Master's Thesis

Protein Labeling Based on Native Chemical Ligation and on Non-Covalent Interaction

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Abstract

Labeling proteins with fluorophores is a convenient technique for protein researches in vivo and in vitro. Expressed protein ligation (EPL) readily provides the addition of unnatural functionality to a recombinant protein framework, by taking advantage of the biosynthesis of N-terminal cysteine and α- thioester protein fragments. A conceptually method is to carry out chemical modification in vitro and then to deliver the modified protein to the desired biological construction. However, for chemical ligation high concentration of reactants required, therefore, chemical modification directly in living cells or cell lysate is inefficient.

In this thesis, to overcome these obstacles, we constructed a novel system for protein labeling by utilizing a coiled-coil tag and intein inspired techniques. The combination of a noncovalent interaction with a covalent ligation is an effective strategy for efficient and stable modification of proteins .[1] The small affinity tags, Acid-a1 and Base-a1, were attached to the reactants to obtain low concentration ligation. This method enables specific labeling in mammalian cell lysate yet, this also has some limitations. In order to develop our strategy, we designed new quencher conjugated peptide fluorophore probe using method using non-covalent interactions rather than covalent intein interactions. Herein, new strategy of noncovalent interaction promoted ligation for protein labeling and experimental results will be explained.

Table of Content

Chapter 1 Introduction

1.1 Protein Visualization

Understanding the function of a protein is essential for gaining insight into many biological processes. Structural properties of a protein can be obtained with various techniques.

Labeling is one of the most common methodologies used for bioanalytical purposes, which can be carried out with radioactive materials or compounds with absorption and/or fluorescence from the ultraviolet to the near-infrared region of the electromagnetic spectrum. Utilization of organic molecules in nonfluorescent labeling, in the ultraviolet and visible regions, is important in several applications. However, in recent years, detection based on fluorescence techniques has received special attention and notable progress has been made in both fluorescence instrumentation and synthesis of new fluorophores.[2] Fluorescence labeling can be extended over a wide range of wavelengths using semiconductor nanocrystals, fluorescent proteins, organic molecules including fluorescent dyes of a variety of colors, luminescent or magnetic labels, sensors for second messengers and other important cellular characteristics.

The organic fluorophores may form covalent or noncovalent linkages with the sample to be analyzed, producing the respective conjugates or complexes that can show fluorescence from short to very long wavelengths, depending on the marker used.

1.2 Protein Tag System

Protein tags are peptide sequences genetically fused to proteins of interest. Often these tags are removable by chemical reagents or by enzymatic means, such as proteolysis or intein splicing. There are

known many protein tag systems: affinity tags, solubilization tags, epitope tags or fluorescent tags. The tags are attached to proteins to improve solubility, to detect localization, or to facilitate expression.

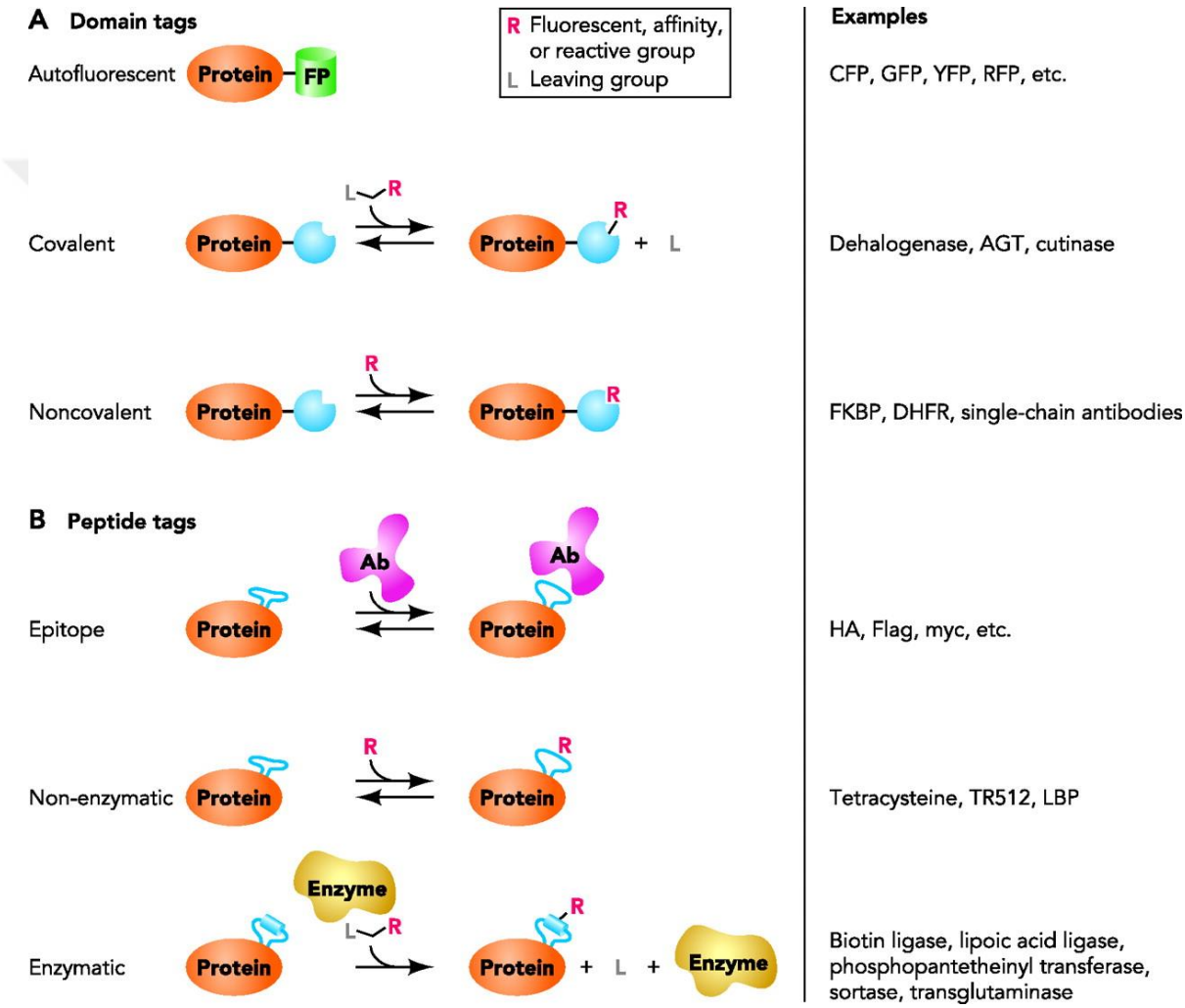


FIGURE 1. Classes of protein domain or peptide tags used for chemical labeling of fusion proteins. *A*: domain tags self-label either covalently or noncovalently. *B*: peptide tags either self-label with varying degrees of reversibility or utilize enzyme-mediated covalent attachment. The standard methods of fluorescent protein or epitope tags are shown for comparison. Examples of tags of each class are listed to the right.

1.3 Protein Labeling Systems

The fluorescent proteins (FP) is a genetically targetable probe that has been used extensively to investigate protein localization. However, FP fluorescence is limited with relatively low intrinsic brightness (near-infrared). Thus, visualization of protein localization in the deep region of tissues is very difficult. Moreover, FP-based indicators have undesirable possibility to form protein aggregates that could affect the function or localization of the target POIs.

Chemical labeling of target proteins is an intriguing solution to these problems, because a broad range of chemicals with different properties might all be targeted to the same labeling motif [3]. Several efforts have been made to chemically label target proteins in live cells, but these approaches have some limitations. Small-molecule fluorescent probes have been widely used to study protein localization as practical alternatives to the use of GFP for imaging. The commercialized representative examples of reported tag proteins are Lumio-tag , HaloTag, SNAP-tag, and DHFR-based tag.

Lumio-tag, using FlAsH or ReAsH, is based upon a fluorescent biarsenical dye and tetracysteine peptide interaction which results in dramatic fluorescence increase. This system can be fused not only to the N or C terminus of a protein, but it can also be incorporated into loops or on the outer surface of a helices with low probability of the tag interfering with target protein function, since tetracysteine motif is sufficiently small. Limitation is the high background fluorescence in biarsenical-labeled cells due to the nonspecific labeling of cysteine-rich proteins.[4]

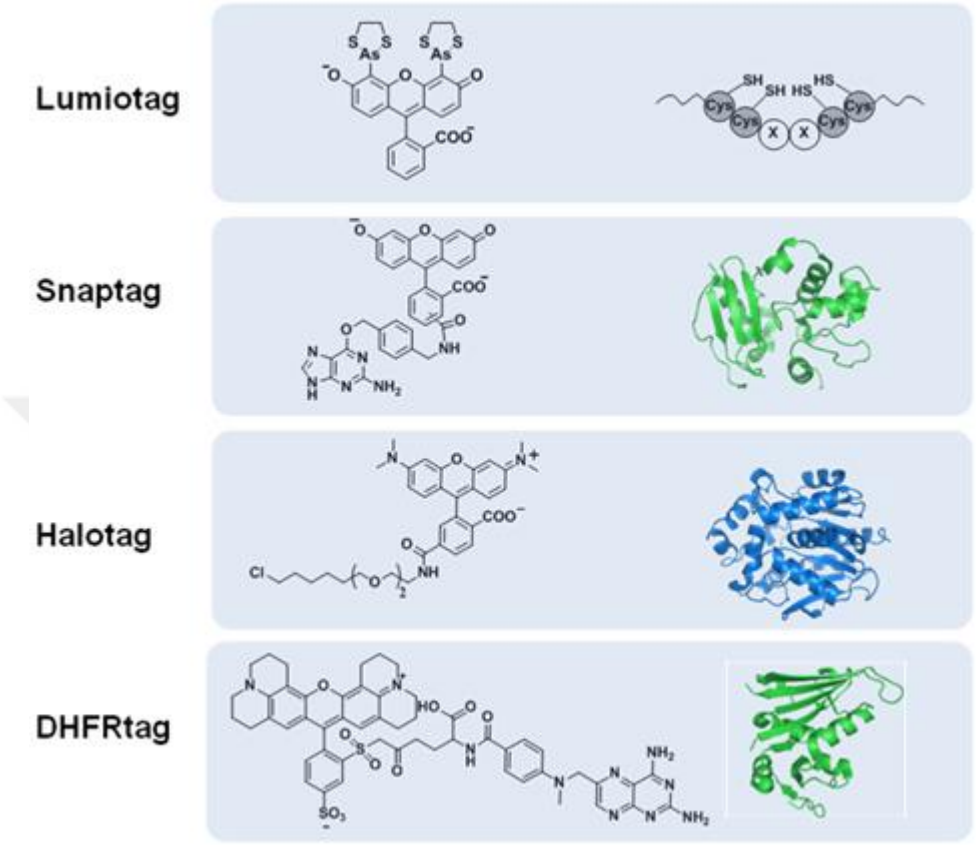


FIGURE 2 Commercialized Tags.

HaloTag is a modified haloalkane dehalogenase designed to react with synthetic haloalkyl ligands covalently, rapidly and specifically. The synthetic ligands comprise a chloroalkane linker attached to a variety of useful molecules, such as fluorescent dyes, affinity handles, or solid surfaces [5]. The binding of HaloTag to the HaloLink resin is essentially irreversible which enables to overcome the equilibrium-based binding limitations associated with affinity tags and provides efficient capture and purification of target protein, even at low expression levels.

SNAP-tag utilizes human O6 -alkylguanine-DNA-alkyltransferase (hAGT) as the protein tagged with synthetic benzylguanine derivatives. SNAP-tag labeling is irreversible and specific but this method can be applied only labeling proteins in hAGT-deficient cell lines, as the benzyl guanine substrates [4].

DHFR-based tag method is established on the noncovalent interaction between *Escherischia coli* dihydrofolate reductase (DHFR) and fluorescently labeled methotrexate. DHFR-deficient CHO cells transiently expressing fusions of eDHFR localized to the plasma membrane or nucleus could be effectively labeled by incubating the cells.[6] Despite the very efficient labeling, DHFR-based tag method has low stability problem.

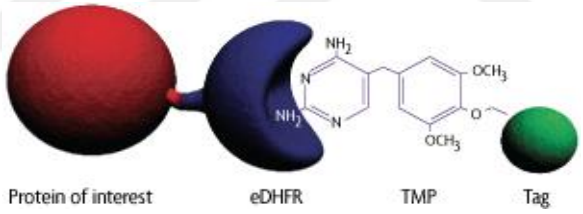


FIGURE 3 DHFR-tagging system.[7]

In addition, the other protein labeling methods have been also reported, for instance, ligase, transglutaminase, hexahistidine, and tetraaspartic acid-based methods.

The advantages of synthetic fluorescent probes are tunable switches for changes in fluorescence characteristics. These probes have intense fluorescence properties and provide a wide range of choices in fluorophores. Meanwhile, the advantages of using protein expression systems are provided by specific expression in cellular components and tissues, convenient experimental procedures, and the potential for

in vivo studies.[8] Combining the advantages of both techniques is expected to provide enhanced methods for exploring molecular functions in biological systems.

1.4 Native Chemical Ligation

Native chemical ligation and its extension, expressed protein ligation, chemically combine two or more peptide/protein fragments into a full-length protein. The N-terminal Cys of one peptide reacts with the C-terminal thioester of another peptide to form a native peptide link. Either recombinant or synthetic methods can be used for preparation of both the thioester peptide and the N-Cys peptide.

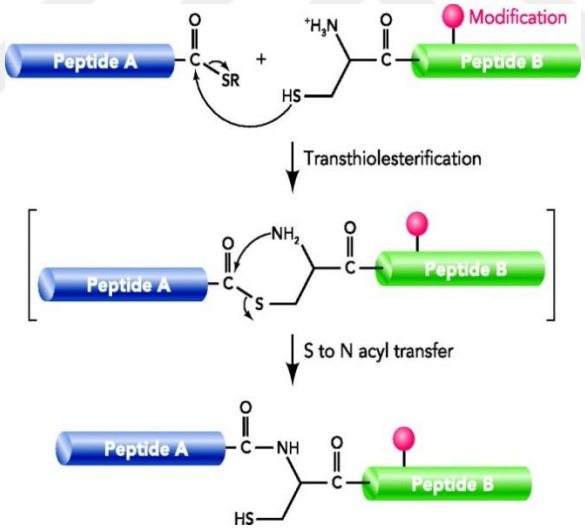


FIGURE 4 Principle of native chemical ligation.[10] Peptide A with a C-terminal thioester group reacts with peptide B with an N-terminal Cys residue through transthioesterification, resulting in a thioester-linked initial product. This product then undergoes an intramolecular rearrangement and gives the final polypeptide that is linked by a native peptide bond. Multiple modifications can be introduced into either peptide through chemical synthesis.

Desired modifications can be introduced in the synthetic peptide fragment by using chemical synthesis and incorporated into the target protein after ligation. This ligation system has been successfully utilized to engage various probes and modifications for studying protein structure and function in vitro [10].

The native chemical ligation method has the power to introduce diverse structures that are synthetically accessible, and multiple modifications can be incorporated in one synthetic peptide. Nonetheless, because peptides longer than 50 residues are difficult to synthesize using solid-phase peptide synthesis, appropriate sites for cleavage and ligation must be chosen carefully, and modifying internal sites in large proteins is cumbersome. The reaction requires relatively high concentration (~ mM), thus it is not practical to apply this technique to cell biology research for the moment. Thus, new methods for increasing reaction efficiency in cells are awaited to solve this problem.

1.5 Coiled Coil

Coiled coils are structural motifs constructed by two or more alpha-helices that associates with each other to form dimer, and more multimeric structure. There can be two, three or four helices in the bundle and these bundles orient in the same (parallel) or in the opposite (antiparallel) direction.

Each strand of a coiled-coil protein may be viewed as a repeated consensus substrings of the form (a-b-c-d-e-f-g)n, where a, b, c,...,g are the seven different structural positions on the coil. The first and fourth position (a and d) are generally apolar or hydrophobic amino acids. When the two strands coil around each other positions a and d are internalized, stabilizing the structure, while positions b, c, e, f, g are exposed on the surface of the protein.

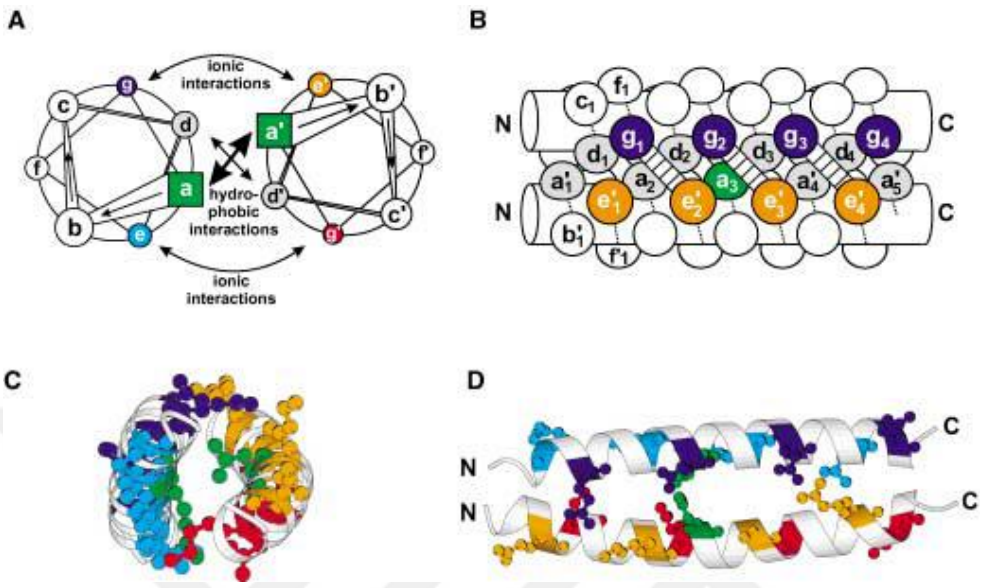


FIGURE 5 A parallel dimeric coiled coil in a schematic representation (A and B) and as ribbon plot of the X-ray structure of the leucine zipper of GCN4. (C and D).[10] Selected side chains are shown as balls and sticks. The helical wheel diagram in (A) and the plot in (C)look down the axis of the α-helices from N-terminus to C-terminus. Panel (B) and (C) provide a side view. The residues are labeled a - g in one helix and a' – g' in the other. The hydrophilic interactions (g and g' in blue and red, respectively ; e and e' in cyan and orange, respectively) within the heptad repeat are shown. In the schematic representations, the hydrophobic core (a/a' and d/d') is shown. For clarity, in the X-ray structure, only the middle a position with the exceptional charged residue is given as a green ball-and-stick model. Parts C and D were generated with molscript.

Leucine-zippers are one of the well-studied subtype of coiled coil construct, in which the amino acid leucine is predominant at the 'd' position of the heptad repeat. They may be shorter than 28 amino acids. Leucine-zipper is proven to be important for the function of the protein.

The designed peptides, Acid-p1 and Base-p1, associate in solution to form a parallel, heterodimeric two-stranded coiled coil. The buried interface of this complex is formed by hydrophobic Leu residues, with the exception of an Asn residue from each strand that is positioned to engage in a buried polar

interaction. Substitution of these buried Asn residues by Leu residues results in a loss of structural uniqueness, as evidenced by a lack of a particular helix orientation in the Acid-Base coiled-coil complex.

Peptide Velcro altered the positions of the Asn residues in the Acid and Base peptides such that a buried polar interaction is only expected to occur when the helices are in an antiparallel orientation. The resulting peptides, Acid-a1 and Base-a1, associate to form a helical heterodimer, confirmed with circular dichroism (CD) and equilibrium sedimentation centrifugation. The helix orientation preference has been measured using covalently linked, disulfide-containing heterodimers in which the constituent peptides are constrained to interact in either a parallel or an antiparallel orientation. [11]

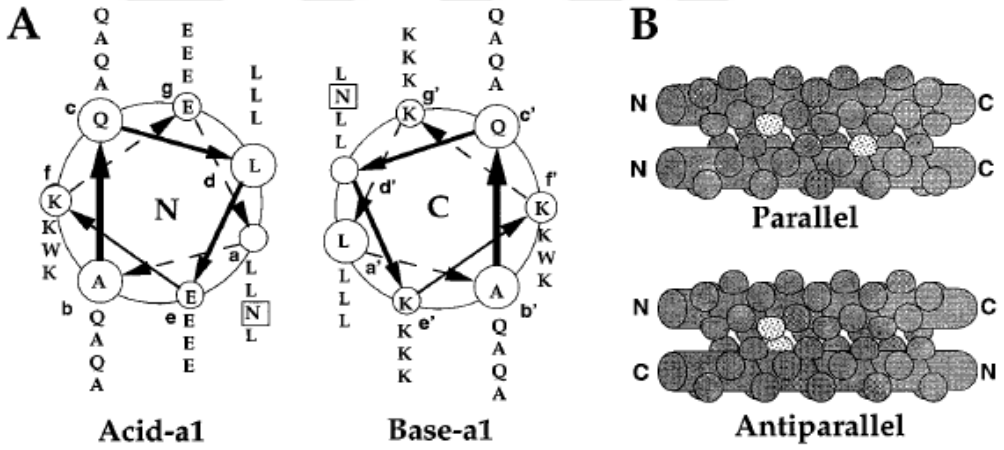


FIGURE 6 (A) Helical wheel representation of the antiparallel Acid-a1-Base-a1 heterodimer. The view is shown looking down the helical axis from the N terminus of Acid-a1 and the C terminus of Base-a1. The peptide sequence of Acid-a1 is Ac-AQLEKELQALEKELAQLEWEN QALEKELAQ-NH2. The sequence for Base-a1 is Ac-AQLKKKLQANKKKLAQLKWKLQALK KKLAQ-NH2. **(**B**)** Schematic view of parallel and antiparallel complexes of Acid-a1 and Base-a1, with the Asn residues represented by stippled semicircles. In the antiparallel complex, the two Asn residues can interact, but in the parallel complex, the Asn residues are separated by more than three turns of helix.

Although both the parallel and antiparallel heterodimers form stable, helical structures, the antiparallel heterodimer is the predominant species at equilibrium when the heterodimers are allowed to undergo thiol-disulfide exchange. In addition, the antiparallel heterodimer is more stable to chemical denaturation than the parallel counterpart by approximately 2.3 kcal/mol. These results demonstrate that a single buried polar interaction in the interface between the helices of a coiled coil is sufficient to determine the relative orientation of its constituent helices.[11]

Chapter 2 Principle of Labeling System and Design of Peptide Probe

2.1 Basis of Concept

The fusion of a fluorescent protein to target proteins has revealed a wealth of information on many biological systems over the last decade. Compared with fluorescent proteins, small molecule probes offer a wider range of property and functionality, since they can be derivatized with fluorophores, haptens, or reactive chemical groups. To attach small molecule probes to proteins, new methods have emerged in recent years involving fusion of protein or peptide tags followed by either noncovalent or covalent binding of small molecule probes to the tags. These probes are designed with functional groups at positions that will not interfere with binding to the tag. A different set of techniques allow specific protein labeling with functional groups with single amino acid precision [9]. These techniques include chemical modification, native chemical ligation, and genetic incorporation of unnatural amino acids. These new approaches differ from earlier biochemical techniques in allowing greater precision of labeling, either in location on the protein or in time, and in allowing use in or on live cells. Basically our new design;

Firstly, native chemical ligation mediated covalent tag system is established with C-terminal thioester and N-terminal cysteine residue.

Coiled coil interaction mediated non-covalent tag system is used to accelerate covalent ligation. Acid-T and Base-T peptides was utilized for this purpose. Covalent attachment of chemical probes has the advantage of greater irreversibility compared with noncovalent binding.

Second part of our design is protein of Interest fused with recombinant tag. Photo Active Yellow Protein (PYP) has chosen as model protein of interest because; small size of PYP (125 amino acids(14 kDa)), soluble, monomeric, can be expressed well in *E. coli*. and PYP is involved with negative

phototaxis of *halorhodospira halophile.* For labeling 6-Carboxy Fluorescein (6-CF) is combined to the substrate as marker fluorescent material.

Basic mechanism; ligation starts with noncovalent interaction of Basea1-Acida1 affinity tags and then transthioesterification occurs between C-terminal Cys and N-terminal thioester. Afterwards intermediate formation, internal nucleophilic attack leads spontaneous rearrangement and finally fluorescent labeling was obtained through natural peptide bond. This system offers stable and specific N-terminal labeling and does not require free thiol substitution.

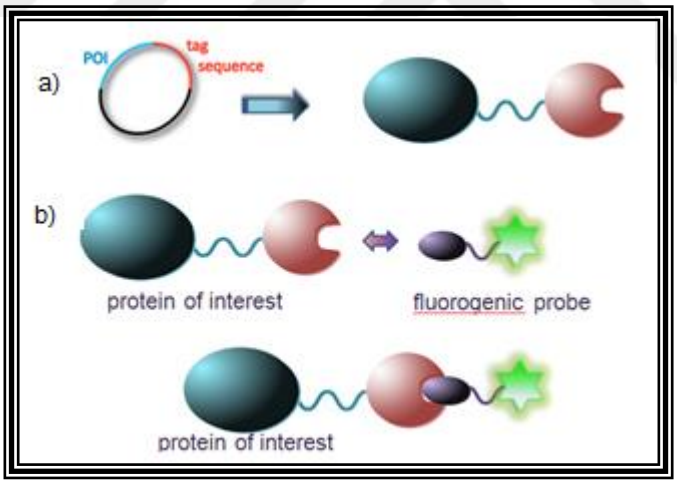


FIGURE 7 Demonstration of fusion tag system and fluorescent labeling. a) DNA encoding protein of interest (POI) fused to a receptor domain. b) Small molecule probe consisting of a ligand coupled to a detectable tag.

2.2 Previous Study

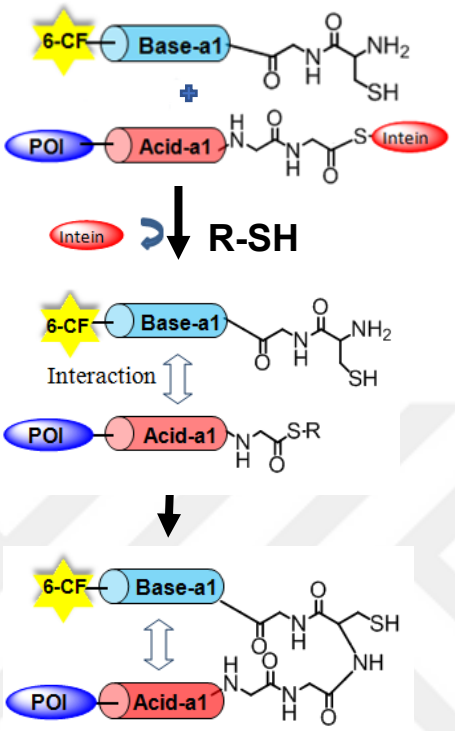


FIGURE 8 Previous strategy developed in our lab. Principle of protein labeling based on noncovalent coiled-coil interaction and intein-mediated ligation.

In a previous study conducted by our group, intein-coiled coil-coupled labeling system was developed. In this system, in order to attain efficient protein labeling, affinity tags, coiled coil, were introduced into the reactants. This ensured that the local concentrations of the reactants would increase. As a result, it is expected that the ligation reaction driven by the intein-derived thioester would occur at low concentration. The coiled coil, Acid-a1 and Base-a1, which form an antiparallel coiled coil, were chosen because these peptides are small (30 amino acids) and the interaction mechanism is well-known. In this labeling method, interaction of the coiled coil was combined with the intein-mediated ligation for protein labeling. Maltose binding protein (MBP) was employed as a model POI and Acid-a1 was fused to its C terminus (MBP-Acid). The intein was further connected to the C terminus of MBP-Acid to generate a fusion protein MBPAcid-In, which could form a thioester in the C terminus of MBP-Acid. As

a labeling reagent, fluorescein-conjugated Basea1 (F-Base), which contains Cys at its N terminus, was prepared with Fmoc solid phase method. Acid-a1 with C-terminal thioester (Acid-T) was also synthesized for interaction analysis and model ligation with F-Base[1].

The main point of this strategy is intein, which is essential for generating thioester at the protein's C terminus. First, intein is removed in the presence of free thiol and Acid-T and F-Base forms an antiparalel coiled coil, which is non-covalent. Covalent bond formation follows non-covalent interaction, labeling was completed after internal rearrangement of covalent bond. However, this method has limitations that only C-terminal labeling is enabled and a large amount of free thiol is required.

2.3 Novel Design Strategy

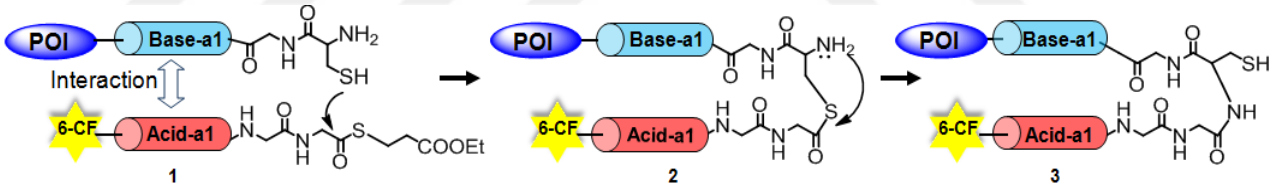


FIGURE 9 Novel Design Basic components of design.

Chapter 3 Results and Discussion

The protein was isolated from E. coli and the peptides were obtained by chemical synthesis with F-moc solid protocol. Labeling reaction is conducted by using peptide probe, Acid-T without fluorophore. Ligation reaction was confirmed by SDS PAGE. The upper band in the gel is represented as a labeled BASE-PYP, while the lower band is unlabeled BASE-PYP.

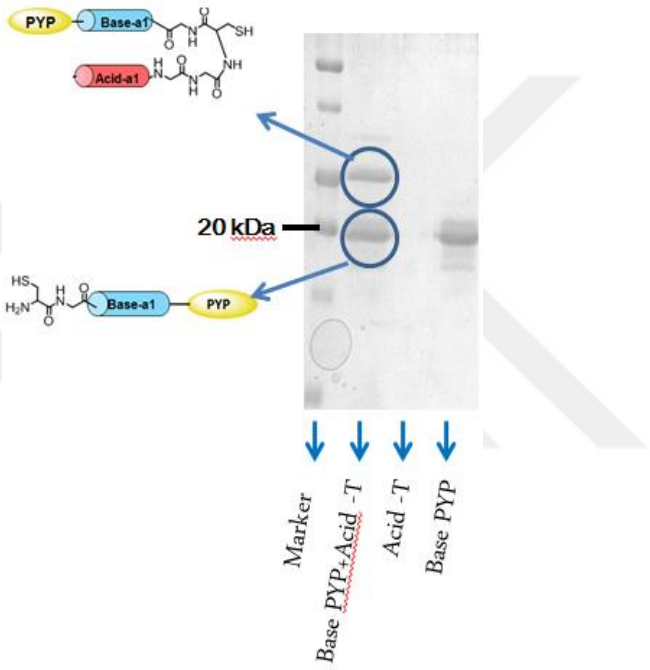


FIGURE 10 Labeling reaction of BASE-PYP with Acid-T.

To investigate the significance of thioester, Acid a1 affinity tag without thioester was used as a negative control reagent. As shown in the SDS page, peptide probe without thiolester does not lead to labeling reaction, judging from the result that no upper band was obtained. This proves that thioester and the following covalent bond formation are important for this labeling reaction.

FIGURE 11 Labeling reaction of BASE-PYP with Acid-T1 without thioester.

To verify the labeling kinetics, time course experiments were conducted. Results showed that labeling is almost completed within 1-2h

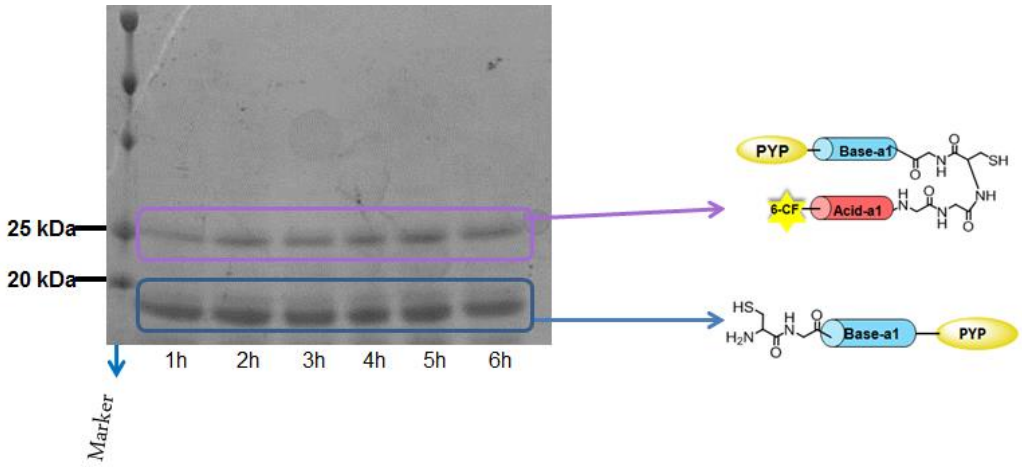


FIGURE 12 Time course experiments of labeling reaction of BASE-PYP with 6CF-Acid-T.

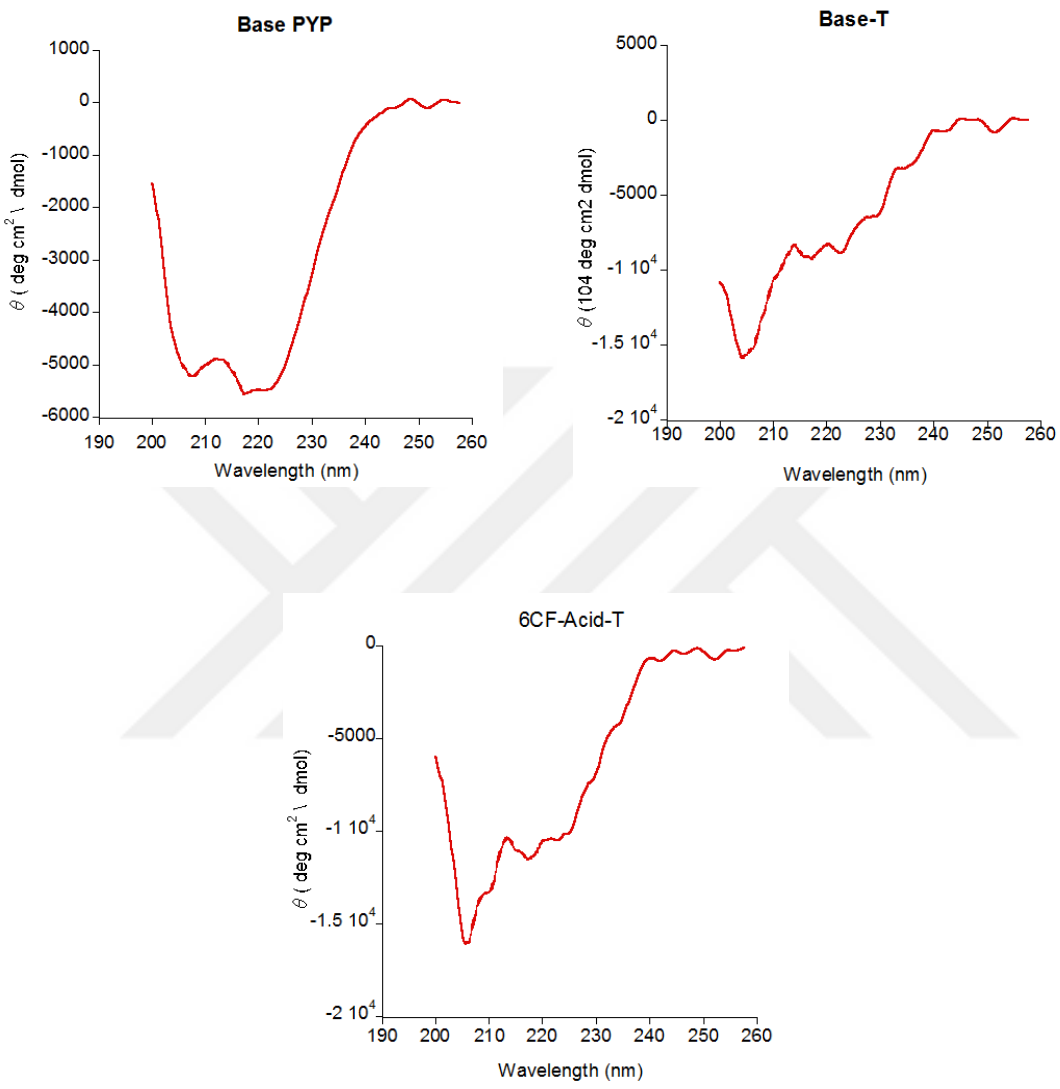
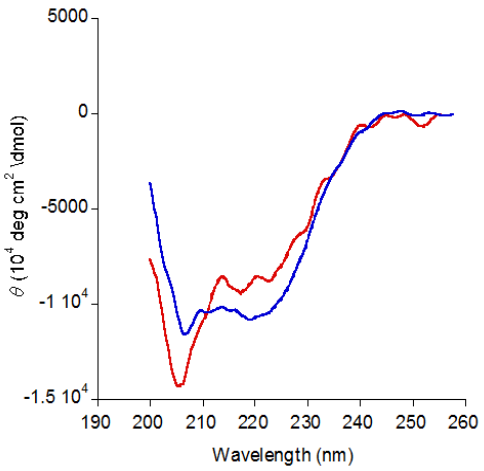


FIGURE 13 CD spectra of BASE-PYP, BASE-T, and 6CF-Acid-T.

A



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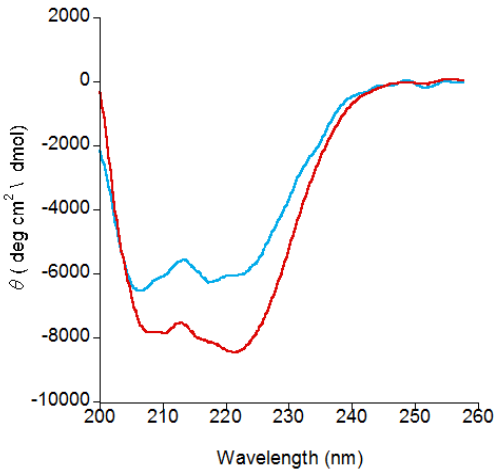


FIGURE 14 A) CD spectra of BASE-PYP (blue) and 6-CF-AcidT (red). B) Spectral change of BASE-PYP in the presence of 6-CF-Acid-T. Blue line represents the sum of the spectrum of Base PYP (blue) and 6-CF-AcidT (red) in (A). Red line shows the spectrumof BASE-PYP complexed with 6-CF-Acid-T.

To confirm coiled coiled formation, circular dichroism (CD) analyses were carried out. CD signal is very sensitive to the secondary structure of polypeptides and proteins. Thus, CD can be used to follow conformational changes of secondary structure. CD spectra of Base PYP (POI), Base-T and 6-CF-AcidT (peptide probe) was taken. In comparison with the simulated spectrum of the simple sum between BASE-PYP and 6-CF-Acid-T, the actual spectrum of the complex displays more negative signal intensity. Thus, the spectral difference is the proof of secondary structure as a result of non-covalent interaction.

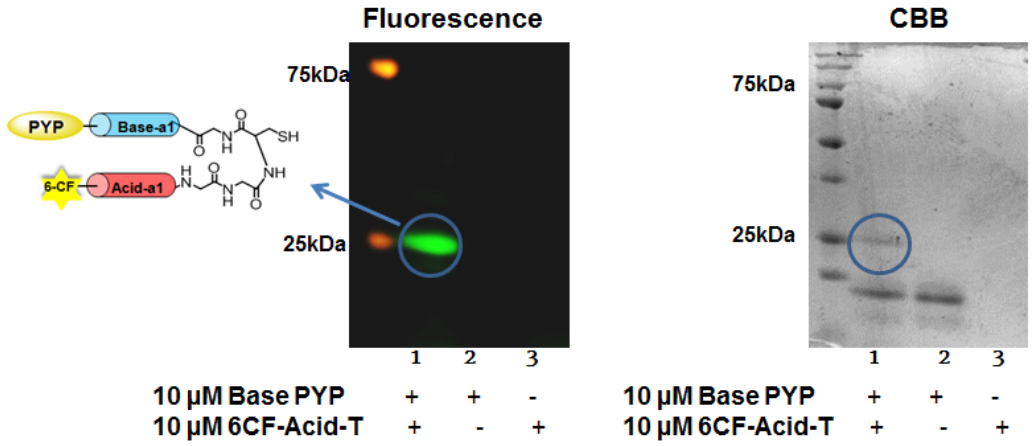


FIGURE 15 Labeling reaction of POI with fluorophore conjugated peptide probe. Reaction is controlled with SDS-PAGE . Figure is illustration of both fluorescent and CBB dyed version of SDS-PAGE.

The labeling reaction of BASE-PYP with a fluorophore-conjugated peptide, 6CF-Acid-T, was analyzed by SDS-PAGE. A fluorescent band was detected in the position, where the protein appeared. This result demonstrate that the labeling reaction occurs and BASE-PYP was covalently conjugated with the peptide probe, because the SDS-PAGE denatures the protein structure and destroy the noncovalent interaction.

Finally, the labeling reaction in cell lysate was conducted to verify the specificity of the conjugation between BASE-PYP and 6-CF-Acid-T. A single fluorescent band was observed in the position of the molecular weight of BASE-PYP. This result demonstrates that the labeling reaction is extremely specific.

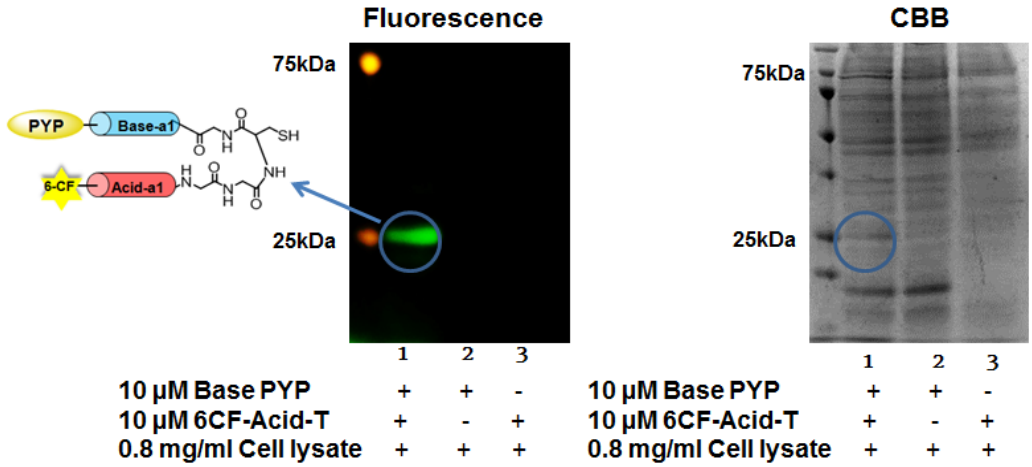


FIGURE 16 Labeling reaction in cell lysate . Specificity is verified by SDS-PAGE. . Figure is illustration of both fluorescent and CBB dyed versions.

In conclusion, we develop a novel protein labeling method by combining coiled coil-based noncovalent interaction and native chemical ligation. By virtue of the specific and stable characteristics of protein labeling and the small size of the protein tag, this technique offers an attractive tool for the detection of proteins in cell lysate.

Chapter 4 Experimental Procedures

4.1 Base－PYP Isolation

- *Protein expression and purification:* E. coli, BL21(DE3) (Novagen), was transformed with pET-His-Base-PYP and grown to an OD600 of 0.6–1.0 in Luria-Bertani medium supplemented with ampicillin (100 μg / L medium) glucose (2 g/L medium) at 37 °C. At this point, the temperature was lowered to 25°C and the protein was expressed overnight by adding isopropyl-β-D-thiogalactopyranoside to the medium with a final concentration of 100 μM. The cells were harvested by centrifugation at 5000 rpm for 10 min; resuspended in column buffer (50 mM sodium phosphate, 300 mM NaCl, 10 mM imidazole, pH 7.5); and lysed by sonication at 4°C (during the purification, the sample was kept at 4°C). The supernatant of the cell lysate was obtained by centrifugation at 10000 rpm for 30min and passed through Ni-NTA column chromatography (Qiagen). Next the resin was washed with washing buffer buffer(50 mM sodium phosphate, 300 mM NaCl, 20 mM imidazole, pH 7.5) and eluted with elution buffer (50 mM sodium phosphate, 300 mM NaCl, 250 mM imidazole, pH 7.5), in accordance with the manufacturer's protocol. For further purification, the elutant was passed through a size exclusion column (GE Healthcare). Then, the protein with N-terminal Cys was generated by protease cleavage reaction; 44 μM Base-PYP reacted with 0.1 U enterokinase enzyme in cleavage buffer(supplied by company) at 20^{o}C for 18h. Reaction is terminated and purified by benzamidine coloumn (GE Healthcare).and the sample was further purified by Ni-NTA column chromatography.The resin was washed with column buffer containing 10 mM imidazole, and reaction mixture eluted. Finally, the buffer exchange was conducted by ultracentrifugation to keep the purified protein in the assay buffer (10mM HEPES, 1 mM TCEP (pH 7.5)). The purity and size of the protein were assessed by SDS-PAGE.

4.2 Peptide Probe Synthesis

Acid-T: (Ac-Acid-a1-G_3-S-$(CH_2)_2$-COOEt)

Ac-
AQLEKELQALEKELAQLEWENQALEKELAQG
GG-S-$(CH_2)_2$-COOEt

CF-Acid-T: (6-CF-Acid-a1-G_3-S-$(CH_2)_2$-COOEt)

6-CF-
AQLEKELQALEKELAQLEWENQALEKELAQG
GG-S-$(CH_2)_2$-COOEt

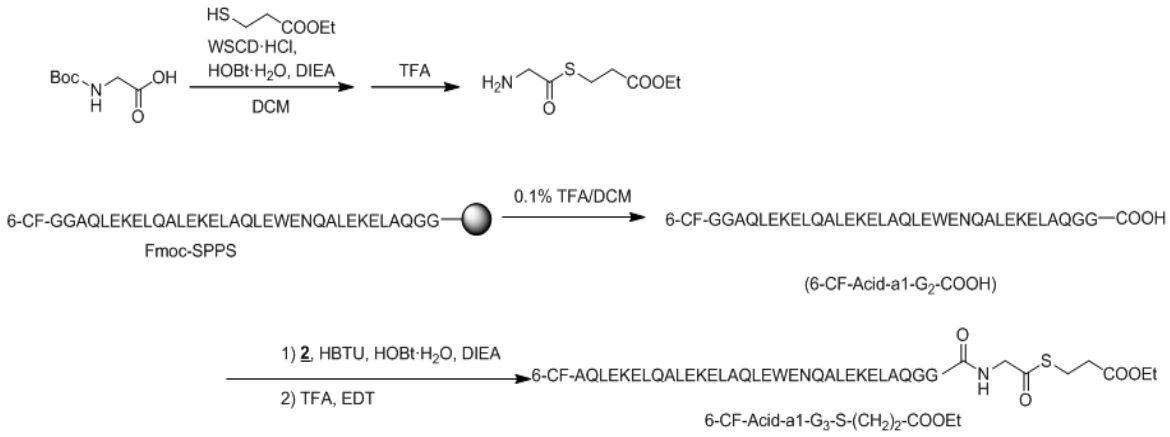


FIGURE 18 Synthesis of peptides.

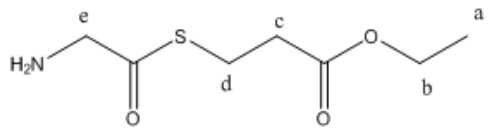
-*Peptide synthesis:* Peptides were synthesized by standard Fmoc solid-phase method. To prepare peptide probes, 6CF-Acid-T and Acid-T, containing thioester, a thiol compound was reacted with peptide probes, 6CF-Acid-a1 and Acid-T, with carboxylic acid at the C-terminus in liquid phase.

4.3 Labeling Experiments

-*Protein labeling experiments:* Acid-T(10 µM) or 6CF-Acid-T (10 µM) and Base-PYP (10 µM) was mixed and incubated in the assay buffer (10 mM HEPES, 1 mM TCEP, pH 7.4) at 25 °C. Protein labeling was confirmed with 15% SDS-PAGE. Control experiment was carried out similar. Acid-a1-G2 (10 µM) and Base-PYP (10 µM) was mixed and incubated in the assay buffer (10 mM HEPES, 1 mM TCEP, pH 7.4) at 25 °C.

-*CD spectroscopy:* The CD spectra of the complex between F-Base (10 µM) and Acid-T (10 µM, between AcidT (10 µM) and Base-PYP (10µM) or 6CF-AcidT (10 µM) and Base-PYP (10µM) were recorded in 2 mM HEPES buffer (pH 7.5) containing 100 µM TCEP at 25°C. The measurement was performed in a cuvette with a path length of 0.2 cm

Chapter 5 Compound Data



^{1}H-NMR(400 MHz, $CDCl_3$)

δ1.26 (t, *J* = 6.8 Hz, 3H, a) 2.64 (t, *J* = 7.2 Hz, 2H, d) 3.24(t, *J* = 6.8 Hz, 2H, c) 4.12 (s, 2H, e) 4.16 (q, *J* = 7.2 Hz, b)

MS (ESI+) m/z Calcd for [M+H]$^+$: 192.07

Found : 192.08

Base-a1

MS (MALDI+) m/z

Calcd for [M+H]$^+$: 3734.70

Found : 3734.47

Acid-a1-G_2

MS (MALDI+) m/z

Calcd for [M+H]$^+$: 3681.2

Found : 3681.4

Acid-a1-G_3-S-$(CH_2)_2$-COOEt

MS (MALDI+) m/z

Calcd for [M+H]$^+$: 3854.17

Found : 3854.28

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Gulcihan Gulseren

2011