an iminium ion via acid catalysis [10]. In addition to its common usage in synthesis of indole alkaloids and isoquinoline, it can be used for N-terminal tryptophan (W) peptide ligation. Using acetic acid alone as the solvent, peptides with unprotected N-terminal tryptophan (W) could be ligated to peptides with C-terminal aldehyde in 24 hours [10], as shown in Figure 2.

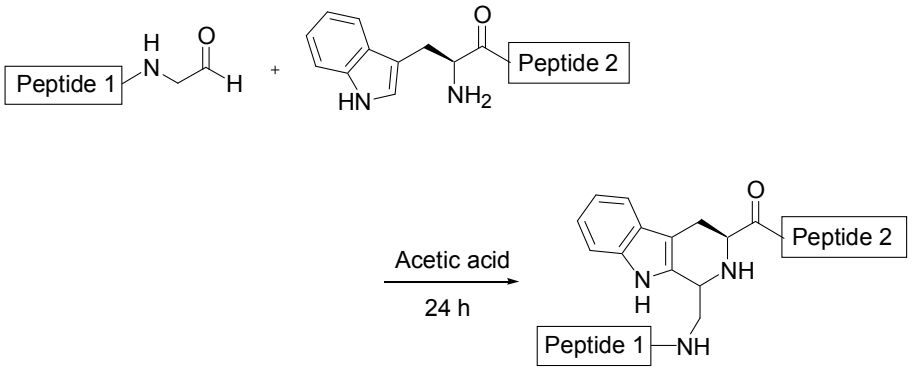


Figure 2. Pictet-Spengler reaction for N-terminal tryptophan peptide ligation.

The advantage of this method is that a peptide ligation product with a stable C-C

bond can be formed in one step. In addition, internal tryptophan residues on the same peptide are not affected which indicates the N-terminal selectivity of the reaction. Nevertheless, the peptide segment with a C-terminal aldehyde group has to be chemically synthesized for the ligation reaction which can be complicated [10].

2.1.1.3 Native Chemical Ligation

Native chemical ligation is a very useful approach for ligation of two unprotected peptides with a N-terminal cysteine and a C-terminal thioester, respectively (Figure 3). The thiol group of the N-terminal cysteine residue first nucleophilically attacked the C-terminal thioester to form a thioester-linked intermediate. Under the same reaction conditions, the intermediate rearranged spontaneously and rapidly by an intramolecular S→N acyl shift to form a native peptide bond [11].

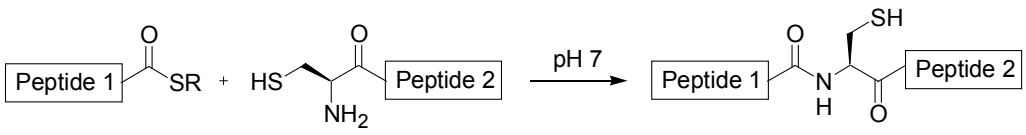


Figure 3. Native chemical ligation.

Since native chemical ligation is highly chemoselective, it is generally applicable

to ligation of unprotected peptides with all common functional groups. Other advantages of this reaction include one-step, rapid, and tolerant to solubilizing agents such as urea. However, the peptide segment carrying the C-terminal thioester has to be chemically synthesized to equip the thioester leaving group. It should be noted that the prevention of oxidation of the thiol of the N-terminal cysteine is important as the formation of disulfide linkage greatly reduce the reactivity of the thiol group towards thioester [11].

2.1.1.4 α-Ketoacid-Hydroxylamine Amide Ligation

α-Ketoacid-hydroxylamine amide ligation involved the decarboxylative condensation of an α-keto carboxylic acid and a N-alkylhydroxylamine to form an amide bond [12], as shown in Figure 4.

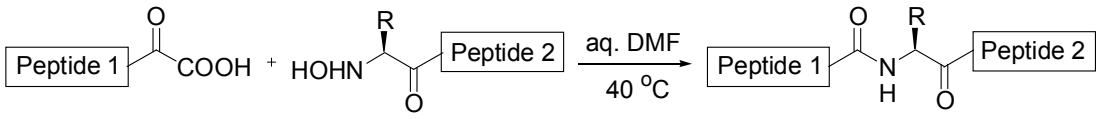


Figure 4. α-Ketoacid-hydroxylamine amide ligation.

This ligation method could proceed in both polar protic and aprotic solvents, usually aqueous dimethylformamide (DMF). In addition to requiring no catalysts

and reagents, the reaction could readily tolerate unprotected peptide segments and it produced water and carbon dioxide as by-products only. Although the reaction is convenient and chemoselective, the peptide segment with C-terminal α-ketoacid or N-terminal hydroxylamine has to be prepared chemically before performing the ligation reaction [12].

2.1.1.5 Biomimetic Transamination

This N-terminal peptide modification method involved the introduction of a reactive aldehyde or ketone group to the N-terminus of a peptide by pyridoxal-5-phosphate (PLP), which is a cofactor effecting many metabolic transformations under enzymatic control. The carbonyl handle was further modified by hydroxylamines or hydrazines for oxime formation (Figure 5) [13]. It should be highlighted that oxime and hydroxylamine groups are bioorthogonal to most amino acid residues [1].

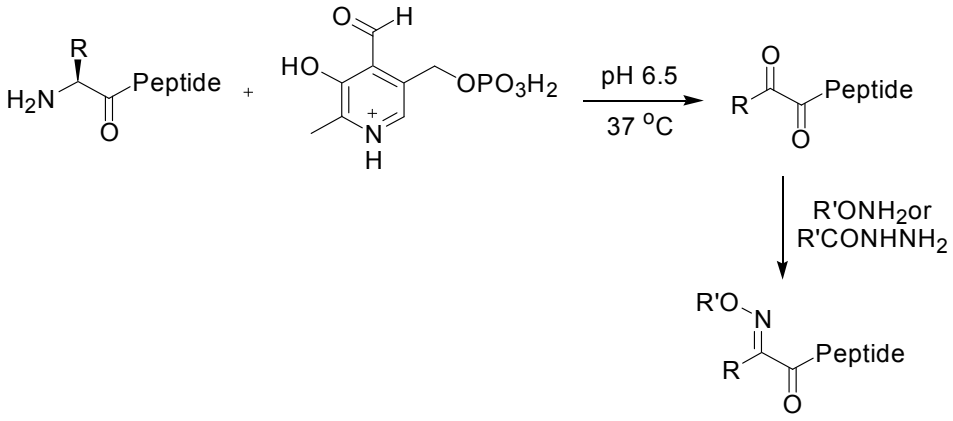


Figure 5. N-terminal peptide modification by a PLP-mediated biomimetic transamination followed by oxime formation.

Optimization of the above reaction revealed that most N-terminal amino acids could undergo the PLP-mediated transamination and gave the desired oxime products, especially glycine (G), alanine (A), glutamic acid (E) and asparagine (N). No modification was found on internal residues including lysine. Some amino acids such as aspartic acid (D), cysteine (C) and serine (S) underwent elimination to give the same oxime product derived by N-terminal alanine (A). Glutamine (Q) could undergo the transamination but was resistant to oxime formation. N-terminal amino acids tryphophan (W), histidine (H), lysine (K) and proline (P) gave covalent PLP adducts as the major products. The latter two adducts could still react with hydroxylamines to give the oxime products [13]. By careful choice of N-terminal residues, this PLP-mediated biomimetic transamination can be a powerful method for site-specific peptide and protein

modification.

2.1.1.6 Ketene Method

It was found by Wong and Che that oxidation of alkynes such as phenylacetylene by a manganese porphyrin [Mn(2,6-Cl_2TPP)Cl] [H_2(2,6-Cl_2TPP) = *meso*-tetrakis(2,6-dichlorophenyl)porphyrin] and oxone could generate ketenes which then reacted with amines or ammoniums to form amides (Figure 6). This protocol was modified for N-terminal α-amino group ligation of peptides [14].

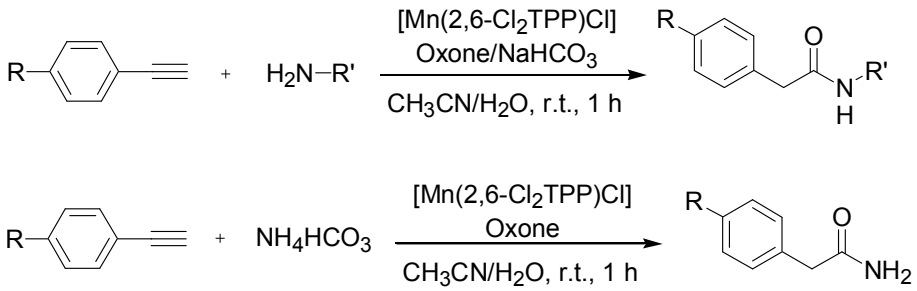


Figure 6. Oxidative amide synthesis by [Mn(2,6-Cl_2TPP)Cl].

Hydrogen peroxide (H_2O_2) was used instead of oxone for the sake of reducing salt concentration and facilitating the MS analysis of reaction mixtures. $NaHCO_3$ buffer was used to maintain a pH of 8.3 in the reaction medium. LC-MS/MS analysis revealed that an amide linkage was formed by the N-terminal α-amino group of the peptide YTSSSKNVVR and the alkyne with the side chains of

tyrosine, threonine, serine and lysine remained intact (Figure 7). This was the first example of acylation of peptides at N-terminal α-amino group without the modification of lysine in $NaHCO_3$ buffer [14].

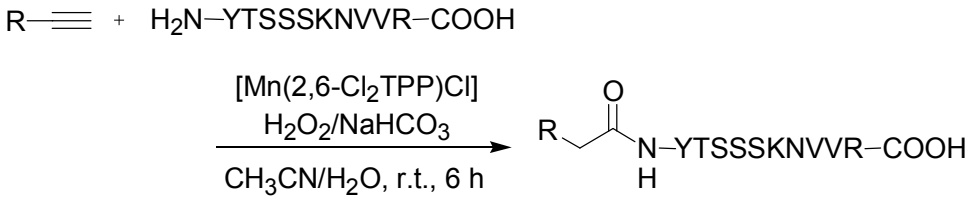


Figure 7. N-terminal α-amino group modification of peptide by [Mn(2,6-Cl_2TPP)Cl] and H_2O_2 in $NaHCO_3$ buffer.

Modifications of other five peptides GEQRKDVYVQLYL, SSCSSCPLSK, STSSSCNLSK, HDMNKVLDL and TYGPVFMSL for N-terminal acylations with phenylacetylene were also successfully achieved. Since the method is an oxidative reaction, intramolecular and intermolecular disulfide linkages were observed at cysteine residues. Sulfoxides were also formed due to the oxidation of methionine residues. Reducing agents such as N-methylmercaptoacetamide and dithiothreitol were suggested to reduce the oxidized cysteines and methionines back to free their free forms [14].

Based on the success of the N-terminal α-amino group modification of peptide

mentioned above, Wong and Che developed a method of employing an isolated ketene for highly selective N-terminal modifications of peptides and proteins under mild conditions followed by a copper-catalyzed click chemistry for functionalizations with azides (Figure 8) [14].

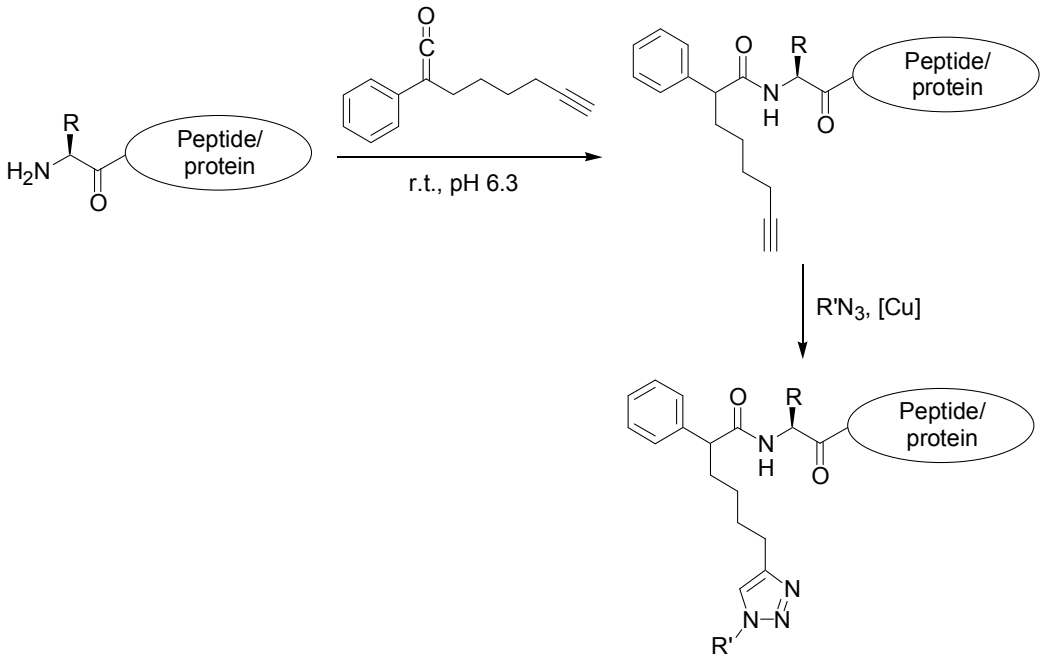


Figure 8. Modification of N-terminal α-amino groups of peptides and proteins by ketenes.

A library of 20 unprotected peptides XSKFR (where X represents the 20 natural amino acids) was modified by an alkyne-functionalized ketene at room temperature at pH 6.3. Excellent N-terminal selectivity (ratio of the modified N-terminal α-amino group to the modified ε-amino group of lysine = >99:1) was

observed in 13 out of the 20 peptides and moderate-to-high N-terminal selectivity (ratio of the modified α-amino group to the modified ε-amino group = 4:1 to 48:1) was observed in 6 out of the 7 remaining peptides, which included N-terminal arginine, lysine, cysteine, phenylalanine, leucine and isoleucine. For the peptide HSKFR which has a N-terminal histidine residue, a low N-terminal selectivity of 1:1 was observed [14].

When an alkyne-functionalized N-hydroxysuccinimide (NHS) ester was used to replace the alkyne-functionalized ketene for comparison, the N-terminal α-amino group selectivity dropped significantly (from 30:1 to 5:1) for most peptides. Excellent N-terminal selectivity (>99:1) was observed in the peptide with N-terminal tryptophan only [14].

To extend the scope of study, proteins including insulin, lysozyme, BCArg and RNaseA were modified using the alkyne-functionalized ketene. Compared to the formation of products with multi-site modifications by NHS ester, N-terminal modifications of the proteins were achieved using the ketene under the conditions of pH 6.3 at room temperature or pH 9.2 at 37 °C. The N-terminal modified proteins were further functionalized by an azide with a dansyl

fluorophore by a copper-catalyzed click chemistry [14].

As shown in above examples, there is a lack of general methods to chemoselectively modify all the N-terminal amino acids. There is a continuous interest in developing new approaches for site-specific peptide modification.

2.1.2 Oxidations of Amino Acids in Peptides and Proteins

Oxidative modification of amino acids in peptides and proteins plays a key role in aging and development of diseases. Free radical oxidation can cause damage to tissues which in turn causes cardiovascular diseases, cancers, neurological diseases, and many other problems. Oxidation of proteins can alter their three-dimensional structures. This changes their biological activities, inhibits normal protein interactions, and even causes cross-linking between proteins [15].

Sources of oxidative species which can cause peptide and protein oxidation include the by-products of the oxidative stress reactions in living cells, the reactive oxygen intermediates formed from metabolic reactions in cells, and the oxidizing compounds presence in a solution. Examples of chemical agents that can oxidize peptides and proteins include hydrogen peroxide (H_2O_2), superoxide

anion ($O_2^{-}\cdot$), singlet oxygen ($^1O_2$), hydroperoxyl radical ($HO_2\cdot$), hydroxyl radical ($\cdot OH$), periodate ion (IO_4^-), ozone (O_3), peroxynitrite ($ONOO^-$), some metal salts and metal-chelating compounds, and ultraviolet and gamma radiations [15].

The amino acid side chains are one of the potential oxidation sites in a peptide or protein molecule. Under exposure of oxidizing agents, aromatic amino acids primarily undergo addition reactions. On the other hand, aliphatic amino acids are usually oxidized to peroxy intermediates, which undergo hydrogen atom abstraction or elimination reactions to form hydroxyls, carbonyls, or peroxides [15].

Cysteine (C) and methionine (M) are the two most oxidation-sensitive amino acids. Upon oxidation, they undergo different sulfur oxidation reactions to form cysteine disulfides, methionine sulfone, methionine sulfoxide and other sulfonate products (Figures 9 and 10). Performic acid can oxidize cysteine and methionine in peptide and protein molecules to more stable products. Therefore, it is sometimes used intentionally before amino acid analysis and hydrolysis. Being the amino acids which are the most sensitive to oxidation, cysteine and methionine are usually served as an early oxidative damage indicator in protein

molecules [15].

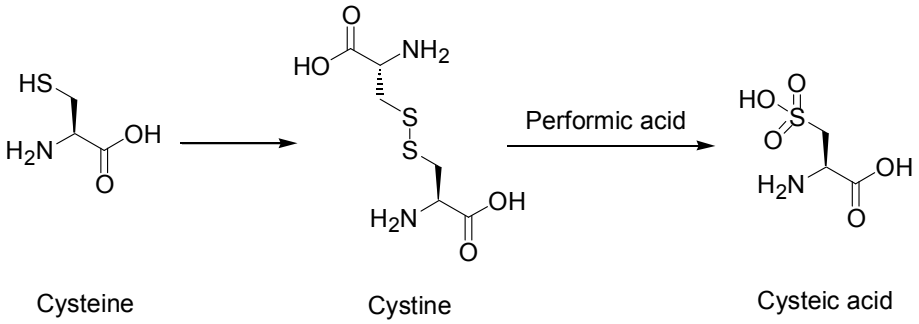


Figure 9. Oxidation of cysteine.

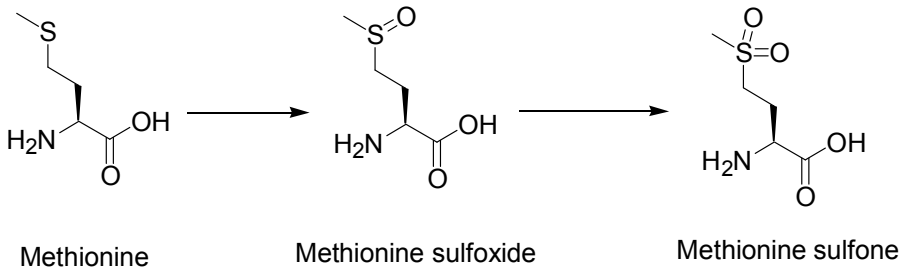


Figure 10. Oxidation of methionine.

The phenolic group of tyrosine (Y) can easily undergo chlorination, hydroxylation, nitrosation, or formation of tyrosine-tyrosine cross-linkage in the presence of oxidizing agents (Figure 11) [15]. The reaction which leads to formation of tyrosine dimer was studied extensively to improve the manufacture of phenolic polymer resins [16]. The conversion of tyrosine to tyrosine dimer by oxidation also inspired a group of researchers to study the protein-protein interactions of nickel-chelated six-His tagged fusion proteins under oxidative

environments [17].

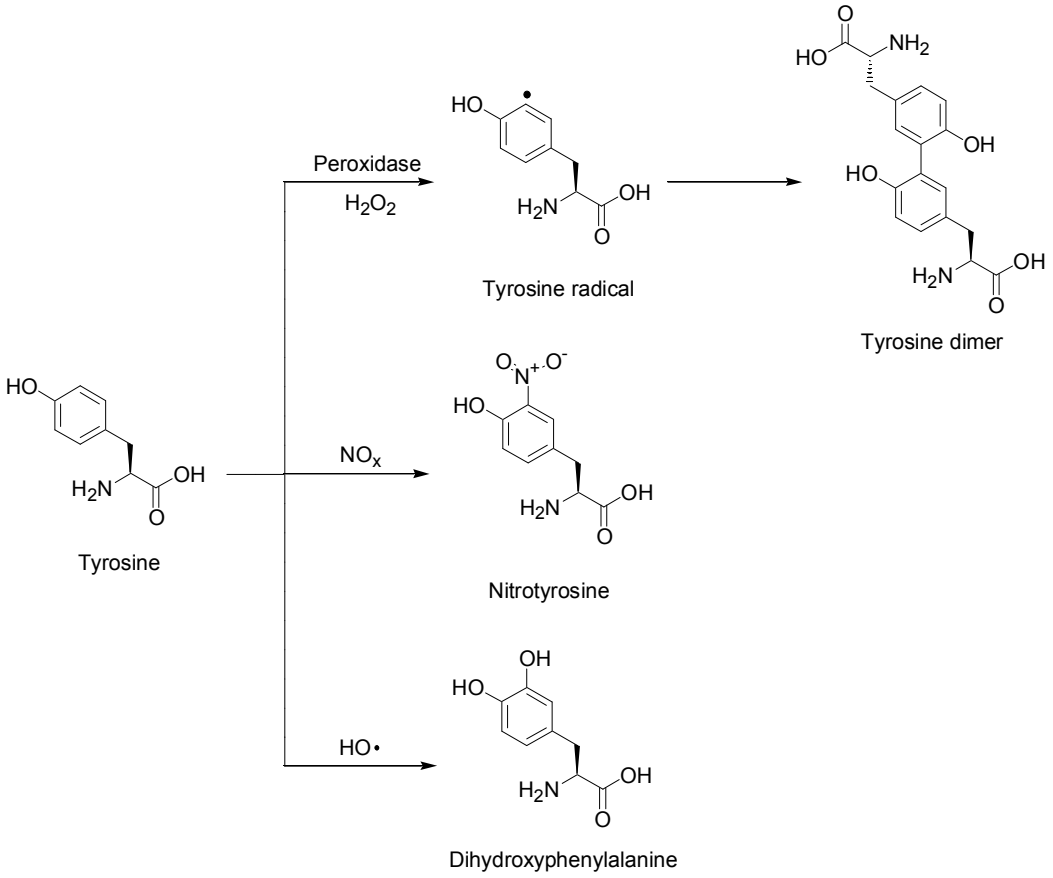


Figure 11. Oxidation of tyrosine.

Nitrogen-containing side chains of amino acids such as lysine (K), proline (P) and arginine (R) can be oxidized to form chloramines or become deaminated to give carbonyl or hydroxyl products (Figures 12-14) [15]. One example was to use Fe-EDTA-mediated human serum albumin oxidation to modify a number of amino acids such as lysine, histidine, arginine and proline to give an extensive formation of aldehydes and ketones [18].

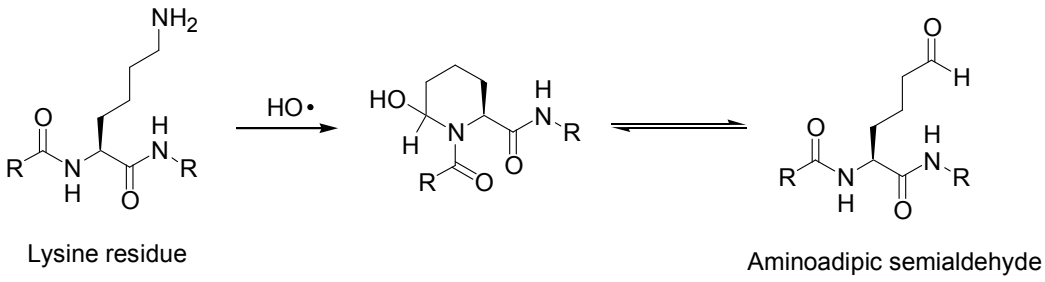


Figure 12. Oxidation of lysine.

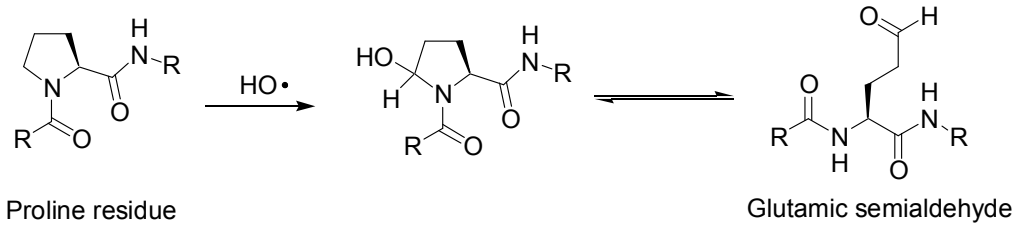


Figure 13. Oxidation of proline.

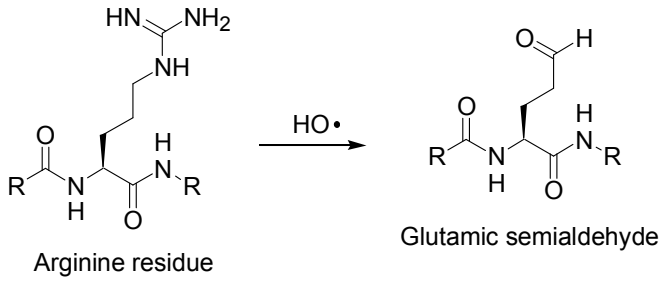


Figure 14. Oxidation of arginine.

Considerable effort has been spent on the studies of oxidative damages to peptides and proteins. Nevertheless, methods using oxidation for peptide modification are still limited due to the formation of complex oxidized products through uncontrolled oxidation reactions.

2.1.3 Oxone

Potassium peroxymonosulfate ($KHSO_5$) is an oxidizing agent which is potentially explosive and was once difficult to isolate and characterize. Now, it is commercially available under the trade name of Oxone or Caroat which is the stable triple salt ($2KHSO_5 \cdot KHSO_4 \cdot K_2SO_4$) of potassium peroxymonosulfate. Oxone is a water-soluble white crystalline solid which is non-toxic and cheap. The active oxidant is the anionic peroxymonosulfate (HSO_5^-), which only accounts for about 50% of the triple salt [19].

Oxone is an inexpensive but versatile reagent widely used in synthetic chemistry. It has a variety of applications including oxidation of C-H bonds, oxidation of alcohols, oxidation of aldehydes, oxidation of amines, oxidation of phenols, olefin functionalizations, halogenations, and many other miscellaneous reactions [19]. It was reported that oxone on silica gel or alumina could be used for selective oxidations of primary and secondary amines to their hydroxylamines at elevated temperatures with or without solvents. It was observed that the over-oxidation of primary amines to their corresponding oximes could occur (Figure 15) [20].

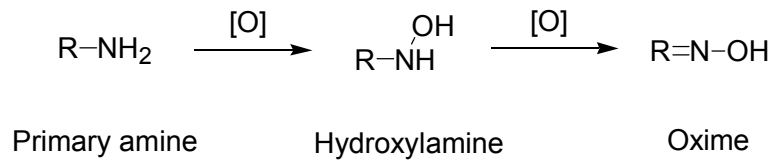
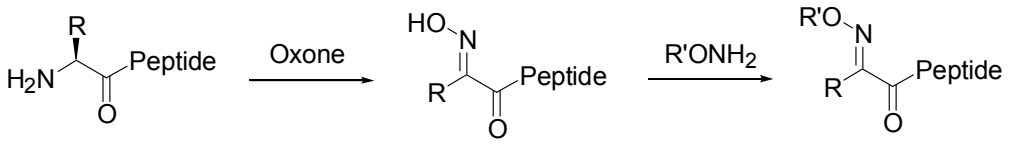


Figure 15. Oxidation of a primary amine to an oxime.

Disposal of the by-products of oxone do not pose immediate threats to aquatic ecosystem. The aqueous components of an oxone reaction should be converted to non-harmful sulfate salts by quenching with sodium bisulfite ($NaHSO_3$) and neutralization by sodium bicarbonate ($NaHCO_3$) since they are acidic and oxidizing. The relatively easy handling of the oxone after reaction makes it attractive to synthesis in large scales [19].

2.2 Objective

As mentioned above, oxone could be used for selective oxidations of primary and secondary amines. Along with our ongoing effort on the development of bioconjugation reactions [21], we would like to study whether oxone could be used as a convenient and chemoselective reagent for efficient and mild modification of N-terminal α-amino group of peptides [22]. The oxime product formed could be followed by a subsequent oxime exchange reaction [23] with *O*-substituted hydroxylamines for functionalizations, as shown in Scheme 1 below.



Scheme 1. N-terminal α-amino group modification by an oxime formation/exchange reaction sequence.

2.3 Results and Discussion

2.3.1 Optimization of Oxidation of N-Terminal α-Amino Group of Peptide

Different oxidants in aqueous solvents were used for selection of oxidants and optimization of reaction conditions for oxidation of N-terminus of peptide **1**. As shown in entries 4 and 6 of Table 1, oxone (200 μM, 2 equiv.) in PBS (25 mM, pH 8.0) and $NaHCO_3$ (1.5 mM, pH 8.3) solutions gave oxime-modified peptide **2** with excellent conversions, respectively. We found that 99% substrate conversion of oxime-modified peptide **2** was achieved for the N-terminal modification of peptide **1** YTSSSKNVVR (100 μM) with oxone (200 μM) and $NaHCO_3$ solution (1.5 mM, pH 8.3) at 25 ^{o}C for 1 hour (Figure 16). In PBS buffers at pH 7.1 and pH 9.3, 70% and 40% conversions were obtained (entries 3 and 5). No conversion to oxime-modified peptide **2** was observed under acidic pHs (entries 1 and 2).

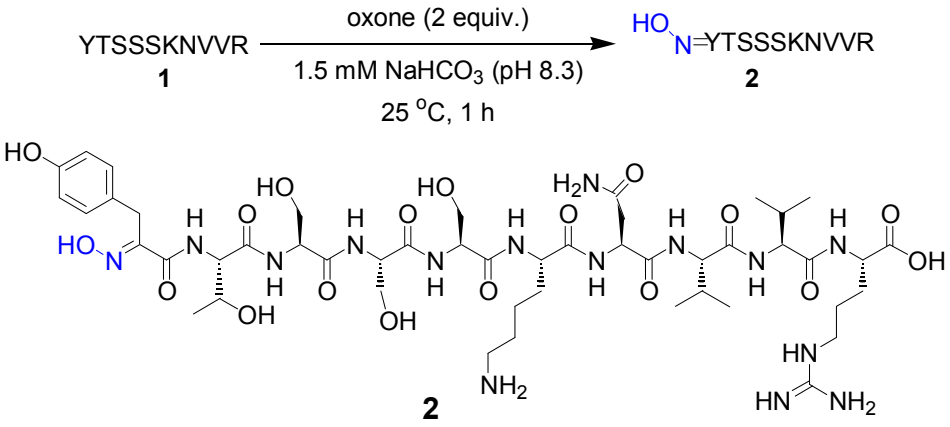


Figure 16. N-terminal α-amino group modification of peptide **1** using oxone in $NaHCO_3$ solution (1.5 mM, pH 8.3).

In $NaHCO_3$ solution (1.5 mM, pH 8.3), *m*-chloroperoxybenzoic acid (*m*-CPBA, 200 μM, 2 equiv.) oxidized the N-terminus of peptide **1** with 29% conversion (entry 7). By increasing the concentration of the oxidant to 2 mM, 85% conversion was obtained (entry 9). It should be noted that no oxime-modified peptide **2** was observed when H_2O_2, $NaIO_4$, $PhI(OAc)_2$, quinoline *N*-oxide, 2,6-dichloropyridine *N*-oxide or 2-bromopyridine *N*-oxide was used as the oxidant in $NaHCO_3$ solution (1.5 mM, pH 8.3) (entries 11-23).

Table 1. Screening conditions for N-terminal modification of peptide **1** by oxime formation[a]

Entry	Oxidant	Oxidant (equiv.)	Aqueous solvent	N-terminal modified peptide (%)[b]
1	oxone	2	PBS (pH 5.1)	0
2	oxone	2	PBS (pH 6.0)	0
3	oxone	2	PBS (pH 7.1)	70
4	oxone	2	PBS (pH 8.0)	96
5	oxone	2	PBS (pH 9.3)	40
6	oxone	2	$NaHCO_3$ (pH 8.3)	99
7	*m*-CPBA	2	$NaHCO_3$ (pH 8.3)	29
8	*m*-CPBA	5	$NaHCO_3$ (pH 8.3)	80
9	*m*-CPBA	10	$NaHCO_3$ (pH 8.3)	85
10	*m*-CPBA	20	$NaHCO_3$ (pH 8.3)	85
11	H_2O_2	5	$NaHCO_3$ (pH 8.3)	0
12	H_2O_2	10	$NaHCO_3$ (pH 8.3)	0
13	H_2O_2	20	$NaHCO_3$ (pH 8.3)	0
14	$NaIO_4$	5	$NaHCO_3$ (pH 8.3)	0
15	$NaIO_4$	10	$NaHCO_3$ (pH 8.3)	0

16	$NaIO_4$	20	$NaHCO_3$ (pH 8.3)	0
17	$PhI(OAc)_2$	2	$NaHCO_3$ (pH 8.3)	0
18	$PhI(OAc)_2$	5	$NaHCO_3$ (pH 8.3)	0
19	$PhI(OAc)_2$	10	$NaHCO_3$ (pH 8.3)	0
20	$PhI(OAc)_2$	20	$NaHCO_3$ (pH 8.3)	0
21	quinoline *N*-oxide	2	$NaHCO_3$ (pH 8.3)	0
22	2,6-dichloropyridine *N*-oxide	2	$NaHCO_3$ (pH 8.3)	0
23	2-bromopyridine *N*-oxide	2	$NaHCO_3$ (pH 8.3)	0

[a] Reactions were carried out with peptide **1** (100 μM) and oxidant in PBS (25 mM, pH 5.1-9.3) or $NaHCO_3$ solution (1.5 mM, pH 8.3) at 25 ^{o}C for 1 h. [b] Conversions were determined by LC-MS for all PBS solutions and by ESI-MS for $NaHCO_3$ solution.

ESI-MS/MS analysis of the crude reaction mixture showed that only the N-terminal α-amino group of tyrosine (Y) was modified while other amino acid residues such as serine (S), threonine (T), lysine (K), arginine (R) and asparagine (N) present in the peptide segment remained intact, as shown by the presence of their corresponding y ions in the MS spectrum in Figure 17.

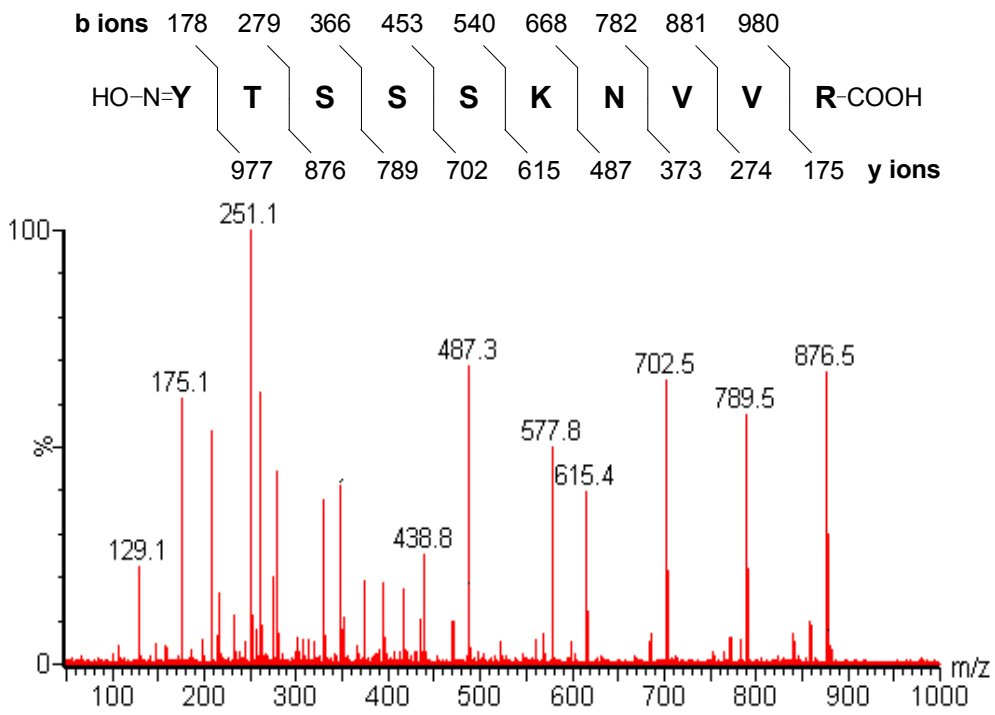


Figure 17. MS/MS spectrum of N-terminal oxime-modified peptide **2** (ESI source, doubly charged ion of m/z = 577.8).

The N-terminal selectivity of the oxidation can be explained by the difference in pK_a values of the amino groups of lysine and N-terminus. The pK_a value of lysine (K) ε-amino group is about 10, which is higher than that of N-terminal α-amino group (about 8) [1]. Under the reaction conditions (pH 8.3), the lysine (K) ε-amino group is largely protonated (~99%) and thus resistant to oxidation. On the contrary, about 50% of the N-terminal α-amino group remains in its free amine form, making it susceptible towards oxidation by oxone.

2.3.2 Time Courses of Oxidation of N-Terminal α-Amino Group of Peptide

Figures 18 and 19 showed the time course experiments for N-terminal α-amino group modification of peptide **1** by different oxone concentrations at 25 ^{o}C and 4 ^{o}C, respectively. As shown in Figure 18, over 99% conversions were achieved in 1 hour at 25 ^{o}C when 2-10 equivalents of oxone were used. Overall conversions of 20-55% were found in 1 hour with 1-5 equivalents of oxone when the time course experiments were performed at 4 ^{o}C (Figure 19). Using 2 equivalents of oxone as an example, there was about 60% difference in the conversions between the reaction performed at 25 ^{o}C and the one performed at 4 ^{o}C. If partial oxidation to the oxime-modified peptide is desired, reducing agents such as sodium sulfite (Na_2SO_3) can be added to the reaction mixture to quench the oxone.

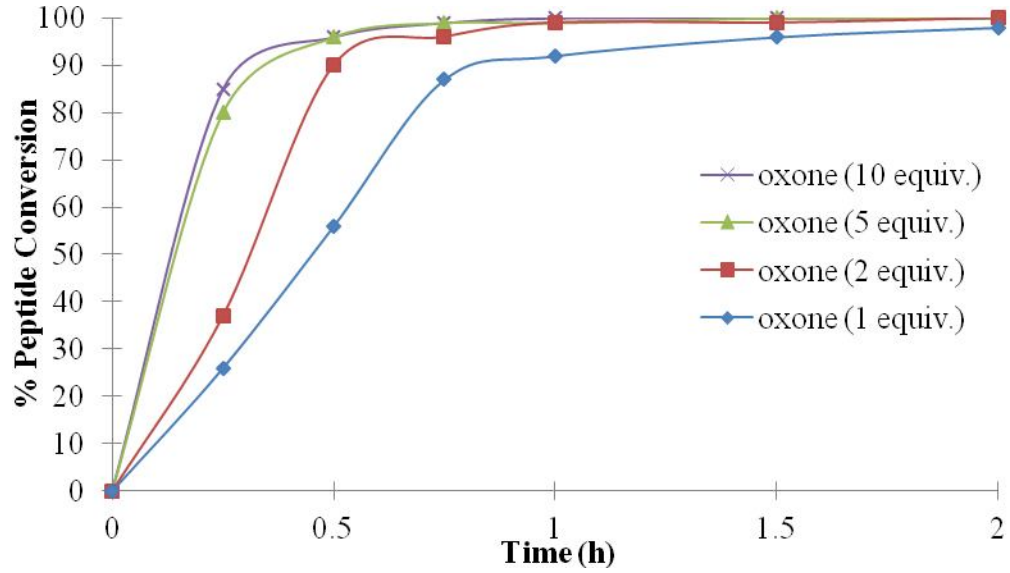


Figure 18. Time course of N-terminal α-amino group modification of peptide **1** (100 μM) with various concentrations of oxone in $NaHCO_3$ solution (1.5 mM, pH 8.3) at 25 °C.

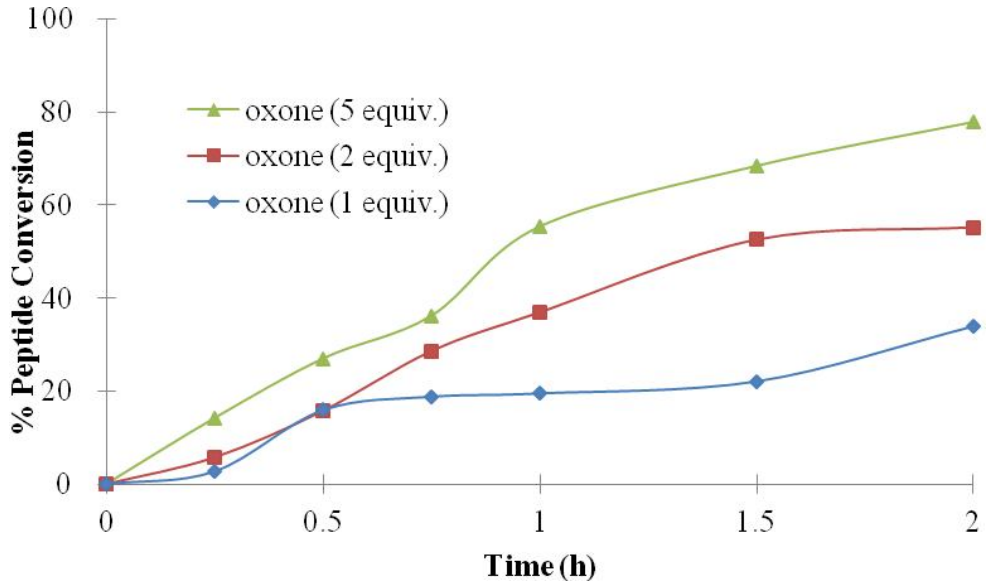


Figure 19. Time course of N-terminal α-amino group modification of peptide **1** (100 μM) with various concentrations of oxone in $NaHCO_3$ solution (1.5 mM,

pH 8.3) at 4 °C.

2.3.3 Model Reaction of Oxidation of N-Terminal α-Amino Group by Oxone

A model reaction with commercially available L-tyrosine methyl ester **3** was performed to provide support for the structure of the oxime-modified peptides (Figure 20). 91% conversion and 67% isolated yield were obtained for the corresponding oxime-modified product **4**.

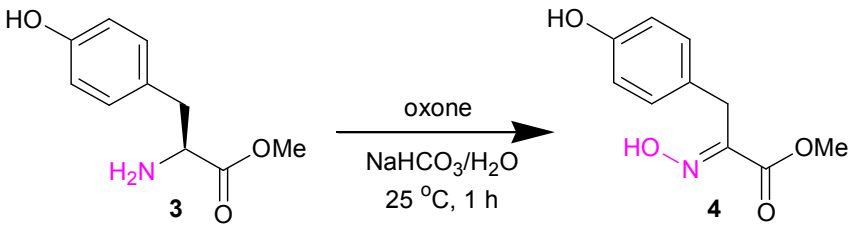


Figure 20. A model study using L-tyrosine methyl ester **3**.

2.3.4 Screening Reactions of Peptides Carrying 20 Different N-Terminal Amino Acids

The scope of the N-terminal modification was extended to a number of unprotected peptides XSKFR where X is one of the 20 natural amino acids. These peptides incorporated nucleophilic lysine (K) and serine (S) moieties were chosen to study the chemoselectivity of the oxidation reaction. As shown in Table 2, N-terminal modifications were found in all peptides XSKFR with conversions

ranging from 21 to 99% (entries 1-21 and 23 in Table 2). ESI-MS/MS analysis revealed that only the N-terminal amino acid of the peptides was modified.

Table 2. N-terminal α-amino group modification of peptides (XSKFR) carrying each of the 20 amino acids at the N-terminus by oxime formation[a]

Entry	Peptide	N-terminal α-amino group modified XSKFR (%)[b]	Both N-terminal α-amino group and side chain modified XSKFR (%)[b]
1	QSKFR	99	-
2	RSKFR	91	-
3	YSKFR	87	-
4	HSKFR	87	-
5	ESKFR	86	-
6	FSKFR	85	-
7	NSKFR	85	-
8	ASKFR	80	-
9	ISKFR	80	-
10	LSKFR	80	-
11	VSKFR	80	-
12	GSKFR	67	-

13	KSKFR	52	-
14	DSKFR	45	-
15	SSKFR	40	-
16	TSKFR	21	-
17	PSKFR	99[c]	-
18	WSKFR	40	38[d]
19[e]	WSKFR	16	6[d]
20[f]	WSKFR	38	41[d]
21	MSKFR	-	55[g]
22	CSKFR	-	0[h]
23[i]	CSKFR	-	99[j]

[a] Reactions were carried out with XSKFR (100 μM), oxone (200 μM) in $NaHCO_3$ solution (1.5 mM, pH 8.3) at 25 ^oC for 1 h. [b] Conversions were determined by ESI-MS. [c] Pyrrolidine ring of proline (P) was oxidized to nitrone. [d] Both N-terminal α-amino group and indole of tryptophan (W) were oxidized to give a 1,3-dihydro-indol-2-one-oxime derivative. [e] *m*-CPBA (200 μM) was used instead of oxone. [f] *m*-CPBA (500 μM) was used instead of oxone. [g] Both N-terminal α-amino and thioether groups of methionine (M) were oxidized to give a sulfone-oxime derivative. [h] Thiol group of Cys (C) was oxidized to

sulfonic acid. [i] Oxone (500 μM) was used. [j] N-terminal α-amino and thiol group of Cys (C) were oxidized to oxime and sulfonic acid, respectively.

The pyrrolidine ring of N-terminal proline (P) of PSKFR was oxidized to nitrone (entry 17 in Table 2) (Figure 21) [24]. For the peptide carrying N-terminal tryptophan (W), both N-terminal α-amino group and indole of tryptophan were oxidized to give a 1,3-dihydro-indol-2-one-oxime derivative (entries 18-20 in Table 2) (Figures 22 and 23).

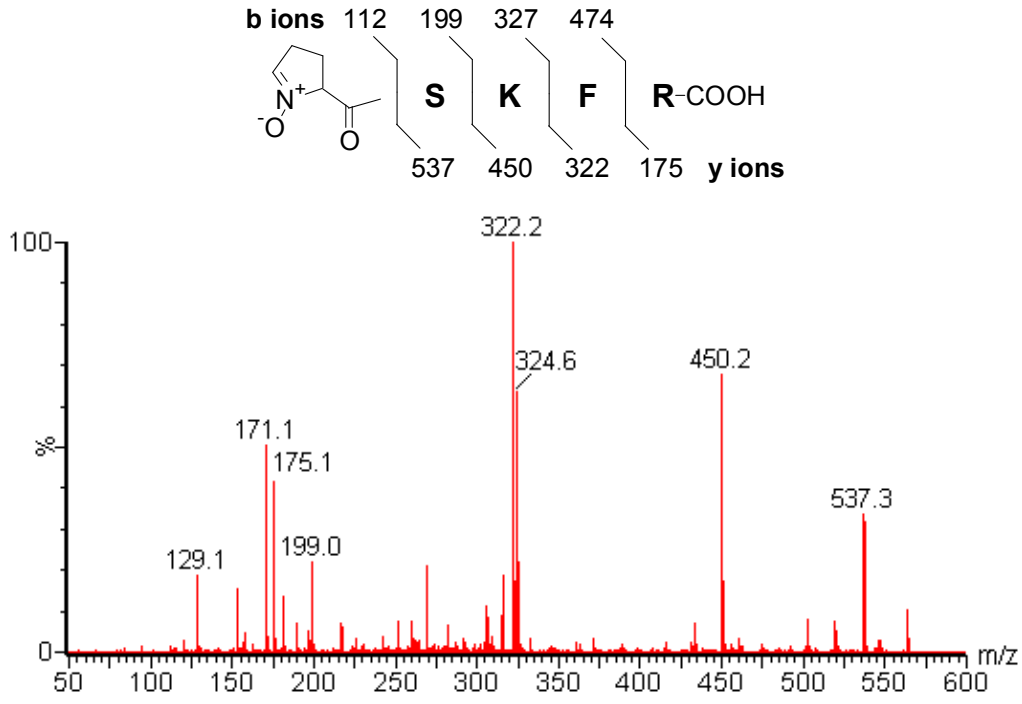


Figure 21. MS/MS spectrum of N-terminal modified peptide PSKFR (ESI source, doubly charged ion of m/z = 324.6).

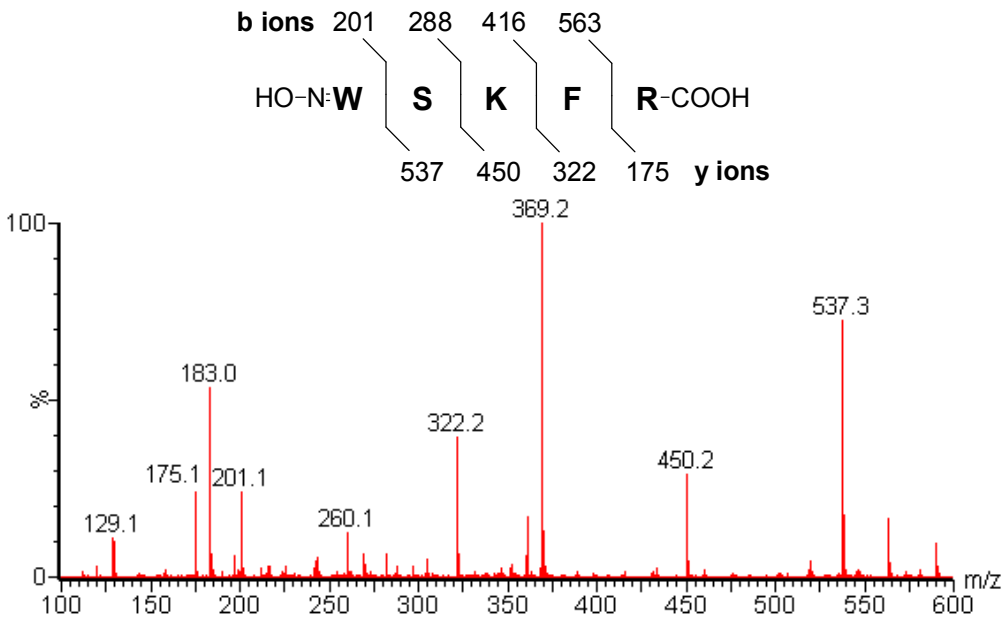


Figure 22. MS/MS spectrum of N-terminal oxime-modified peptide WSKFR (ESI source, doubly charged ion of m/z = 369.2).

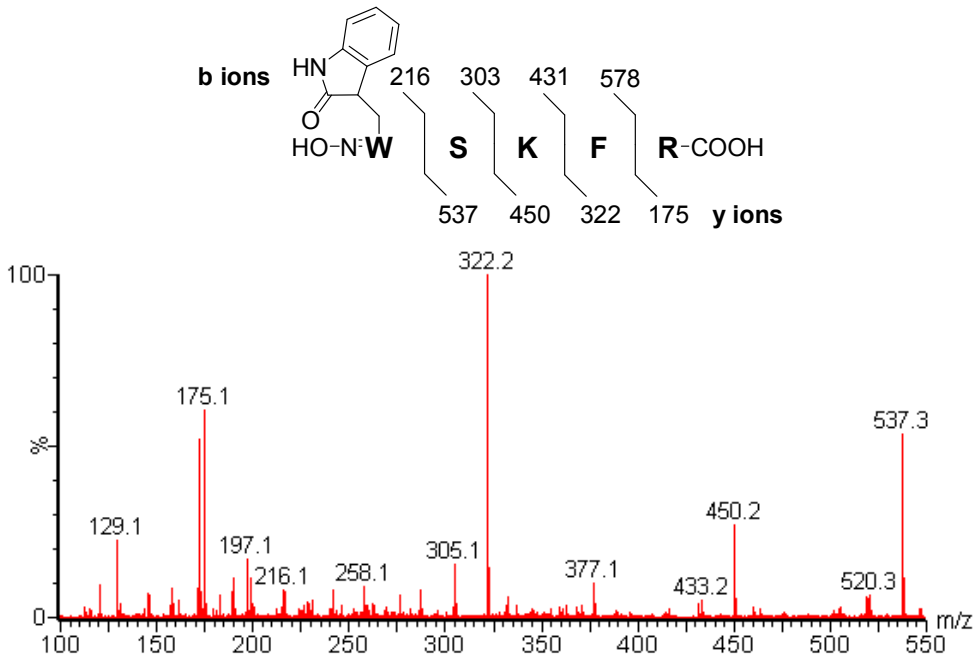


Figure 23. MS/MS spectrum of N-terminal modified peptide WSKFR (ESI source, doubly charged ion of m/z = 377.1).

For the peptide carrying N-terminal methionine (M), both N-terminal α-amino and thioether groups of methionine were oxidized to give a sulfone-oxime derivative (entry 21 in Table 2) (Figures 24 and 25).

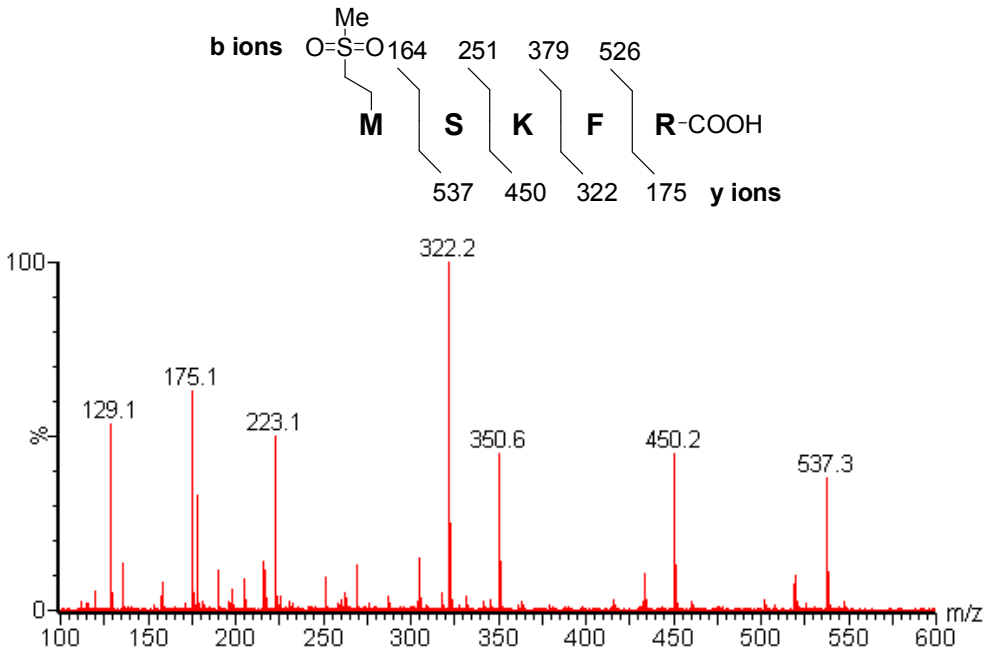


Figure 24. MS/MS spectrum of N-terminal modified peptide MSKFR (ESI source, doubly charged ion of m/z = 350.6).

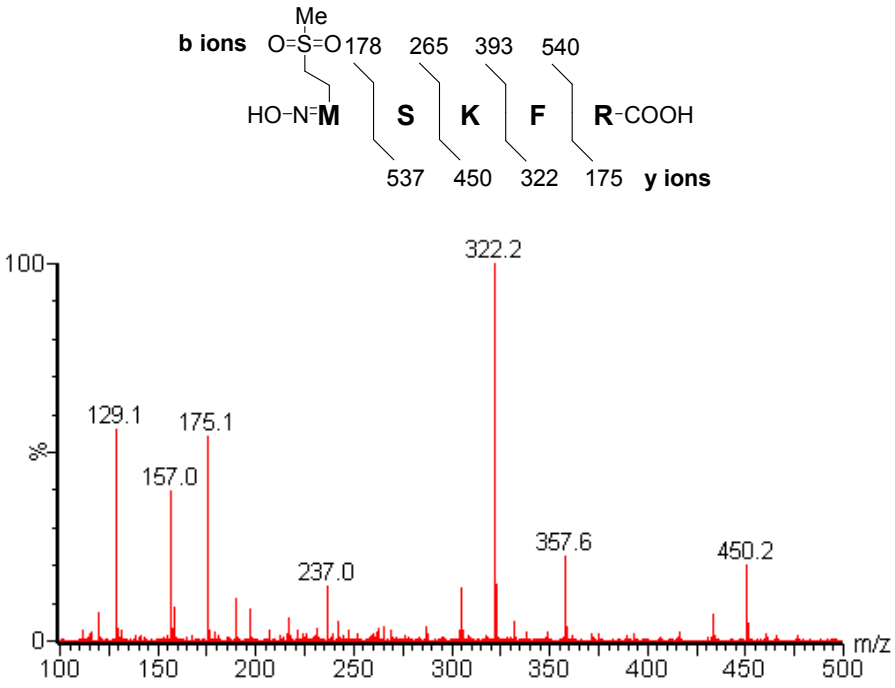


Figure 25. MS/MS spectrum of N-terminal modified peptide MSKFR (ESI source, doubly charged ion of m/z = 357.6).

Only the thiol group in CSKFR was oxidized to give sulfonic acid when 200 μM of oxone was used (entry 22 in Table 2) (Figure 26). When the oxone concentration was increased to 500 μM, the N-terminal α-amino and thiol group of cysteine (C) were oxidized to oxime and sulfonic acid, respectively (entry 23 in Table 2) (Figure 27).

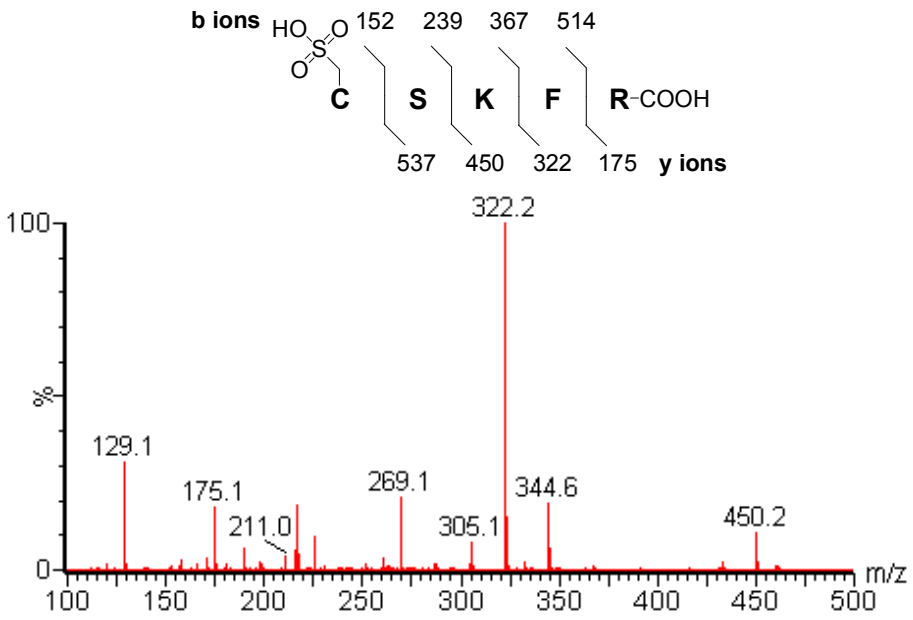


Figure 26. MS/MS spectrum of N-terminal modified peptide CSKFR (ESI source, doubly charged ion of m/z = 344.6).

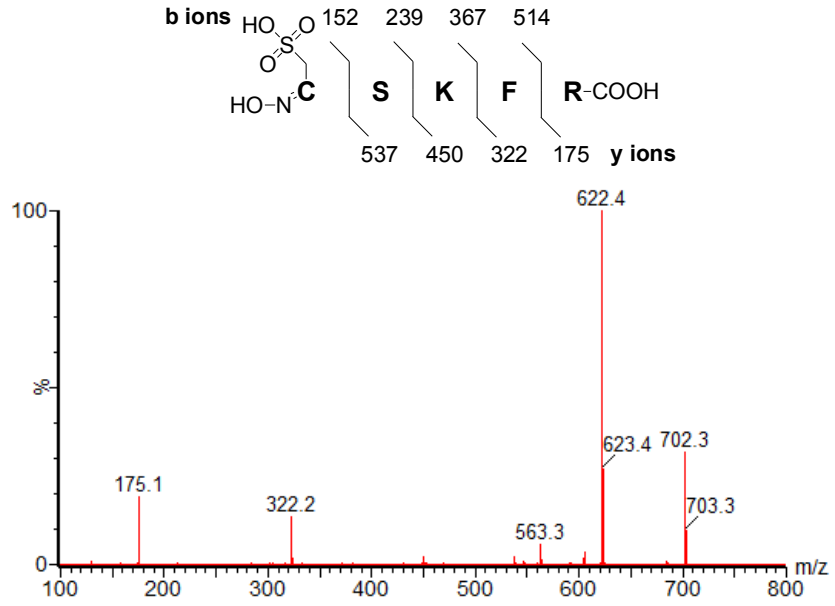


Figure 27. MS/MS spectrum of N-terminal modified peptide CSKFR (ESI source, singly charged ion of m/z = 702.3).

The common methods for N-terminal peptide and protein modification are applicable to a few N-terminal amino acids only, for examples, modifications of N-terminal serine (S) or threonine (T) by periodate oxidation [8] and N-terminal cysteine (C) by native chemical ligation [11], respectively. It should be highlighted that our N-terminal α-amino group modification of peptides by using oxone provides a convenient and chemoselective method for introduction of an oxime handle to the N-terminus of virtually all the 20 natural amino acids via a simple and mild oxidation reaction.

2.3.5 Peptide Functionalization by an Oxime Exchange Reaction

An oxime exchange reaction [23] between the oxime-modified peptide **2** and *O*-substituted hydroxylamines was studied and performed for sequential peptide functionalization, as shown in Figure 28. The mechanism of the oxime exchange reaction is shown in Figure 29 [25].

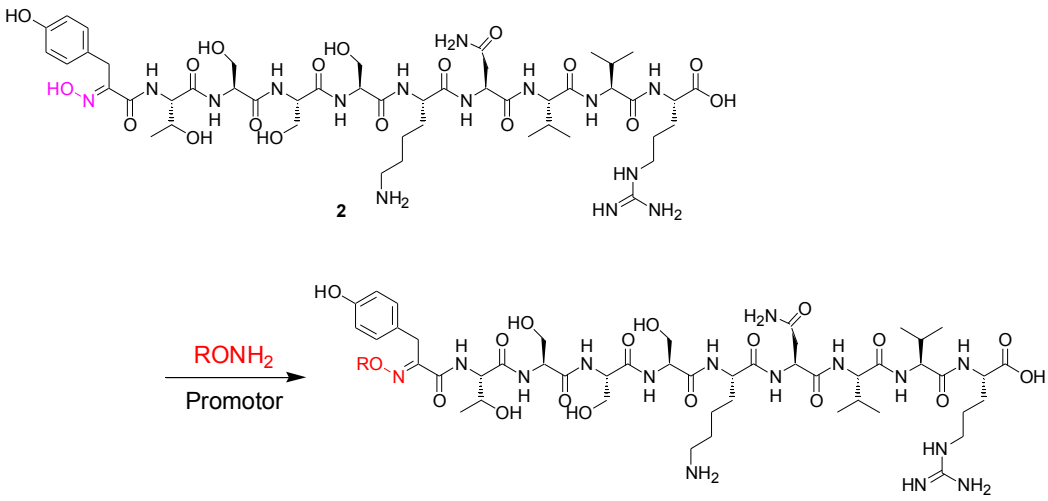


Figure 28. Oxime exchange reaction between the oxime-modified peptide **2** and *O*-substituted hydroxylamines using different promoters.

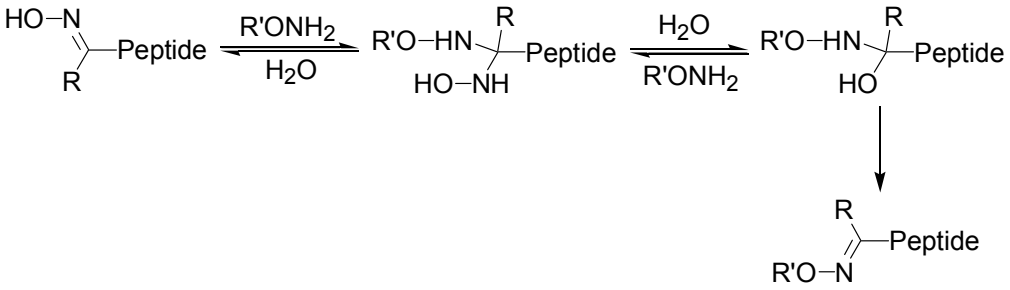


Figure 29. Mechanism of the oxime exchange reaction between the oxime-modified peptide and *O*-substituted hydroxylamine.

Different promoters, amounts of hydroxylamine, and reaction temperatures were investigated for optimization. Methoxylamine and *O*-benzylhydroxylamine were used as the hydroxylamines for the investigation. The MS spectra for the formation of the hydroxylamine-functionalized peptides were shown in Figures 30 and 31 below.

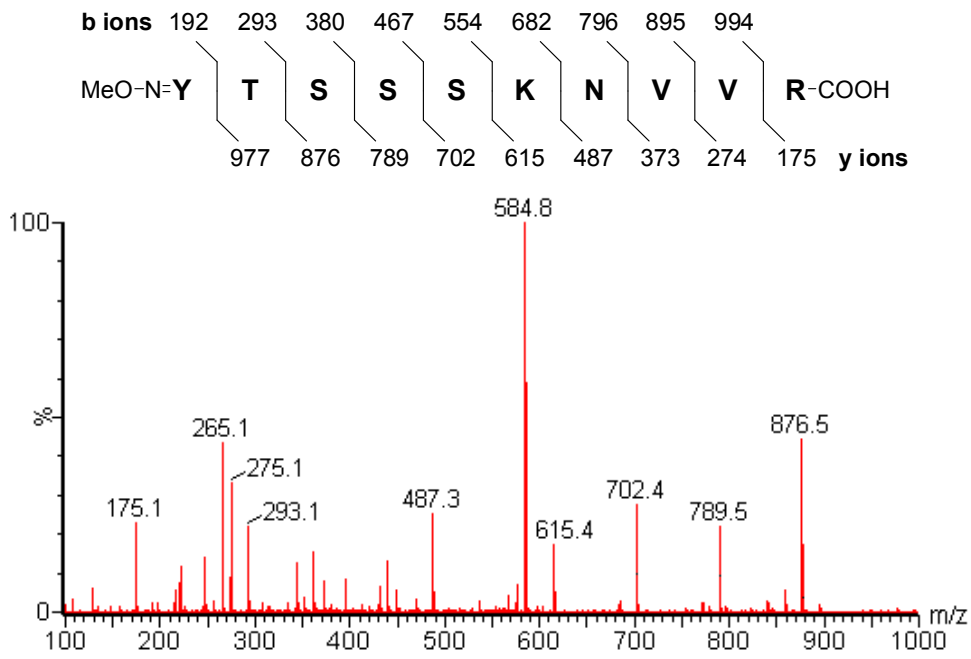


Figure 30. MS/MS spectrum of N-terminal methoxylamine-functionalized peptide YTSSSKNVVR (ESI source, doubly charged ion of m/z = 584.8).

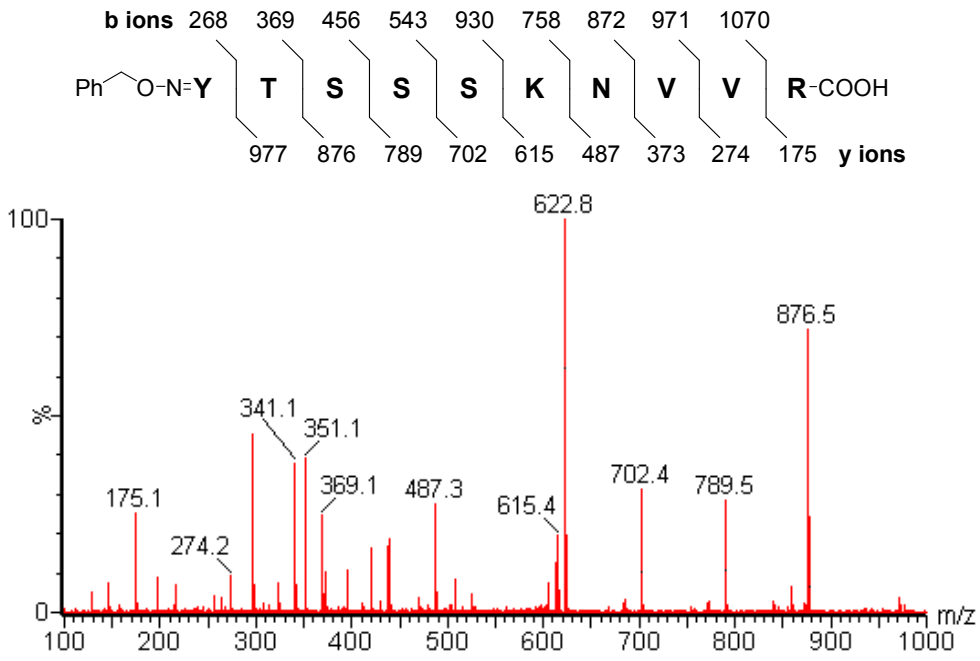


Figure 31. MS/MS spectrum of N-terminal *O*-benzylhydroxylamine-functionalized peptide YTSSSKNVVR (ESI source, doubly charged ion of m/z = 622.8).

In general, conversions increased with increased amounts of hydroxylamine (Table 3). It was observed that the transoximation of oxime-modified peptide **2** with *O*-benzylhydroxylamine could be achieved by using trifluoroacetic acid (0.65 equiv.) as the promoter [23] in 50 ^{o}C for 20 hours to give *O*-benzyloxime-modified peptide in 98% conversion (entry 1 in Table 3). In addition, the oxime exchange reaction progressed well with aniline and *p*-anisidine as the promoters under neutral reaction conditions [26], giving *O*-benzyloxime-modified peptides in up to 96% and 93% conversions,

respectively (entries 9 and 23 in Table 3).

Table 3. Screening conditions for transoximation of oxime-modified YTSSSKNVVR

Entry[a]	Promotor	Promotor (equiv.)	$RONH_2$	$RONH_2$ (equiv.)	Temperature (^oC)	Conversion of peptide 2 (%)[b]
1	CF_3COOH	0.65	$PhCH_2ONH_2$	20	50	98
2	CF_3COOH	0.65	$PhCH_2ONH_2$	50	50	97
3[c]	CF_3COOH	0.65	$PhCH_2ONH_2$	50	50	33
4	CF_3COOH	0.13	$PhCH_2ONH_2$	50	50	46
5	aniline	100	$MeONH_2$	1000	25	3
6	aniline	100	$MeONH_2$	1000	40	80
7	aniline	100	$PhCH_2ONH_2$	1000	25	2
8	aniline	100	$PhCH_2ONH_2$	1000	40	82
9	aniline	100	$PhCH_2ONH_2$	1000	50	96
10[b]	aniline	100	$PhCH_2ONH_2$	1000	50	25
11	aniline	100	$PhCH_2ONH_2$	100	50	23
12	aniline	100	$PhCH_2ONH_2$	500	50	62
13	aniline	200	$PhCH_2ONH_2$	200	50	6

14	aniline	200	$PhCH_2ONH_2$	500	50	29
15	aniline	200	$PhCH_2ONH_2$	1000	50	55
16	aniline	500	$PhCH_2ONH_2$	200	50	3
17	aniline	500	$PhCH_2ONH_2$	1000	50	52
18	aniline	1000	$PhCH_2ONH_2$	200	50	0
19	aniline	1000	$PhCH_2ONH_2$	500	50	25
20	aniline	1000	$PhCH_2ONH_2$	1000	50	46
21	*p*-anisidine	100	$PhCH_2ONH_2$	100	50	10
22	*p*-anisidine	100	$PhCH_2ONH_2$	500	50	37
23	*p*-anisidine	100	$PhCH_2ONH_2$	1000	50	93
24[b]	*p*-anisidine	100	$PhCH_2ONH_2$	1000	50	21

[a] Unless otherwise specified, the reaction time was 20 hours. [b] Determined by ESI-MS. [c] 4 hours.

Incorporation of functionalized hydroxylamines such as dansyl (**5a**) and polyethylene glycol (PEG) (**5b**, average molecular weight = 500) groups onto oxime-modified peptide **2** was performed and functionalized peptides **6a** and **6b** were obtained in 99% conversions (Figure 32). The MS spectra of the N-terminal dansyl-functionalized and PEG-functionalized peptides **6a** and **6b** were shown in

Figures 33 and 34, respectively. Functionalization of peptides with fluorophores allows molecular imaging for biological studies. PEGylation of peptides facilitates the alteration of its solubility and bioavailability such as reducing their degradations by the proteolytic enzymes in the body [27]. It is forecasted that various functionalities can be introduced to peptides via our N-terminal modification method for studies and applications in the field of chemical biology.

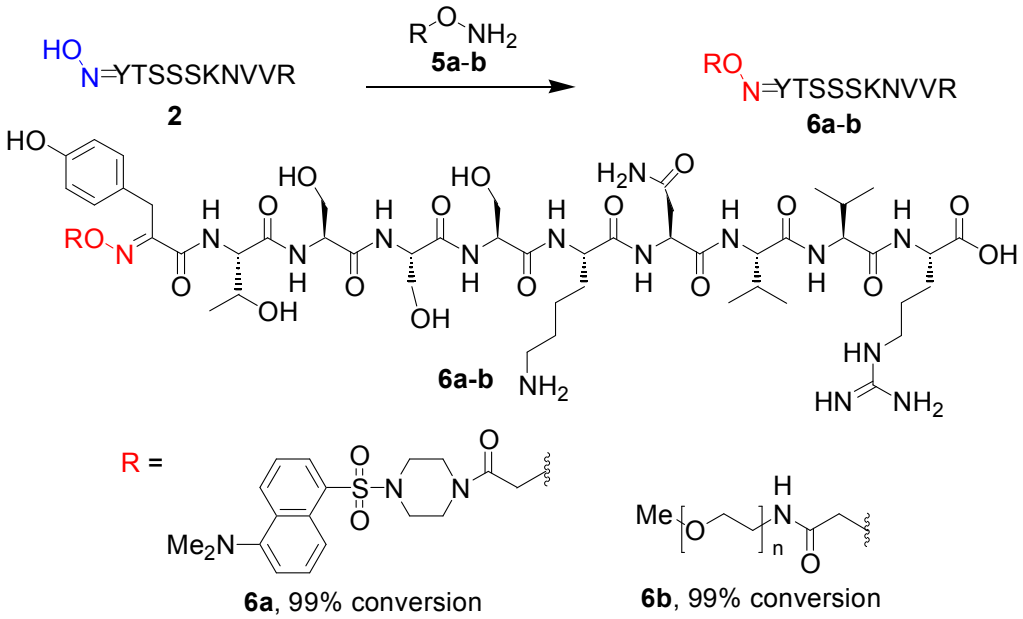


Figure 32. Oxime exchange of oxime-modified peptide **2** by functionalized hydroxylamines **5a-b**.

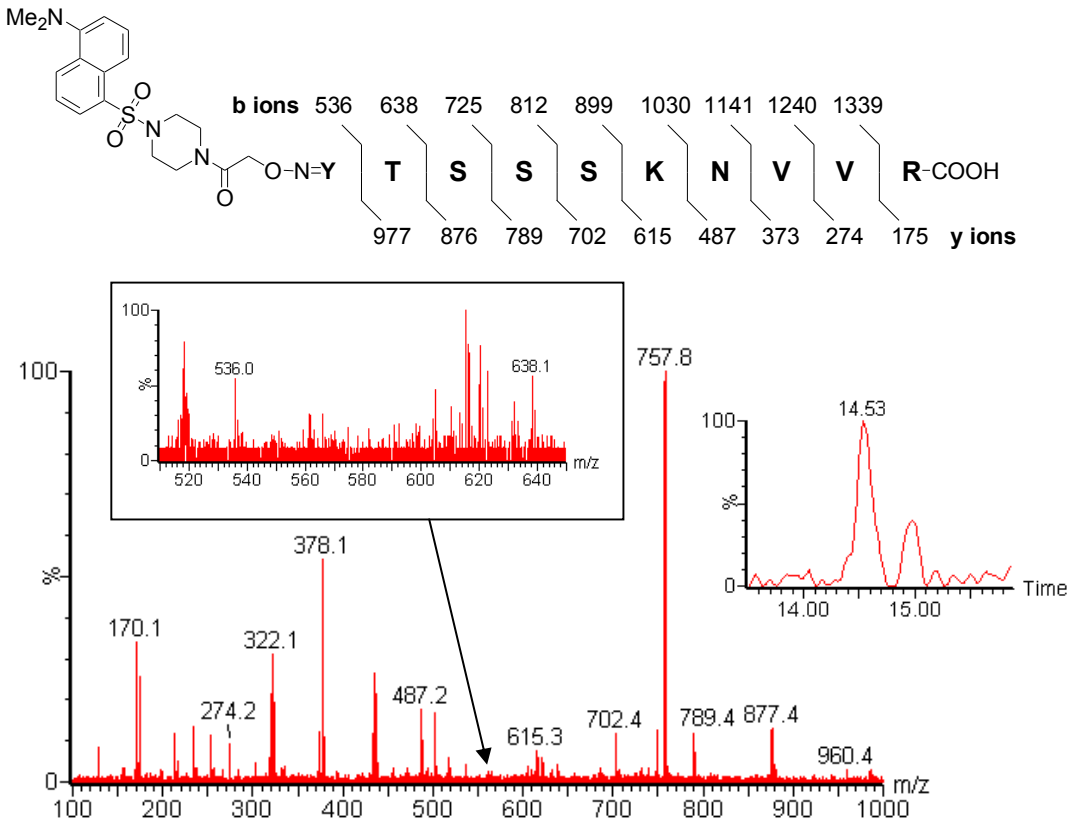


Figure 33. MS/MS spectrum of N-terminal dansyl-functionalized peptide YTSSSKNVVR (ESI source, doubly charged ion of m/z = 757.8) and the XIC chromatogram at t = 14.53 min (inset).

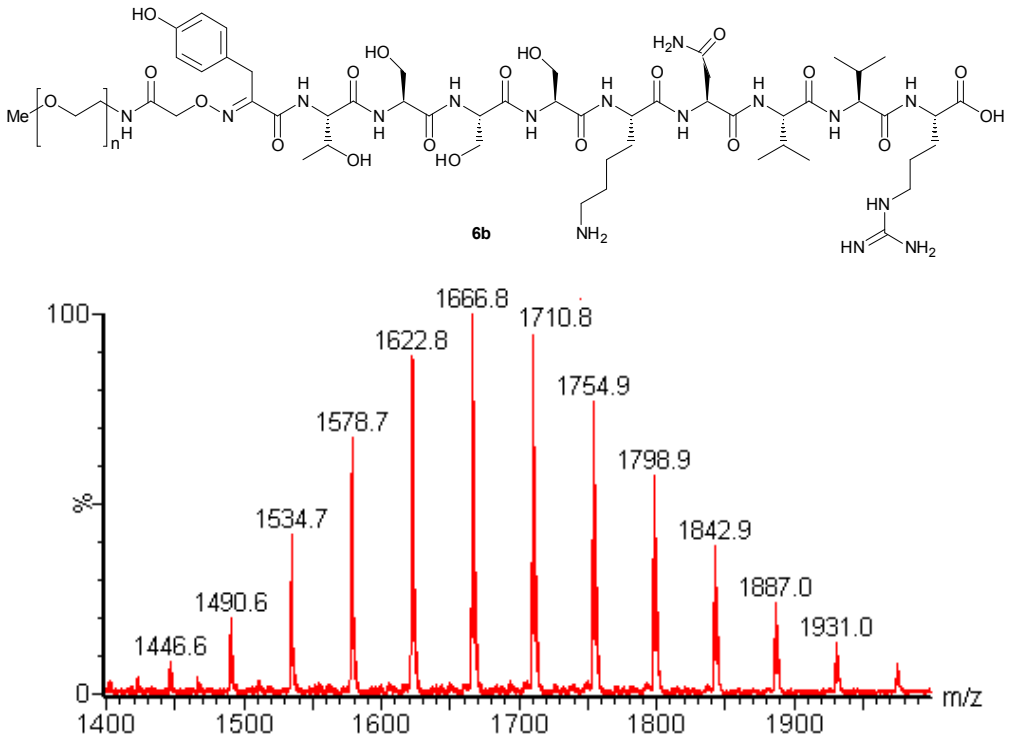


Figure 34. MALDI-TOF spectrum of N-terminal PEG-functionalized peptide YTSSSKNVVR (ESI source, singly charged ions of m/z = 1446.6-1931.0).

This work on N-terminal α-amino group modification of peptides by an oxime formation/exchange reaction sequence was published in *Chemical Communications* [28].

2.4 Conclusion

In conclusion, we have developed a highly selective method for N-terminal modification of peptides by an oxime formation/exchange reaction sequence. The present work demonstrates the potential of oxone as a chemoselective oxidizing reagent for N-terminal modification of peptides.

2.5 Suggestions for Future Research

In the present work, a highly selective method for N-terminal modification of peptides by an oxime formation/exchange reaction sequence was developed. The direction for future research will be to extend the method to N-terminal α-amino group modification of protein molecules. Since proteins are more sensitive to pHs and oxidizing agents, the reaction conditions for oxone oxidation of proteins have to be studied and optimized to avoid the alternation of the protein structure after modification.

The reaction conditions for the sequential oxime exchange for protein functionalization should be studied as well. This is because the presence of highly acidic trifluoroacetic acid will likely denature protein molecules. Although excellent conversions can also be achieved with aniline and *p*-anisidine as the promoters under neutral reaction conditions, the reactions require 1000 equivalent of the *O*-substituted hydroxylamine which has to be dissolved in organic solvents. Other methods for oxime exchange should be investigated to minimize the use of organic solvents in the reaction as the presence of organic solvents may alter the protein structures.

2.6 Experimental Section

2.6.1 General Procedure

All reagents were commercially available and used without further purification. Milli-Q® water used as reaction solvent in peptide modification and LC-MS was deionized using a Milli-Q® Gradient A10 system (Millipore, Billerica, USA). Flash column chromatography was performed using silica gel 60 (230-400 mesh, ASTM). ^{1}H and ^{13}C NMR spectra were recorded on Bruker DPX-400, DPX-600, Varian Unity Inova 400 NB and 500 NB spectrometers. The chemical shifts are expressed in ppm and coupling constants are given in Hz. Data for ^{1}H NMR are recorded as follows: chemical shift (δ, ppm), multiplicity (s, singlet; br s, broad singlet; d, doublet; dd, doublet of doublet; t, triplet; td, triplet of doublets; m, multiplet), coupling constant (Hz), integration. Data for ^{13}C NMR are reported in terms of chemical shift (δ, ppm). Low resolution mass spectra (MS) and high resolution mass spectra (HR-MS) were obtained on Waters Micromass Q-Tof 2™ with positive ESI in terms of mass to charge ratio (m/z).

ESI-MS Analysis of Peptide Modification

The mass spectra were performed over a m/z range of 100-2000 on Waters Micromass Q-Tof 2™ with positive ESI, and the raw spectra were deconvoluted

by the MassLynx 4.1 Transform Program (Waters, Manchester, UK). Desolvation and source temperatures were 150 °C and 80 °C respectively. Operating conditions optimized for the detection of reaction mixture were the following: capillary voltage 3 kV, sample cone voltage 30 V, extraction voltage 4 V and collision cell voltage 10 eV.

LC-MS Analysis of Peptide Modification

Mass spectrometry analysis was performed using the ESI source of Q-Tof 2™ (Waters-Micromass, Manchester, UK) in the positive ion mode. The CapLC® system (Waters, Manchester, UK) was equipped with a Poroshell 300SB-C18 column (1.0 mm ID × 75 mm , 5μm) with ZORBAX Poroshell guard column (1.0 mm ID × 17 mm, 5 μm) (Agilent-Technologies Inc., Wilmington, USA). Mobile-phase A was made of 0.5% formic acid in Milli-Q® water. Mobile-phase B was made of 0.5% formic acid in acetonitrile. 2 μL of sample was injected with a flow rate of 40 μL/min at 25 ^{o}C. The initial conditions for separation were 3% B for 3 min, followed by a linear gradient to 70% B by 30 min, 3% B by 31 min then to 3% B by 45 min. The mass spectrometer was performed over a m/z range of 200-2000, and the raw spectra were deconvoluted by the MassLynx 4.1 Transform Program (Waters, Manchester, UK). Desolvation and source

temperatures were 150 °C and 80 °C respectively. Operating conditions optimized for the detection of reaction mixture were the following: capillary voltage 3 kV, sample cone voltage 30 V, extraction voltage 4 V and collision cell voltage 10 eV.

MALDI-TOF-MS Analysis of Peptide Modification for PEG-modified Peptide 6b

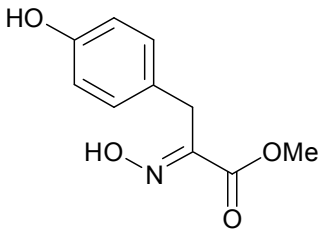
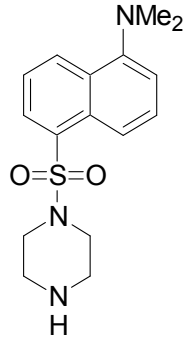
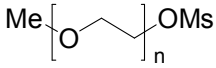
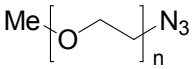
An aliquot of 10 μL of sample was mixed with 10 μL of 10 mg/mL CHCA matrix in acetonitrile/water (1:1) with 0.1% TFA. An aliquot of 1 μL of the sample-matrix mixture was spotted onto the stainless steel target plate and air-dried. The target plate was then mounted onto a MALDI Micro-MX Time-of-Flight mass spectrometer (Waters, Milford, MA) for analysis. The laser of the MALDI source was a 337 nm pulse laser (Model 337Si-63, Spectra Physics, Mountain View, CA) operating at a pulse frequency of 10 Hz. The mass spectrometer was operated in positive and reflectron mode. The flight tube and reflectron voltage of the TOF mass analyzer were set at +12 kV and −5.2 kV, respectively. The extraction voltage and extraction delay were set at 2 kV and 500 ns respectively. The mass spectrometry analysis was performed over a m/z range of 500-2000.

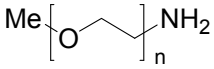
Calculation of Peptide Conversion

The crude reaction mixture of unmodified peptides (peptide) and modified peptides (product) was subjected to LC-MS or ESI-MS analysis. After data processing by MassLynx 4.1 Transform Program, peptide conversion at different time intervals was determined by measuring the relative peak intensities of peptide and product in the mass spectrum as follows:

$$Peptide\ Conversion\ (\%) = \left(1 - \frac{Relative\ Intensity\ of\ Peptide}{Relative\ Intensities\ of\ Peptide\ and\ Product}\right) \times 100\%$$

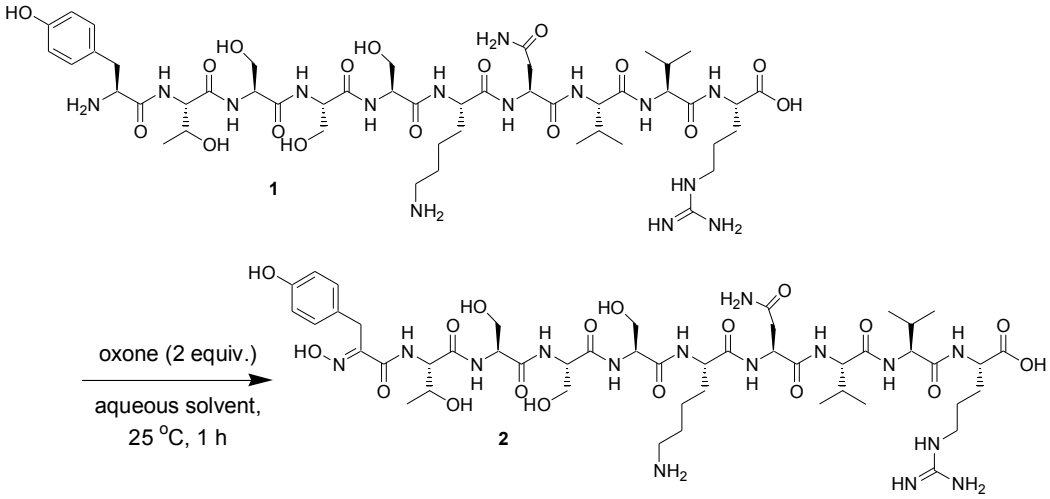
2.6.2 Literature References of Compounds

 **4**	N. Kotoku, H. Tsujita, A. Hiramatsu, C. Mori, N. Koizumi and M. Kobayashi, *Tetrahedron*, 2005, **61**, 7211-7218.
 N-Dansylpiperazine	V. Sashuk, D. Schoeps, and H. Plenio, *Chem. Commun.*, 2009, **7**, 770-772.
 **5bb**	(a) B. C. Mei, K. Susumu, I. L. Medintz and H. Mattoussi, *Nat. Protoc.*, 2009, **4**, 412-423; (b) K. Susumu, B. C. Mei and H. Mattoussi, *Nat. Protoc.*, 2009, **4**, 424-436.
 **5bc**	(a) B. C. Mei, K. Susumu, I. L. Medintz and H. Mattoussi, *Nat. Protoc.*, 2009, **4**, 412-423; (b) K. Susumu, B. C. Mei and H. Mattoussi, *Nat. Protoc.*, 2009, **4**, 424-436.

 **5bd**	(a) B. C. Mei, K. Susumu, I. L. Medintz and H. Mattoussi, *Nat. Protoc.*, 2009, **4**, 412-423; (b) K. Susumu, B. C. Mei and H. Mattoussi, *Nat. Protoc.*, 2009, **4**, 424-436.

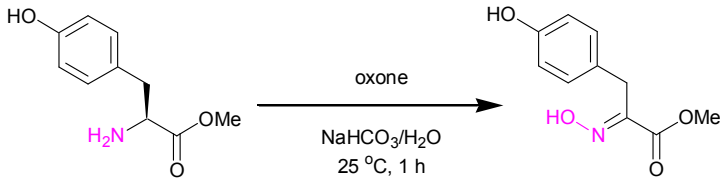
2.6.3 Experimental Procedure

Procedure for Oxidative N-Terminal Modification of Peptide 1



A mixture of peptide **1**, YTSSSKNVVR, (10 μL of 1 mM in H_2O), oxone (2 μL of 10 mM in water), and $NaHCO_3$ (5 μL of 30 mM in water, 1.5 mM) in water (83 μL) was stirred at 25 °C for 1 hour. The resulting mixture (10 μL) was diluted with 90 μL water and 1% formic acid in acetonitrile (100 μL). The diluted mixture was analyzed by ESI-MS analysis for determination of the peptide conversion. Site-specific modified peptide **2** was determined by ESI-MS/MS analysis.

Procedure for Model Study of N-Terminal Modification of L-Tyrosine Methyl Ester



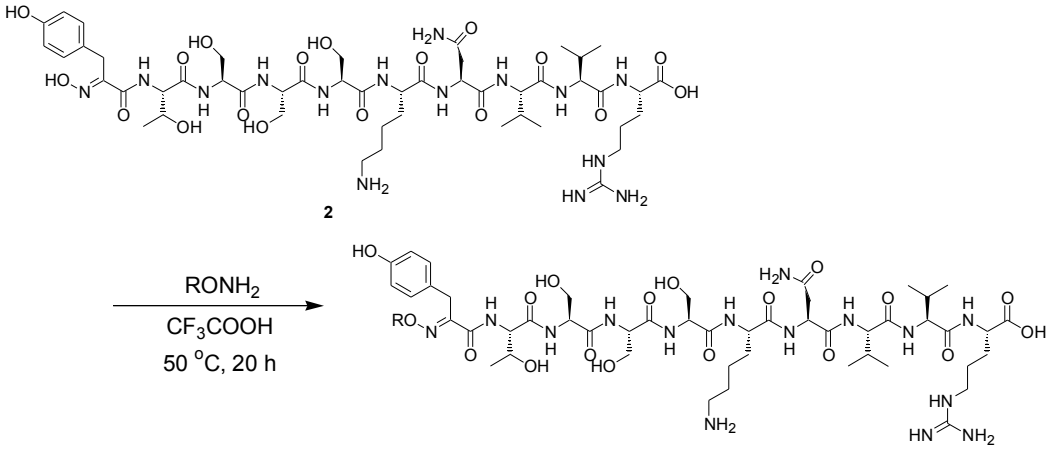
3 **4**

To a round-bottom flask containing L-tyrosine methyl ester **3** (0.1 mmol) and $NaHCO_3$ (1.5 mmol) in water (10 mL) was added oxone (0.2 mmol). After stirring at 25 °C for 1 hour, the reaction mixture was extracted with dichloromethane (15 mL × 3). The combined organic layer was dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography using ethyl acetate/n-hexane to give product **4** in 67% isolated yield.

Characterization Data of 4

White powder; analytical TLC (silica gel 60) (80% ethyl acetate/n-hexane), R_f = 0.69; ^{1}H NMR (400 MHz, CD_3OD) δ 7.07 (d, J = 8.5 Hz, 2H), 6.66 (d, J = 8.5 Hz, 2H), 3.82 (s, 2H), 3.76 (s, 3H); ^{13}C NMR (100 MHz, CD_3OD) δ 164.81, 155.78, 151.11, 129.79, 127.29, 115.00, 51.50, 29.08; ESI-MS *m/z* 210 $[M+H]^+$.

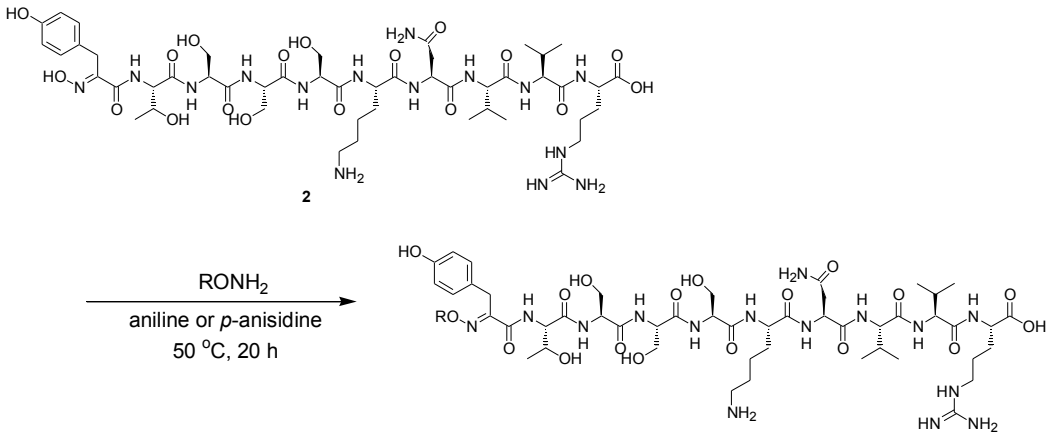
Procedure for Transoximation of Oxime-modified YTSSSKNVVR using Trifluoroacetic Acid



2

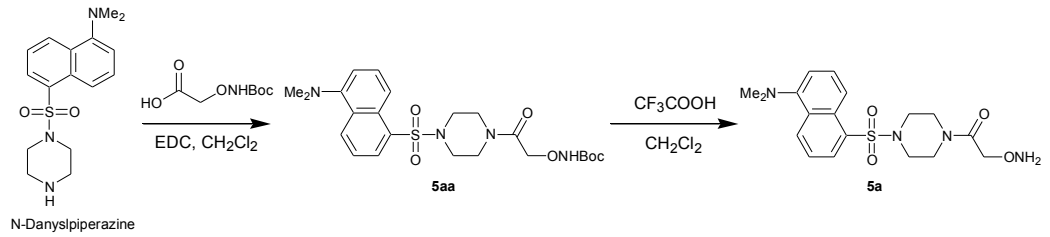
A mixture of oxime-modified YTSSSKNVVR **2** (50 μL of crude reaction mixture), 10 mM methylhydroxylamine or *O*-benzylhydroxylamine in water (2 μL), trifluoroacetic acid (5 μL) in water (43 μL) was stirred at 50 ^{o}C for 20 hours. The resulting mixture was added with 1% formic acid in acetonitrile (100 μL). The diluted mixture was analyzed by ESI-MS for determination of the oxime-modified peptide conversion.

Procedure for Transoximation of Oxime-modified YTSSSKNVVR using Aniline or *p*-Anisidine



A mixture of oxime-modified YTSSSKNVVR **2** (50 μL of crude reaction mixture), 10 mM methylhydroxylamine or *O*-benzylhydroxylamine in water (2 μL), 200 mM aniline or *p*-anisidine in acetonitrile (5 μL) in water (43 μL) was stirred at 50 °C for 20 hours. The resulting mixture was added with 1% formic acid in acetonitrile (100 μL). The diluted mixture was analyzed by ESI-MS/MS for determination of the oxime-modified peptide conversion.

Procedure for Synthesis of 5a



A mixture of N-dansylpiperazine (0.11 mmol), (Boc-aminooxy)acetic acid (0.13 mmol) and N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride

(EDC, 0.17 mmol) in dichloromethane (10 mL) was stirred at room temperature under nitrogen atmosphere for overnight. The reaction mixture was washed by water (50 mL) and then dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel using methanol/dichloromethane as eluent to give **5aa** with 91% yield.

Characterization Data of 5aa

Pale green powder; analytical TLC (silica gel 60) (10% methanol/dichloromethane), R_f = 0.64; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.59 (d, *J* = 8.5 Hz, 1H), 8.35 (d, *J* = 8.5 Hz, 1H), 8.20 (dd, *J* = 7.5 Hz, 1H), 7.85 (s, -NH), 7.55 (dd, *J* = 8.0 Hz, 2H), 7.19 (d, *J* = 8.5 Hz, 1H), 4.57 (s, 2H), 3.42-3.45 (m, 2H), 3.20-3.22 (m, 4H), 2.89 (s, 5H), 1.43 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.71, 156.22, 152.11, 132.41, 131.30, 130.99, 130.47, 130.31, 128.46, 123.32, 119.45, 115.57, 82.19, 73.50, 73.46, 65.99, 45.68, 45.57, 45.43, 44.48, 41.42, 28.31, 15.41; ESI-MS m/z 493 $[M+H]^+$; HRMS (ESI) for $C_{23}H_{33}N_4O_6S$, calculated 493.2121, found 493.2115.

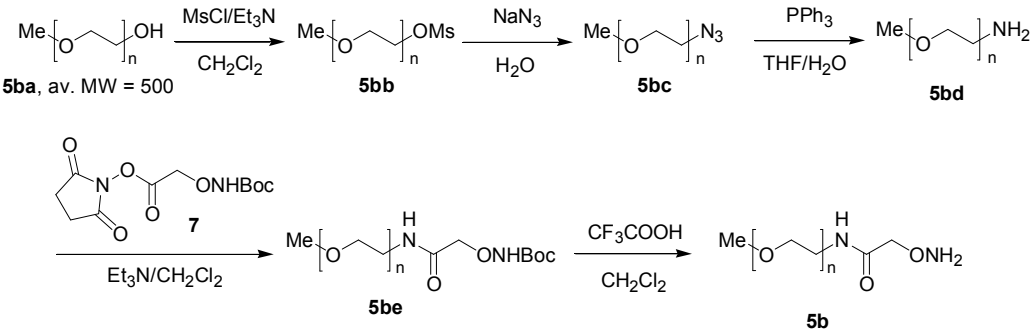
A solution of **5aa** (0.10 mmol) and trifluoroacetic acid (2 mL) in

dichloromethane (4 mL) was stirred at room temperature for 30 minutes. The reaction mixture was concentrated under reduced pressure and triturated by dichloromethane twice (10 mL × 2). The residue was purified by flash column chromatography on silica gel using methanol/dichloromethane as eluent to give **5a** with 90% yield.

Characterization Data of 5a

Pale green powder; analytical TLC (silica gel 60) (10% methanol/dichloromethane), R_f = 0.54; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.59 (d, *J* = 8.5 Hz, 1H), 8.39 (d, *J* = 8.5 Hz, 1H), 8.20 (dd, *J* = 7.5 Hz, 1H), 7.53 (dd, *J* = 8.0 Hz, 2H), 7.20 (d, *J* = 8.0 Hz, 1H), 4.57 (d, *J* = 8.5 Hz, 2H), 3.63-3.66 (m, 2H), 3.53-3.56 (m, 2H), 3.15-3.18 (m, 4H), 2.89 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.68, 156.74, 151.93, 131.12, 130.92, 128.33, 123.29, 119.56, 115.52, 71.86, 45.87, 45.53, 44.82, 41.48, 21.69, 15.74; ESI-MS m/z 393 $[M+H]^+$; HRMS (ESI) for $C_{18}H_{25}N_4O_4S$, calculated 393.1597, found 393.1609.

Procedure for Synthesis of 5b



Poly(ethylene glycol) methyl ether 500 **5ba** (20 mmol) was dissolved in dry dichloromethane (20 mL) and triethylamine (72 mmol) under nitrogen atmosphere. Methanesulfonyl chloride (71 mmol) was added at 0 ^{o}C. After the addition, the ice bath was removed. The reaction mixture was allowed to warm up to room temperature slowly and stirred for 4 hours. Water (100 mL) was then added to quench the reaction. The crude reaction mixture was washed with 1 M HCl solution (20 mL), saturated $NaHCO_3$ solution (20 mL), and brine (20 mL), and the organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated to dryness under reduced pressure to obtain **5bb** with 95% yield.

5bb (11 mmol) and sodium azide (27 mmol) were dissolved in water (12 mL) under nitrogen atmosphere. The mixture was refluxed for 18 hours. After the reaction, the resulting mixture was extracted with dichloromethane (50 mL × 3). The combined organic layer was washed with brine (50 mL), dried over

anhydrous Na_2SO_4, filtered and evaporated to dryness under reduced pressure to afford **5bc** with 82% yield.

A mixture of **5bc** (5 mmol) and triphenylphosphine (6 mmol) was dissolved in tetrahydrofuran (15 mL) under nitrogen atmosphere. The mixture was stirred for 3 hours, followed by addition of water (0.21 mL) and allowed to stir for 18 hours. After the reaction, 1 M HCl solution (15 mL) was added to the crude reaction mixture and washed with diethyl ether (20 mL × 3). The aqueous layer was cooled to 0 ^{o}C, neutralized by careful addition of KOH (36 mmol), and extracted with dichloromethane (20 mL × 3). The combined organic layer was washed with brine (20 mL), dried over anhydrous Na_2SO_4, filtered and evaporated to dryness under reduced pressure to give **5bd** with 85% yield.

5bd (0.5 mmol), (Boc-aminooxy)acetic acid N-hydroxysuccinimide ester **7** (0.6 mmol), triethylamine (0.75 mmol) were dissolved in dry dichloromethane (5 mL) under nitrogen atmosphere. The mixture was stirred at room temperature for 24 hours. After addition of water (5 mL), the resulting mixture was extracted with dichloromethane (20 mL × 3). The combined organic layer was dried over

anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure to obtain **5be** with 95% yield.

Characterization Data of 5be

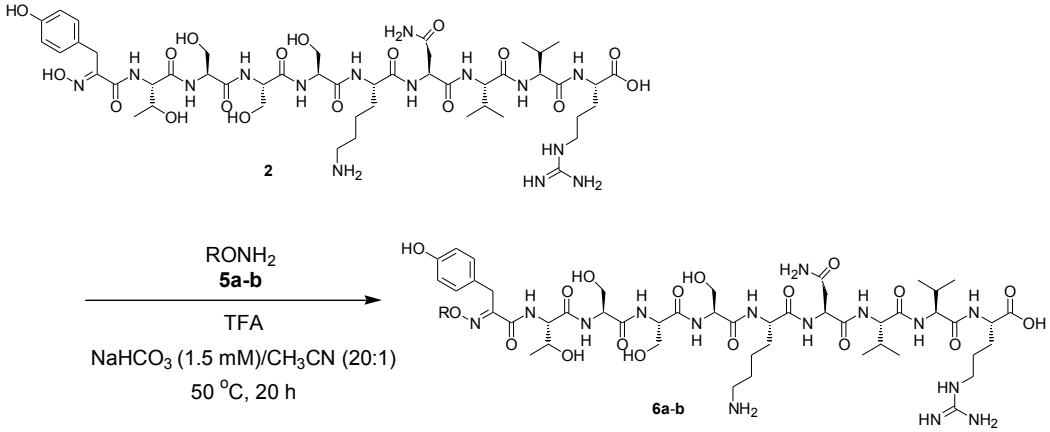
Pale yellow liquid; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.07 (br s, 1H), 4.31 (s, 2H), 3.63-3.68 (m, 34H, PEG), 3.59 (t, J = 5.5 Hz, 2H), 3.55 (t, J = 5.5 Hz, 2H), 3.47-3.51 (m, 2H), 3.38 (s, 3H), 1.48 (s, 9H).

To a solution of **5be** (0.15 mmol) in dichloromethane (1 mL) in an ice bath was dropwise added trifluoroacetic acid (1 mL). The mixture was then warmed to room temperature and stirred for 30 minutes. After the reaction, the solvent was evaporated to nearly dryness under reduced pressure and diluted with water (5 mL). The aqueous solution in an ice bath was neutralized by slow addition of solid $NaHCO_3$ until no CO_2 evolution was observed. The solution was then extracted with dichloromethane (20 mL × 3). The combined organic layer was dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure to give **5b** with 83% yield.

Characterization Data of 5b

Pale yellow liquid; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.16 (br s, 1H), 4.15 (s, 2H), 3.64-3.67 (m, 35H, PEG), 3.60 (t, J = 5.0 Hz, 2H), 3.55 (t, J = 5.0 Hz, 2H), 3.50-3.53 (m, 2H), 3.38 (3H, s).

Procedure for Transoximation of Oxime-modified YTSSSKNVVR with Functionalized Hydroxylamines using Trifluoroacetic Acid



A mixture of oxime-modified YTSSSKNVVR **2** (50 μL of crude reaction mixture), 10 mM aminooxy-compound **5a** or **5b** in acetonitrile (2 μL), trifluoroacetic acid (5 μL) and water (43 μL) was stirred at 50 ^{o}C for 20 hours. The resulting mixture was added with 1% formic acid in acetonitrile (100 μL). The diluted mixture was analyzed by LC-MS or MALDI-TOF for determination of the N-terminal functionalized peptides conversions.

2.6.4 MS and NMR Spectra

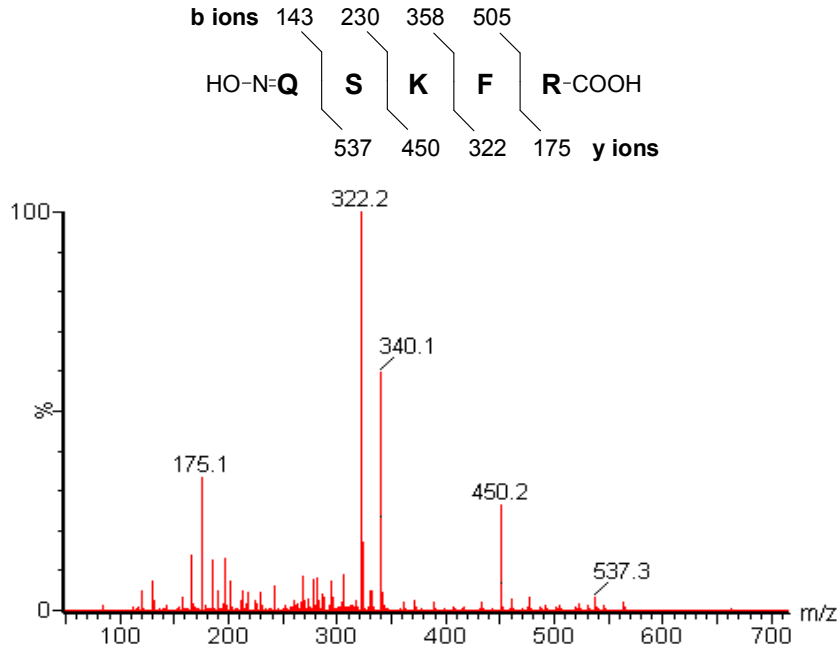


Figure S1. MS/MS spectrum of N-terminal oxime-modified peptide QSKFR (ESI source, doubly charged ion of m/z = 340.1).

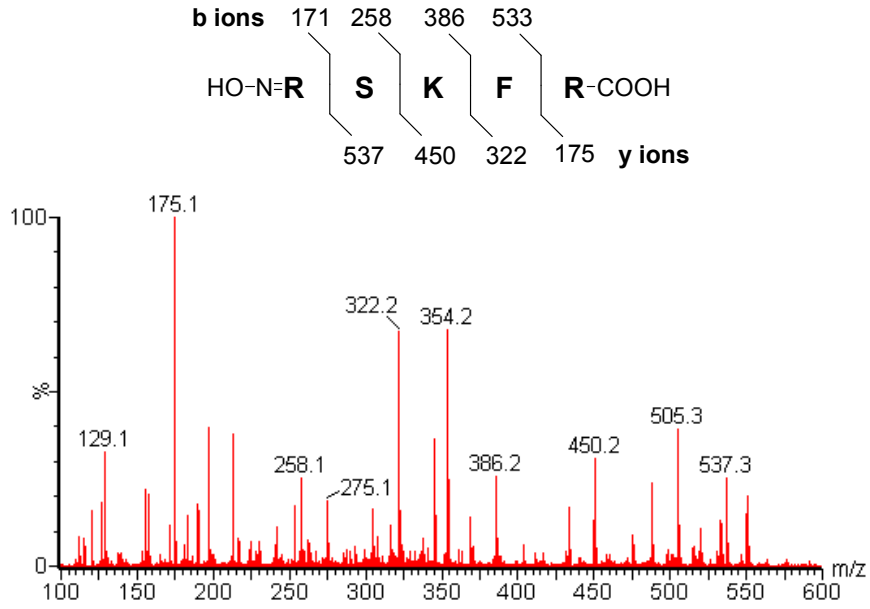


Figure S2. MS/MS spectrum of N-terminal oxime-modified peptide RSKFR (ESI source, doubly charged ion of m/z = 354.2).

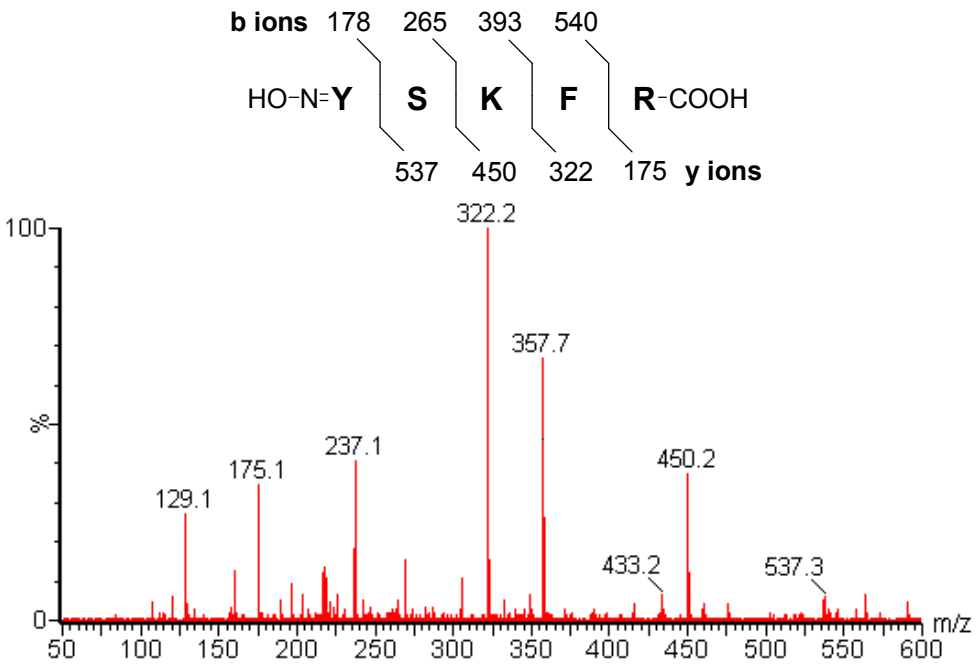


Figure S3. MS/MS spectrum of N-terminal oxime-modified peptide YSKFR (ESI source, doubly charged ion of m/z = 357.7).

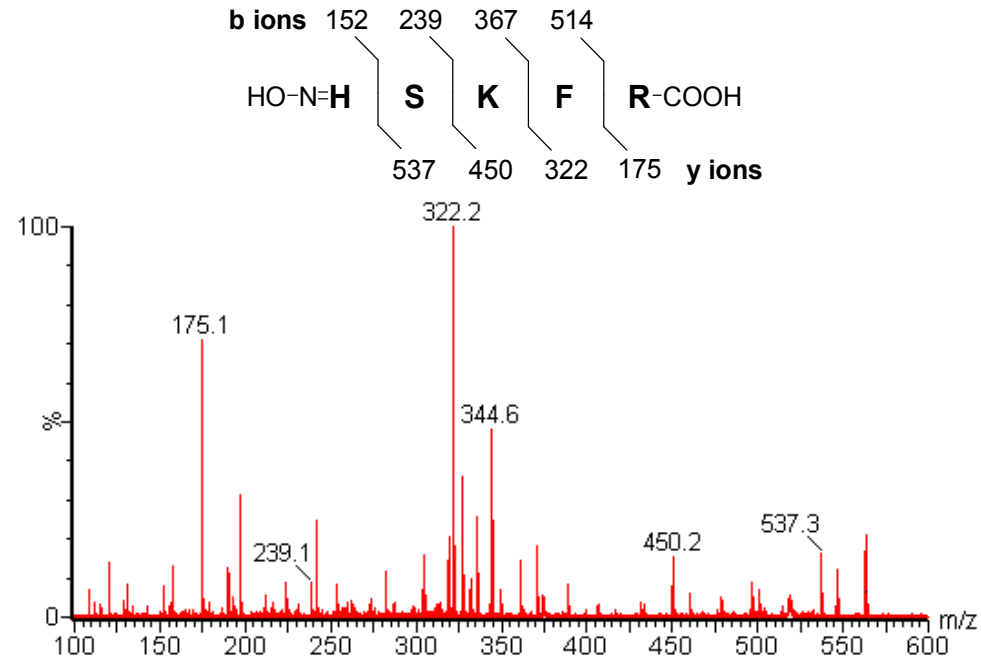


Figure S4. MS/MS spectrum of N-terminal oxime-modified peptide HSKFR (ESI source, doubly charged ion of m/z = 344.6).

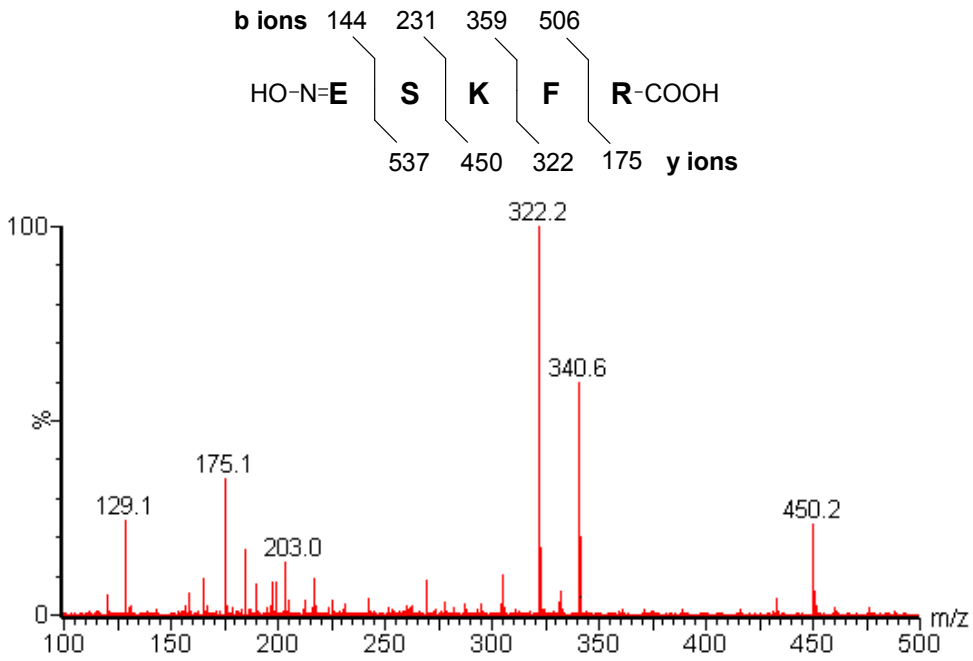


Figure S5. MS/MS spectrum of N-terminal oxime-modified peptide ESKFR (ESI source, doubly charged ion of m/z = 340.6).

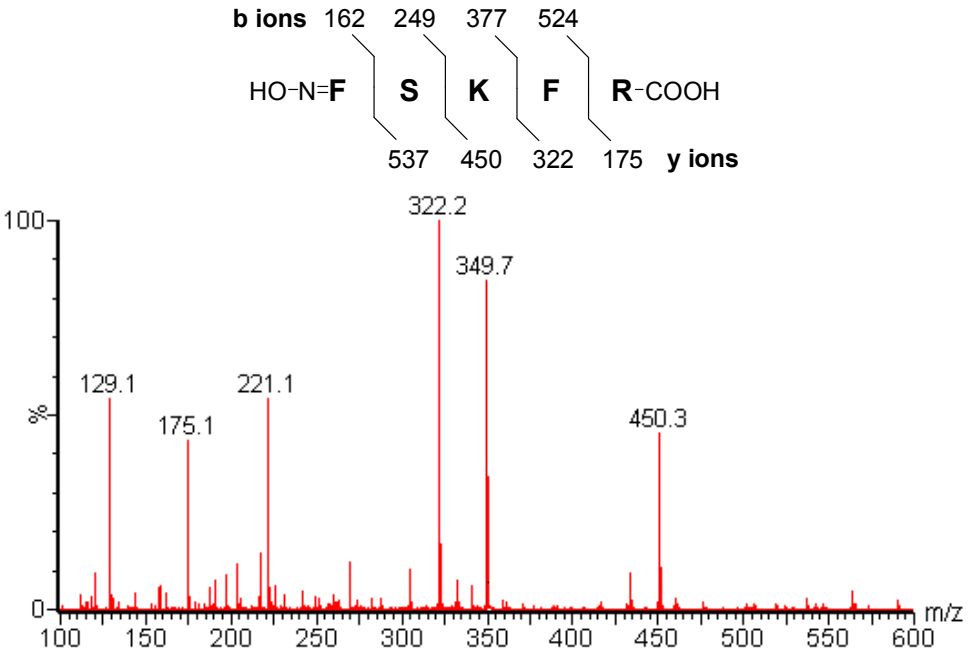


Figure S6. MS/MS spectrum of N-terminal oxime-modified peptide FSKFR (ESI source, doubly charged ion of m/z = 349.7).

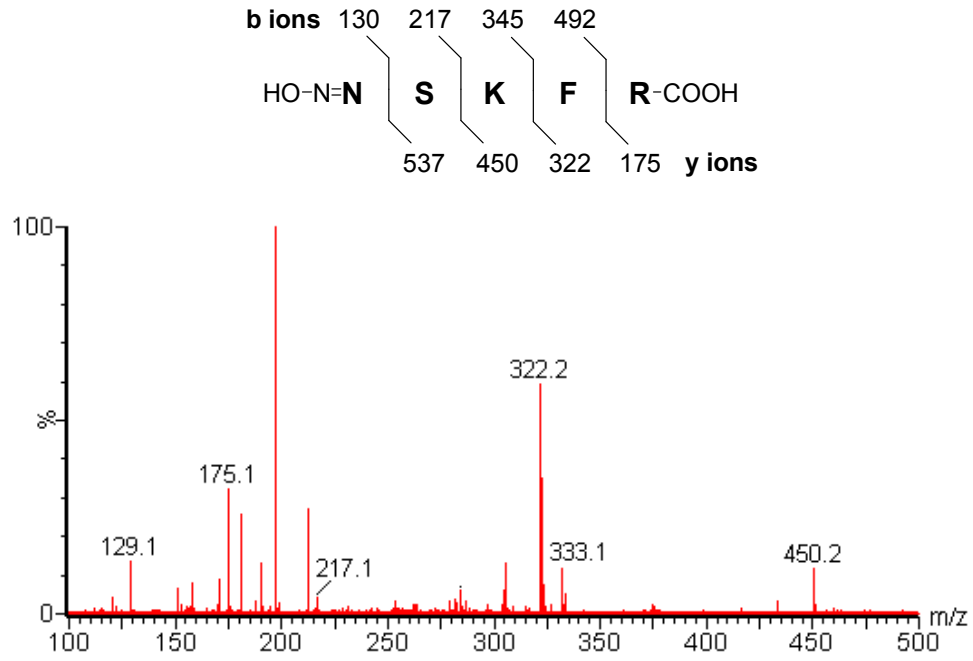


Figure S7. MS/MS spectrum of N-terminal oxime-modified peptide NSKFR (ESI source, doubly charged ion of m/z = 333.1).

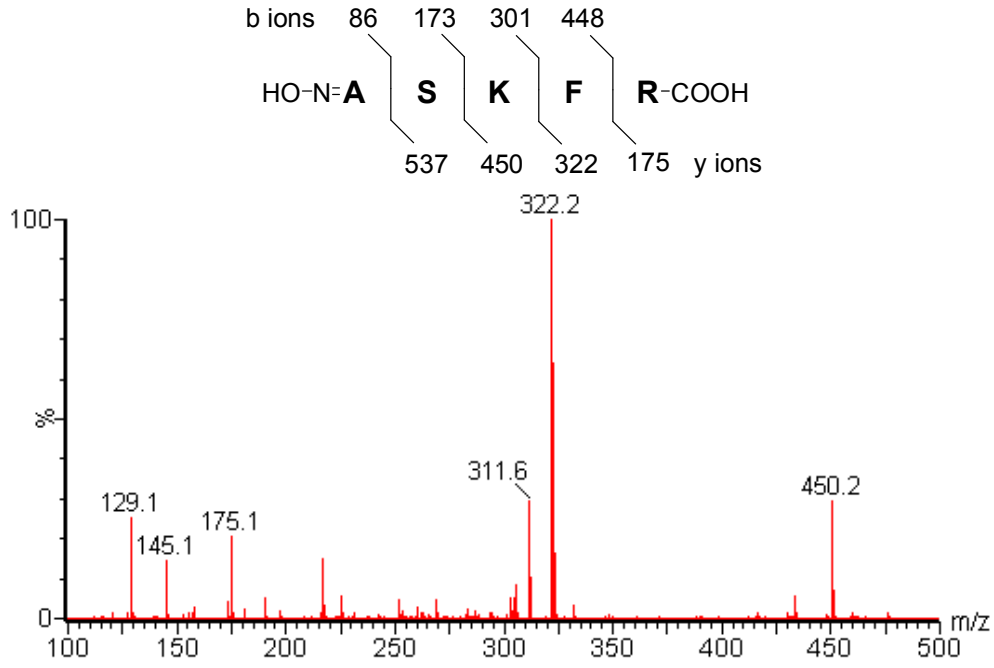


Figure S8. MS/MS spectrum of N-terminal oxime-modified peptide ASKFR (ESI source, doubly charged ion of m/z = 311.6).

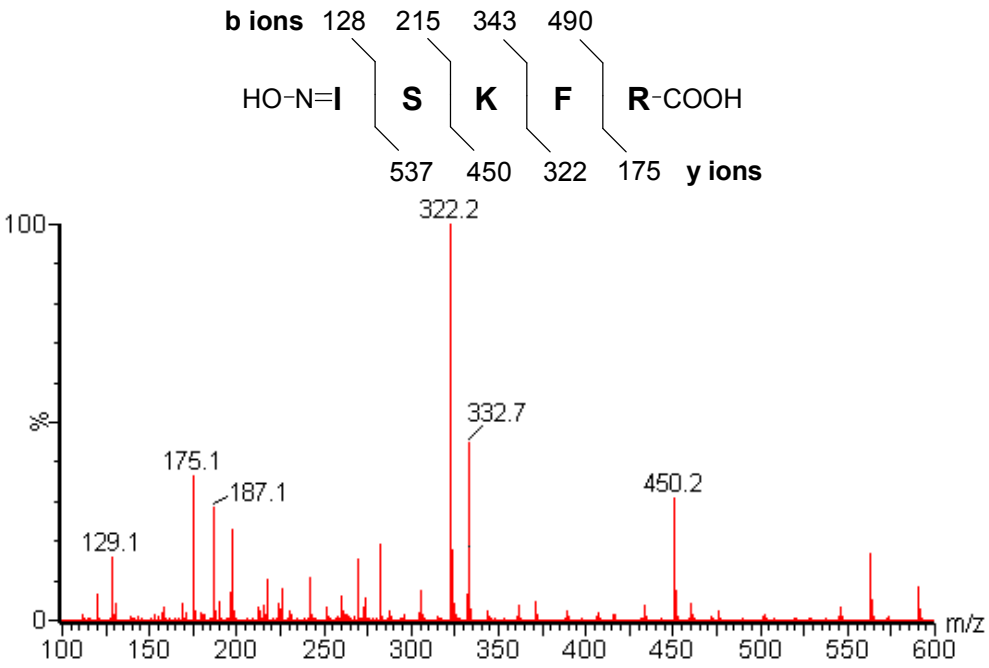


Figure S9. MS/MS spectrum of N-terminal oxime-modified peptide ISKFR (ESI source, doubly charged ion of m/z = 332.7).

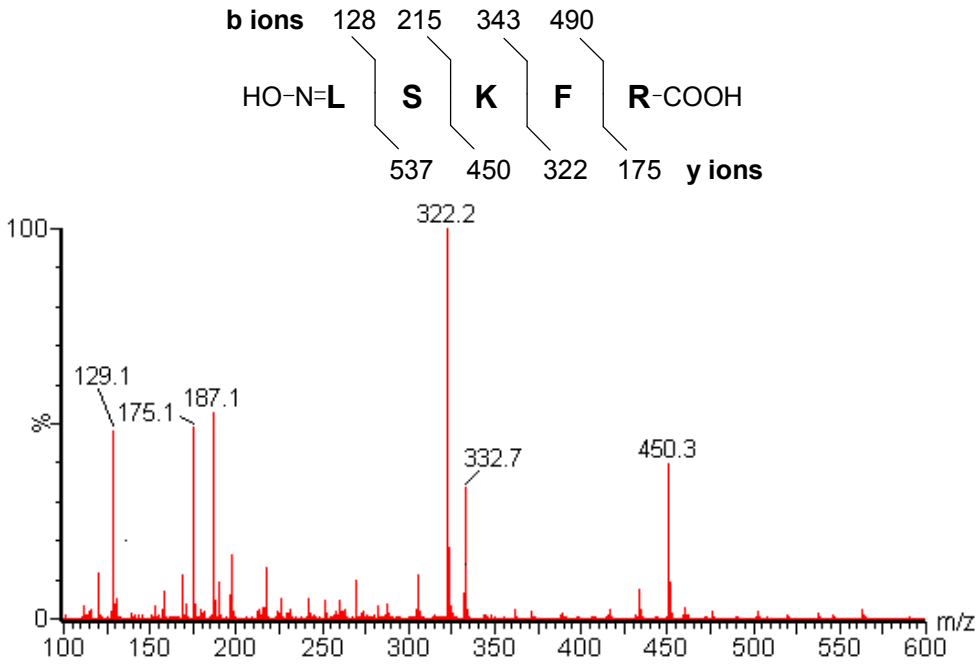


Figure S10. MS/MS spectrum of N-terminal oxime-modified peptide LSKFR (ESI source, doubly charged ion of m/z = 332.7).

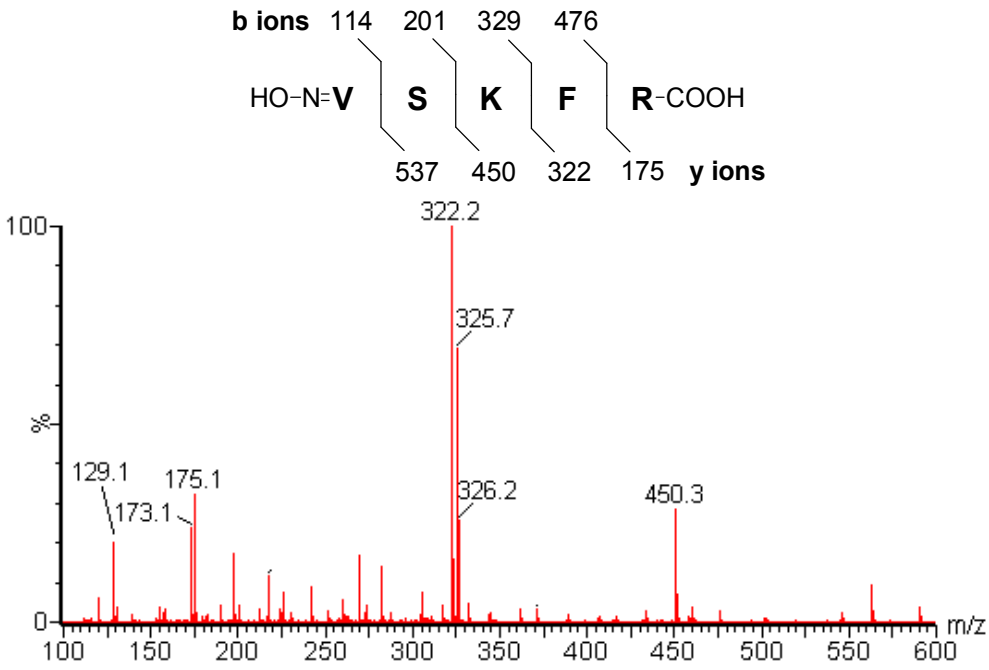


Figure S11. MS/MS spectrum of N-terminal oxime-modified peptide VSKFR (ESI source, doubly charged ion of m/z = 325.7).

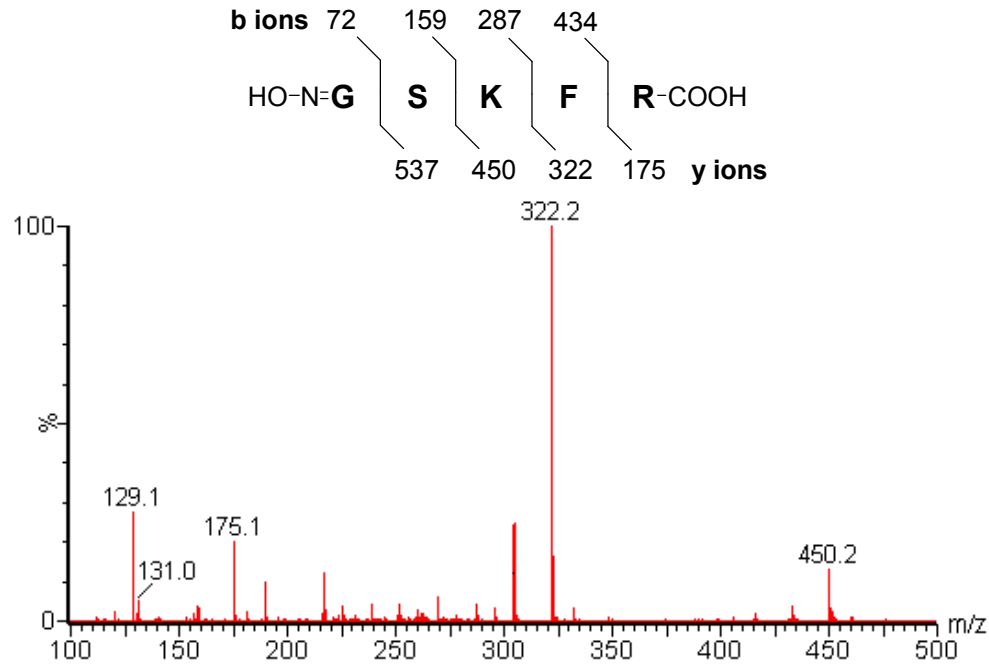


Figure S12. MS/MS spectrum of N-terminal oxime-modified peptide GSKFR (ESI source, doubly charged ion of m/z = 304.6).

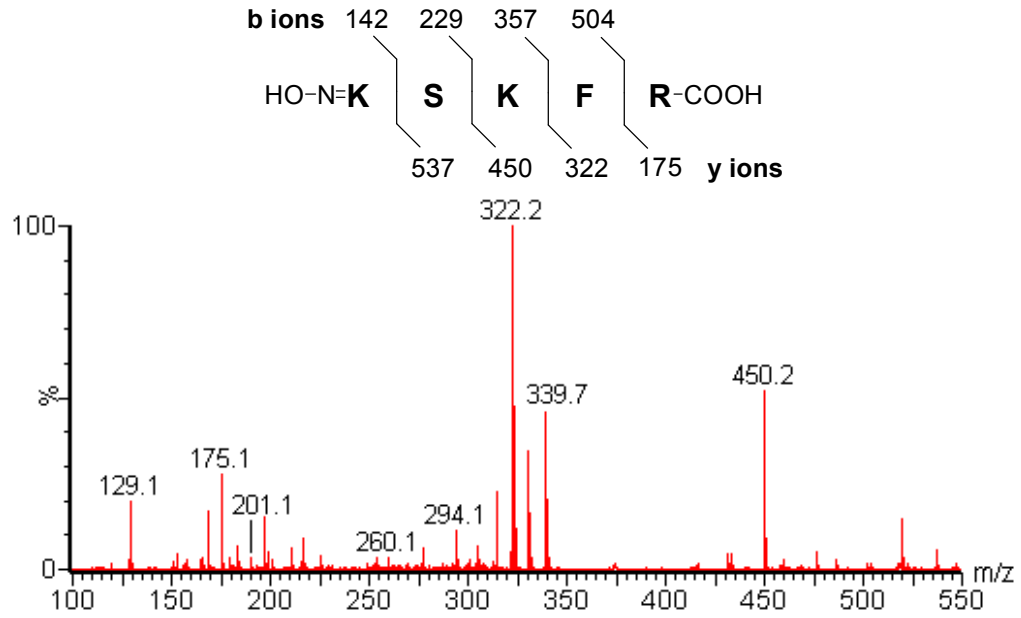


Figure S13. MS/MS spectrum of N-terminal oxime-modified peptide KSKFR (ESI source, doubly charged ion of m/z = 339.7).

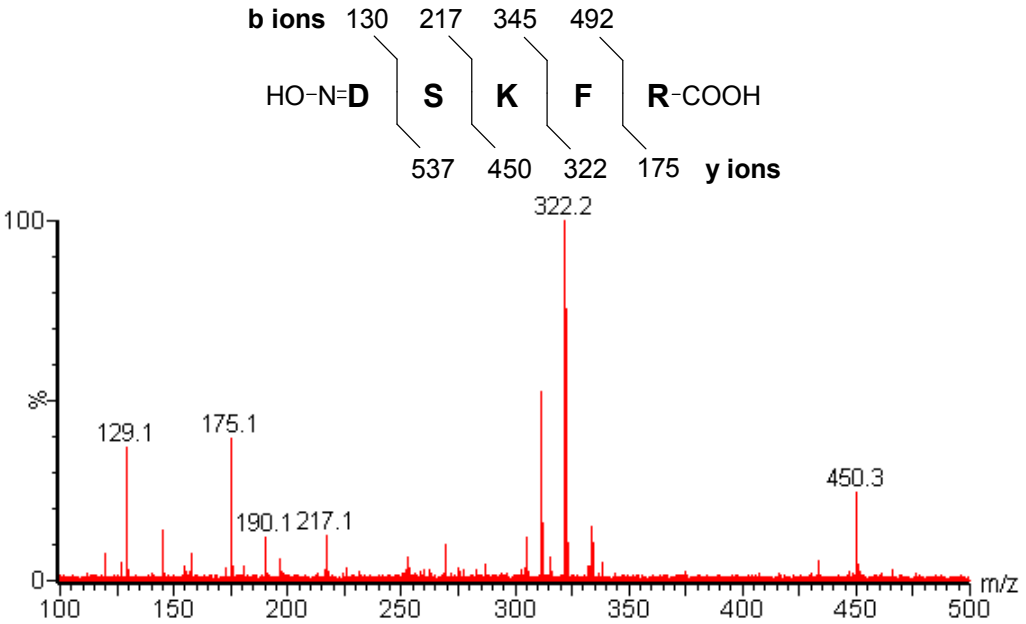


Figure S14. MS/MS spectrum of N-terminal oxime-modified peptide DSKFR (ESI source, doubly charged ion of m/z = 333.6).

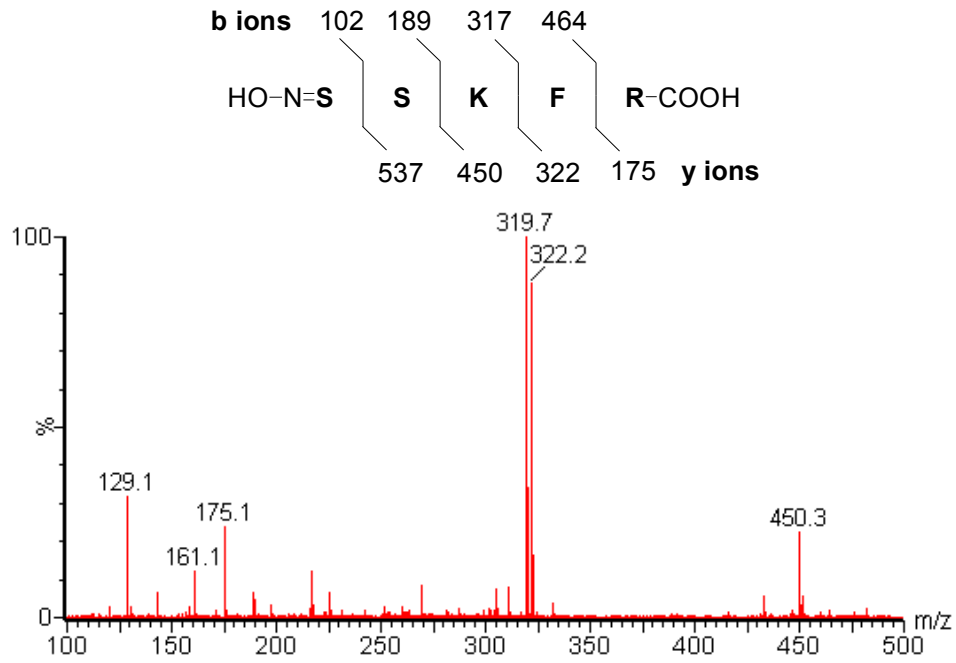


Figure S15. MS/MS spectrum of N-terminal oxime-modified peptide SSKFR (ESI source, doubly charged ion of m/z = 319.7).

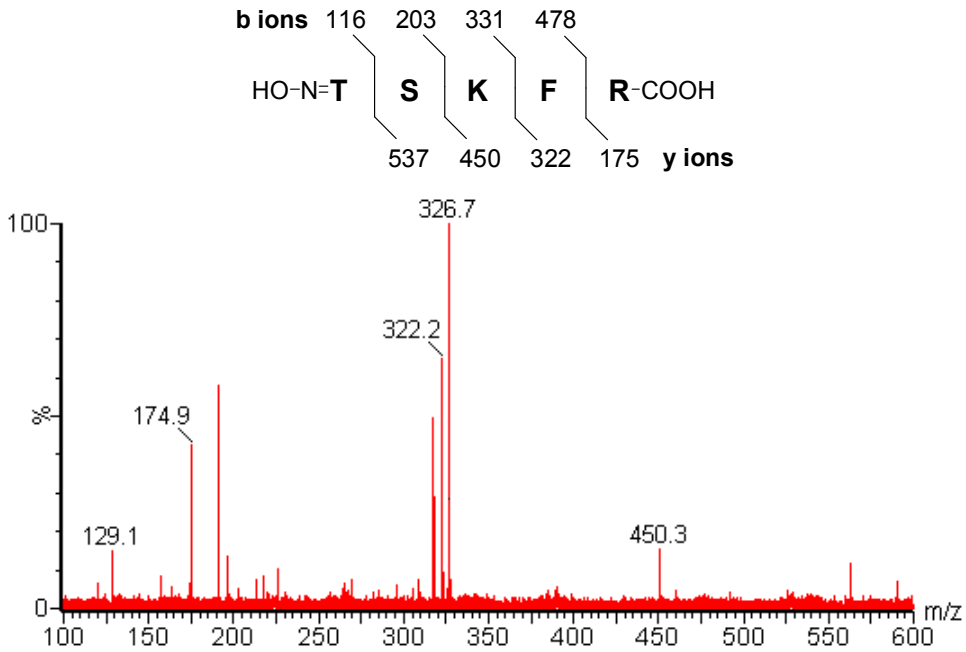
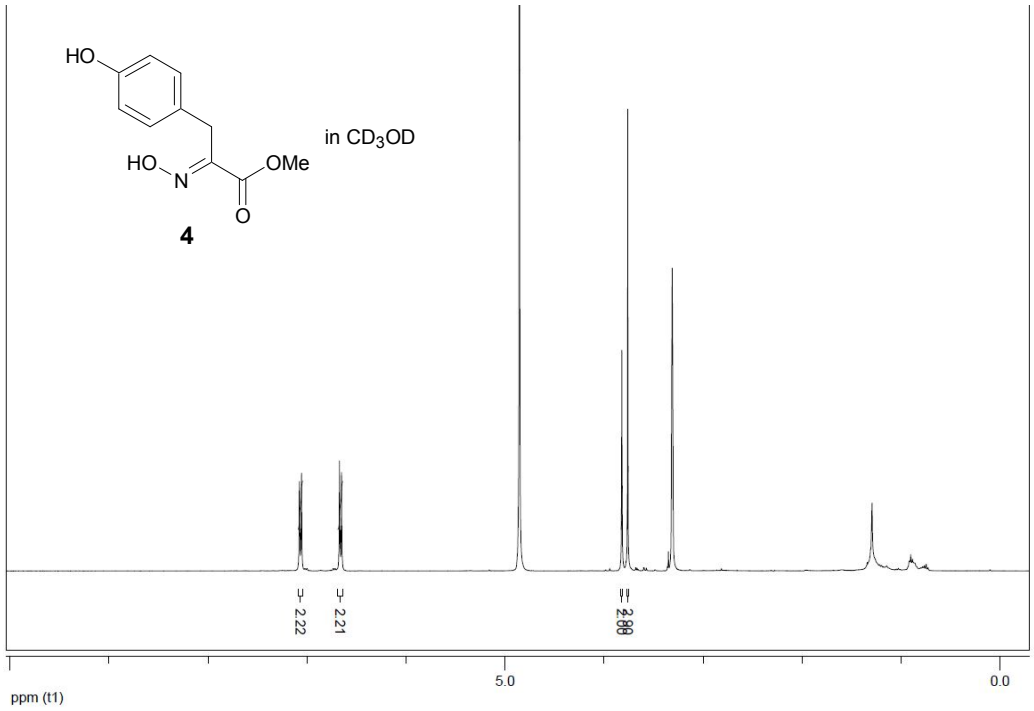
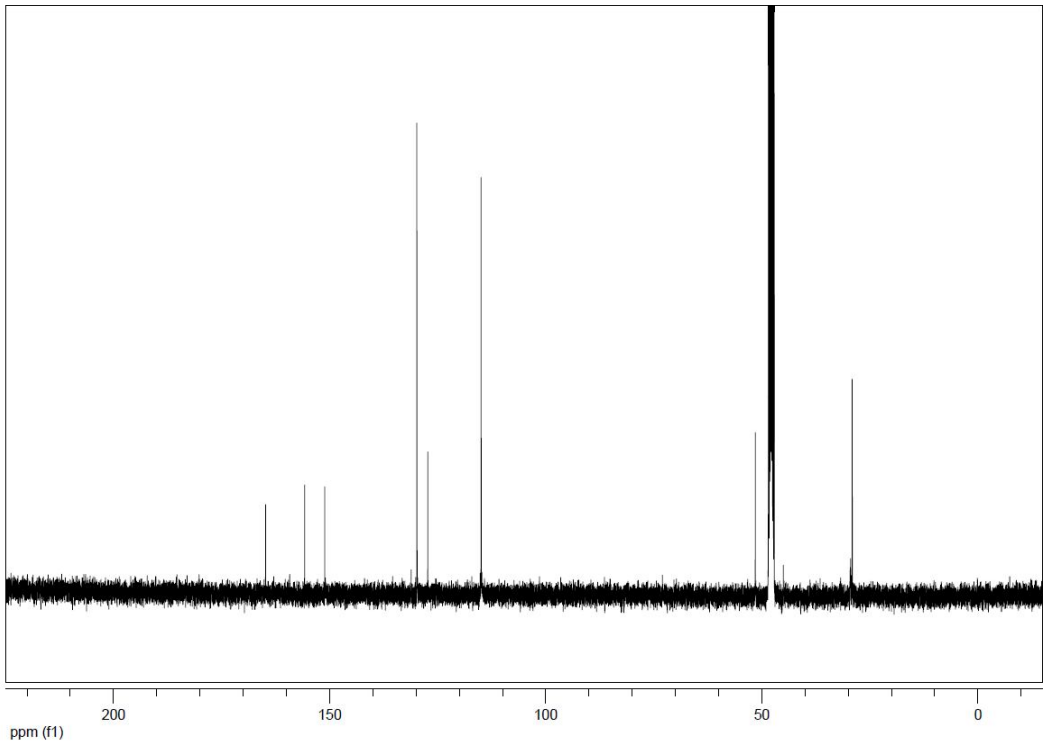


Figure S16. MS/MS spectrum of N-terminal oxime-modified peptide TSKFR (ESI source, doubly charged ion of m/z = 326.7).

NMR Spectra of 4





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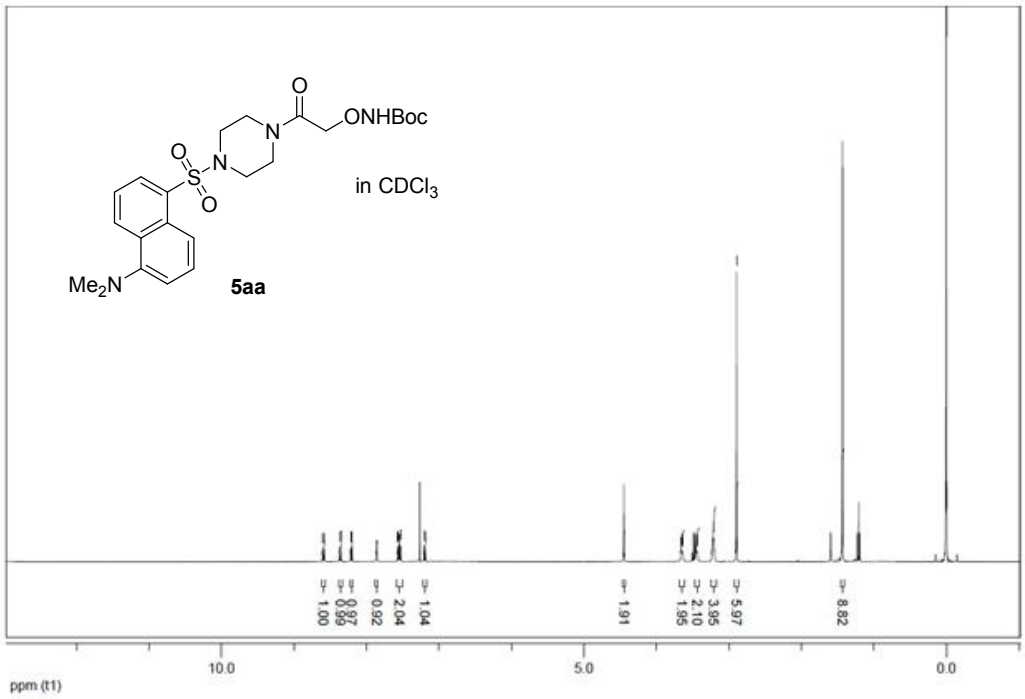
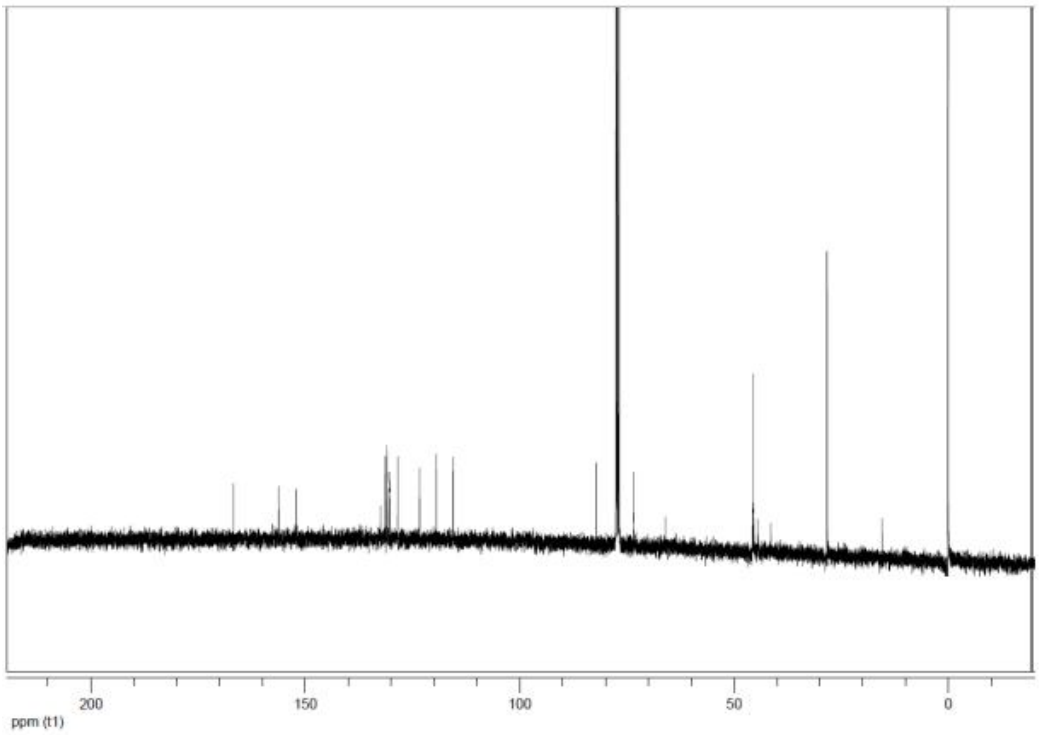
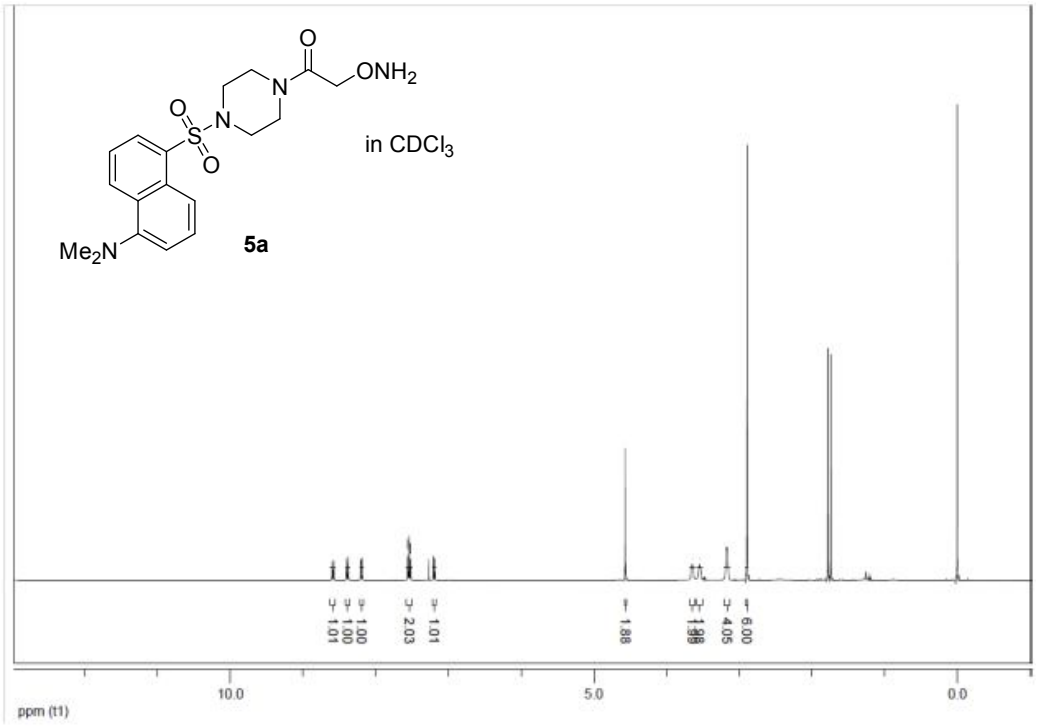
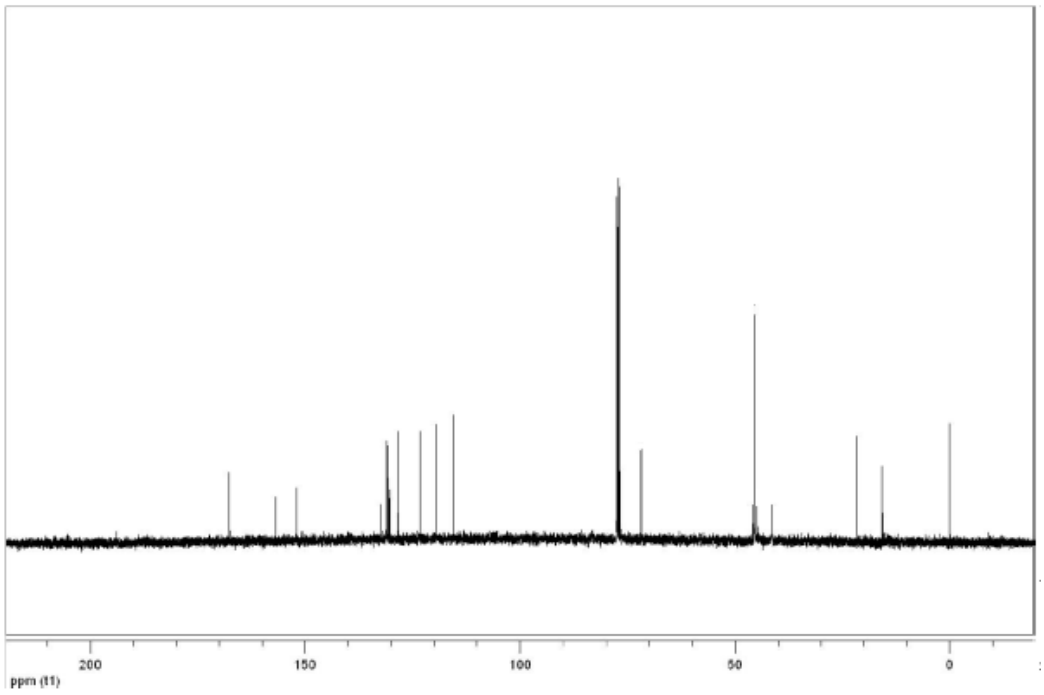


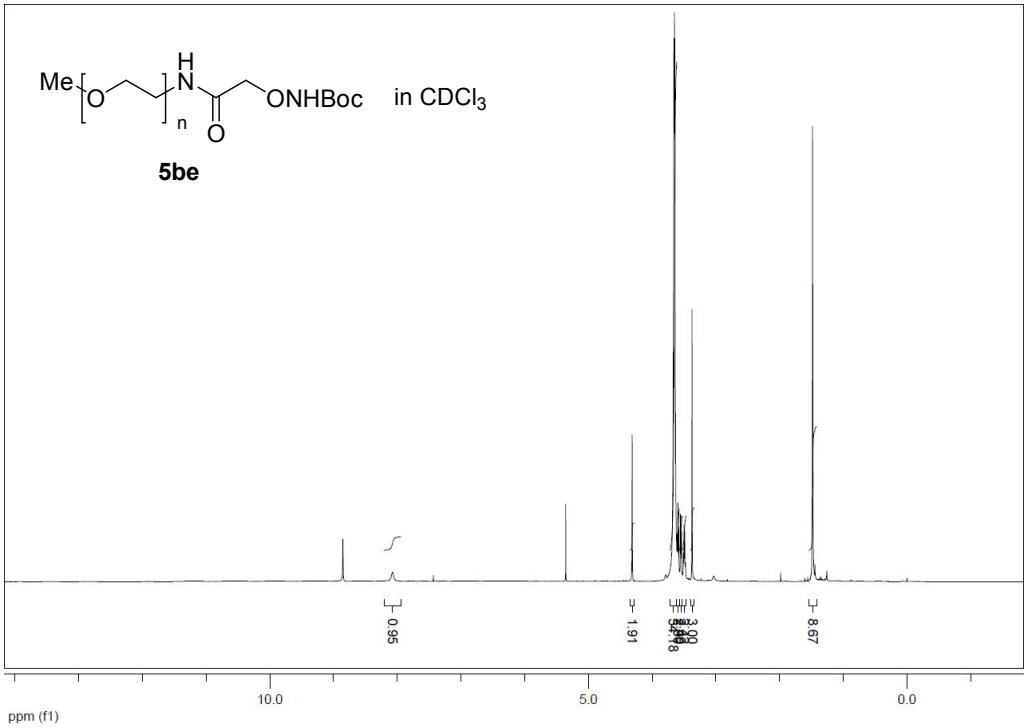


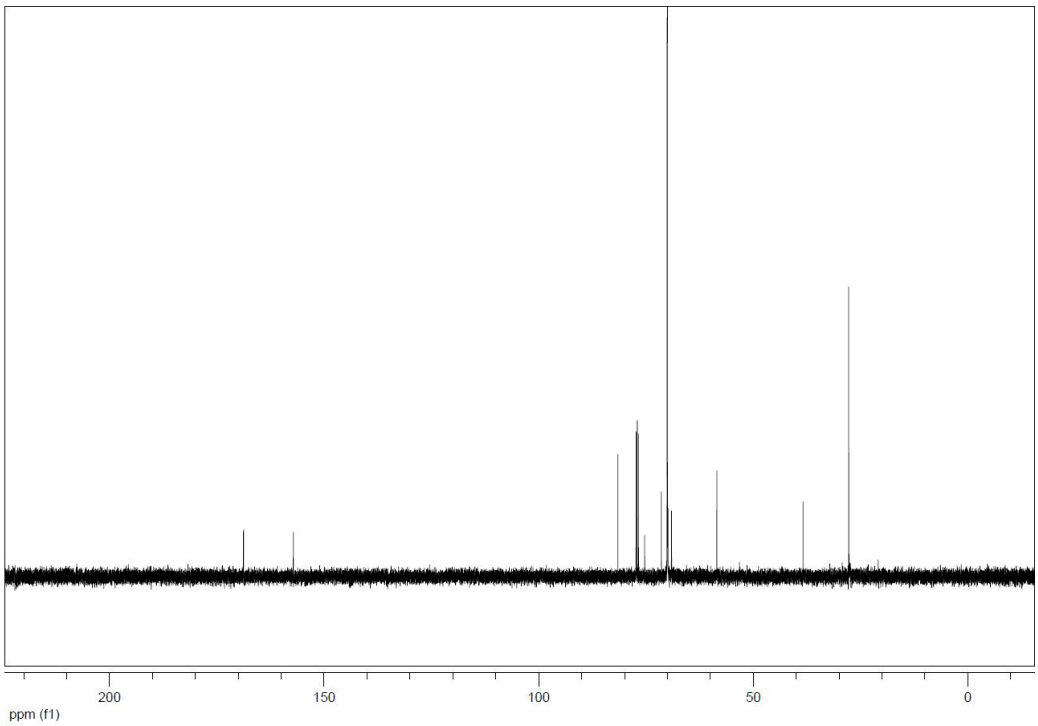
NMR Spectra of 5a





NMR Spectra of 5be





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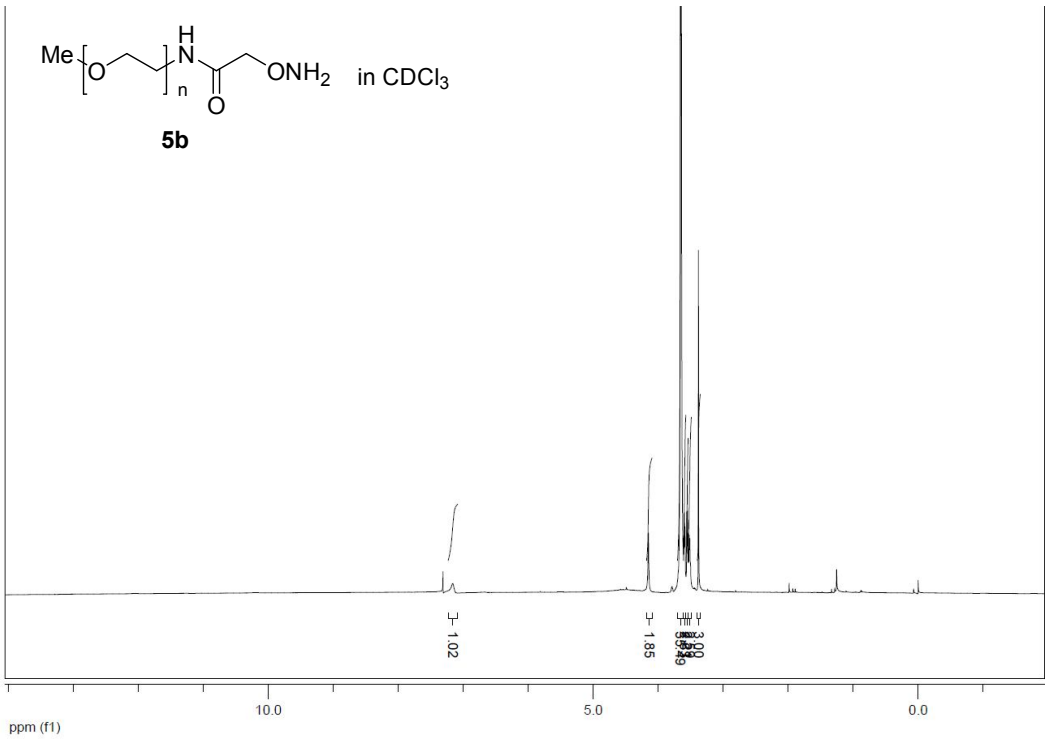

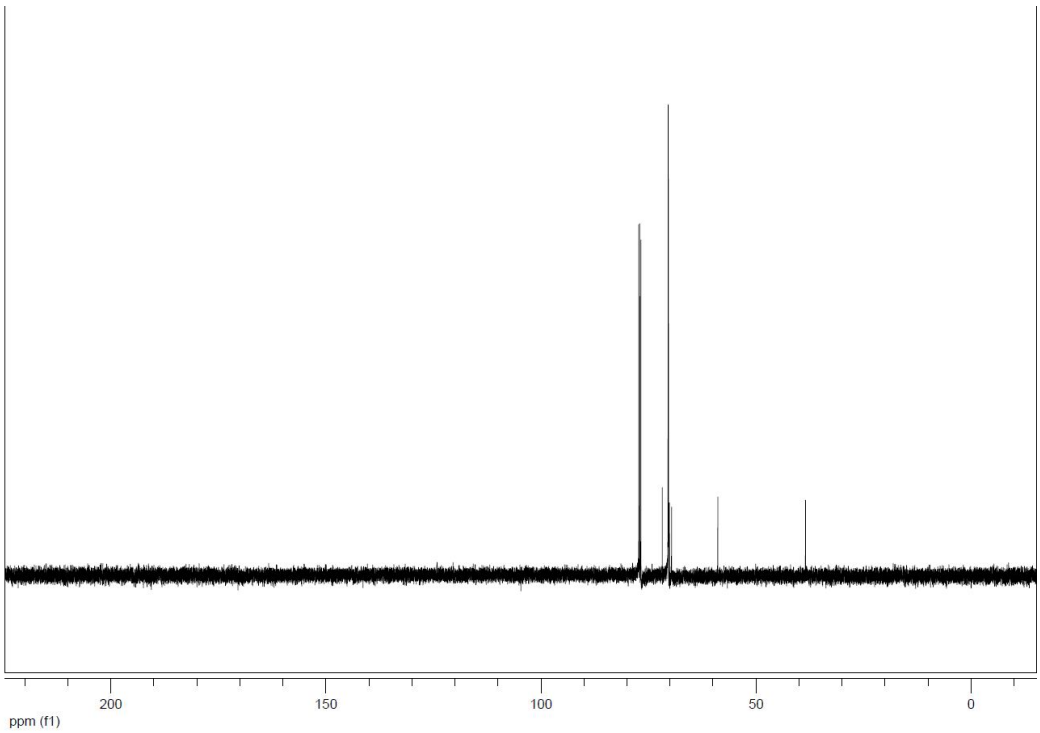

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CHAPTER 3: FLUORESCENT DETECTION OF FORMALDEHYDE BY A GOLD-CATALYZED THREE-COMPONENT COUPLING REACTION

3.1 Introduction

3.1.1 Characteristics of Formaldehyde

Formaldehyde is a highly reactive organic compound naturally found in environment. It is a colourless gas with a pungent and irritating odor. It exists in air from photochemical oxidations of products of combustion processes such as cigarette smoking, vehicle exhaust and incineration. In industry, formaldehyde is usually used for manufacture of resins and plastics, and production of other chemicals. Commercially, formaldehyde is commonly available as a 37% aqueous solution [1].

3.1.2 Occurrence in Food

Formaldehyde is present in food mainly due to natural occurrence, use as food additive, or release from food packaging materials.

Formaldehyde is a natural metabolite in various living organisms, and thus

present in many fresh and preserved foods as a normal component. Typical foods containing formaldehyde include mushrooms, meats, marine products, and many fruits and vegetables with a base content of 1-98 mg/kg in average [2].

Upon ingestion and absorption, formaldehyde is quickly converted to non-toxic formic acid by formaldehyde dehydrogenase and several other enzymes. Formic acid is excreted through urine in form of methanol or further transformed to carbon dioxide which is eliminated by exhalation. Formaldehyde can also be broken down for synthesis of larger molecules in tissues. Because of its rapid metabolism, the concentration of free formaldehyde is normally very low in living organisms [3].

However, formaldehyde may be inappropriately or illegally added to foods. Recently, formaldehyde was found to be deliberately used in food processing in Mainland for bleaching, protein coagulation and preservation purposes, causing a serious hazard to public health. Common incriminated food items include shelled shrimp, soya bean sticks, mung bean vermicelli and hydrated food such as tripe, chicken paws, etc. This practice results in high levels of the chemical in foods with extreme cases of over 4250 mg/kg. According to the announcement of the

Ministry of Health in 2008, usage of synthetic formaldehyde in foods for preservative or bleaching purposes is an illegal practice in China [2]. According to the Preservatives in Food Regulation (Cap. 132BD) under the Public Health and Municipal Services Ordinance (Cap. 132), formaldehyde is prohibited to be added to food as a preservative in Hong Kong [4].

Besides natural occurrence and intentional addition, researches showed that formaldehyde could migrate from packaging materials to the contained water, especially those bottles made from polyethylene terephthalate (PET). Although the amount of formaldehyde migration depended on the quality of the PET, in general only ppb levels were found in the contained water which should cause no adverse health effects on the consumers [5].

3.1.3 Health Impacts on Human

If exposure to formaldehyde is excessive, it can cause adverse health effects on us both acutely and chronically.

Acute toxicity is defined by the harmful health effects caused by a single exposure to a certain substance. Formaldehyde can affect those organs and

tissues of first contact with it because of its high biological reactivity and water solubility. There were many cases showing that formaldehyde caused acute poisoning, irritation problems, dermal allergy and allergic asthma to the patients who were accidentally exposed to a large amount of formaldehyde [6].

Chronic toxicity is defined by the harmful health effects caused by a long-term exposure to a certain substance [6]. According to the International Agency for Research on Cancer (IARC), formaldehyde is carcinogenic to humans to cause nasopharyngeal cancer. The agency also suggested that there is strong but not sufficient evidence for the relationship between occupational formaldehyde exposure and leukemia [7]. In 2005, the World Health Organization (WHO) drew a conclusion revealing that formaldehyde is not carcinogenic through oral exposure [8]. In addition to carcinogenicity, long-term exposure to formaldehyde can induce neurotoxicity, reproductive toxicity, hematotoxicity, genotoxicity and damages to pulmonary functions. Since formaldehyde can increase the formation of reactive oxygen species which damage chromosomes and deoxyribonucleic acids, it is suspected to be related to genotoxicity [6].

3.1.4 Acceptable Values for Intake

Owing to the adverse health effects, the WHO established a tolerable daily intake (TDI) of 0.15 mg/kg/day for formaldehyde. TDI estimates the amount of a certain substance in food or water that can be ingested daily over lifetime without any appreciable health risks [9]. The United States Environmental Protection Agency (USEPA) also determined an oral reference dose (RfD) of 0.2 mg/kg /day. RfD provides a numerical estimation of a daily oral exposure of a certain substance for the human population that is unlikely to cause adverse effects over lifetime [10].

As mentioned before, our body can quickly metabolize formaldehyde into harmless formic acid. Also, formaldehyde may be bound with marcomolecules in food which makes it inactive. Therefore, consumption of foods containing natural formaldehyde is unlikely to cause acute adverse effects to us [6].

3.1.5 Recent Analytical Methods for Formaldehyde

3.1.5.1 Liquid Chromatography

To ensure food safety and quality, testing is indispensable. A large number of liquid chromatography (LC) methods have been developed to measure

formaldehyde contents in food. Owing to its high reactivity, formaldehyde is often derivatized with other substances and then separated from other carbonyl compounds by LC. The most well known method is to react the formaldehyde extracted from food samples with 2,4-dinitrophenylhydrazine (2,4-DNPH) (Figure 1). The amount of the hydrazone formed after derivatization is determined by high performance liquid chromatography (HPLC) with ultraviolet detection at 360 nm. This method was used to determine formaldehyde contents in different foods and beverages such as beer, fruit juice and mushroom. The limit of detection could be as low as 0.12 ppb [11].

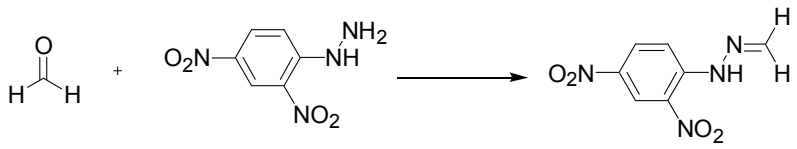


Figure 1. Reaction between formaldehyde and 2,4-DNPH.

Besides 2,4-DNPH, other derivatizing reagents are developed for formaldehyde determination. One of the methods used the reaction between formaldehyde, ammonia and ethyl 3-oxobutanoate to give diethyl 2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate which was then analyzed by HPLC, as shown in Figure 2. This method was used to quantify formaldehyde in spirits. The limit of detection was found to be 0.25 ppm [12].

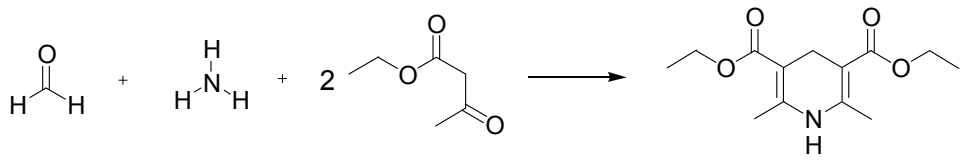


Figure 2. Reaction between formaldehyde, ammonia and ethyl 3-oxobutanoate.

Another method employed dimedone as the derivatizing reagent for formaldehyde and the derivative was analyzed by LC-MS (Figure 3). The authors used this method to determine formaldehyde contents in raw and cooked shiitake mushrooms. The limit of quantification was 50 ppm [13].

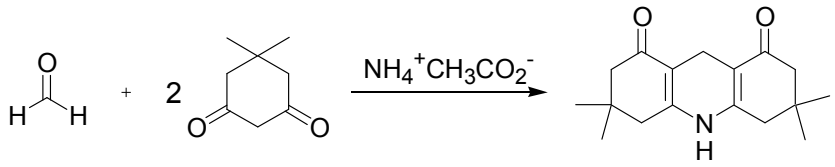


Figure 3. Reaction between formaldehyde and dimedone.

3.1.5.2 Gas Chromatography

Gas chromatography (GC) is also a very common technique for instrumental determination of formaldehyde in food. In addition to HPLC, the derivative of formaldehyde and 2,4-DNPH can be analyzed by GC-MS as well. Formaldehyde contents in squid, squid products, flour and beer were analyzed and the limit of

detection was found to be as low as 0.1 ppm [14].

Another modified method employed 2,2,2-trifluoroethylhydrazine for derivatization. The conditions were optimized to achieve a limit of detection of 0.1 ppm. This method was applicable to fermented foods such as kimchi, soybean paste, soy sauce and bean-paste soup [15].

One method used pentafluorobenzyl-hydroxyl-amine hydrochloride as the derivatizing reagent to detect formaldehyde in different fish species with different processing conditions such as fresh, deep frozen, roasted and boiled by GC-MS. The limit of detection and limit of quantification of this method were 17 ppb and 28 ppb, respectively. These values showed the good sensitivity of the method [16].

3.1.5.3 Fluorescence Spectrometry

A number of fluorescent methods have been developed for determination of formaldehyde in foods owing to the high sensitivity of fluorescence. One recently developed method used formaldehyde as the catalyst for the oxidation reaction of pyronine Y by sodium periodate in phosphoric acid. Fluorescence

intensities of pyronine Y decreased with increased concentrations of formaldehyde. The limit of detection was 0.02 ppb which indicated an excellent sensitivity of the analytical method. Formaldehyde contents in fish, acaleph and vermicelli were determined by this method [17].

Another method employed the reaction between formaldehyde, acetoacetanilide and ammonia for a spectrofluorometric determination of the analyte by a flow injection system. The established system could analyze 15 samples in one hour. The limit of detection was 0.09 ppb. The method was applicable to water samples such as tap water and river water. Note that most cations, anions and organic compounds commonly found in water, such as sodium, nitrate and acetaldehyde, did not interfere the formaldehyde determination [18].

Hantzsch reaction between formaldehyde and cyclohexane-1,3-dione was used to detect formaldehyde in foods. A flow injection system with fluorometer was adapted for the derivatization and the reaction was accelerated by microwave irradiation. Under optimized conditions, the limit of detection could be as low as 0.02 ppb and the sample throughput was 28 injections per hour. This method was used to determine formaldehyde contents in dried mushroom, vermicelli, bean

curd, alcoholic beverage and fruit beverage without any sample pretreatment processes [19].

Fluorescence energy transfer (FET) is a process in which the emission of the donor molecule is absorbed by the acceptor molecule [20]. This phenomenon can be applied in formaldehyde determination. A water-soluble terbium(III)-sodium hexametaphosphate (Tb-SHMP) chelate complex was synthesized to enhance the fluorescence intensity of the terbium(III) ion. Formaldehyde was reacted with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (AHMT) under alkaline conditions at room temperature to form 6-mercapto-5-triazole[4,3-b]-S-tetrazine (Figure 4) [20].

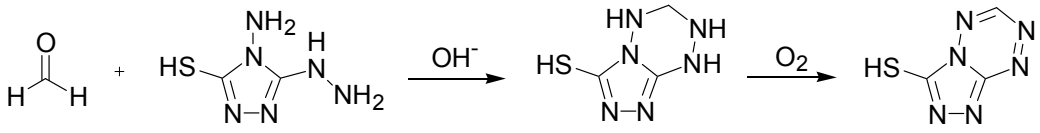


Figure 4. Reaction between formaldehyde and AHMT.

Owing to the spectral overlap between the emission of Tb-SHMP complex and the absorption of 6-mercapto-5-triazole[4,3-b]-S-tetrazine, a method for determination of formaldehyde was established using Tb-SHMP chelate complex as the donor and 6-mercapto-5-triazole[4,3-b]-S-tetrazine as the acceptor. The

limit of detection was 7 ppb and the method was used for water samples [20]. Another similar method developed by the same research group used terbium(III) cerium phosphate ($CePO_4:Tb^{3+}$) nanocrystal as the donor instead of Tb-SHMP chelate complex. The method was also used to determine formaldehyde contents in water samples and its limit of detection was 0.165 ppt [21].

3.1.5.4 UV-Visible Spectrophotometry

A number of spectrophotometric methods have been developed for formaldehyde detection in foods. One example was to use phloroglucinol as the chromogenic agent to derivatize formaldehyde under mild conditions (Figure 5). An orange-coloured compound was produced and its absorbance at 474 nm was measured to quantify formaldehyde concentrations. Since the coloured product was unstable, a flow injection system was used to control the reaction time and measurement. The limit of detection was 23 ppb and food samples such as bean curd stick, preserved jerryfish and dry mushroom were analyzed [22].

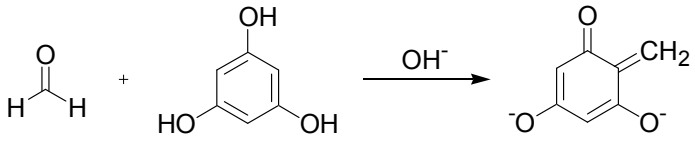


Figure 5. Reaction between formaldehyde and phloroglucinol.

Another spectrophotometric method was developed based on an oxidation reaction. The analyte, formaldehyde, acted as the catalyst of the oxidative reaction of rhodamine B by potassium bromate in the presence of sulfuric acid. The reaction completed in 6 minutes and formaldehyde concentrations were measured by the decrease in absorbance of the rhodamine B at 515 nm. This method had a limit of detection of 2.9 ppb and was applicable to many food samples including sea cucumber, bamboo shoot, tripe, mushroom and shrimp [23].

One recently developed method also made use of the catalytic effect of formaldehyde. Formaldehyde speeds up the oxidation of eosin Y by potassium bromate in phosphoric acid medium. The reaction was monitored by spectrophotometer and the decrease in absorbance of eosin Y at 518 nm was used to quantify formaldehyde concentrations. The limit of detection was 10 ppb. Shrimp, bean curd sheet and shiitake mushroom were analyzed by this method to determine their formaldehyde contents [24].

One colorimetric method was developed for fast detection of formaldehyde in foods. The reaction between formaldehyde and acetylacetone produced

3,5-diacetyl-l-1,4-dihydrolutidine which was yellow in colour. The reaction completed in 6 minutes. Formaldehyde concentrations could be estimated by comparing the colours of the reaction mixtures with the reference colour card prepared by different formaldehyde standards (Figure 6). The limit of detection of this method was 0.8 ppm. Food samples such as mushroom, sea cucumber, cuttlefish, melon seeds and flour were analyzed by this method. Due to the very little sample preparation, fast reaction time and high selectivity, this method was suitable for semi-quantitative on-site analysis of formaldehyde contents in foods [25].

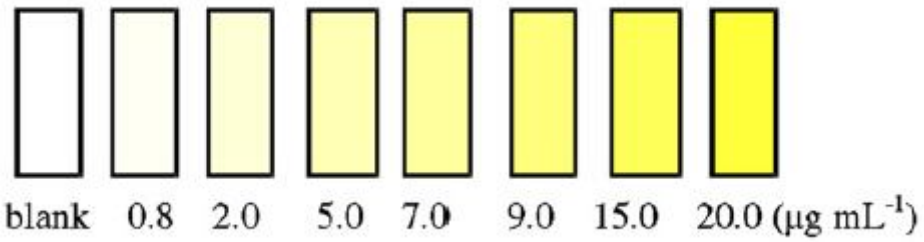


Figure 6. Reference colour card for semi-quantitative formaldehyde determination. (Reproduced from the author's article.)

As shown above, a lot of analytical methods have been developed for measurement of formaldehyde contents in foods. However, most of these methods require sophisticated operation skills and expensive instrumentations

such as HPLC and GC. Some of them may be susceptible to interference by other carbonyl-containing compounds or require very tedious sample pre-treatment for heterogeneous food matrix. Results from spectrophotometric methods can be interfered by the colours of the food samples. A comparison between the traditional instrumental analysis and the fluorescent fast detection of formaldehyde (the present work) is shown in the table below.

Table 1. Comparison between formaldehyde detection by traditional instrumental analysis and fluorescent fast detection

Traditional instrumental analysis	**Fast detection by fluorescent probes**
Expensive analytical instruments	Expensive analytical instruments are not required
Tedious sample preparation	Relatively simple sample preparation
Relatively low specificity to the target analyte	High specificity of the fluorescent probe to the target analyte
Easily interfered by other chemicals	Not interfered by the colours of foods

Currently, the majority of rapid detection kits available in the market are targeting the formaldehyde in air as well as biomedical samples. A colorimetric

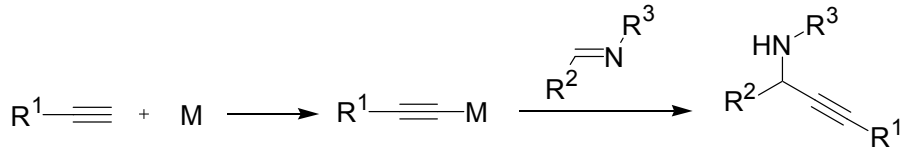
testing kit which allows rapid detection of formaldehyde in food was recently developed by Global Complex Co., Ltd. However, result of this colorimetric method (red/pink colour as read-out) could be easily interfered by natural color of food. More importantly, its selectivity and dynamic range is not mentioned clearly. Therefore, there is a pressing need to develop a simple and inexpensive method for rapid detection of formaldehyde in foods with high sensitivity and specificity.

3.1.6 Three-component Coupling Reaction of an Aldehyde, an Amine and an Alkyne for Propargylamine Synthesis

In recent years, transition metal-catalyzed three-component coupling reaction of aldehydes, amines and alkynes has become a convenient approach for propargylamine synthesis. Propargylamines are versatile synthetic intermediates for further modifications to nitrogen-containing heterocycles, natural products and therapeutic drug molecules [26].

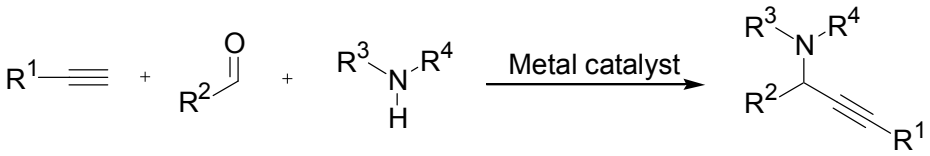
Traditionally, propargylamines were synthesized by mixing an imine with a metal acetylide (Scheme 1) which was formed by reacting a terminal alkyne with strong bases such as butyllithium in a stoichiometric amount. The use of a large

amount of reagents and its high sensitivity to moisture made this process unpopular [26].



Scheme 1. Traditional approach for synthesis of propargylamines, where M represents a metal.

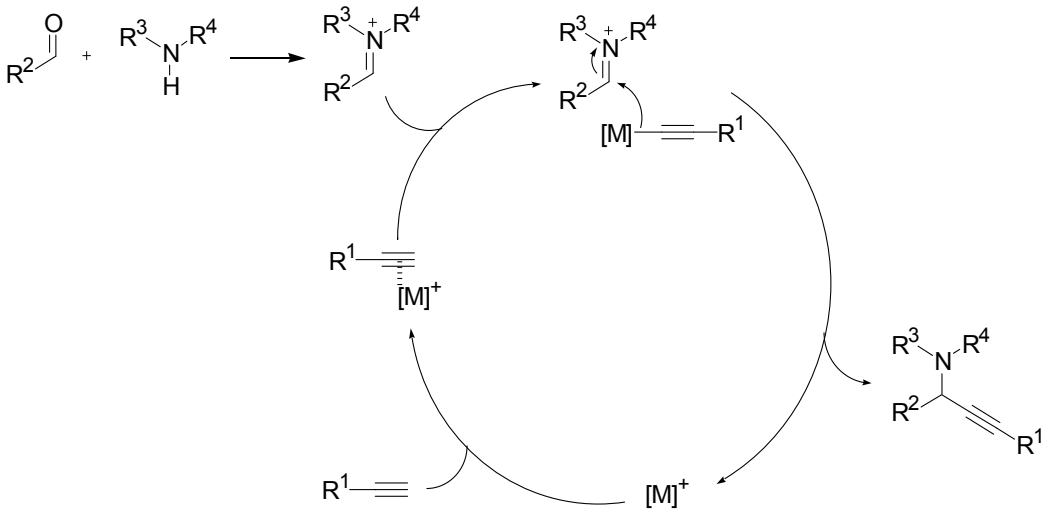
Around a decade ago, the direct three-component coupling of an aldehyde, an amine and an alkyne catalyzed by a transition-metal (known as A3-coupling) was developed through the *in situ* formation of an imine or iminium ion from the aldehyde and amine (Scheme 2) [26].



Scheme 2. The three-component coupling reaction of an aldehyde, an amine and an alkyne catalyzed by a transition-metal for propargylamine synthesis.

It is proposed that an intermediate π-metal-alkyne complex is formed first via

abstraction of the alkyne proton. The metal acetylide then reacts with the imine or iminium ion generated from the aldehyde and amine to yield the propargylamine. The metal catalyst is regenerated after the coupling (Scheme 3) [26].



Scheme 3. Tentative mechanism of a A3-coupling reaction of an aldehyde, an amine and an alkyne catalyzed by a transition-metal.

The first example of solid phase A3-coupling reaction using a catalytic amount of CuCl was performed by Dyatkin and Rivero in 1998. They immobilized aryl alkynes on polymer support (Rink resin) and used different substituted aldehydes and secondary amines for propargylamine synthesis under the catalysis of CuCl. The products were cleaved from the resin support by trifluoroacetic acid (TFA) (Figure 7) [27].

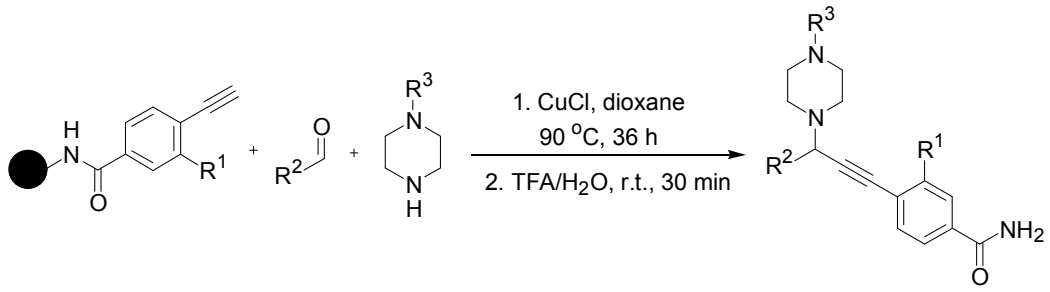


Figure 7. A3-coupling reaction performed using resin-supported aryl alkynes.

Following the success of the first A3-coupling reaction, a number of important advancements have been made. Various metal catalysts such as Cu(I), Cu(II), Au(I), Au(III) and Ag(I) salts were found to afford good yields of propargylamines, and their recyclabilities in A3-coupling were studied. Water was found to be the best solvent for A3-coupling in general, though some organic solvents such as toluene and DMF were also suitable in some situations. Many substrates including aromatic and aliphatic aldehydes, aromatic and aliphatic alkynes, secondary amines, tert-butyl amine, and aniline were studied and the formations of the corresponding propargylamines were successful. Solvent-free and microwave-assisted approaches were also developed in recent years [28]. Note that secondary amines were employed in most of these advancements. Only a few successful methods of using primary amines to synthesize propargylamines by A3-coupling reactions were reported [26].

A3-coupling reactions are now becoming more popular for the synthesis of propargylamines which are key intermediates for synthesis of many heterocycles. By using different available starting materials, diversified classes of propargylamines can be synthesized.

One example was to use CuI as the catalyst to synthesize the propargylamines, followed by an intramolecular acetylene hydroarylation catalyzed by Pd to form 3-benzazepine scaffold (Figure 8). Some 3-benzazepine derivatives have potential effects on treatment of certain neurologic disorders as they are known to be inhibitors of glycine transporter GlyT1 [29].

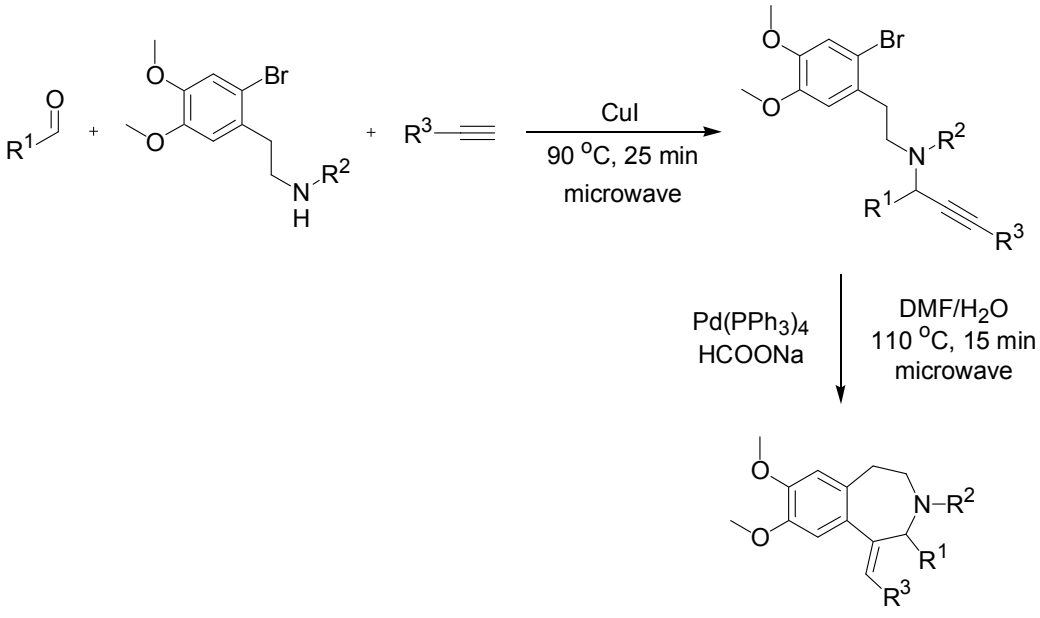


Figure 8. CuI-catalyzed A3-coupling reaction as the key step for 3-benzazepine

synthesis.

One more example employed A3-coupling for modification of artemisinin (Figure 9). Artemisinin is a natural product and their derivatives are known to be effective in treatment of *Plasmodium falciparum* malaria and certain cancers. A Au(III) salen complex was prepared and used as the catalyst by Wong and Che. Several propargylamine-modified artemisinin derivatives were synthesized and their sensitive endoperoxide group remained intact after reactions. These artemisinin derivatives showed promising cytotoxic effects against human hepatocellular carcinoma cell line (HepG2) [30].

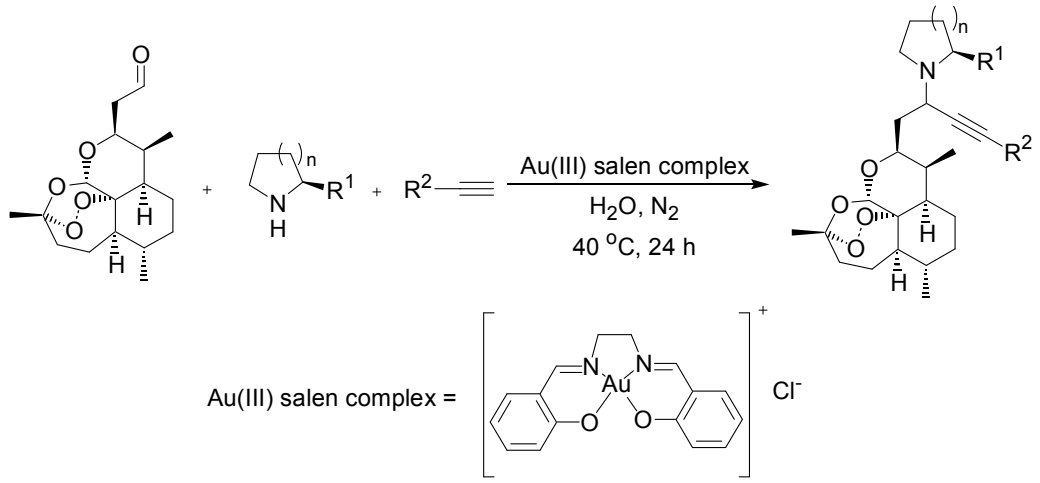


Figure 9. Gold(III) salen complex-catalyzed A3-coupling reaction for modification of artemisinin.

As shown above, A3-coupling reaction is a convenient approach for synthesis of propargylamines for further modifications to nitrogen-containing heterocycles, therapeutic drug molecules and natural products.

3.2 Objective

Previous research works by our group showed that steric effect can affect the product formation in A3-coupling reaction. In that work, 1,2:3,4-di-*O*-isopropylidene-*α*-D-*galacto*-hexodialdo-1,5-pyranose (the sugar aldehyde), pyrrolidine (the amine) and phenylacetylene (the alkyne) were coupled to form propargylamine catalyzed by a cyclometallated gold(III) complex, as shown in Figure 10 below. Because of the favourable front side attack of the gold acetylide, the propargylamine shown on the upper side of Figure 10 was the major product with a dr value of 97:3 which indicated a high diastereoselectivity [31].

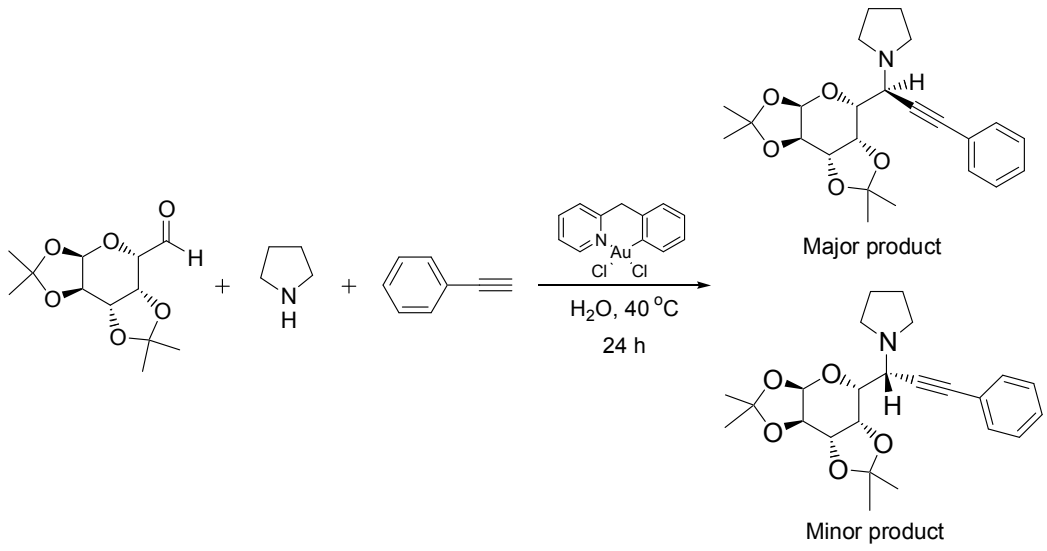


Figure 10. A3-coupling of a sugar aldehyde, pyrrolidine and phenylacetylene.

As formaldehyde is the smallest aldehyde in nature, it is expected that the selectivity can be achieved by increasing the steric bulkiness around the amino group of the secondary amine, By this multi-component reaction, formaldehyde can be incorporated to fluorophores through propargylamine formation for detection purpose.

The work is a collaboration with Guangdong Entry-Exit Inspection and Quarantine Bureau (GDCIQ). GDCIQ is an administrative and law-enforcement department directly under General Administration of Quality Supervision, Inspection and Quarantine of the People's Republic of China (AQSIQ). The duties of GDCIQ include entry-exit health quarantine, animal and plant quarantine, certification, survey, inspection, and administration and supervision of import-export commodities to safeguard the public health and promote the foreign economy and trade in Guangdong [32].

The title of our collaboration is the development of a fluorescent probe for fast detection of formaldehyde in food. We focus on the research of the formaldehyde detection method in Hong Kong. GDCIQ is responsible for the method validation using food samples in the later stage.

3.3 Results and Discussion

3.3.1 Synthesis of Compounds

Considering that aldehyde is attacked by an amine to form the iminium salt in A3-coupling reaction, we planned to use amine as the formaldehyde probe. Inspired by the significant effect of steric hindrance on the formation of propargylamines [31], commercially available 2,2,6,6-tetramethylpiperidine **1a** was chosen for its four bulky methyl groups. We started our work with the reaction of formaldehyde (1 mmol), amine **1a** (1.1 mmol), commercially available phenylacetylene **2a** (1.5 mmol) and bis-cyclometallated gold(III) complex **3a** (0.01 mmol) [33b] in water (1 mL) at 40 ^{o}C for 24 hours (Figure 11). This reaction was performed using the reaction conditions and reagent ratio of our previously published A3-coupling reaction [33] to see if the desired propargylamine **4a** could be formed. After reaction, the product was extracted by dichloromethane and then purified by flash column chromatography. An isolated yield of 74% was obtained for propargylamine **4a** which indicated that the gold(III) complex-catalyzed A3-coupling reaction of an aldehyde, an amine and an alkyne is applicable for formaldehyde detection.

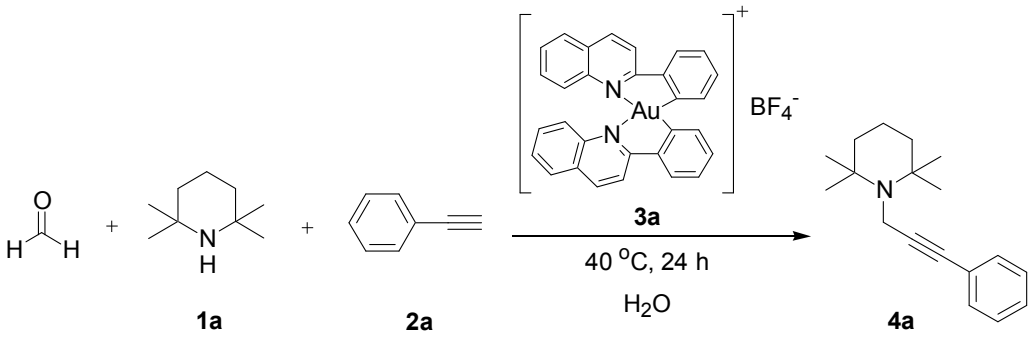


Figure 11. Gold(III) complex-catalyzed A3-coulping reaction of formaldehyde, amine **1a** and alkyne **2a**.

A reaction time of 24 hours is too long for fast detection of formaldehyde. Therefore, the reaction time was shortened to 1 hour for further investigations. Formaldehyde (1 mmol), amine **1a** (1.1 mmol), alkyne **2a** (1.5 mmol) and bis-cyclometallated gold(III) complex **3a** (0.1 mmol) in water (1 mL) was reacted for 1 hour (Figure 12). The reaction temperature was increased to 50 ^{o}C and the amount of catalyst was increased to 0.1 mmol to improve the yield for compensation of the reduced reaction time. An isolated yield of 6% of propargylamine **4a** was obtained which showed formaldehyde could still react with the amine and alkyne to form the product in 1 hour.

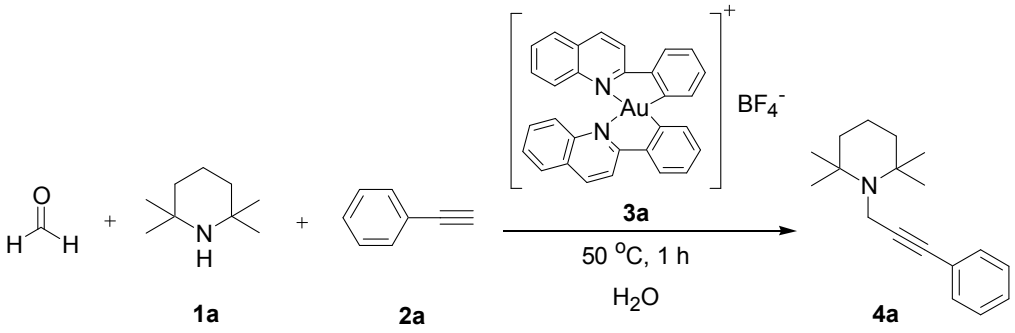


Figure 12. Gold(III) complex-catalyzed A3-coulping reaction of formaldehyde, amine **1a** and alkyne **2a**.

Since a yield of 6% was too low, the reaction conditions of the A3-coupling reaction for formaldehyde detection should be optimized. To facilitate the screening of different reaction parameters, a model reaction was designed (Figure 13) and a HPLC-based assay was employed.

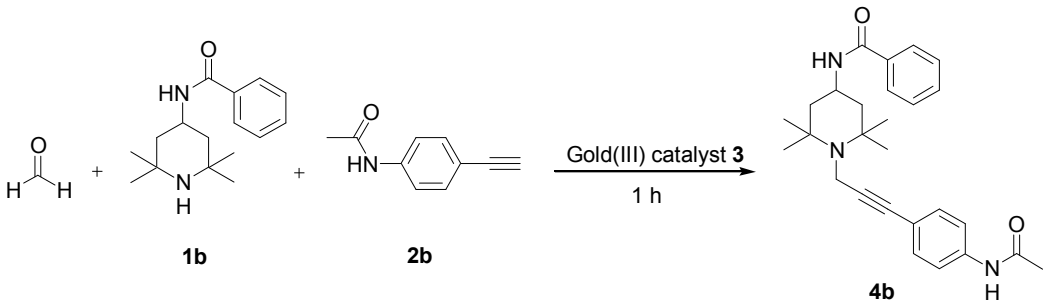


Figure 13. Model reaction for the optimization of reaction conditions for formaldehyde detection.

Amine **1b** was synthesized by reacting 4-amino-2,2,6,6-tetramethylpiperidine

and benzoyl chloride for overnight with an isolated yield of 84%. The phenyl ring provided by benzoyl chloride enhances the UV absorbance of amine **1b** to facilitate HPLC analysis. Since phenylacetylene is relatively volatile which may vaporize during HPLC analysis, a solid alkyne **2b** was synthesized by reacting 4-ethynylaniline and acetic anhydride for 6 hours with an isolated yield of 95%. After that, formaldehyde was reacted with amine **1b** and alkyne **2b** using a cyclometallated gold(III) complex **3c** [34] for 24 hours to get the propargylamine **4b** with an isolated yield of 36% (Figure 14). Details of the model reaction and the HPLC-based assay will be discussed in Section 3.3.3.

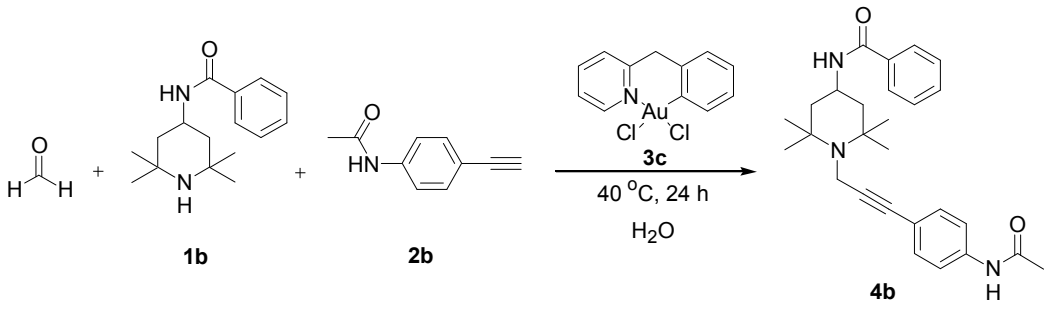


Figure 14. A3-coupling reaction for synthesis of propargylamine **4b**.

3.3.2 Studies on Selectivity of the Formaldehyde Probe

Mass spectrometry (MS) was used to study the selectivity of the formaldehyde probe due to its high accuracy and high sensitivity. When formaldehyde (0.1 mmol) was used as the aldehyde to react with the amine **1a** (0.11 mmol) and

phenylacetylene **2a** (0.15 mmol) catalyzed by bis-cyclometallated gold(III) complex **3a** (0.01 mmol) in water at 50 ^{o}C for 1 hour (Figure 12), the desired propargylamine **4a** was formed as determined by ESI-MS. When the same molar amounts of acetaldehyde, isobutyraldehyde, benzaldehyde and acetone were used under the same reaction conditions respectively, ESI-MS analysis showed no corresponding propargylamines were formed (Figure 15).

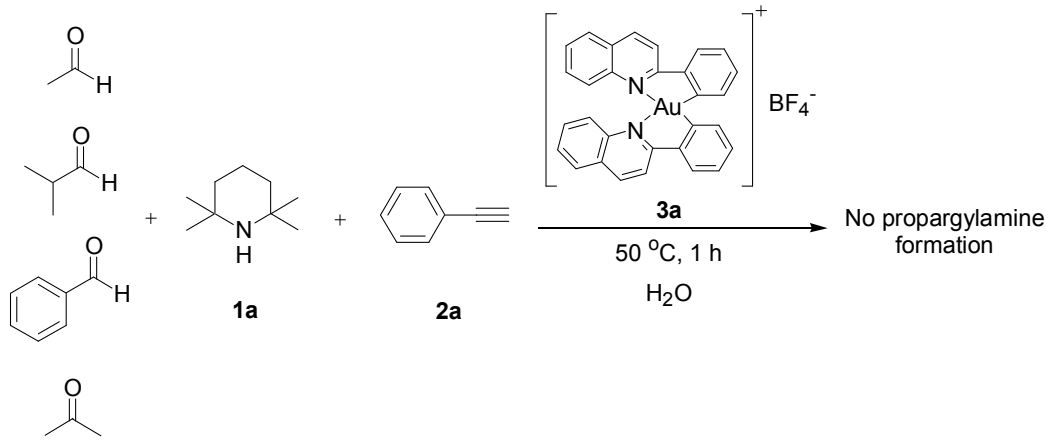


Figure 15. Reaction for study of the selectivity of amine **1a** to different aldehydes and acetone.

Another commercially available but less sterically bulky cis-2,6-dimethylpiperidine was also used as the amine to study its selectivity to formaldehyde (Figure 16). ESI-MS analysis showed that only formaldehyde could give the corresponding propargylamine (Figure 17) while acetaldehyde

gave no propargylamine product under the same reaction conditions. Due to the more convenient functionalization, 2,2,6,6-tetramethylpiperidine **1a** was used for further investigations.

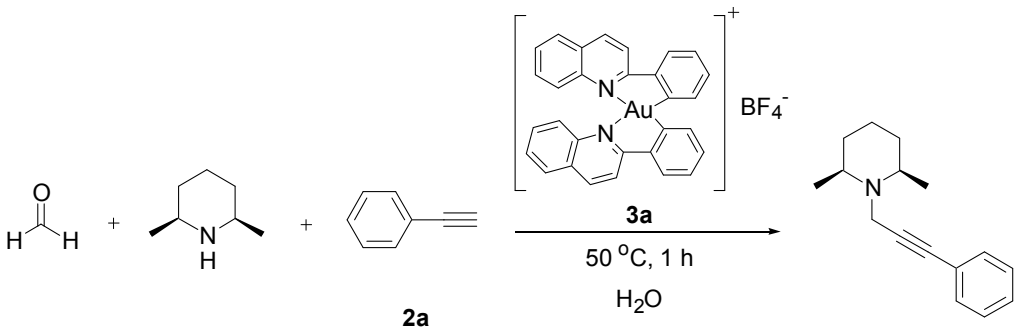


Figure 16. Reaction for study of the selectivity of cis-2,6-dimethylpiperidine to formaldehyde.

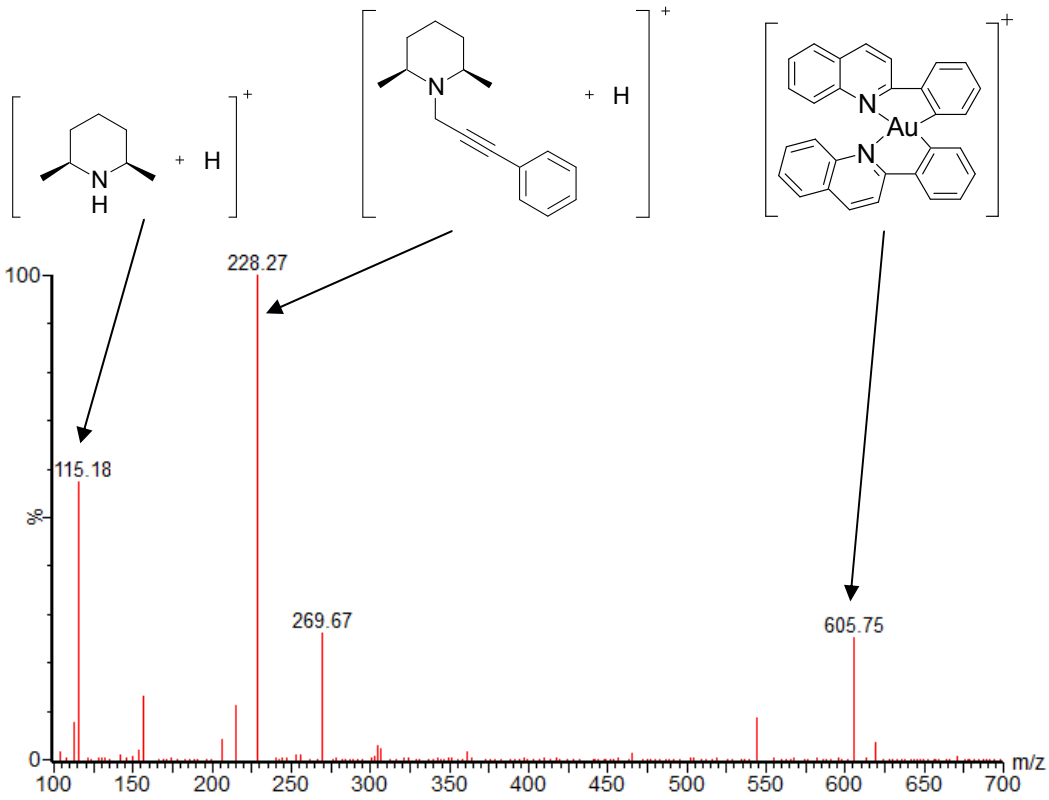


Figure 17. MS spectrum of formation of product by cis-2,6-dimethylpiperidine as the formaldehyde probe.

3.3.3 Optimization of A3-Coupling Reaction Conditions

After identifying the probe for exclusive formaldehyde detection, a model reaction using amine **1b** and alkyne **2b** (Figure 13) was designed, as mentioned in Section 3.3.1. A HPLC-based assay was employed to screen the effects of different parameters on the yield of propargylamine **4b**, such as catalysts, solvents, temperatures and reagent ratios.

For the HPLC conditions, reverse phase gradient with an XTerra MS C18

Column were used. The mobile phase consisted of water and acetonitrile. The gradient was optimized to separate compounds **1b**, **2b**, **3a** and **4b** in the reaction mixture within 18 minutes. Figure 18 showed a typical HPLC chromatogram for the model reaction. All the four compounds with UV absorption **1b**, **2b**, **3a** and **4b** were separated by the HPLC gradient.

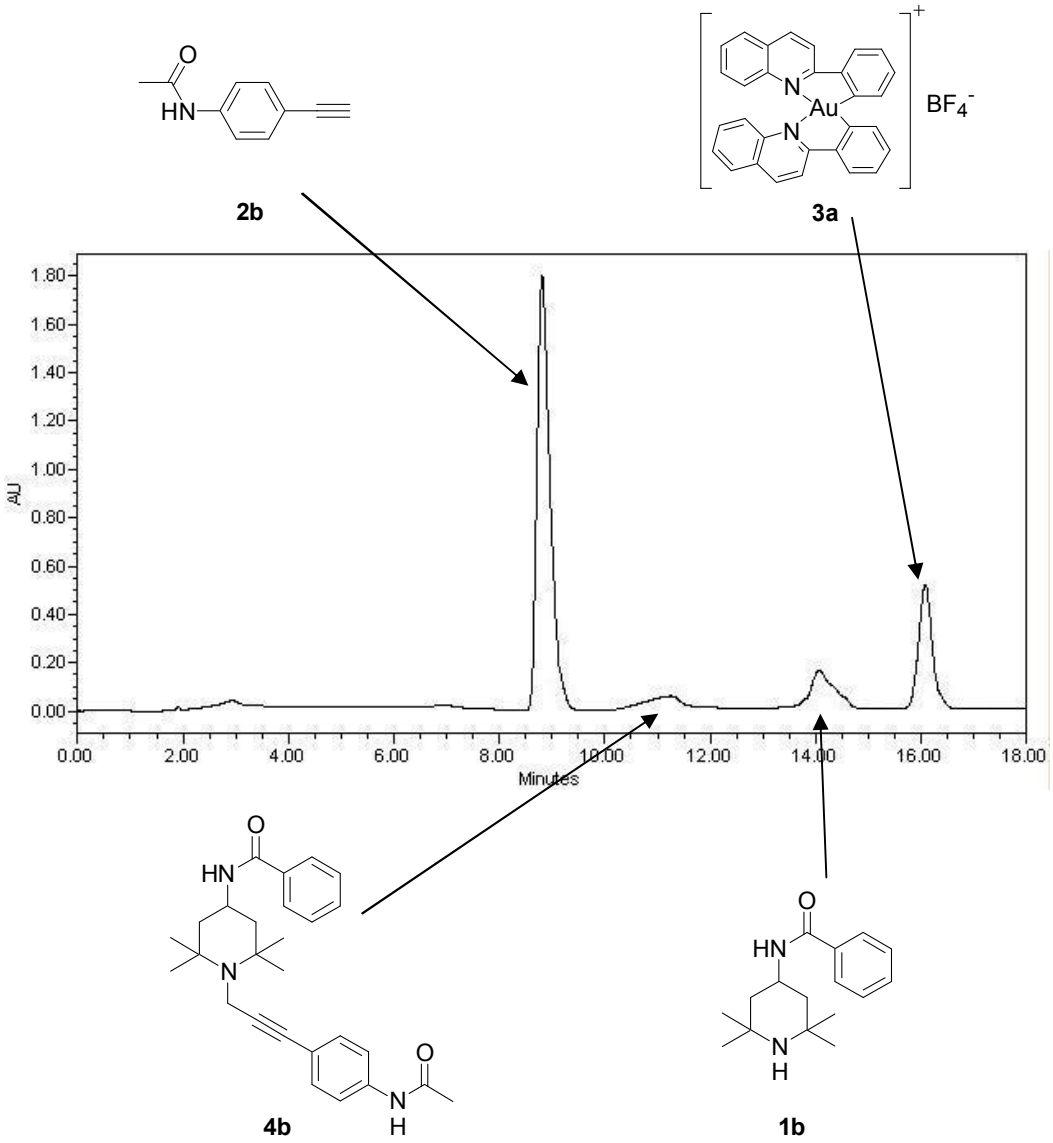


Figure 18. Typical HPLC chromatogram for study of the model reaction.

In order to quantify the amount of propargylamine **4b** formed in the model reaction, a calibration curve of propargylamine **4b** was constructed (Figure 19). The dynamic range of the curve was from 1 ppm to 200 ppm of propargylamine **4b**. The coefficient of determination (R^2) was calculated to be 0.9996 which indicated an excellent linearity. This showed that the curve was very reliable to be used for quantification of propargylamine **4b** by the HPLC assay.

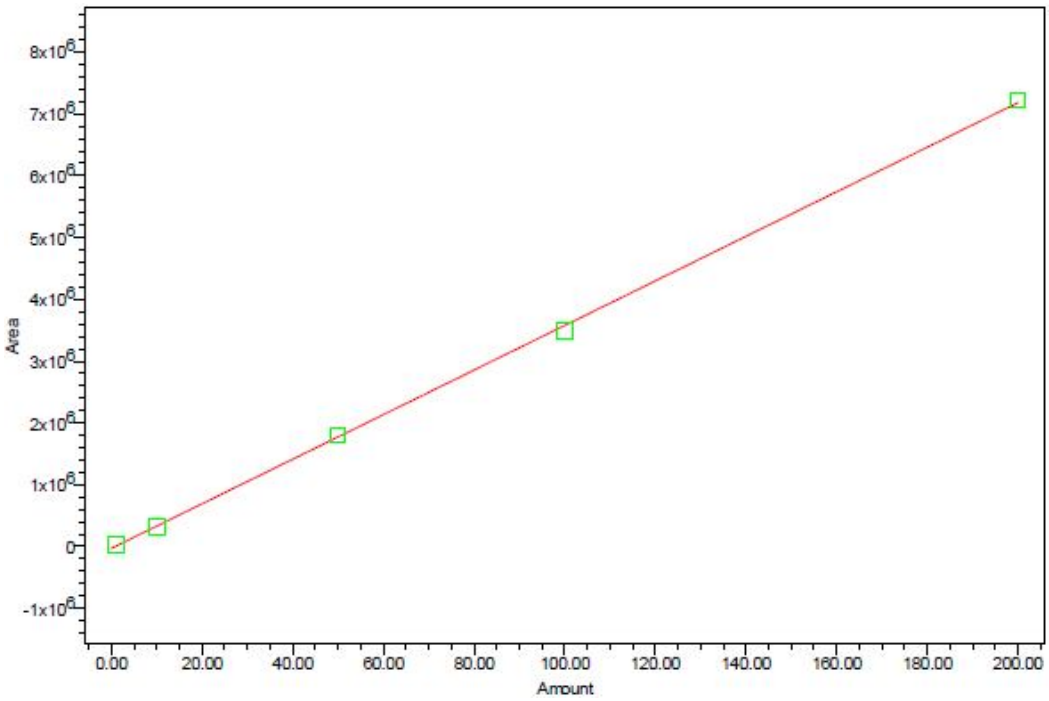
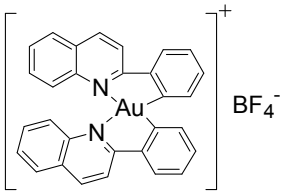
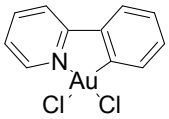
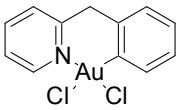


Figure 19. Calibration curve for quantification of propargylamine **4b** formed in the model reaction by HPLC. The x-axis is formaldehyde concentration and y-axis is peak area.

Three cyclometallated gold(III) complexes **3a-c** commonly used by our research group [31 and 33] were synthesized and screened to observe their catalytic activities on the formation of propargylamine **4b** by the three-component coupling reaction of formaldehyde, amine **1b** and alkyne **2b**. After reacting for 1 hour at 50 ^{o}C, the reaction mixtures were diluted and injected into HPLC to separate different components and determine the yields of propargylamine **4b**. As shown in Table 2, bis-cyclometallated gold(III) complex **3a** gave the best yield (4.0%, entry 1) among the three catalysts studied. This result is consistent with our previous work. We found that 60% isolated yield of propargylamine could be obtained even with 0.1 mol % of **3a** at 40 ^{o}C for 24 hours, and 1 mol % of **3a** could give propargylamine in 25% yield at room temperature for 24 hours. Such high reactivity of **3a** can be explained by its distorted square planar geometry with elongated bond lengths which generate a highly reactive gold(III) reaction centre for alkyne activation upon ligand dissociation [33b].

Table 2. Screening conditions for effect of different cyclometallated gold (III) complexes on the yield of propargylamine **4b** in the model reaction[a]

Entry	Cyclometallated gold(III) complex	Yield of 4b[b] (%)
1	 **3a**	4.0
2	 **3b**	1.1
3	 **3c**	2.2

[a] Reactions were carried out with formaldehyde (20 μmol, in form of an aqueous solution), amine **1b** (22 μmol), alkyne **2b** (30 μmol) and cyclometallated gold(III) complexes **3a-c** (4 μmol) in water at 50 ^{o}C for 1 hour. [b] Determined by HPLC.

After choosing the most effective bis-cyclometallated gold(III) catalyst **3a**, the effect of different solvents on the yield of propargylamine **4b** was evaluated using the established HPLC assay. Formaldehyde was added in form of 100 μL aqueous solution. As shown in Table 3, some polar solvents which are miscible with water (entries 2-5) gave less propargylamine **4b** when compared to using

water alone (entry 1) or even no product was formed as determined by HPLC. This result suggested that using a co-solvent system with water and a polar miscible solvent may gave poor yields in the bis-cyclometallated gold(III) complex-catalyzed three-component coupling reaction of an aldehyde, an amine and an alkyne for propargylamine synthesis when compared to using water as the only solvent. On the other hand, organic solvents which are immiscible with water (ethyl acetate and 1,2-dichloroethane, entries 7-8) gave better yields of propargylamine **4b** when compared to using water alone (entry 1). Using 1,2-dichloroethane as the solvent gave 3 times higher yield (12.1%, entry 8) than using water (3.8%, entry 1). Owing to the higher yield, 1,2-dichloroethane was chosen to be the solvent of the reaction for screening of other parameters.

Table 3. Screening conditions for effect of solvents on the yield of product **4b** in the model reaction[a]

Entry	**Solvent[b]**	**Yield of 4b[c] (%)**
1	Water	3.8
2	Acetonitrile	-
3	Dimethylformamide	-
4	Tetrahydrofuran	1.6

5	1,4-Dioxane	2.2
6	Toluene	1.1
7	Ethyl Acetate	5.4
8	1,2-Dichloroethane	12.1

[a] Reactions were carried out with formaldehyde (20 μmol, in form of an aqueous solution), amine **1b** (22 μmol), alkyne **2b** (30 μmol) and bis-cyclometallated gold(III) complex **3a** (4 μmol) at 50 ^{o}C for 1 hour. [b] 600 μL of solvent was used. [c] Determined by HPLC.

After choosing the best solvent 1,2-dichloroethane for the A3-coupling reaction, the effect of the ratio of 1,2-dichloroethane to water was evaluated. This is because water is usually used as the solvent to extract formaldehyde from food samples. Therefore, water is inevitable to be present in the detection process. Different ratios of 1,2-dichloroethane to water were used in the model reaction (Table 4). The reaction mixtures were stirred vigorously to ensure proper mixing of the two immiscible layers. After reaction, 1,2-dichloroethane was added to the reaction mixtures to make up the organic layer to a final volume of 450 μL, respectively, for easy comparison after HPLC analysis. In general, the yields of propargylamine **4b** increased as the ratio of 1,2-dichloroethane to water increased,

and the best yield (11.4%) was achieved when the ratio of 1,2-dichloroethane to water was 9:1 (entry 9). The reason for this observation may be the higher solubility of the reagents amine **1b**, alkyne **2b** and bis-cyclometallated gold(III) complex **3a** in a larger volume of organic solvent, i.e. 1,2-dichloroethane, and thus the reaction proceeded more readily. Another possible reason may be the higher partition of formaldehyde to a larger volume of 1,2-dichloroethane layer which made the reaction proceeded better in the organic layer.

Table 4. Screening conditions for effect of ratio of 1,2-dichloroethane to water on the yield of product **4b** in the model reaction[a]

Entry	1,2-Dichloroethane (μL)	Water (μL)	Ratio	Yield of 4b[b] (%)
1	50	450	1:9	2.2
2	100	400	1:4	3.1
3	150	350	3:7	3.7
4	200	300	2:3	2.5
5	250	250	1:1	3.7
6	300	200	3:2	4.2
7	350	150	7:3	4.5
8	400	100	4:1	8.8

9	450	50	9:1	11.4

[a] Reactions were carried out with formaldehyde (20 μmol, in form of an aqueous solution), amine **1b** (22 μmol), alkyne **2b** (30 μmol) and bis-cyclometallated gold(III) complex **3a** (4 μmol) at 50 ^{o}C for 1 hour. [b] Determined by HPLC.

To study the effect of temperatures on the yield of the model reaction, four temperatures ranging from 25 to 60 ^{o}C were chosen (Table 5). No product **4b** was formed when the reaction was carried out at room temperature (entry 1) for 1 hour. 40 ^{o}C and 60 ^{o}C gave similar yields (entries 2 and 4) but 50 ^{o}C gave the best yield (12.1%, entry 3) among the four temperatures studied. The lower yield at 60 ^{o}C (4.1%) compared to that at 50 ^{o}C (12.1%) may be due to the escape of formaldehyde from the reaction mixture at high temperature.

Table 5. Screening conditions for effect of temperatures on the yield of product **4b** in the model reaction[a]

Entry	**Temperature (^{o}C)**	**Yield of 4b[b] (%)**
1	25	-
2	40	3.6
3	50	12.1

4	60	4.1

[a] Reactions were carried out with formaldehyde (20 μmol, in form of an aqueous solution), amine **1b** (22 μmol), alkyne **2b** (30 μmol) and gold(III) complex **3a** (4 μmol) in 1,2-dichloroethane for 1 hour. [b] Determined by HPLC.

After identifying the optimal reaction temperature, different amounts of amine **1b**, alkyne **2b** and bis-cyclometallated gold(III) complex **3a** were screened to study their effects on the yield of propargylamine **4b** (Table 6). Generally speaking, the yield of propargylamine **4b** increased when the amounts of **1b**, **2b** and **3a** used for reaction increased, with up to 56% yield was achieved in 1 hour using 5 equivalents of the bis-cyclometallated gold(III) complex **3a** with respect to formaldehyde (entry 12). It should be noted that poor yield (8%) was obtained when the same molar amounts of **1b**, **2b** and **3a** were used (entry 10). A set of experiments was performed using formaldehyde in excess with amine **1b** as the limiting reagent (entries 17-25). However, the yields of propargylamine **4b** were not as good as the one mentioned above (56%, entry 12). Therefore, we decided to use a large excess of reagents to react with the limiting amount of formaldehyde in samples as the detection mode.

Table 6. Screening conditions for effect of reagents ratios on the yield of product **4b** in the model reaction[a]

Entry	Amine 1b (μmol)	Alkyne 2b (μmol)	Gold(III) complex 3a (μmol)	Yield of 4b[b] (%)
1	10	15	2	-
2	10	50	10	18
3	10	50	30	19
4	10	100	1	5
5	10	100	2	-
6	10	150	2	-
7	10	200	1	1
8	50	10	10	19
9	50	50	30	41
10	50	50	50	8
11	50	100	2	-
12	50	100	50	56
13	50	150	2	-
14	100	50	50	40
15	100	150	2	6

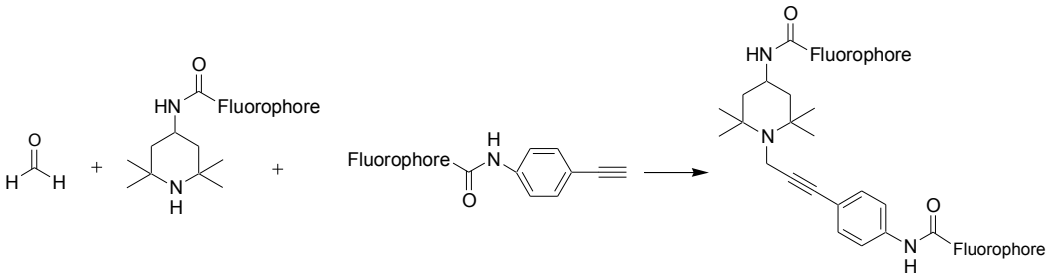
16	100	100	50	53
17[c]	10	15	1	3
18[c]	10	100	1	9
19[c]	10	100	2	16
20[d]	20	30	2	4
21[d]	20	200	2	23
22[d]	20	200	4	46
23[e]	10	15	1	5
24[e]	10	100	2	28
25[f]	20	30	2	12

[a] Unless otherwise specified, reactions were carried out with formaldehyde (10 μmol, in form of an aqueous solution) in 1,2-dichloroethane at 50 ^{o}C for 1 hour. [b] Determined by HPLC. [c] 100 μmol of formaldehyde was used. [d] 200 μmol of formaldehyde was used. [e] 300 μmol of formaldehyde was used. [f] 600 μmol of formaldehyde was used.

3.3.4 Fluorescent Detection of Formaldehyde

As mentioned before, our goal is to detect formaldehyde in food samples by fluorescent means due to the high sensitivity. Therefore, different amines and

alkynes with fluorophores were synthesized and used for reaction to see whether product with two fluorophores was formed, as shown in Scheme 4.



Scheme 4. General scheme of the three-component coupling reaction of formaldehyde, a fluorescent amine and a fluorescent alkyne for dual-fluorophore propargylamine synthesis.

Amine **1c** was synthesized by reacting 4-amino-2,2,6,6-tetramethylpiperidine and commercially available 7-(diethylamino)coumarin-3-carboxylic acid using N,N,N',N'-tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) as the coupling reagent (Figure 20). After washing with water and purification by flash column chromatography, amine **1c** with a coumarin fluorophore was obtained with 91% yield.

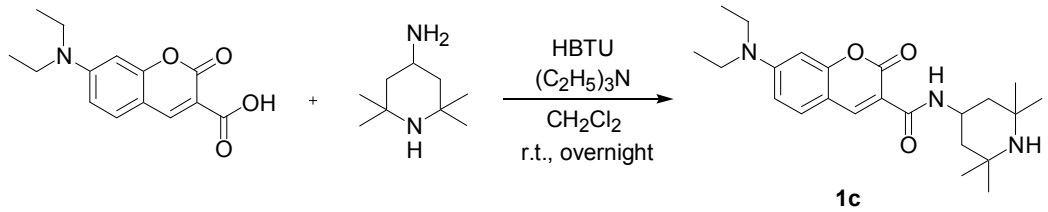


Figure 20. Synthesis of amine **1c**.

Alkyne **2c** was synthesized by reacting 7-(diethylamino)coumarin-3-carboxylic acid and 4-ethynylaniline using HBTU as the coupling reagent (Figure 21). After washing with water and purification by flash column chromatography, alkyne **2c** with a coumarin fluorophore was obtained with 79% yield.

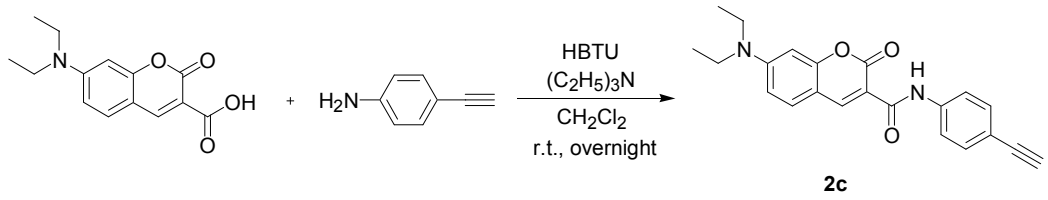


Figure 21. Synthesis of alkyne **2c**.

Alkyne **2d** was synthesized by reacting 4-ethynylaniline and dansyl chloride (Figure 22). Purification of the reaction mixture by flash column chromatography offered alkyne **2d** with a dansyl fluorophore with 69% yield.

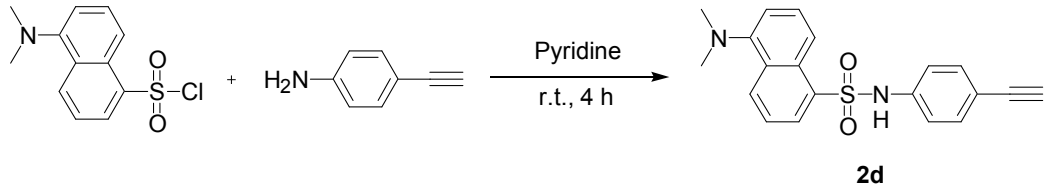
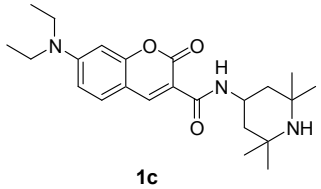
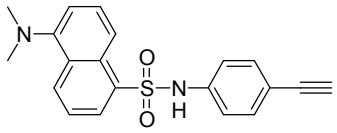
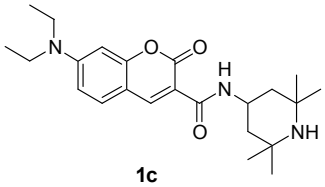
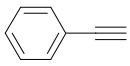
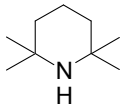
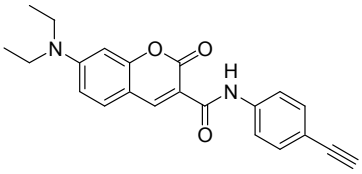
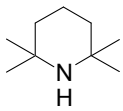
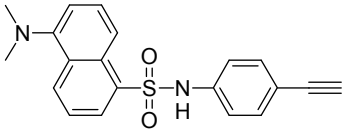


Figure 22. Synthesis of alkyne **2d**.

After synthesis, amines **1c** and alkynes **2c-d** with fluorophores (coumarin and dansyl) were used in different combinations for the three-component coupling reaction for formaldehyde detection (Table 7). As determined by ESI-MS, the corresponding propargylamines were formed in different combinations (Figures 23-26). Coumarin has an excitation wavelength at 409 nm and an emission wavelength at 473 nm which gives blue fluorescence. Dansyl has an excitation wavelength at 337 nm and an emission wavelength 492 nm which gives green fluorescence. The results showed that different fluorophores could be used for the reaction for formaldehyde detection, and one fluorophore or two fluorophores could be used according to the needs. Due to the relatively easy synthesis of coumarin amine **1c** and alkyne **2c**, coumarin was used as the fluorophore for further investigations.

Table 7. Screening of different fluorescent amines and alkynes on the formation of fluorophore product via propargylamine formation[a]

Entry	Amine	Alkyne	Formation of product[b]
1	 **1c**	 **2d**	Yes
2	 **1c**	 **2a**	Yes
3	 **1a**	 **2c**	Yes
4	 **1a**	 **2d**	Yes

[a] Reactions were carried out with formaldehyde (20 μmol, in form of an aqueous solution), amine **1a** and **1c** (150 μmol), alkyne **2a**, **2c**-**d** (300 μmol) and gold(III) complex **3a** (150 μmol) in 1000 μL 1,2-dichloroethane at 50 ^{o}C for 1 hour. [b] Determined by ESI-MS.

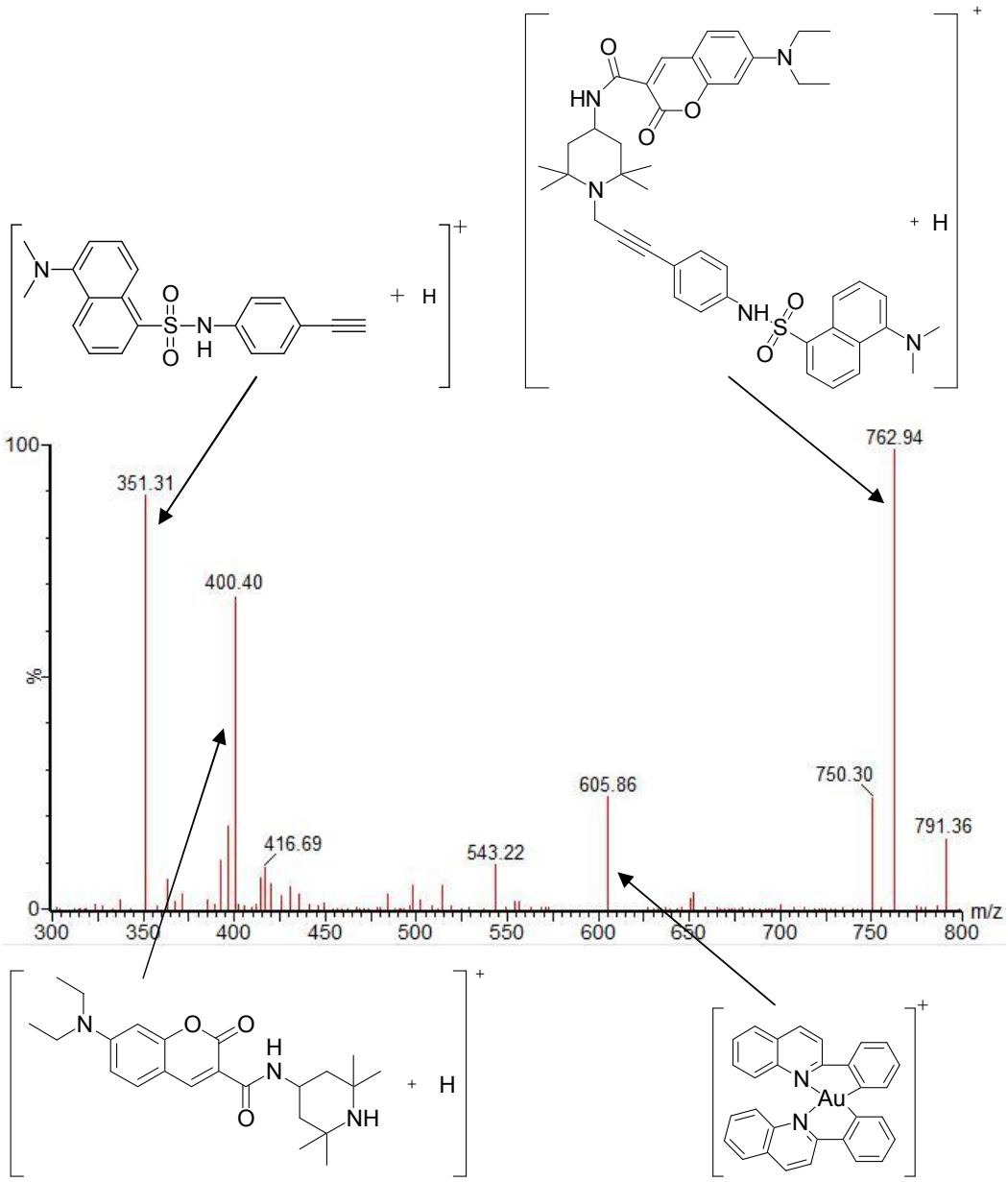


Figure 23. MS spectrum of product formation from the coumarin amine and dansyl alkyne.

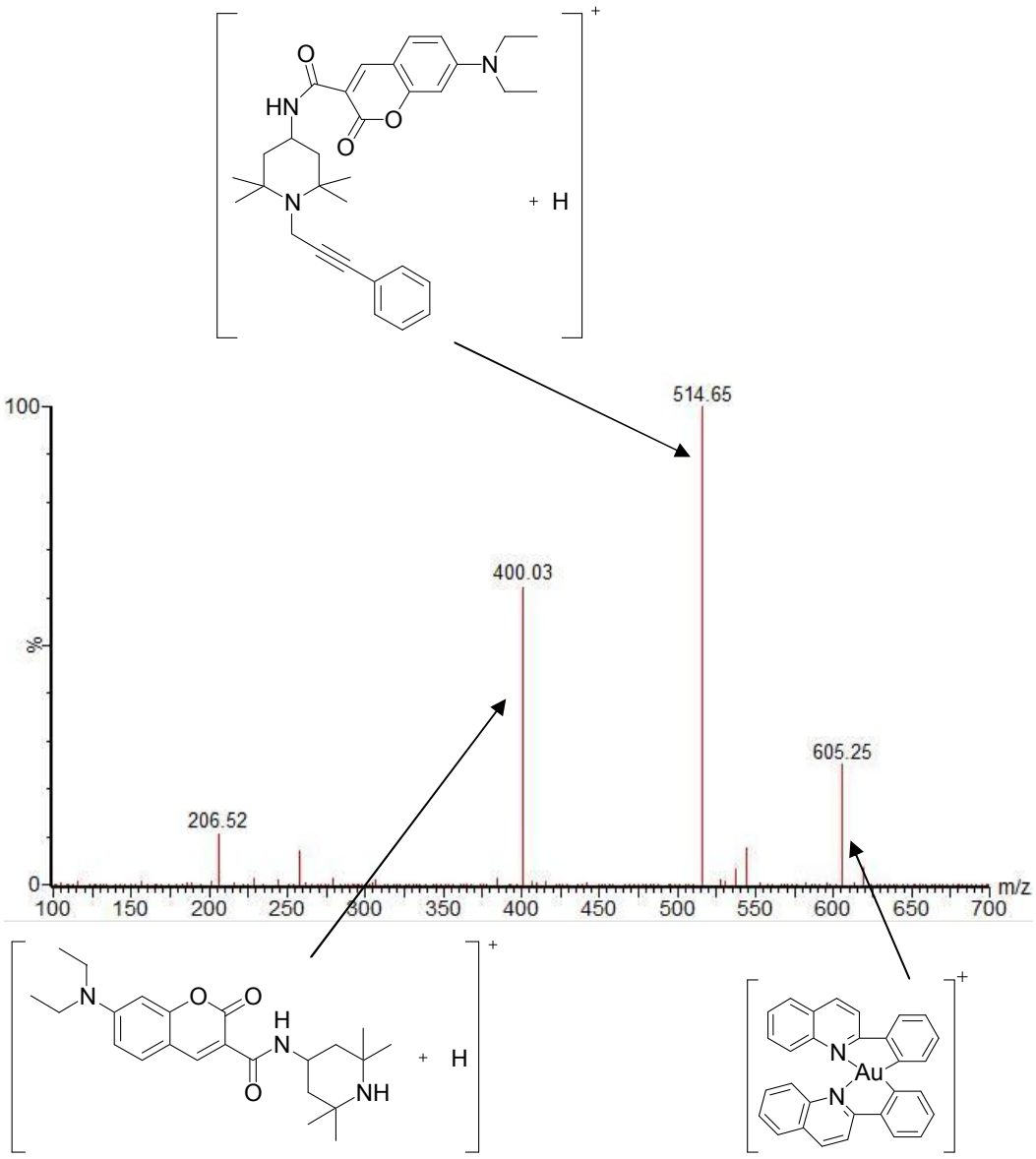


Figure 24. MS spectrum of product formation from the coumarin amine and phenylacetylene.

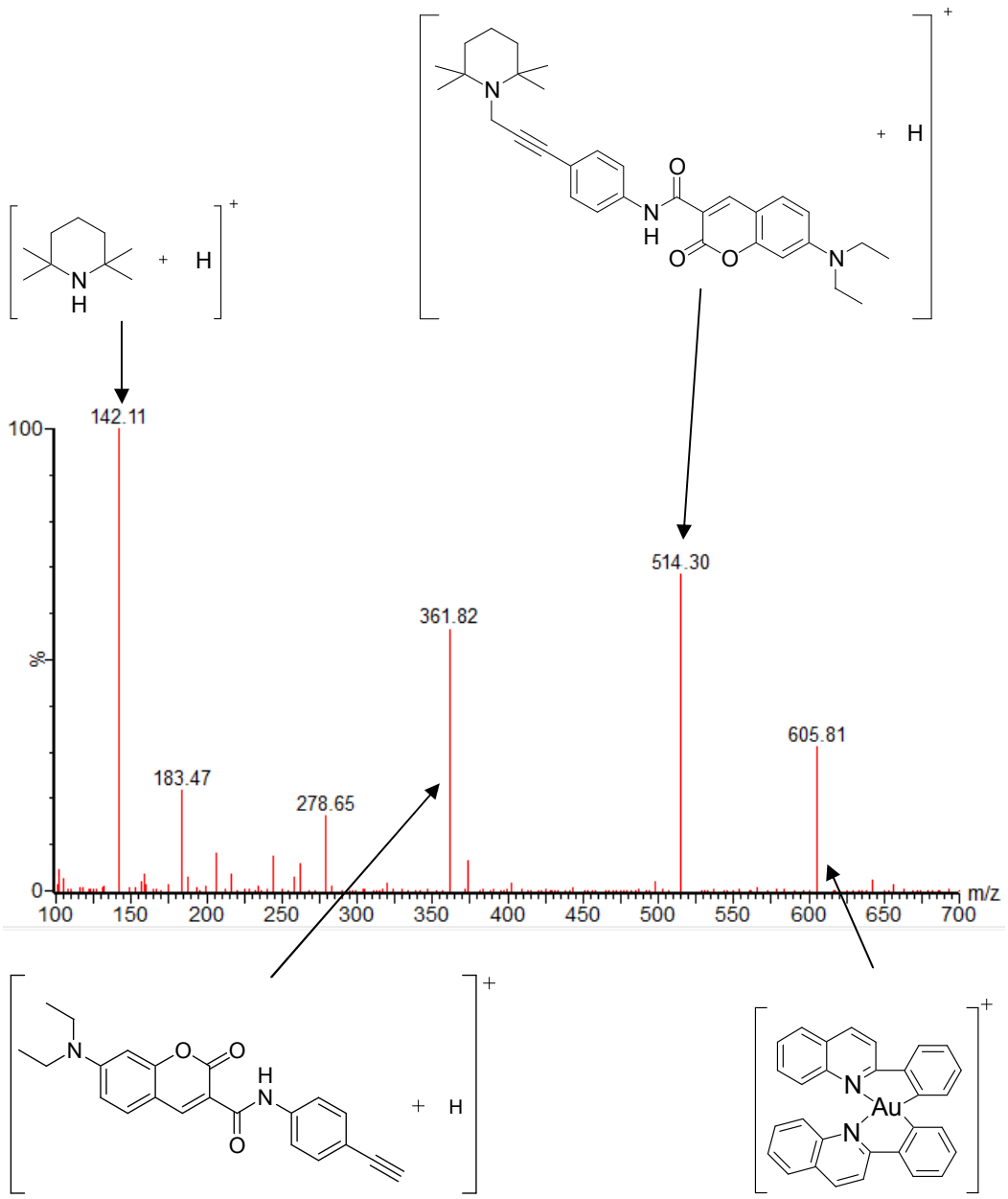


Figure 25. MS spectrum of product formation from 2,2,6,6-tetramethylpiperidine and coumarin alkyne.

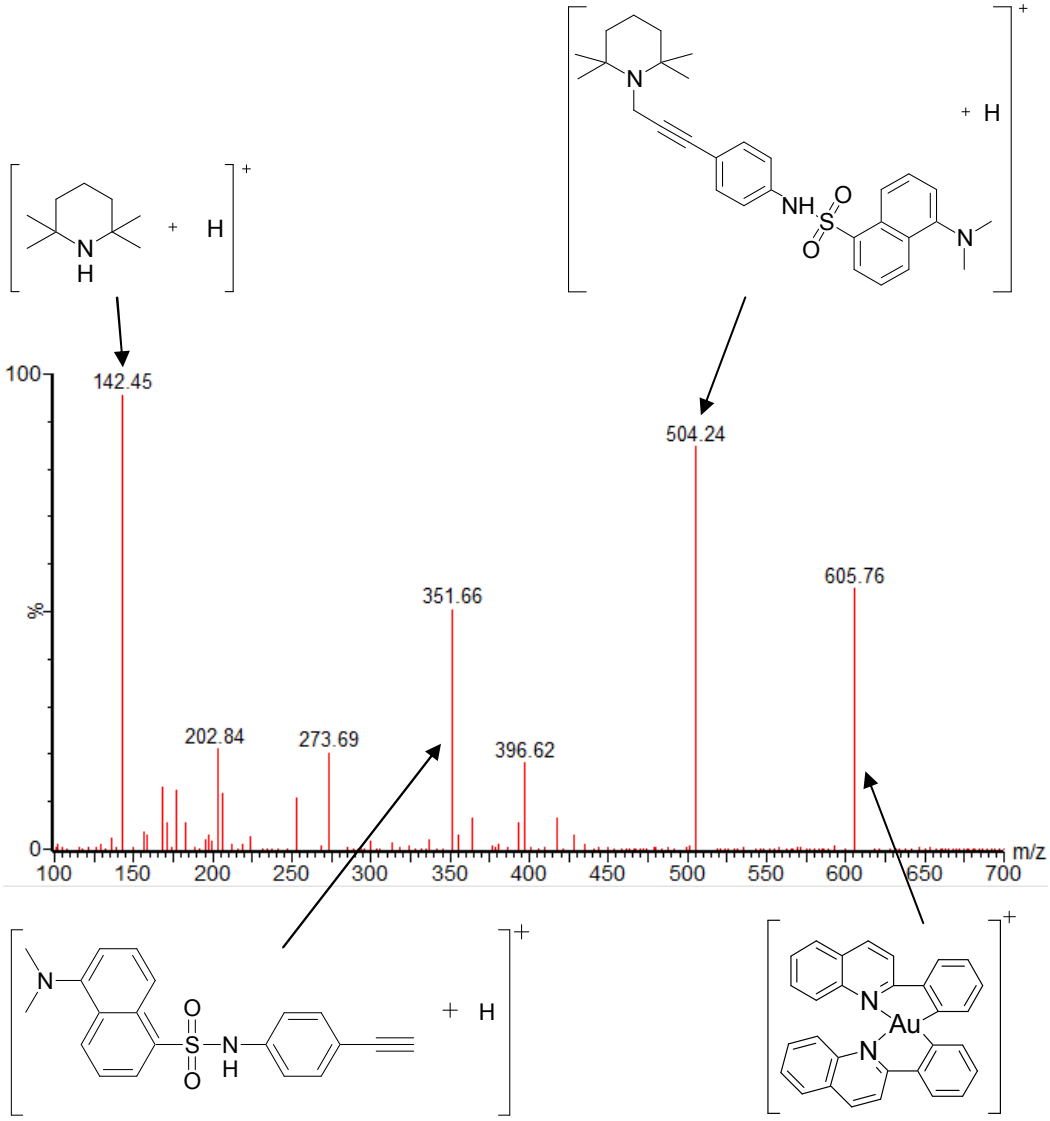


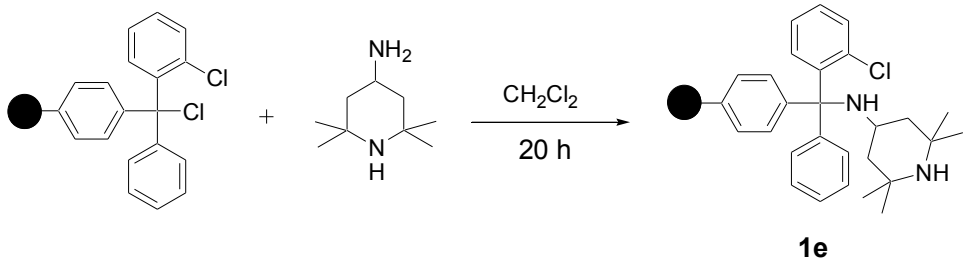
Figure 26. MS spectrum of product formation from 2,2,6,6-tetramethylpiperidine and dansyl alkyne.

3.3.5 Formaldehyde Detection on Resin Support

Since the formaldehyde concentration is to be determined by the fluorescent intensity of the propargylamine formed via the gold(III) complex-catalyzed three-component coupling reaction, excess fluorescent reagents have to be removed after reaction and before measurement to reduce the background fluorescent signal. The washing step is important especially when a large excess of reagents are used to increase the yield of product. To facilitate the washing step, the reaction system mentioned above was transferred to a solid support using solid phase organic synthesis (SPOS).

Commercially available polymer-bound 2-chlorotrityl chloride resin (200-400 mesh) was chosen as the solid support. Commercially available 4-amino-2,2,6,6-tetramethylpiperidine was attached to the resin to form the formaldehyde probe **1e** according to literature procedure [35], as shown in Figure 27. After reaction and endcapping any remaining reactive 2-chlorotrityl groups with HPLC-grade methanol, the resin was washed with dichloromethane, dimethylformamide and then dichloromethane again to remove the unreacted 4-amino-2,2,6,6-tetramethylpiperidine [35]. After drying, the amine-functionalized resin could be used as the formaldehyde probe. The

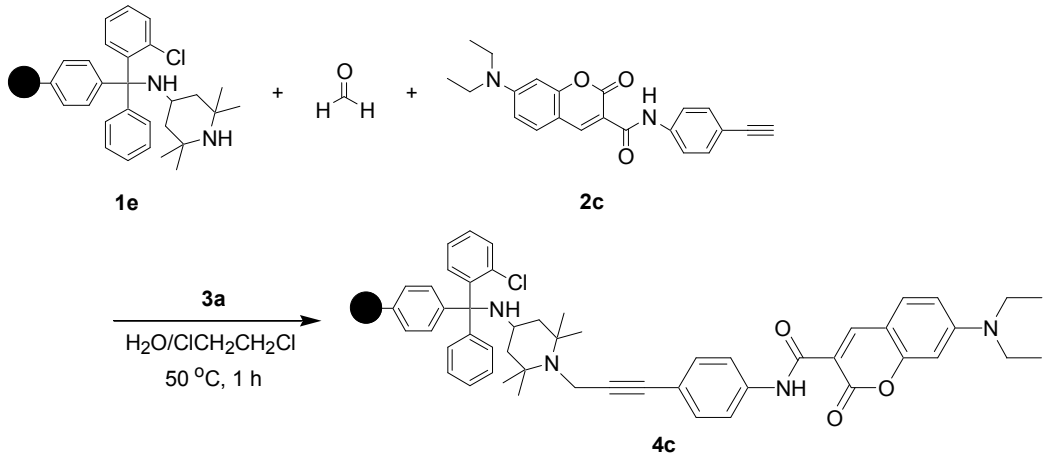
theoretical loading of the amine on the resin is about 98% [35].



1e

Figure 27. Synthesis of formaldehyde probe **1e**.

Since the resin beads swell in dichloromethane, the substituent 1,2-dichloroethane was used as the solvent to tolerate the 50 ^{o}C reaction temperature. Using excessive coumarin-linked alkyne **2c** (0.02 mmol) and bis-cyclometallated gold(III) catalyst **3a** (0.01 mmol), product **4c** was formed in 1 hour (Figure 28).



1e **2c**

4c

Figure 28. A3-coupling reaction of formaldehyde, a solid supported amine **1e**

and a coumarin-linked alkyne **2c** via propargylamine formation.

After washing the resin beads with literature procedure [35], the formation of product could be observed by the blue fluorescence on the resin beads under irradiation of a Spectroline® handheld dual wavelength UV lamp (Model: ENF-280C/FBE) at 365 nm due to the attachment of the coumarin fluorophore to the resin beads. The brightness of the resin was related to the formaldehyde concentrations used, as shown in Figure 29.

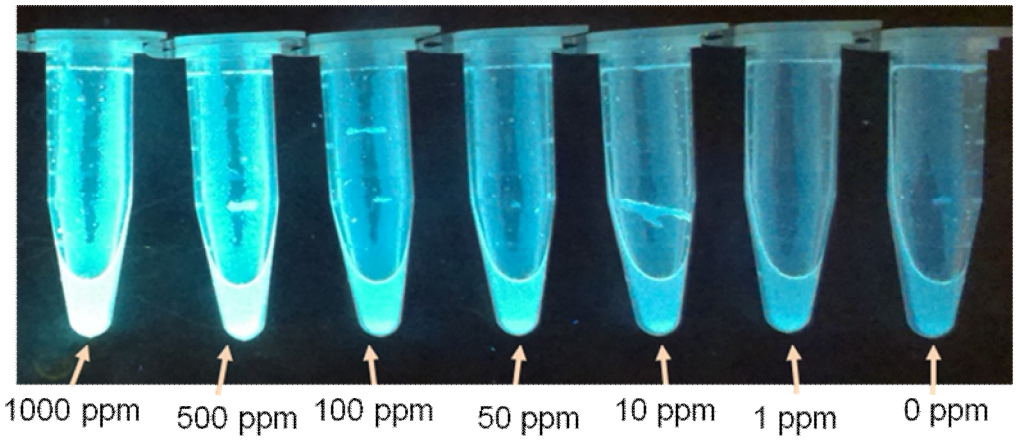


Figure 29. Product **4c** in 500 µL of dichloromethane under irradiation of UV lamp at 365 nm.

To confirm product formation, propargylamine **5a** was cleaved from the resin by a mixture of 1:1 trifluoroacetic acid and dichloromethane [35] (Figure 30).

ESI-MS analysis showed that propargylamine **5a** was formed (Figure 31). The cleavage mixture was neutralized by saturated sodium bicarbonate ($NaHCO_3$) and purified by flash column chromatography to give propargylamine **5a** with 4% yield. A fluorescent spectrum was obtained for propargylamine **5a** which showed a maximal emission wavelength at 461 nm (Figure 32).

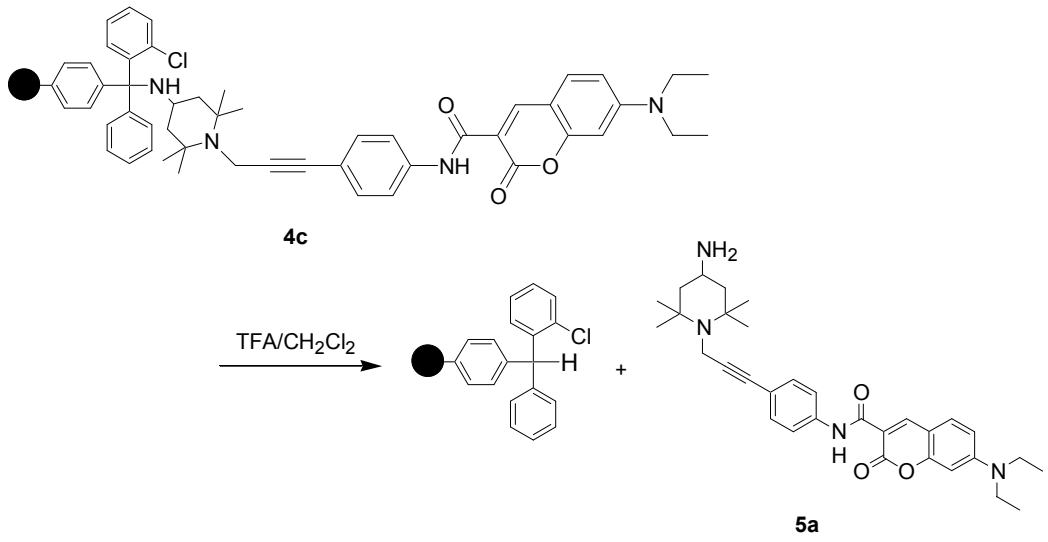


Figure 30. Cleavage of propargylamine **5a** from the resin **4c**.

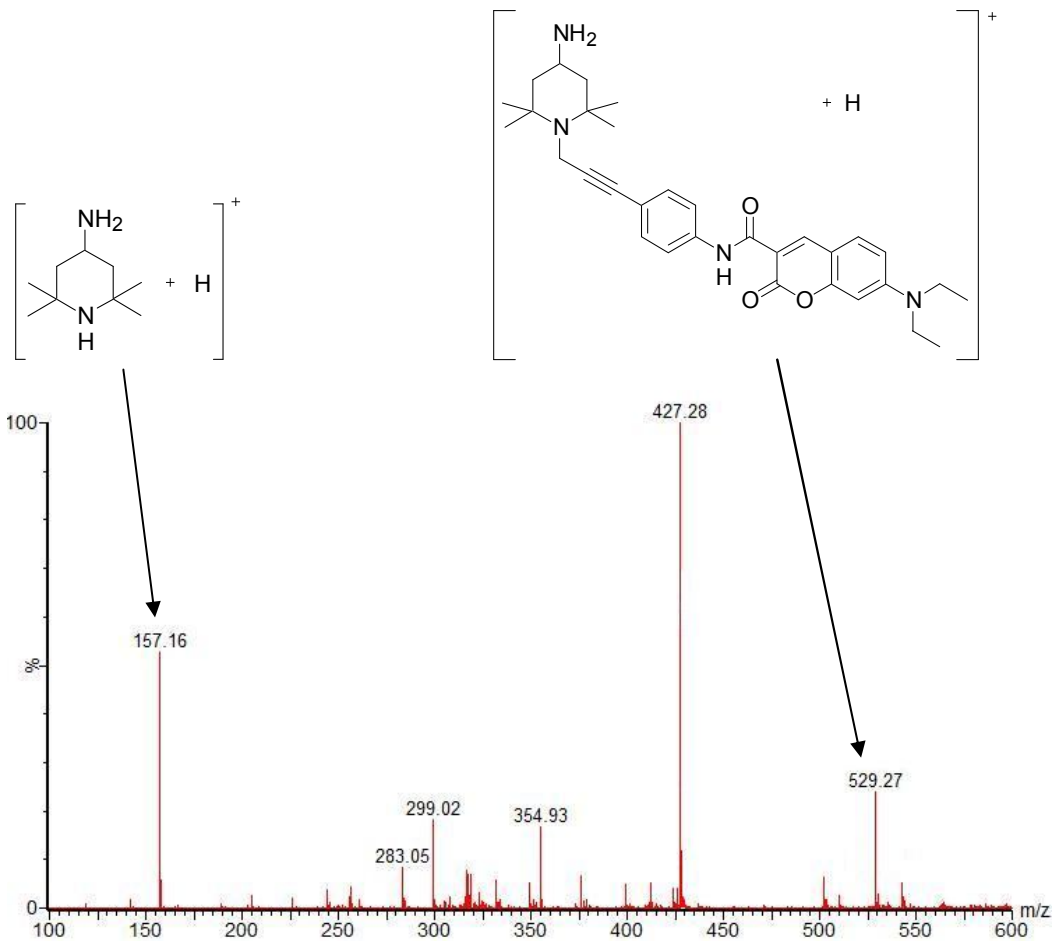


Figure 31. MS spectrum of cleaved product from the resin beads.

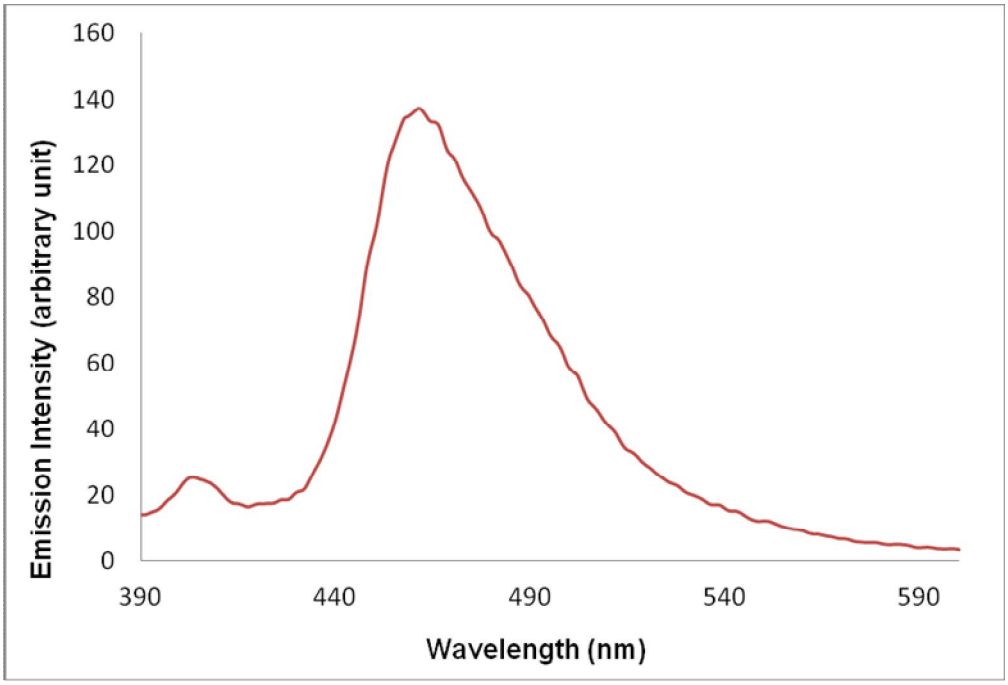


Figure 32. Fluorescent spectrum of propargylamine **5a**.

The selectivity experiments were performed using the resin-supported formaldehyde probe **1e** to observe whether product was formed when acetaldehyde, isobutyraldehyde and benzaldehyde was used, respectively. After cleavage of the reaction mixtures, ESI-MS analysis showed no propargylamine was formed when 18400 ppm of acetaldehyde, 30000 ppm of isobutyraldehyde and 26500 ppm of benzaldehyde were used, respectively.

The competition experiment was also performed to study whether the presence of other aldehydes inhibited the formation of product **4c** from formaldehyde. Thus, 5000 ppm of formaldehyde, acetaldehyde, isobutyraldehyde and benzaldehyde

were added to the same reaction container and allowed to react. After the washing and cleavage steps, it was found that only propargylamine **5a** from formaldehyde was formed by ESI-MS analysis. Hence, other aldehydes present in the reaction mixture did not inhibit the formation of product from formaldehyde.

To study the linearity between different formaldehyde concentrations and fluorescence intensities, formaldehyde solutions ranging from 0 to 500 ppm were used in the solid-supported reaction to react with amine **1e** (0.019 mmol), alkyne **2c** (0.02 mmol) and gold(III) complex **3a** (0.01 mmol) in 600 μL of 1,2-dichloroethane at 50 ^{o}C. After 1 hour of reaction and washing step, 2.0 mg of the resin beads from different reaction mixtures were accurately weighed and transferred to a microplate for measurement of fluorescent intensity by a microplate reader (Figure 33). Figure 34 showed the calibration curve obtained. An excellent linearity ($R^2 = 0.99$) was obtained in the calibration curve.

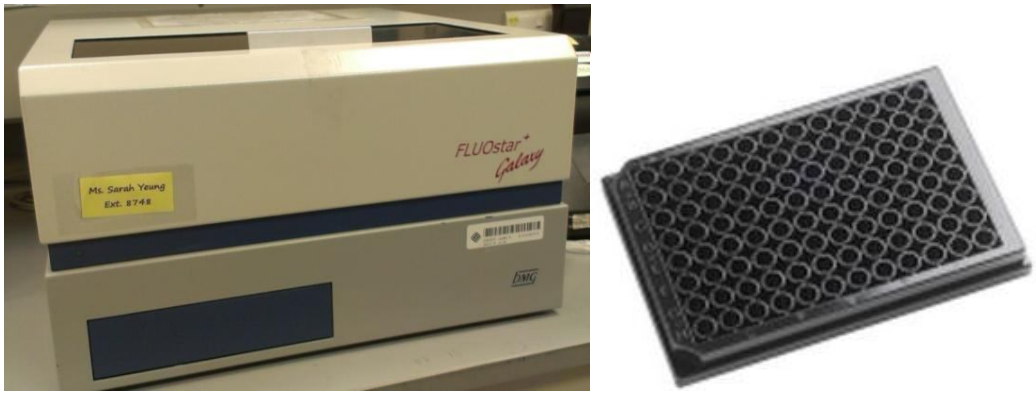


Figure 33. Photo of a microplate reader (left) and a microplate (right).

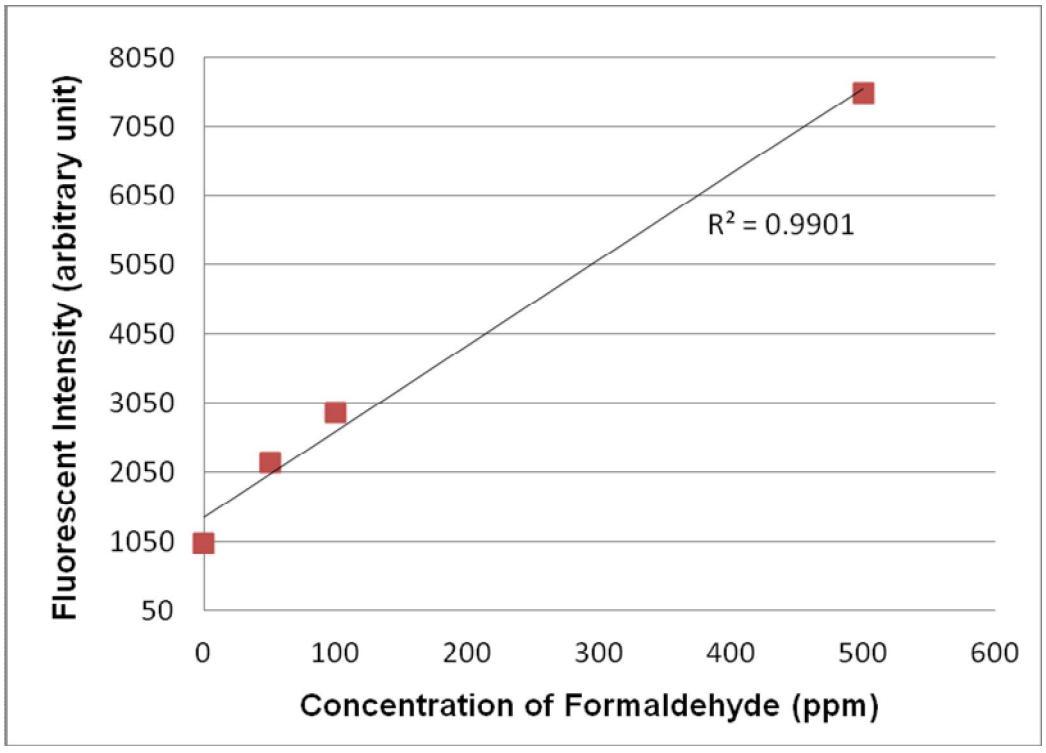


Figure 34. Linearity between 0-500 ppm formaldehyde concentrations and the fluorescence intensities of the resin beads.

However, there are some limitations to use the solid-supported amine probe to detect formaldehyde. Firstly, the washing step is quite time-consuming and

troublesome. In order to remove all the excess reagents thoroughly after reaction, large volumes of solvents have to be used and the washing process does need time. Secondly, resin is not a very good solid support for our application. As shown in Figure 34 above, the fluorescent intensity for 0 ppm of formaldehyde is pretty high (about 1050 arbitrary unit). The limit of detection of this method is 100 ppm based on a signal-to-noise ratio of 3:1 for calculation. This value is much higher than those fluorescent methods reported in recent literature, which are usually at ppb levels. A limit of detection of 100 ppm is only suitable for the detection of foods with heavy addition of formaldehyde for preservation or bleaching functions. The reason behind the high limit of detection may be the high background value. The resin beads have auto fluorescence in the blue region, as shown in the rightmost 0 ppm tube in Figure 29. This natural phenomenon leads to a high background fluorescent intensity as the coumarin fluorophore also gives emission signal in the blue region. As a result, the limit of detection becomes very high. Moreover, the calibration curve shown in Figure 34 with excellent linearity is difficult to be reproduced. Hence, it is a challenging task to quantify formaldehyde contents by measuring the fluorescent intensities on the resin support. Possible methods for improvements will be discussed later.

3.3.6 Application to Mushroom Samples

To determine the applicability of the bis-cyclometallated gold(III) complex-catalyzed three-component coupling reaction for detection of formaldehyde in foods, water extract of dried Shiitake mushroom was used as the formaldehyde source. The optimized conditions of the model reaction mentioned in Figure 13 were used. It was found that the desired propargylamine **4b** was formed after 1 hour of reaction at 50 ^{o}C, as confirmed by ESI-MS. The formaldehyde concentration in the water extract of the mushroom sample was determined by HPLC, which was about 3.1 ppm. The 2,4-DNPH method mentioned in Section 3.1.5.1 was also used for the same water extract which gave a concentration of 9.6 ppm. The value of formaldehyde content determined by our present method is consistent with the well-established 2,4-DNPH method but has some degree of deviation. One possible reason is due to the incomplete conversion of formaldehyde to the desired product in our method. In the 2,4-DNPH method, all the formaldehyde essentially reacts with the hydrazine to form the hydrazone which is then analyzed by HPLC. In our method, the conversion of formaldehyde to the propargylamine is incomplete (only 56%) even under the best reaction conditions. This explains the deviation in formaldehyde concentrations determined by the two methods.

To determine the applicability of the solid phase version of the bis-cyclometallated gold(III) complex-catalyzed three-component coupling reaction for detection of formaldehyde in foods, water extract of dried Shiitake mushroom was used as the formaldehyde source. It was found that the desired propargylamine **5a** was formed on the resin beads after 1 hour of reaction at 50 ^{o}C, and the cleaved product was confirmed by ESI-MS (Figure 35). As mentioned before, it is difficult to quantify formaldehyde content based on the fluorescent intensity on the resin beads. Hence this step was skipped. Further improvement on this method will be discussed in later section.

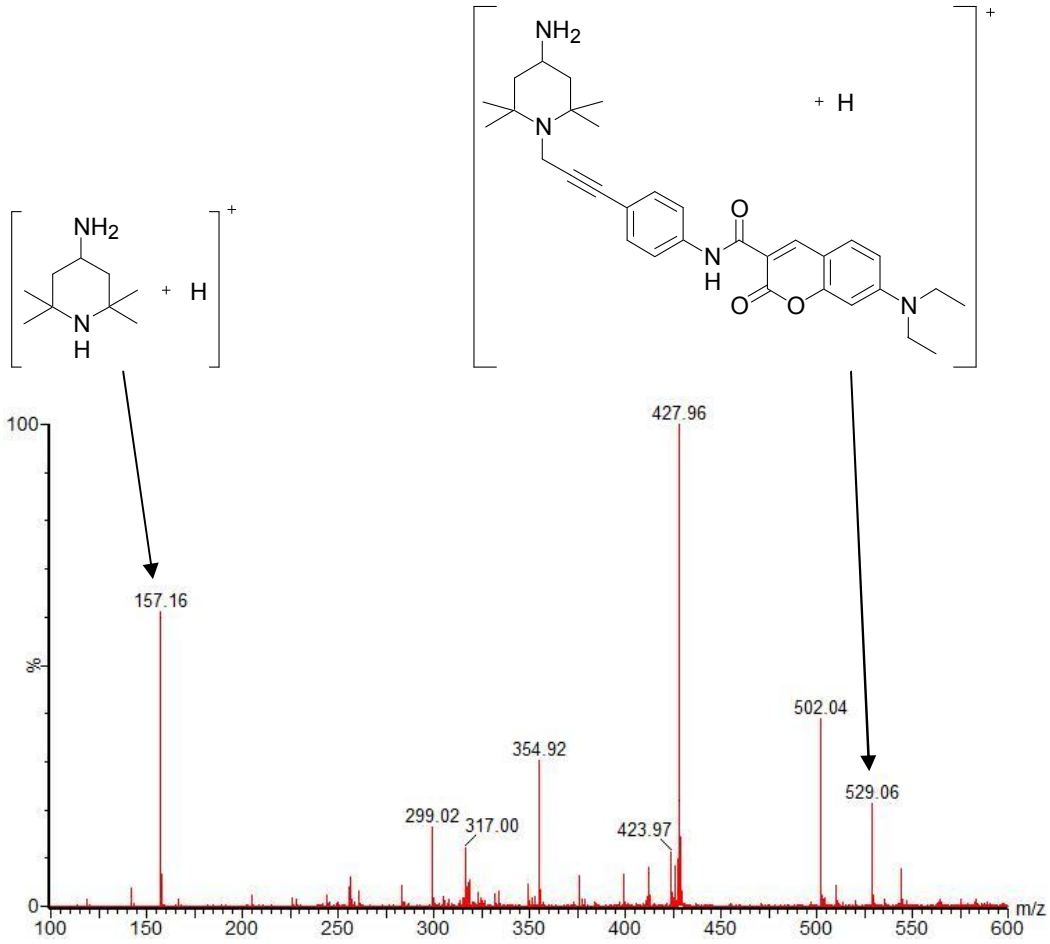


Figure 35. MS spectrum of cleaved product from the resin beads using mushroom sample as the formaldehyde source.

A demonstration on the use of the resin beads is shown in the two figures below.

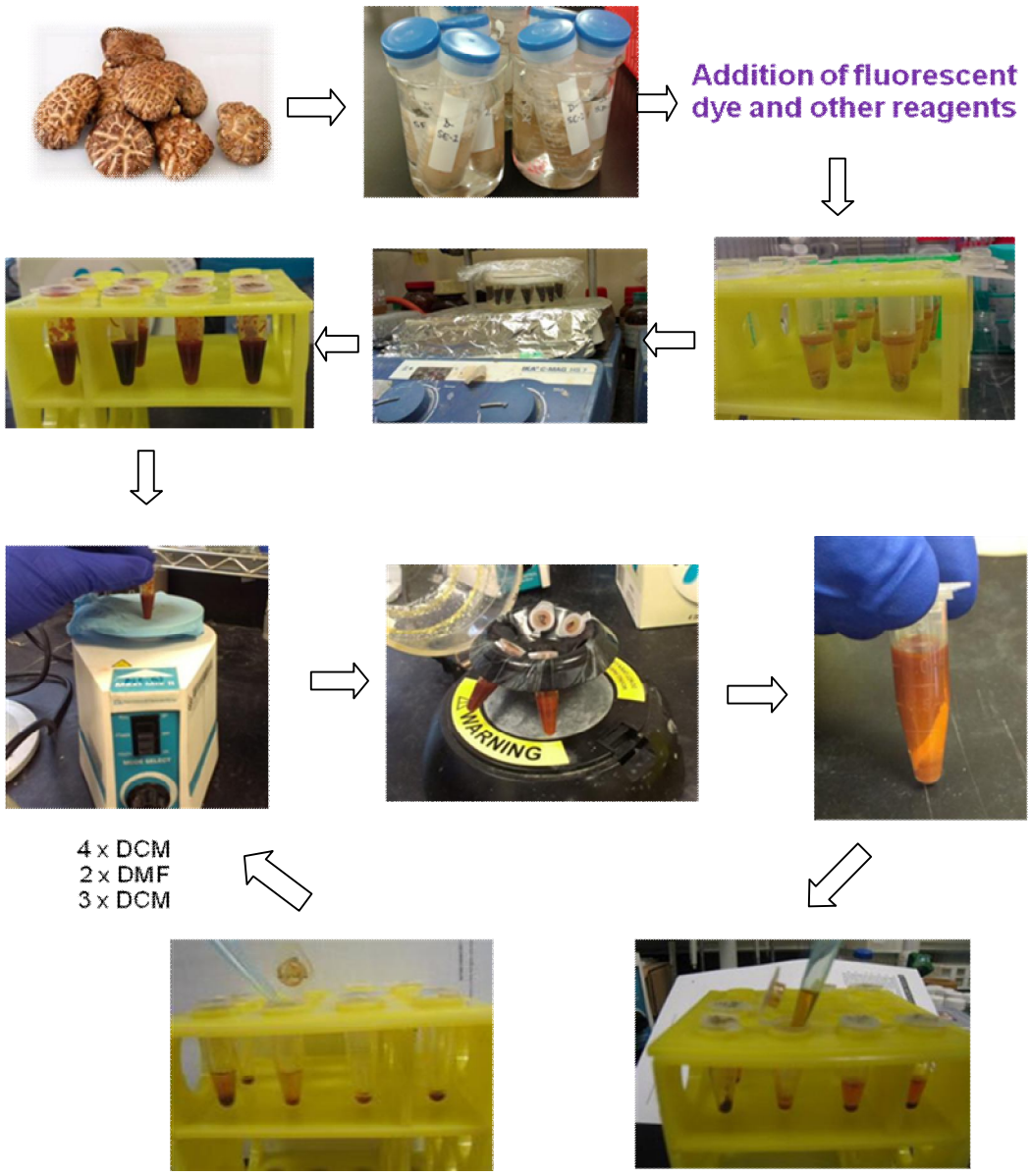


Figure 36. Procedures for using the resin beads. Dried shiitake mushrooms were homogenized and added with water for formaldehyde extraction. The water extract was mixed with the fluorescent dye and other reagents for A3-coupling reaction. After reaction, the reaction mixtures were washed with different solvents to remove excessive reagents.

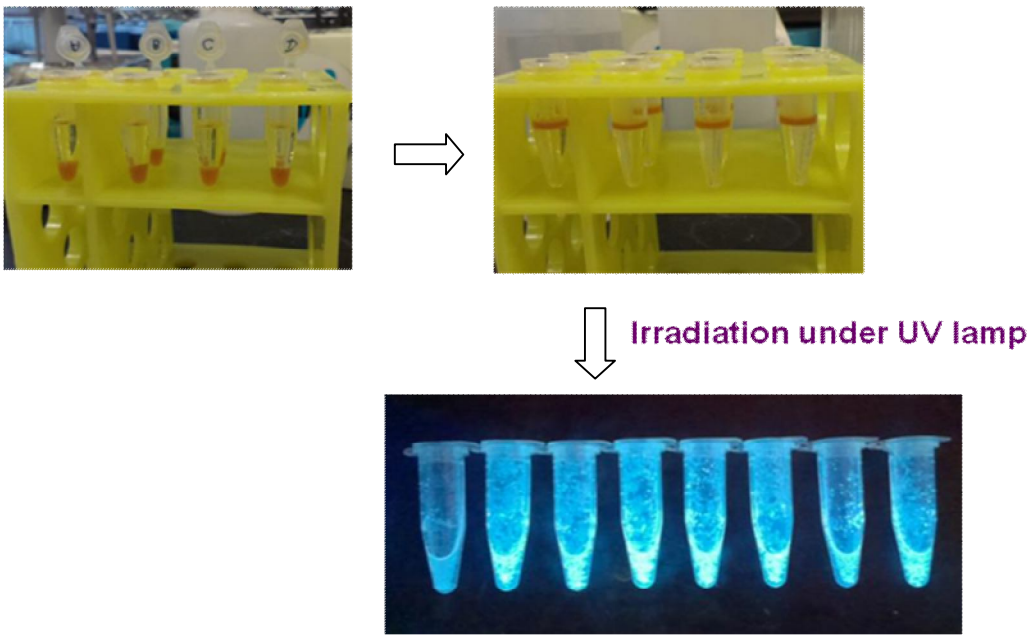


Figure 37. Emission of blue fluorescent from the resin beads under irradiation of a handheld UV lamp.

We are currently applying a joint Chinese patent with GDCIQ for the fluorescent detection of formaldehyde mentioned above.

3.4 Conclusion

A bis-cyclometallated gold(III) complex-catalyzed synthesis of propargylamine via a three component coupling reaction of an aldehyde, an amine and an alkyne for detection of formaldehyde in food was developed. The probe has excellent selectivity on formaldehyde which is a significant advantage compared to those current fast detection methods. The reaction was optimized to allow formaldehyde detection within 1 hour, with up to 56% yield. Different fluorophores such as coumarin and dansyl could be attached for fluorescent detection. The reaction system was immobilized on 2-chlorotrityl chloride resin beads for washing of excess reagents. Measurement of fluorescent intensity on the resin showed excellent linearity ($R^2 = 0.99$). The reaction was applicable to mushroom samples. It is envisioned that our method can be further developed to a convenient fast detection kit for on-site food inspection.

3.5 Suggestions for Future Research

To further simplify the time-consuming washing step in the solid phase version, other solid supports can be used. For example, the formaldehyde probe can be immobilized on glass or paper which can facilitate the washing of excess reagents after reaction.

To further decrease the limit of detection of our method, other fluorophores can be used. Since the resin has auto fluorescence in the blue colour region, using fluorophores with emission wavelengths in red or yellow region can reduce the overlapping in the blue region. As a result, the background fluorescent intensity can be much reduced and the signal-to-noise ratio can be significantly improved at low formaldehyde concentrations. Examples of suitable fluorophores include rhodamine B, Texas Red, eosin, Cy5, and some derivatives of fluorescein and boron-dipyrromethene (BODIPY).

After optimization of the detection method, this novel fluorescent probe for exclusive formaldehyde detection can be further developed into a simple and inexpensive rapid detection kit. This will not only enable the trade to implement front-line quality control, but also assist the related government agencies in

high-throughput on-site food safety inspection. The longer-term goal is to establish a brand new chemical technology platform which allows simple, inexpensive, high-throughput and rapid detection of harmful substances in food by using specially designed fluorescent probes especially for front-line quality control as well as on-site food safety inspection. It is anticipated that this platform will contribute significantly to secure the food safety and hence public health of the local community.

3.6 Experimental Section

3.6.1 General Procedure

All reagents were commercially available and used without further purification. Milli-Q® water used as reaction solvent and HPLC mobile phase was deionized using a Milli-Q® Gradient A10 system (Millipore, Billerica, USA). Flash column chromatography was performed using silica gel 60 (230-400 mesh, ASTM). ^{1}H and ^{13}C NMR spectra were recorded on Bruker DPX-400, DPX-600, Varian Unity Inova 400 NB and 500 NB spectrometers. The chemical shifts are expressed in ppm and coupling constants are given in Hz. Data for ^{1}H NMR are recorded as follows: chemical shift (δ, ppm), multiplicity (s, singlet; br s, broad singlet; d, doublet; dd, double doublet; t, triplet; td, triplet of doublets; m, multiplet), coupling constant (Hz), integration. Data for ^{13}C NMR are reported in terms of chemical shift (δ, ppm). Low resolution mass spectra (MS) and high resolution mass spectra (HR-MS) were obtained on Waters Micromass Q-Tof 2™ with positive ESI in terms of mass to charge ratio (m/z). Microplate reader results were obtained with BMG FLUOstar Galaxy Multi-functional Microplate Reader.

ESI-MS Analysis of Propargylamine Formation in A3-Coupling Reaction

The mass spectrometer was performed over a m/z range of 100-1000 on Waters Micromass Q-Tof 2™ with positive ESI, and the raw spectra were deconvoluted by the MassLynx 4.1 Transform Program (Waters, Manchester, UK). Desolvation and source temperatures were 150 °C and 80 °C respectively. Operating conditions optimized for the detection of reaction mixture were the following: capillary voltage 3 kV, sample cone voltage 30 V, extraction voltage 4 V and collision cell voltage 10 eV.

HPLC Conditions for Model Reaction Study

Waters 2695 HPLC Separation Module equipped with XTerra MS C18 (5 μm, 4.6 mm x 100 mm) Column and Waters 2998 Photodiode Array (PDA) Detector was used for separation. The flow rate was 0.6 mL/min. The injection volume was 10 μL. The PDA detector wavelength was set to 254 nm.

Mobile phase A was made of Milli-Q® water. Mobile phase B was made of acetonitrile. The initial conditions for separation were 30% B for 3 minutes, followed by a linear gradient to 70% B by 3 minutes. The composition was maintained for 4 minutes, followed by a linear gradient to 100% B by 1 minute.

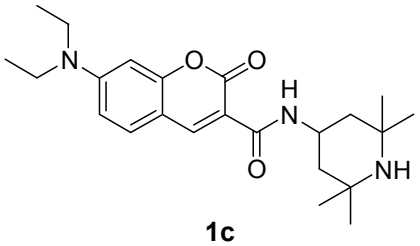
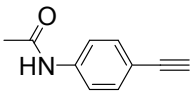
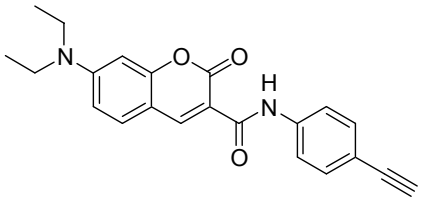
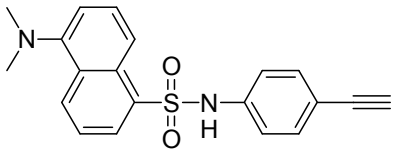
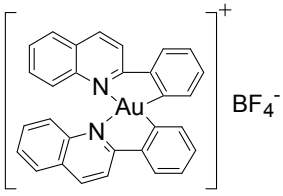
The composition was maintained for 3 minutes, followed by a linear gradient to 30% B by 2 minutes. The composition was maintained for 2 minutes.

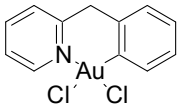
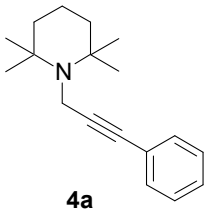
HPLC Conditions for Determination of Formaldehyde Content by the 2,4-DNPH Method

Waters 2695 HPLC Separation Module equipped with XTerra MS C18 (5 μm, 4.6 mm x 100 mm) Column and Waters 2998 Photodiode Array (PDA) Detector was used for separation. The flow rate was 1.0 mL/min. The injection volume was 10 μL. The PDA detector wavelength was set to 360 nm.

Mobile phase A was made of Milli-Q® water. Mobile phase B was made of acetonitrile. The initial conditions for separation were 40% B for 5 minutes, followed by a linear gradient to 0% B by 5 minutes. The composition was maintained for 2 minutes, followed by a linear gradient to 40% B by 1 minute.

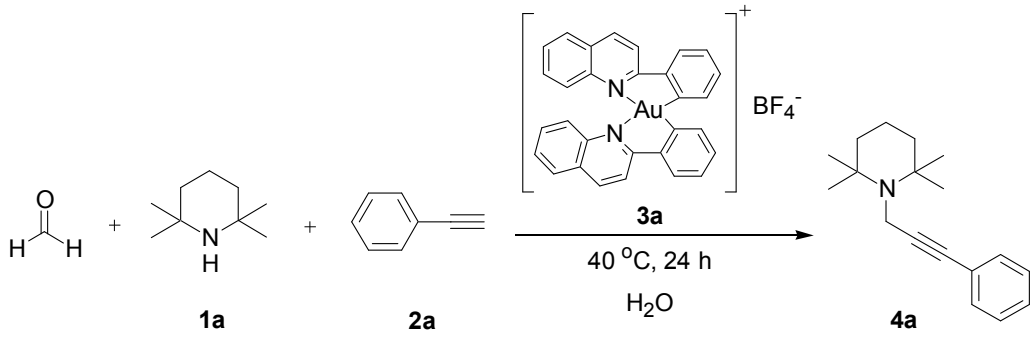
3.6.2 Literature References of Compounds

 **1c**	E. Szabo, P. Hrdlovic and M. Danko, *Dyes Pigments*, 2011, **90**, 129-138.
 **2b**	W. K. Chan, C. M. Ho, M. K. Wong and C. M. Che, *J. Am. Chem. Soc.*, 2006, **128**, 14796-14797.
 **2c**	M. Maldonado-Domínguez, R. Arcos-Ramos, M. Romero, B. Flores-Pérez, R. Santillan, P. G. Lacroix, I. Malfant and N. Farfán, *New J. Chem.*, 2014, **38**, 260-268.
 **2d**	K. K. Y. Kung, G. L. Li, L. Zou, H. C. Chong, Y. C. Leung, K. H. Wong, V. K. Y. Lo, C. M. Che and M. K. Wong, *Org. Biomol. Chem.*, 2012, **10**, 925-930.
 **3a**	H. M. Ko, K. K. Y. Kung, J. F. Cui and M. K. Wong, *Chem. Commun.*, 2013, **49**, 8869-8871.

 **3b**	E. C. Constable and T. A. Leese, *J. Organomet. Chem.*, 1989, **363**, 419-424.
 **3c**	M. A. Cinellu, A. Zucca, S. Stoccoro, G. Minghetti, M. Manassero and M. Sansoni, *J. Chem. Soc., Dalton Trans.*, 1995, 2865-2872.
 **4a**	P. H. Li and L. Wang, *Tetrahedron*, 2007, **63**, 5455-5459.

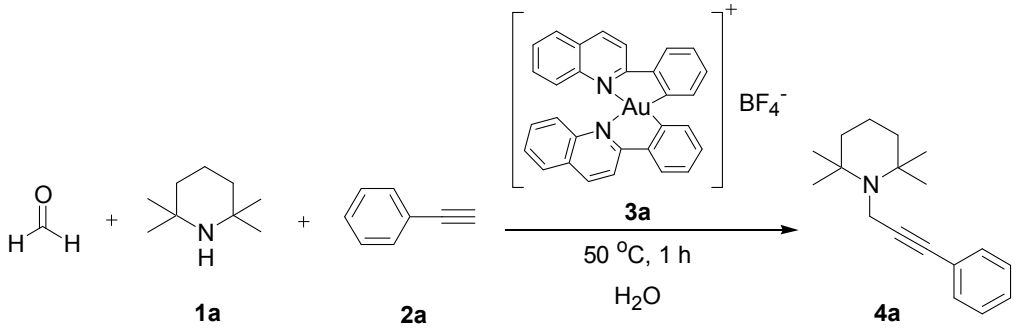
3.6.3 Experimental Procedure

Procedure for Study of Applicability of A3-coupling Reaction for Formaldehyde Detection



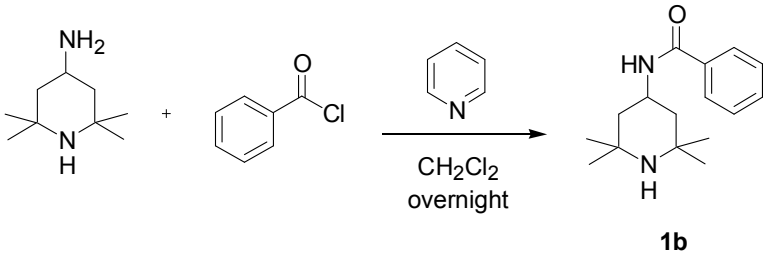
A mixture of formaldehyde (1 mmol), commercially available 2,2,6,6-tetramethylpiperidine **1a** (1.1 mmol), commercially available phenylacetylene **2a** (1.5 mmol) and bis-cyclometallated gold(III) complex **3a** (0.01 mmol) in water (1 mL) was stirred at 40 ^{o}C for 24 hours. After reaction, the mixture was extracted with dichloromethane (15 mL × 3). The combined organic layer was dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography using ethyl acetate/n-hexane as eluent to give propargylamine **4a** in 74% isolated yield.

Procedure for Study of Applicability of A3-coupling Reaction for Formaldehyde Detection in 1 Hour



A mixture of formaldehyde (1 mmol), commercially available 2,2,6,6-tetramethylpiperidine **1a** (1.1 mmol), commercially available phenylacetylene **2a** (1.5 mmol) and bis-cyclometallated gold(III) complex **3a** (0.1 mmol) in water (1 mL) was stirred at 50 °C for 1 hour. After reaction, the mixture was extracted with dichloromethane (15 mL × 3). The combined organic layer was dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography using ethyl acetate/n-hexane as eluent to give propargylamine **4a** in 6% isolated yield.

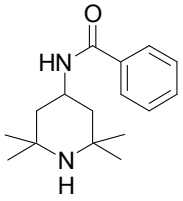
Procedure for Synthesis of 1b



A mixture of 4-amino-2,2,6,6-tetramethylpiperidine (6 mmol), benzoyl chloride

(3 mmol) and pyridine (3 mmol) was stirred in dichloromethane (20 mL) under nitrogen atmosphere at room temperature for overnight. After reaction, the mixture was concentrated under reduced pressure. The residue was purified by flash column chromatography using methanol/ethyl acetate as eluent to give amine **1b** in 84% isolated yield.

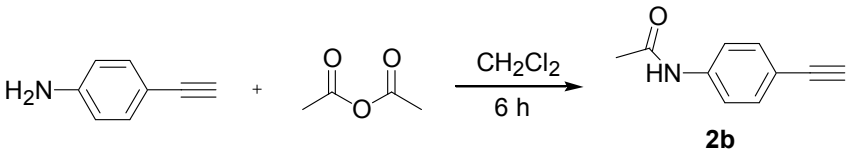
Characterization Data of 1b



1b

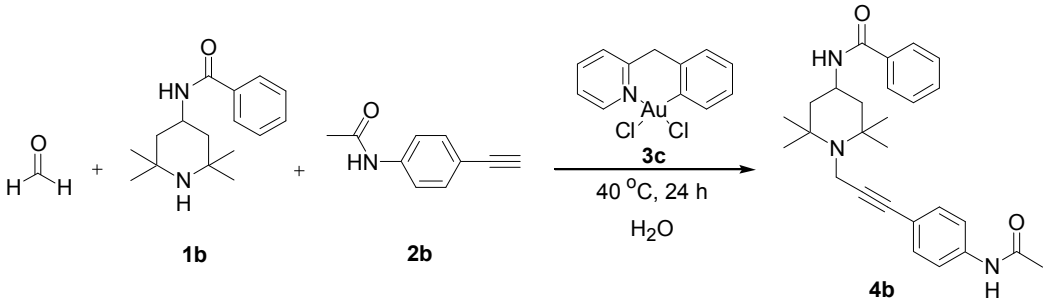
White powder; analytical TLC (silica gel 60) (40% methanol/ethyl acetate), R_f = 0.45; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, *J* = 7.2 Hz, 2H), δ 7.48 (t, *J* = 7.3 Hz, 1H), δ 7.41 (t, *J* = 7.6 Hz, 2H), δ 6.05 (d, *J* = 7.2 Hz, 1H), δ 4.41-4.51 (m, 1H), δ 1.99 (dd, *J* = 6.2 Hz, 2H), δ 1.29 (s, 6H), δ 1.14 (s, 6H), δ 1.04 (t, *J* = 12.2 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.70, 134.75, 131.34, 128.51, 126.75, 51.24, 45.20, 43.05, 34.82, 28.42; ESI-MS m/z 261 $[M+H]^+$; HRMS (ESI) for $C_{16}H_{25}N_2O$, calculated 261.1961, found 261.1960.

Procedure for Synthesis of 2b



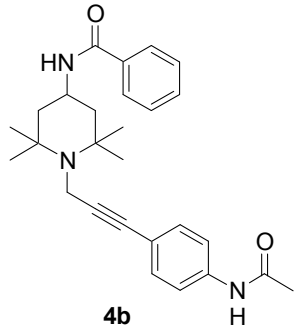
A mixture of 4-ethynylaniline (2.5 mmol) and acetic anhydride (3.3 mmol) in dichloromethane (5 mL) was stirred at room temperature for 6 hours. After evaporation of solvent, the residue was purified by flash column chromatography using ethyl acetate/n-hexane as eluent to afford alkyne **2b** in 95% yield.

Procedure for Synthesis of 4b



A mixture of formaldehyde (1 mmol), amine **1b** (1.1 mmol), alkyne **2b** (1.5 mmol) and cyclometallated gold(III) complex **3c** (0.01 mmol) in water (1 mL) was stirred at 40 ^{o}C for 24 hours. After reaction, the mixture was extracted with ethyl acetate (15 mL × 3). The combined organic layer was dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography using ethyl acetate/n-hexane as eluent to give propargylamine **4b** in 36% yield.

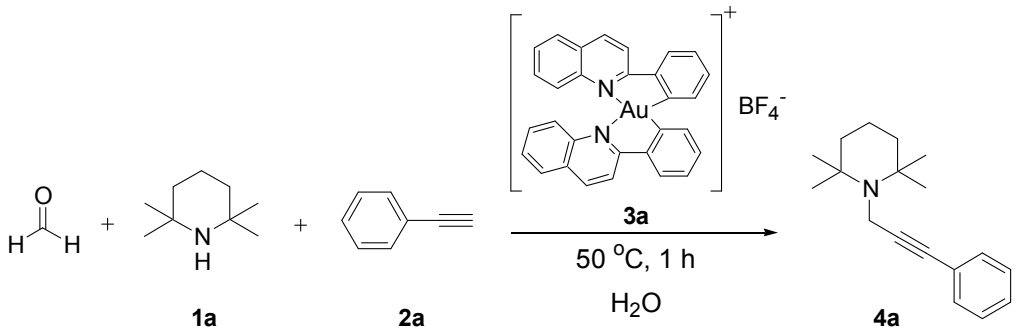
Characterization Data of 4b



4b

White powder; analytical TLC (silica gel 60) (70% ethyl acetate/n-hexane), R_f = 0.57; ^{1}H NMR (400 MHz, CD_3OD) δ 7.81 (d, *J* = 7.2 Hz, 2H), δ 7.50 (d, *J* = 8.1 Hz, 3H), δ 7.44 (t, *J* = 7.0 Hz, 2H), δ 7.29 (d, *J* = 8.1 Hz, 2H), δ 4.36-4.42 (m, 1H), δ 3.63 (s, 2H), δ 2.11 (s, 3H), δ 1.79 (d, *J* = 10.0 Hz, 2H), δ 1.56 (t, *J* = 12.2 Hz, 2H), δ 1.32 (s, 6H), δ 1.25 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.65, 169.76, 139.59, 136.07, 132.82, 132.57, 129.52, 128.36, 120.86, 120.80, 92.69, 82.19, 57.37, 43.35, 34.22, 33.99, 23.89, 22.39; ESI-MS m/z 432 $[M+H]^+$; HRMS (ESI) for $C_{27}H_{34}N_3O_2$, calculated 432.2646, found 432.2649.

Procedure for Study of Selectivity of the Formaldehyde Probe

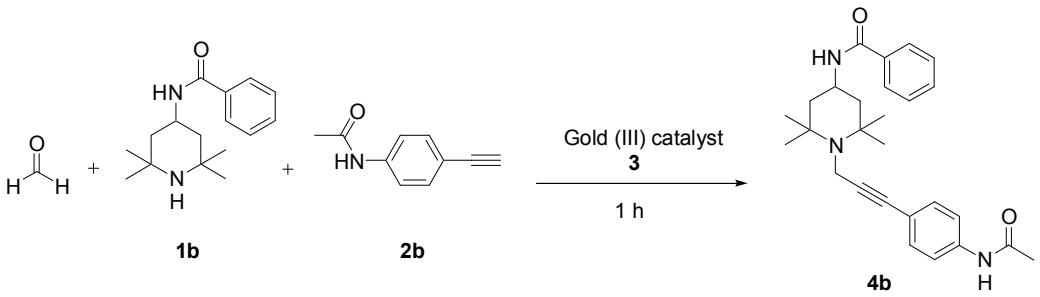


A mixture of formaldehyde (0.1 mmol), 2,2,6,6-tetramethylpiperidine **1a** (0.11 mmol), phenylacetylene **2a** (0.15 mmol) and bis-cyclometallated gold(III) complex **3a** (0.01 mmol) in water (600 μL) was stirred at 50 ^{o}C for 1 hour. After reaction, the mixture was extracted with dichloromethane (600 μL). The organic layer was analyzed by ESI-MS for propargylamine formation.

The reaction was repeated with acetaldehyde, isobutyraldehyde, benzaldehyde and acetone to replace formaldehyde, respectively. ESI-MS analysis of the reaction mixtures showed no propargylamine formation for acetaldehyde, isobutyraldehyde, benzaldehyde and acetone.

The reaction was repeated with commercially available cis-2,6-dimethylpiperidine to replace 2,2,6,6-tetramethylpiperidine. ESI-MS analysis showed that only formaldehyde could give the corresponding propargylamine while acetaldehyde gave no propargylamine under the same reaction conditions.

Procedure for the Model Reaction for Optimization of Reaction Conditions



Effects of Cyclometallated Gold(III) Complexes

A mixture of formaldehyde (20 μmol, in form of an 100 μL aqueous solution), amine **1b** (22 μmol), alkyne **2b** (30 μmol) and cyclometallated gold(III) complex **3a** (4 μmol) in water (600 μL) was stirred at 50 ^{o}C for 1 hour. After reaction, 20 μL of the reaction mixture was mixed with 980 μL acetonitrile for HPLC analysis. The reaction was repeated with cyclometallated gold(III) complexes **3b** and **3c**, respectively.

Effects of Solvents

A mixture of formaldehyde (20 μmol, in form of an 100 μL aqueous solution), amine **1b** (22 μmol), alkyne **2b** (30 μmol) and bis-cyclometallated gold(III) complex **3a** (4 μmol) in water (600 μL) was stirred at 50 ^{o}C for 1 hour. After reaction, 20 μL of the reaction mixture was mixed with 980 μL acetonitrile for HPLC analysis. The reaction was repeated with acetonitrile, dimethylformamide,

tetrahydrofuran, 1,4-dioxane, toluene, ethyl acetate and 1,2-dichloroethane as the solvent, respectively.

Effects of Solvent Ratios

Mixtures of formaldehyde (20 μmol, in form of a 50 μL aqueous solution), amine **1b** (22 μmol), alkyne **2b** (30 μmol) and bis-cyclometallated gold(III) complex **3a** (4 μmol) in different volumes of 1,2-dichloroethane and water were stirred at 50 ^{o}C for 1 hour. After reaction, 1,2-dichloroethane was added to the reaction mixtures to make up the organic layer to a final volume of 450 μL, respectively, for easy comparison after HPLC analysis. Then, the reaction mixtures were mixed by a vortex mixer for 1 minute. 20 μL of the organic layer was mixed with 980 μL acetonitrile for HPLC analysis.

Effects of Temperatures

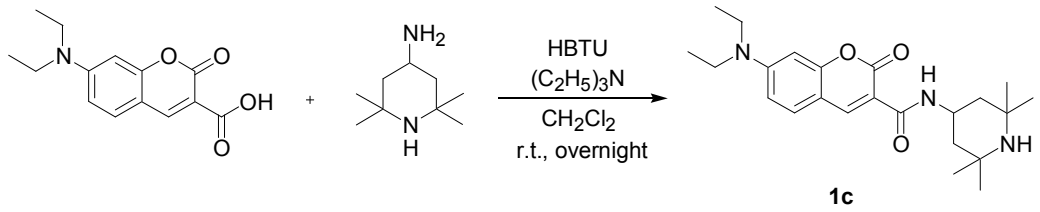
A mixture of formaldehyde (20 μmol, in form of a 50 μL aqueous solution), amine **1b** (22 μmol), alkyne **2b** (30 μmol) and bis-cyclometallated gold(III) complex **3a** (4 μmol) in 1,2-dichloroethane (450 μL) was stirred at room temperature for 1 hour. After reaction, 20 μL of the organic layer was mixed with 980 μL acetonitrile for HPLC analysis. The reaction was repeated at 40 ^{o}C, 50 ^{o}C

and 60 °C, respectively.

Effects of Reagent Ratios

A mixture of formaldehyde (in form of a 50 μL aqueous solution), amine **1b**, alkyne **2b** and bis-cyclometallated gold(III) complex **3a** in different amounts in 1,2-dichloroethane (450 μL) was stirred at 50 °C for 1 hour. After reaction, 20 μL of the organic layer was mixed with 980 μL acetonitrile for HPLC analysis.

Procedure for Synthesis of 1c

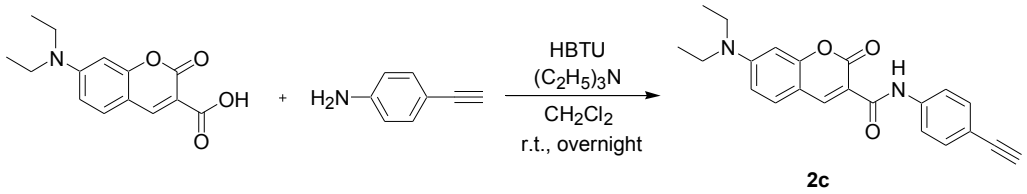


1c

A mixture of 7-(diethylamino)coumarin-3-carboxylic acid (0.5 mmol), 4-amino-2,2,6,6-tetramethylpiperidine (0.6 mmol), triethylamine (1 mmol) and N,N,N',N'-tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) (0.6 mmol) in dichloromethane (20 mL) was stirred under nitrogen atmosphere at room temperature for overnight. After reaction, the mixture was washed with water (15 mL × 3). The organic layer was dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography using methanol/dichloromethane as

eluent to give **1c** in 91% yield.

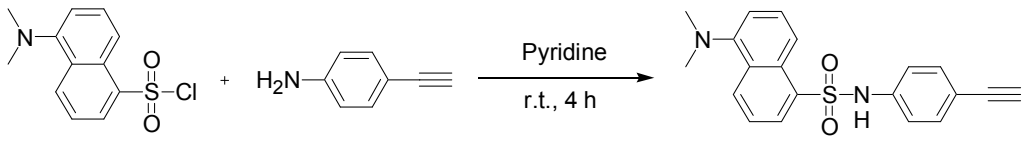
Procedure for Synthesis of 2c



2c

A mixture of 7-(diethylamino)coumarin-3-carboxylic acid (0.5 mmol), 4-ethynylaniline (0.6 mmol), triethylamine (1 mmol) and HBTU (0.6 mmol) in dichloromethane (20 mL) was stirred under nitrogen atmosphere at room temperature for overnight. After reaction, the mixture was washed with water (15 mL × 3). The organic layer was dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography using dichloromethane/n-hexane as eluent to give **2c** in 79% yield.

Procedure for Synthesis of 2d

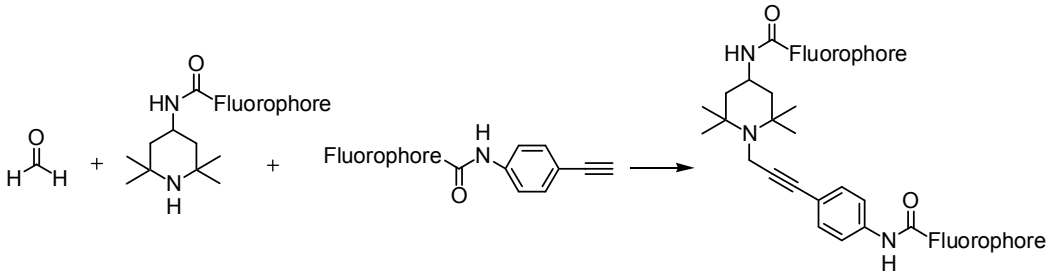


2d

A mixture of dansyl chloride (0.5 mmol), 4-ethynylaniline (0.5 mmol) in pyridine (5 mL) was stirred under nitrogen atmosphere at room temperature for 4 hours.

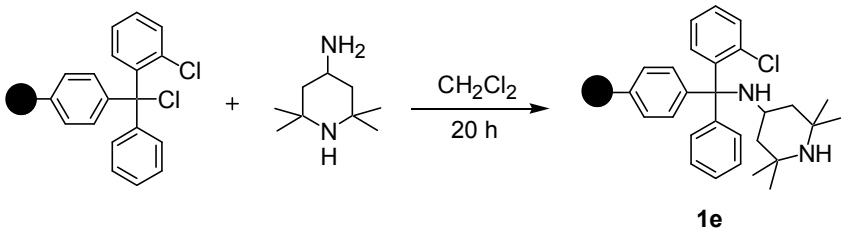
After reaction, the mixture was washed with 2 M HCl (20 mL) and then extracted with ethyl acetate (20 mL × 3). The combined organic layer was dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography using ethyl acetate/n-hexane as eluent to give **2d** in 69% yield.

Procedure for Fluorescent Detection of Formaldehyde



A mixture of formaldehyde (20 μmol, in form of a 50 μL aqueous solution), amine **1a** or **1c** (150 μmol), alkyne **2a**, **2c** or **2d** (300 μmol) and bis-cyclometallated gold(III) complex **3a** (150 μmol) was stirred in 1,2-dichloroethane (1 mL) at 50 ^{o}C for 1 hour. After reaction, the mixtures were analyzed by ESI-MS for the formation of products.

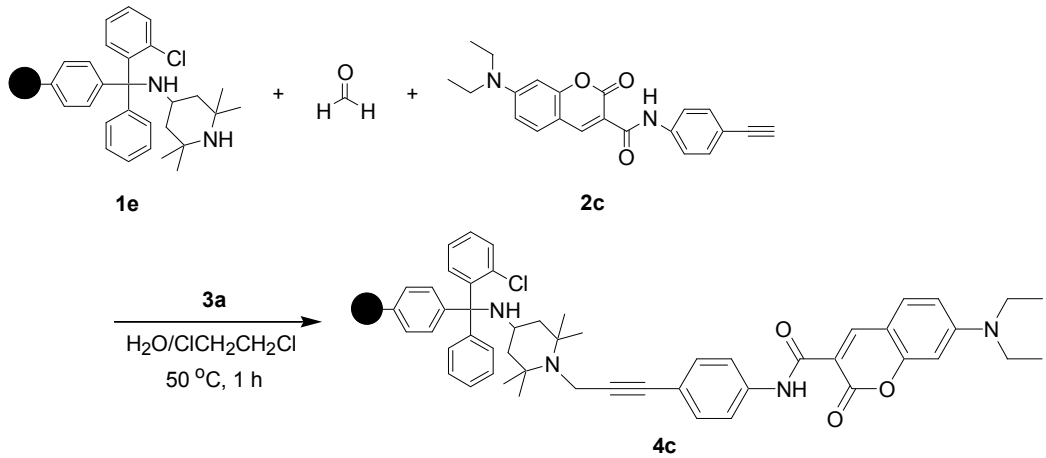
Procedure for Synthesis of Formaldehyde Probe on Resin Support



1e

Commercially available polymer-bound 2-chlorotrityl chloride resin (15 mg) was swollen in dichloromethane (250 μL) for 15 minutes. 4-amino-2,2,6,6-tetramethylpiperidine (0.19 mmol) was added and the reaction vessel was gently shaken for 20 hours. After reaction, 12 μL HPLC grade methanol was added and mixed for 15 minutes to endcap any remaining reactive 2-chlorotrityl groups. The resin was washed three times with 1 mL of dichloromethane, two times with 1 mL of dimethylformamide, and three times with 1 mL of dichloromethane. The resin was then dried for further use.

Procedure for Formaldehyde Detection on Resin Support

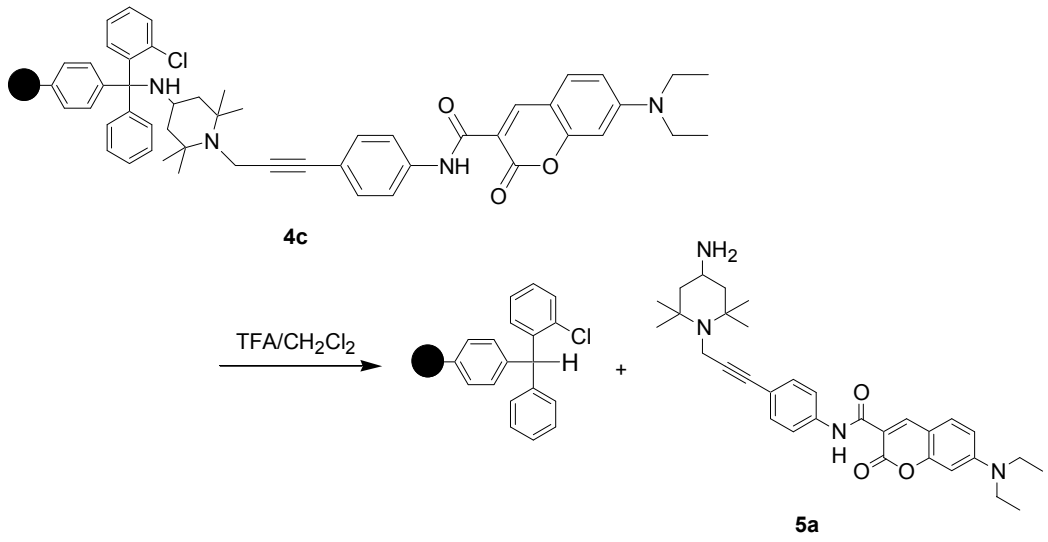


1e **2c** **4c**

A mixture of solid-supported amine **1e** (15 mg), formaldehyde (0.02 mmol, in

form of an 100 μL aqueous solution), coumarin alkyne **2c** (0.02 mmol), bis-cyclometallated gold(III) complex **3a** (0.01 mmol), and 600 μL of 1,2-dichloroethane was gently stirred at 50 ^{o}C for 1 hour. After reaction, the resin was washed four times with 1 mL of dichloromethane, two times with 1 mL of dimethylformamide, and three times with 1 mL of dichloromethane.

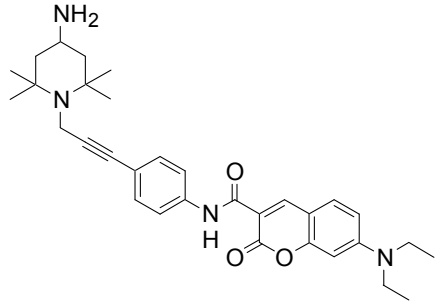
Procedure for Cleavage of Product from Resin Support



Resin **4c** (15 mg) was suspended in 1:1 trifluoroacetic acid and dichloromethane (300 μL) at room temperature for 1 minute for cleavage of propargylamine **5a** from the solid support. The cleaved solution was analyzed by ESI-MS for confirmation of product formation. To provide structural support of propargylamine **5a**, the cleaved solution was neutralized by saturated $NaHCO_3$ solution (5 mL). The organic layer was dried over anhydrous $MgSO_4$, filtered,

and concentrated under reduced pressure. The residue was purified by flash column chromatography using methanol/dichloromethane as eluent to give propargylamine **5a** in 4% yield.

Characterization Data of 5a



5a

Yellow powder; analytical TLC (silica gel 60) (10% methanol/dichloromethane), R_f = 0.42; ^{1}H NMR (400 MHz, DMSO) δ 8.75 (s, 1H), δ 7.73 (d, *J* = 9.1 Hz, 1H), δ 7.68 (d, *J* = 8.6 Hz, 2H), δ 7.33 (d, *J* = 8.6 Hz, 2H),), δ 6.85 (dd, *J* = 4.5 Hz, 1H), δ 6.66 (d, *J* = 2.1 Hz, 1H), δ 3.56 (s, 2H), δ 3.48-3.53 (m, 4H), δ 1.78 (dd, *J* = 6.1 Hz, 4H), δ 1.41 (t, *J* = 6.7 Hz, 3H), δ 1.23 (s, 6H), δ 1.15 (t, *J* = 6.7 Hz, 3H), δ 1.08 (s, 6H); ^{13}C NMR (100 MHz, DMSO) δ 162.08, 160.68, 157.32, 152.77, 148.11, 131.79, 131.69, 119.49, 110.39, 107.82, 95.86, 92.23, 79.20, 78.87, 78.54, 55.00, 54.78, 44.33, 44.24, 32.97, 21.42, 12.21; ESI-MS m/z 529 $[M+H]^+$; HRMS (ESI) for $C_{32}H_{41}N_4O_3$, calculated 529.3173, found 529.3177.

Procedure for Using Water Extract of Mushroom as Formaldehyde Source

The purchased dried mushroom sample was sealed in plastic bags at room temperature without direct exposure to light. The mushroom sample with stems removed was homogenized by a blender. About 2 g of the sample was weighed and stored in a centrifuge tube. Milli-Q® water (20 mL) was added into the tube and the tube was sealed. The tube was placed into an ultrasonic bath for extraction at 40 °C for 1 hour. After that, the sample was centrifuged at 4000 rpm for 10 minutes. The centrifuged sample was filtered with filter paper and funnel. The filtrate was collected by a 50 mL volumetric flask. Milli-Q® water (20 mL) was added into the centrifuge tube again and the tube was placed into an ultrasonic bath for extraction at 40 °C for 30 minutes. The sample was centrifuged at 4000 rpm for 10 minutes. The centrifuged sample was filtered with filter paper and funnel. The filtrate was collected by the 50 mL volumetric flask used previously. The flask was made up with Milli-Q® water and was mixed well. This gave the water extract of the mushroom sample.

A mixture of solid-supported amine **1e** (15 mg), water extract of mushroom (100 μL), coumarin alkyne **2c** (0.02 mmol), bis-cyclometallated gold(III) complex **3a** (0.01 mmol) and 600 μL of 1,2-dichloroethane was gently stirred at 50 °C for 1

hour. After reaction, the resin was washed four times with 1 mL of dichloromethane, two times with 1 mL of dimethylformamide, and three times with 1 mL of dichloromethane. The product was cleaved from the resin support and analyzed by ESI-MS for confirmation of product formation.

For the 2,4-DNPH method, water extract of mushroom sample (5 mL) was transferred to a 10 mL volumetric flask. 8000 ppm 2,4-DNPH in acetonitrile (0.5 mL) and acetonitrile (4 mL) were added into the flask. The flask was made up with Milli-Q® water and was mixed well. The derivatized sample was filtered with PTFE 0.22 μm syringe filter and transferred to HPLC vial for analysis by HPLC.

3.6.4 Raw Data and Spectra

HPLC Chromatograms for Construction of Calibration Curve for the Model Reaction

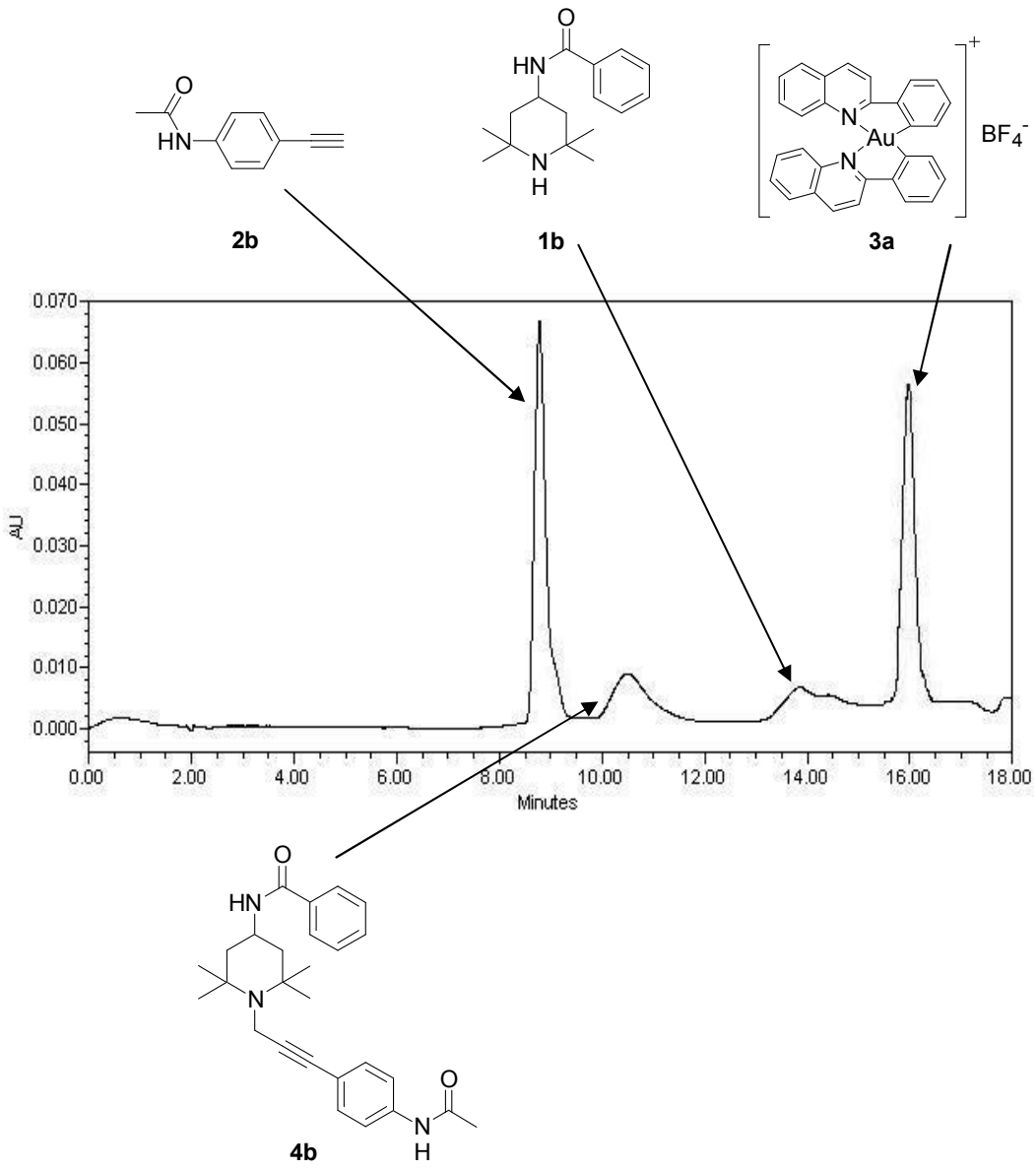


Figure S1. HPLC chromatogram for 1 ppm of **1b**, **2b**, **3a** and **4b**.

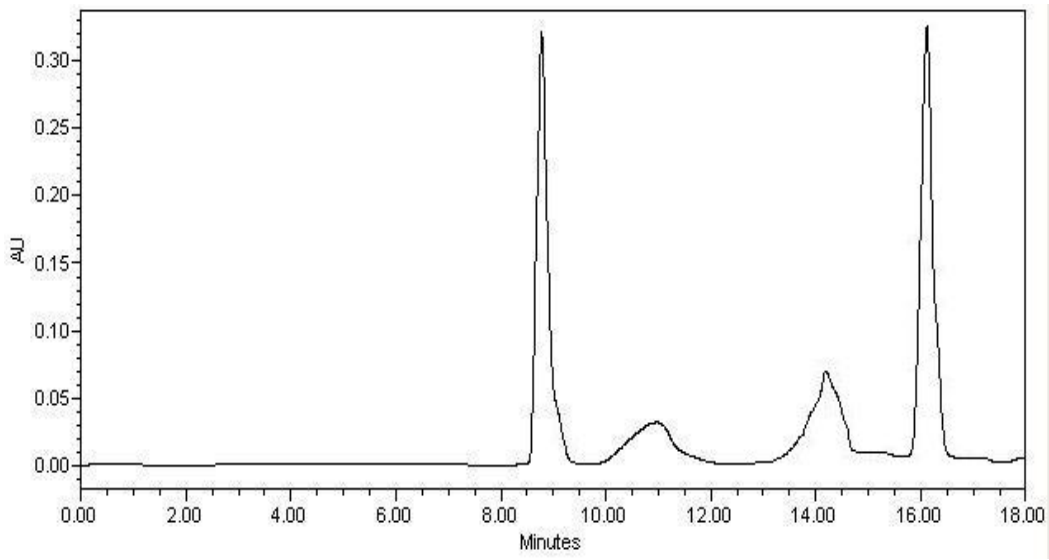


Figure S2. HPLC chromatogram for 10 ppm of **1b**, **2b**, **3a** and **4b**.

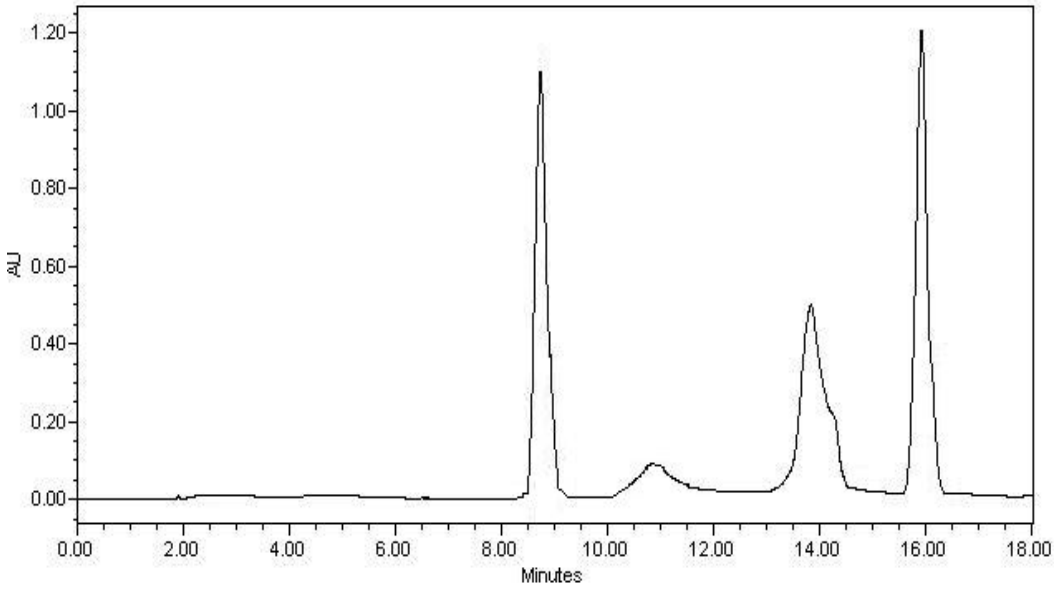


Figure S3. HPLC chromatogram for 50 ppm of **1b**, **2b**, **3a** and **4b**.

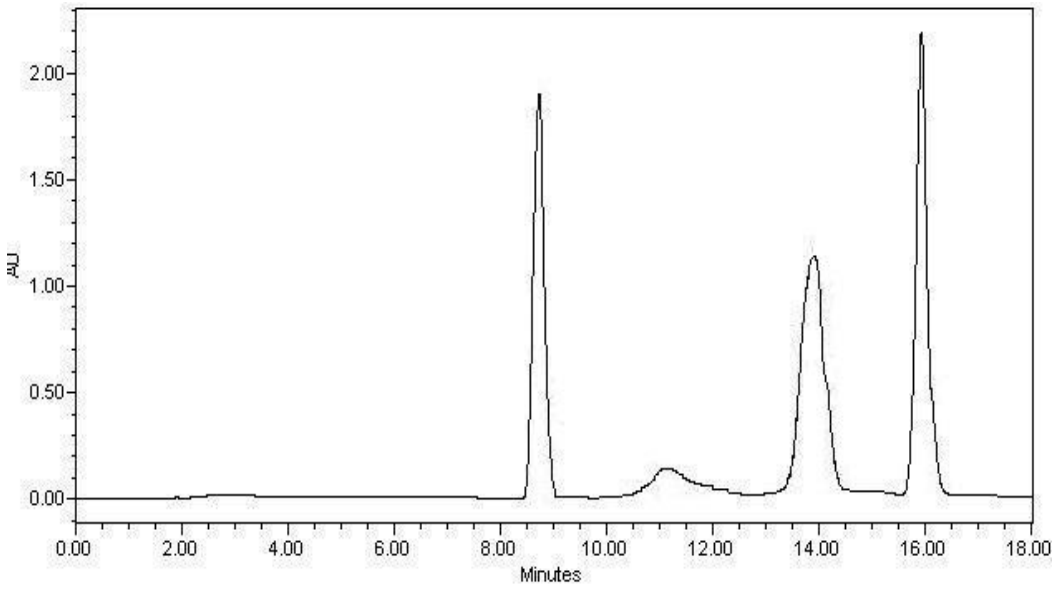


Figure S4. HPLC chromatogram for 100 ppm of **1b**, **2b**, **3a** and **4b**.

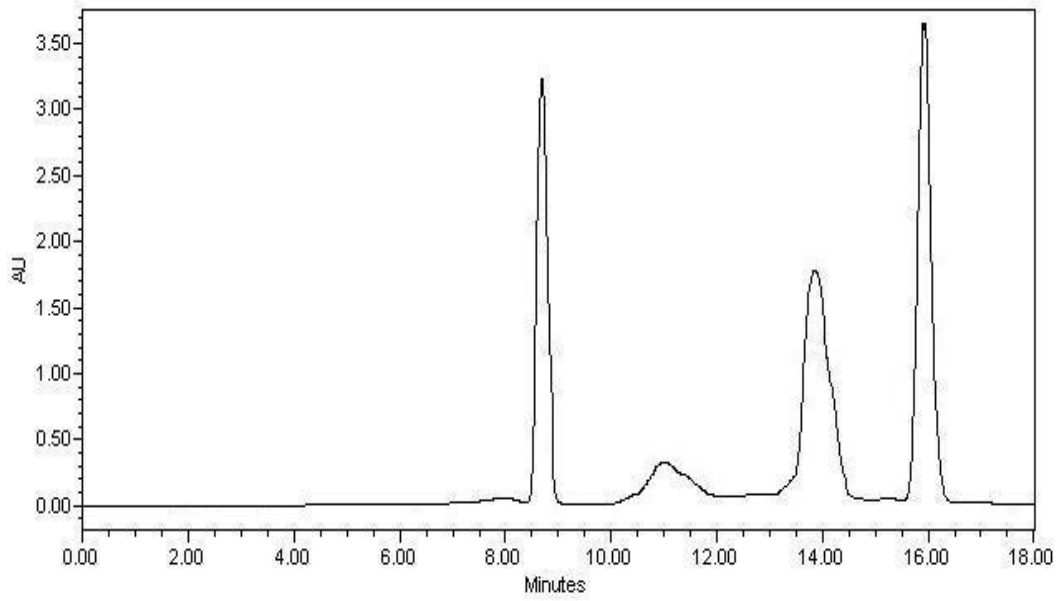


Figure S5. HPLC chromatogram for 200 ppm of **1b**, **2b**, **3a** and **4b**.

Table S1. Data for construction of calibration curve for the model reaction

Concentration of 4b	Peak Area (Determined by HPLC)
1	39195
10	324417
50	1797622
100	3481545
200	7222159

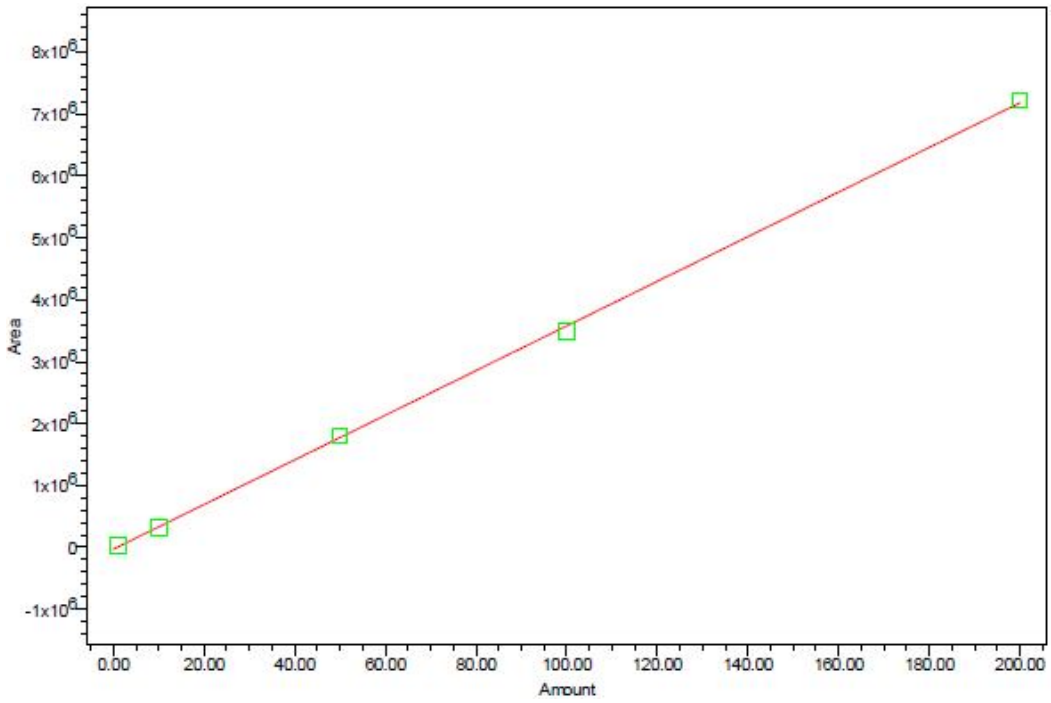


Figure S6. Calibration curve for compound **4b**. The x-axis is formaldehyde concentration and y-axis is peak area.

Measurement of Fluorescent Intensity by Microplate Reader

2.0 mg of the resin beads from different reaction mixtures were accurately weighed and transferred to a microplate for measurement of fluorescent intensity. The fluorescent intensity was measured by BMG FLUOstar Galaxy Fluorescence Microplate Reader. The number of cycle, number of flash and cycle time were set to be 10. The delay was set to be 0.5 second. The excitation wavelength was 355 nm and emission wavelength was 460 nm.

Table S2. Data for construction of calibration curve for the resin beads

Formaldehyde concentration	**Fluorescent intensity (arbitrary unit)**
0	1031
50	2187
100	2939
500	7603

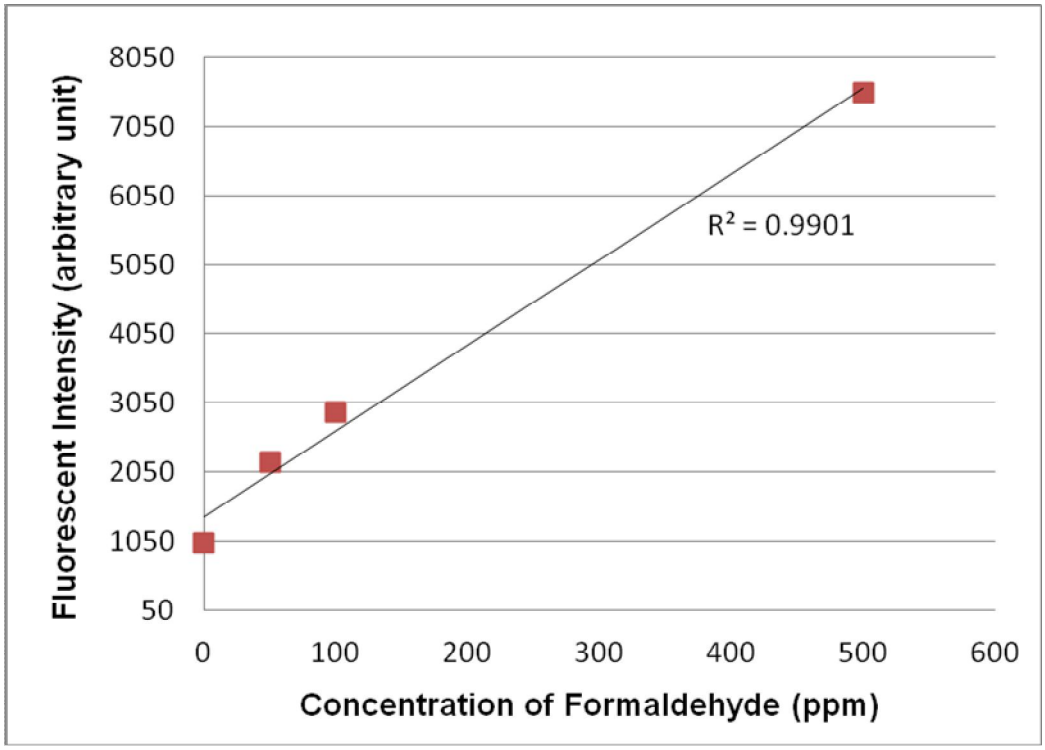


Figure S7. Calibration curve for the resin beads.

HPLC Chromatogram for Determination of Formaldehyde Content by the 2,4-DNPH Method

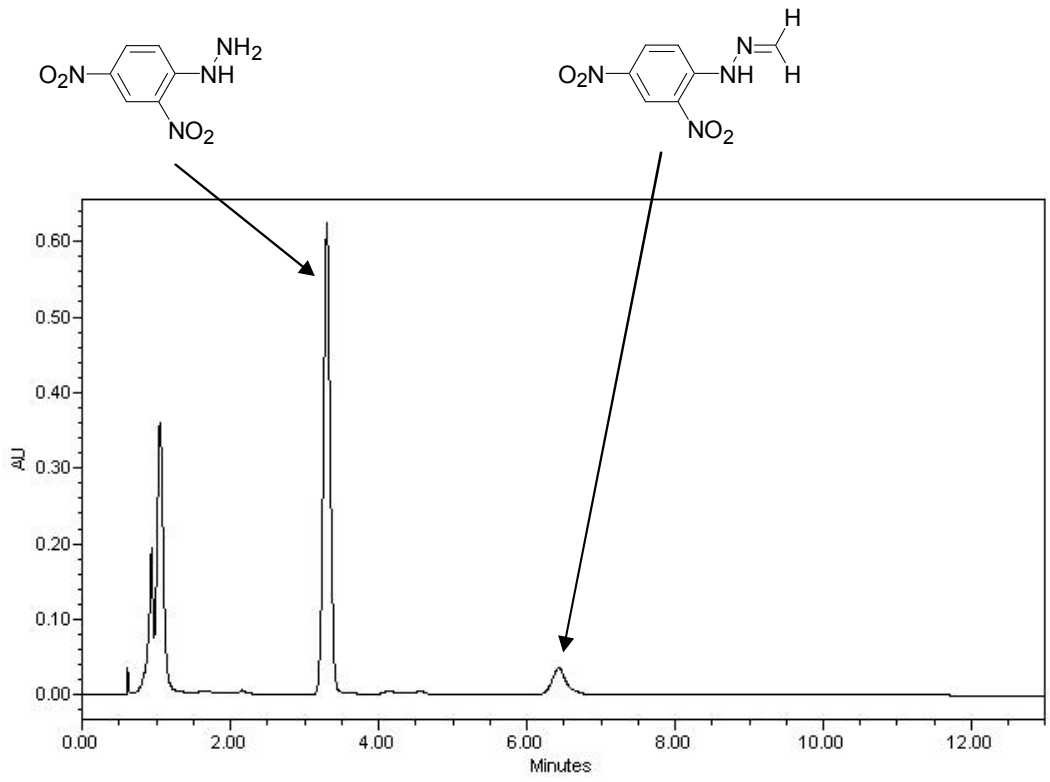
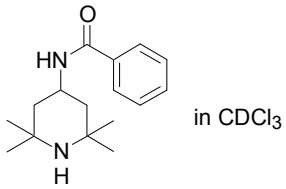


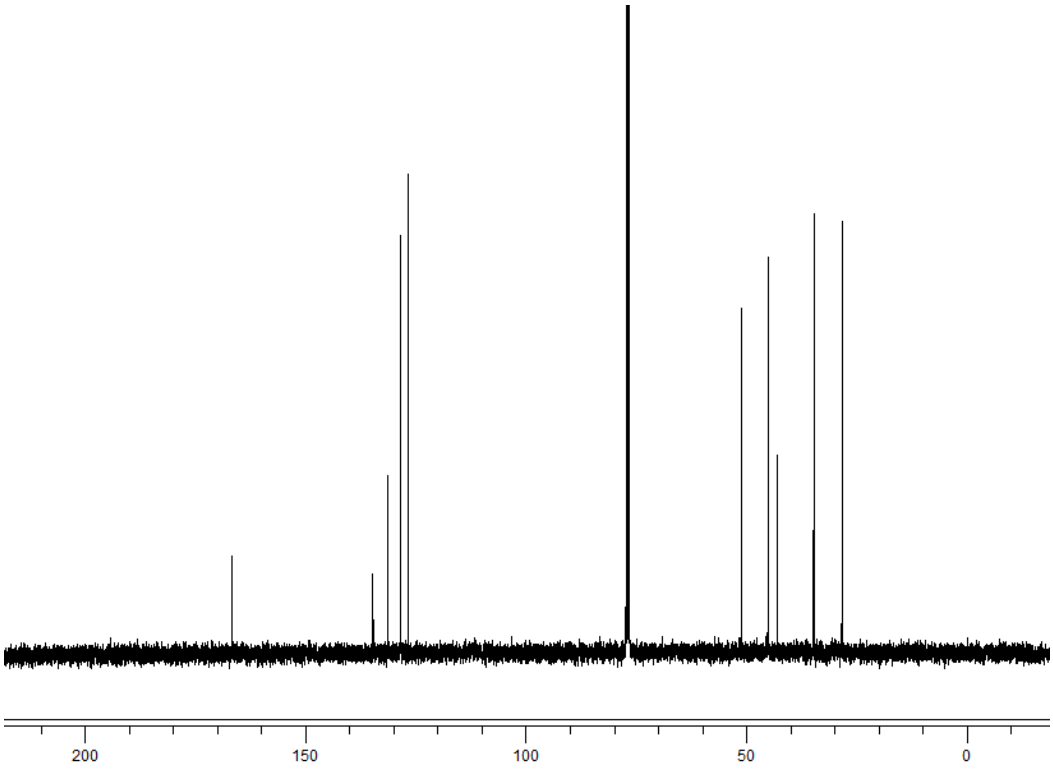
Figure S8. HPLC chromatogram for determination of formaldehyde content in the mushroom sample by the 2,4-DNPH method.

NMR Spectrum of 1b

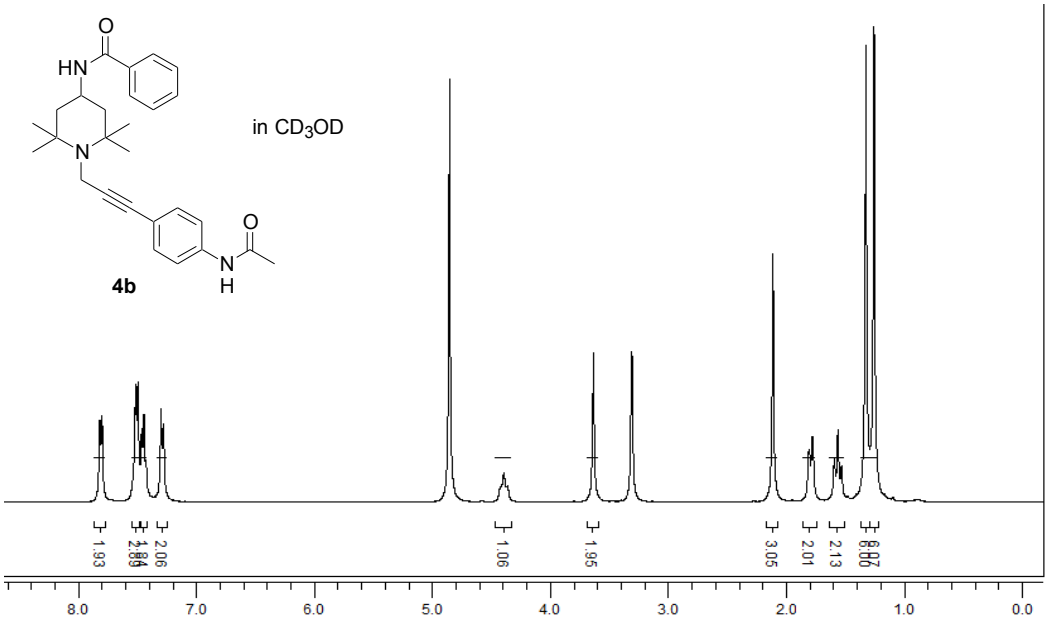


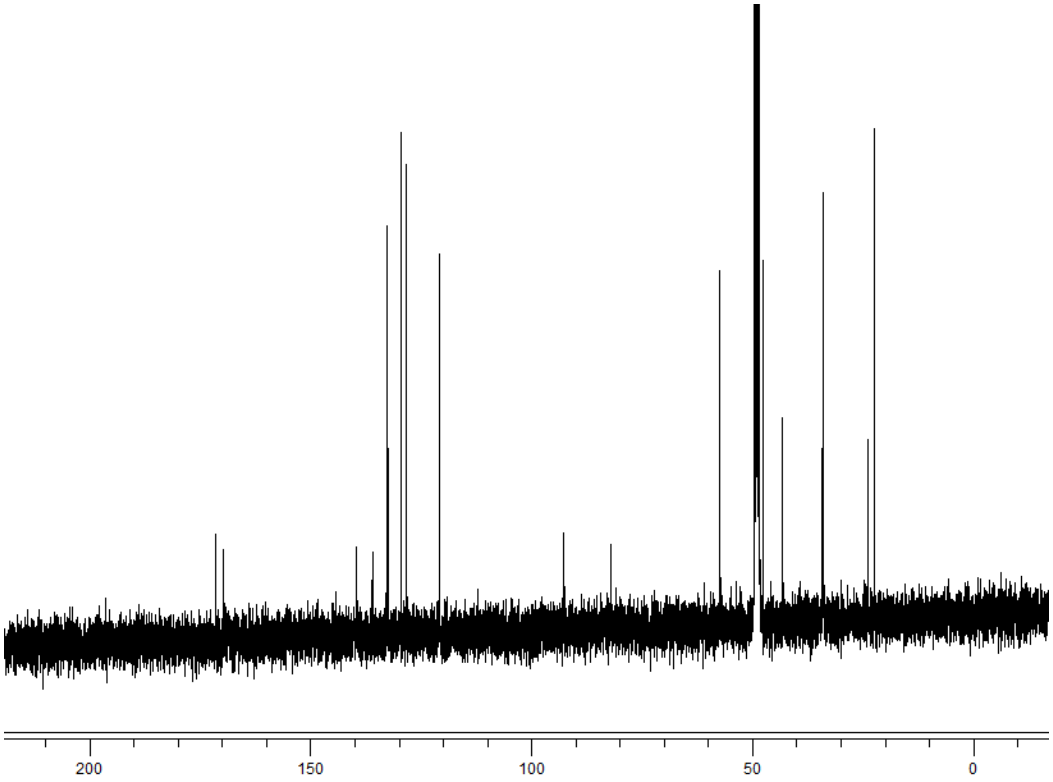
in $CDCl_3$

1b

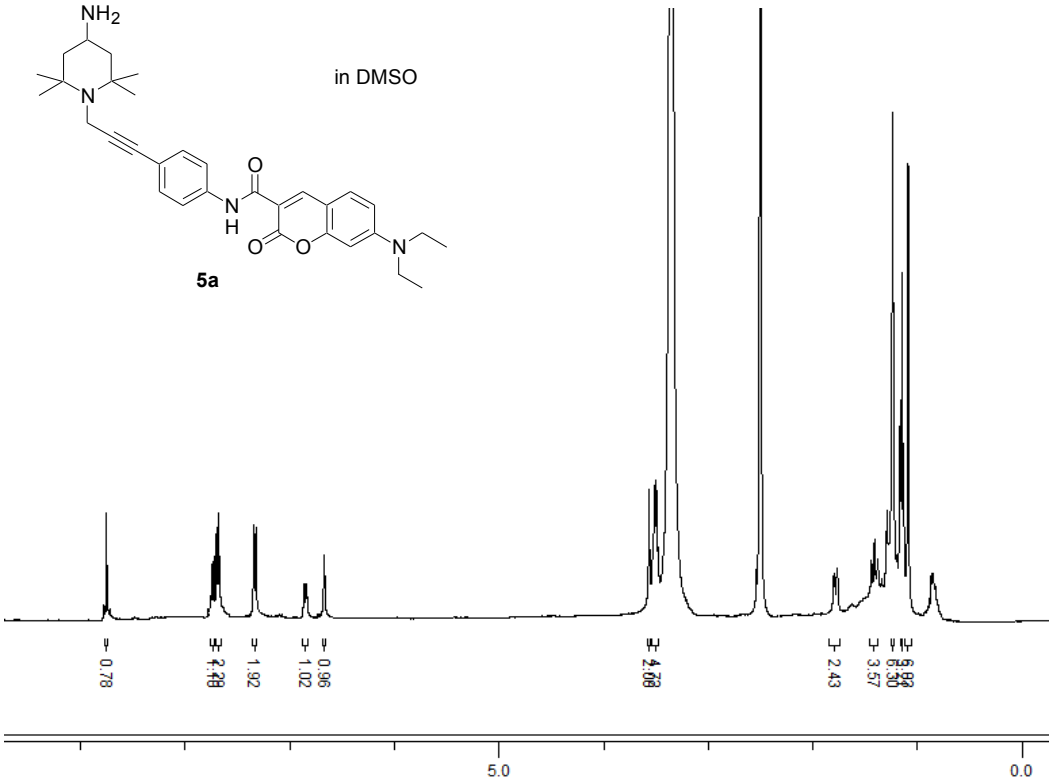


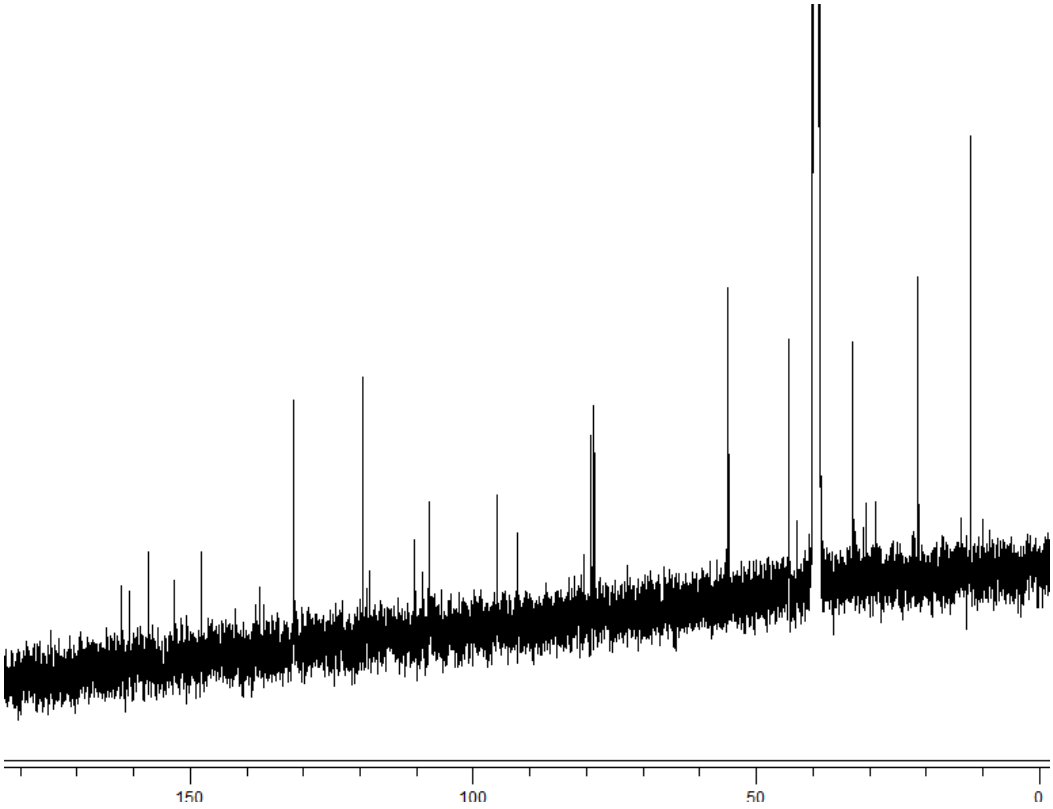
NMR Spectrum of 4b





NMR Spectrum of 5a





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